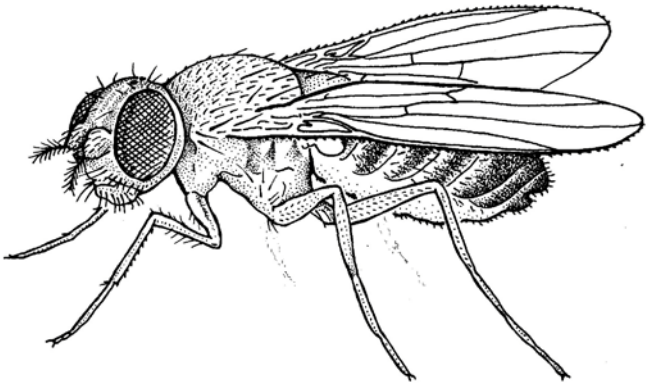
Drosophila Information Service



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Preface

This is the 100th volume of Drosophila Information Service (often called "DIS" by those in the field), which was first printed in March, 1934. For those first issues, material contributed by *Drosophila* workers was arranged by C.B. Bridges and M. Demerec. As noted in its preface, which is reprinted in Dros. Inf. Serv. 75 (1994), Drosophila Information Service was undertaken because, "An appreciable share of credit for the fine accomplishments in *Drosophila* genetics is due to the broadmindedness of the original *Drosophila* workers who established the policy of a free exchange of material and information among all actively interested in *Drosophila* research. This policy has proved to be a great stimulus for the use of *Drosophila* material in genetic research and is directly responsible for many important contributions." Since that first issue, DIS has continued to promote open communication.

The production of this volume of DIS could not have been completed without the generous efforts of many people. Except for the special issues that contained mutant and stock information now provided in detail by FlyBase and similar material in the annual volumes, all issues are now freely-accessible from our web site: www.ou.edu/journals/dis. For early issues that only exist as aging typed or mimeographed copies, some notes and announcements have not yet been fully brought on line. But we intend to fill in those gaps for historical purposes in the future.

We continue to encourage all researchers to consider submitting articles that use *Drosophila* for teaching as well as articles that report new techniques, research results, and interesting new mutations. In the interests of honoring the long-standing philosophy of open exchange of ideas, we sometimes accept articles that have unusual or limited perspectives. We thank the many contributors from around the world who sent material for this issue, and we invite your submissions for future annual issues as well as any suggestions you have for maintaining this as a useful *Drosophila* research community resource.

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List of Contributions

General Announcements

Research Notes

Mutation Notes

Technique Notes

Teaching Notes

Other Reports

Research Notes

Female sterility in *Drosophila melanogaster* bearing TRiP *shRNAi* constructs driven by *traffic-jam-GAL4.*

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The ovary is a sex-specific organ composed of somatic cells and germ cells. In addition to structural roles, ovarian somatic cells are critical regulatory components of the germline stem cell niche, providing instructive signals for proliferation and sexual identity of the germline (Oliver, 2002; Murray *et al.,* 2010). Additionally, follicle cells provide patterning information to the developing oocyte and produce the vitelline membrane and chorion (Spradling, 1993). Emerging tools, such as controllable short hairpins to mediate RNAi (*shRNAi*), are increasing the ease of doing clonal analysis to determine the roles of genes in specific cell types in the context of the whole animal (Perkins *et al.,* 2015).

The binary control system where yeast GAL4 is expressed under the control of tissue-specific promoters to drive expression of other transgenes with upstream activating sequences (UAS) allows for expression in specific cell types (Brand and Perrimon, 1993). The *traffic jam* driver (*tj-GAL4*; FBgn0000964) is expressed in all the somatic cells that contact the germline from gonadogenesis in late embryonic development to adulthood (Li *et al.,* 2003). By using the *tj-GAL4* driver with *UAS-shRNAi* lines from the Transgenic RNAi Project (TRiP) (Perkins *et al.,* 2015), we have screened for genes that are required for female fertility in *tj* expressing cells. We randomly selected 1156 *shRNAi* TRiP lines collection in September of 2012 using the RAND function in excel to explore the frequency of female sterility (although it should be noted that the TRiP collection coverage was not random at that time).

Flies were grown on cornmeal media (LabExpress, Ann Arbor, Michigan; yeast 15 gm, cornmeal 27 gm, sucrose 43 gm, agar 8 gm, 0.4% propionic acid, 0.15% tegosept, and water to 1 L; http://lab-express.com/flyfoodsupplies.htm#flyfood, Flyfood A). Males from the *shRNAi* lines were crossed to *tj-GAL4/CyO, Kr-Gal4 UAS-GFP* virgins. The *tj-GAL4/+ UAS-shRNAi/+* females were collected and mated to w^{1118} */Y (*BL# 3605, FBst0003605) males, grown at 25°C and allowed to lay eggs for 3-5 days. The presence of progeny was scored after 10 days. We found that 184 trans-heterozygous genotypes resulted in female sterility (15.9% of tested lines). Next we were interested in checking if we identified any new female sterile alleles at these loci. Out of 184, 52 lines have been reported to have female sterile alleles in previous studies (Gramates *et al.*, 2017). The remaining 132 are the first female sterile alleles for those loci. Results are summarized in Table 1.

Acknowledgments: We thank members of the Oliver lab for discussions. This work was supported by the Intramural Research Program of the NIH, The National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK).

References: Brand, A.H., and N. Perrimon 1993, Development 118**:** 401-415; Gramates, L.S., S.J. Marygold, G.D. Santos, J.M. Urbano, G. Antonazzo, B.B. Matthews, A.J. Rey, C.J. Tabone, M.A. Crosby, D.B. Emmert, K. Falls, J.L. Goodman, Y. Hu, L. Ponting, A.J. Schroeder, V.B. Strelets, J. Thurmond, P. Zhou, and C. the FlyBase, 2017, Nucleic Acids Res 45**:** D663-D671; Li, M.A., J.D. Alls, R.M. Avancini, K. Koo, and D. Godt 2003, Nat. Cell Biol. 5**:** 994-1000; Murray, S.M., S.Y. Yang, and M. Van Doren 2010, Curr. Opin. Cell Biol. 22**:** 722-729; Oliver, B., 2002, Int. Rev. Cytol. 219**:** 1-60; Perkins, L.A., L. Holderbaum, R. Tao, Y. Hu, R. Sopko, K. McCall, D. Yang-Zhou, I. Flockhart, R. Binari, H.S. Shim, A. Miller, A. Housden, M. Foos, S. Randkelv, C. Kelley, P. Namgyal, C. Villalta, L.P. Liu, X. Jiang, Q. Huan-Huan, X. Wang, A. Fujiyama, A. Toyoda, K. Ayers, A. Blum, B. Czech, R. Neumuller, D. Yan, A. Cavallaro, K. Hibbard, D. Hall, L. Cooley, G.J. Hannon, R. Lehmann, A. Parks, S.E. Mohr, R. Ueda, S. Kondo, J.Q. Ni, and N. Perrimon

2015, Genetics 201**:** 843-852; Spradling, A., 1993 *The Development of* Drosophila melanogaster, edited by M. Bate. Cold Spring Harbor Laborartory Press.

Table1. Genes tested for female sterility using tj-GAL4.

Row number	FlyBase gene name	FlyBase gene identifier	Transgenic RNAi Project identifier	Female Sterility result
1	Actn3	FBgn0015008	JF02279	Yes[1]
2	ago	FBgn0041171	HMS00111	Yes[1]
3	Ald	FBgn0000064	JF02071	Yes[1]
4	alpha-Cat	FBgn0010215	HMS00317	Yes[1]
5	Ama	FBgn0000071	HMS00297	Yes[1]
6	Ance	FBgn0012037	HMS03009	Yes[1]
7	Aos1	FBgn0029512	HM05183	Yes[1]
8	APC10	FBgn0034231	HMS00176	Yes[1]
9	Arp1	FBgn0011745	HM05259	Yes[1]
10	Arpc3A	FBgn0038369	JF02370	Yes[1]
11	ATPsynB	FBgn0019644	JF02899	Yes[1]
12	ATPsynD	FBgn0016120	HMS01078	Yes[1]
13	betaggt-I	FBgn0015000	HMS01165	Yes[1]
14	Cam	FBgn0000253	HMS01318	Yes[1]
15	Ced-12	FBgn0032409	HM05042	Yes[1]
16	CG11247	FBgn0037120	JF02238	Yes[1]
17	CG11317	FBgn0039816	JF02902	Yes[1]
18	CG12050	FBgn0032915	HM05080	Yes[1]
19	CG12128	FBgn0033473	HMS00960	Yes[1]
20	CG13096	FBgn0032050	HMS00206	Yes[1]
21	CG13692	FBgn0031254	HMS00207	Yes[1]
22	CG15534	FBgn0039769	HMS03023	Yes[1]
23	CG17385	FBgn0033934	HMS00617	Yes[1]
24	CG30020	FBgn0050020	JF02532	Yes[1]
25	CG33123	FBgn0053123	HMS01335	Yes[1]
26	CG3803	FBgn0034938	HMS01473	Yes[1]
27	CG4038	FBgn0011824	HMS00979	Yes[1]
28	CG4115	FBgn0038017	HMS01446	Yes[1]
29	CG42307	FBgn0259203	HMS00381	Yes[1]
30	CG5261	FBgn0031912	HMS02170	Yes[1]
31	CG5589	FBgn0036754	HMS00325	Yes[1]
32	CG5660	FBgn0035942	HMS01327	Yes[1]
33	CG7840	FBgn0032014	HMS00426	Yes[1]
34	CG8005	FBgn0035854	HMS02169	Yes[1]
35	CG8728	FBgn0033235	HMS00561	Yes[1]
36	CG8858	FBgn0033698	HMS01128	Yes[1]
37	CG9667	FBgn0037550	HM04069	Yes[1]
38	CG9865	FBgn0034649	HMS01785	Yes[1]
39	cnk	FBgn0021818	HMS00238	Yes[1]

40	croc	FBgn0014143	HMS01122	Yes[1]
41	Cul3	FBgn0261268	HMS01572	Yes[1]
42	CycA	FBgn0000404	JF02472	Yes[1]
43	CycK	FBgn0025674	HMS01003	Yes[1]
44	Cyp4g15	FBgn0030304	JF02914	Yes[1]
45	cype	FBgn0015031	HMS00815	Yes[1]
46	Cyt-c1	FBgn0035600	HMS01057	Yes[1]
47	Dbp80	FBgn0024804	HMS01160	Yes[1]
48	DCTN2-p50	FBgn0021825	HM05084	Yes[1]
49	Deaf1	FBgn0013799	HMS00516	Yes[1]
50	defl	FBgn0036038	JF02801	Yes[1]
51	Drice	FBgn0019972	HMS00398	Yes[1]
52	east	FBgn0261954	HMS00816	Yes[1]
53	eIF-4a	FBgn0001942	HMS00927	Yes[1]
54	Eip75B	FBgn0000568	HMS01530	Yes[1]
55	form3	FBgn0053556	HMS00393	Yes[1]
56	Ggamma30A	FBgn0267252	HMS01362	Yes[1]
57	Hsc70-1	FBgn0001216	HMS00888	Yes[1]
58	Itp-r83A	FBgn0010051	JF01957	Yes[1]
59	jing	FBgn0086655	HMS01493	Yes[1]
60	kni	FBgn0001320	HMS01184	Yes[1]
61	kto	FBgn0001324	HMS01062	Yes[1]
62	l(3)72Ab	FBgn0263599	HMS00994	Yes[1]
63	LanA	FBgn0002526	JF02908	Yes[1]
64	lat	FBgn0005654	JF01915	Yes[1]
65	lin-52	FBgn0029800	HMS01602	Yes[1]
66	lov	FBgn0266129	HMS01126	Yes[1]
67	MED14	FBgn0035145	HMS01049	Yes[1]
68	MED16	FBgn0034707	HMS00978	Yes[1]
69	MED22	FBgn0040339	HMS01047	Yes[1]
70	MED25	FBgn0038760	HMS00256	Yes[1]
71	MED27	FBgn0037359	HMS01050	Yes[1]
72	MED28	FBgn0039337	HMS00458	Yes[1]
73	MED30	FBgn0035149	HMS01601	Yes[1]
74	MED8	FBgn0034503	HMS01275	Yes[1]
75	Mi-2	FBgn0262519	HMS00301	Yes[1]
76	mop	FBgn0036448	HMS00706	Yes[1]
77	msk	FBgn0026252	HMS00020	Yes[1]
78	Mtor	FBgn0013756	HMS00735	Yes[1]
79	mys	FBgn0004657	HMS00043	Yes[1]
80	Nat1	FBgn0031020	HMS01400	Yes[1]
81	ND-23	FBgn0017567	HM05229	Yes[1]
82	Nipped-B	FBgn0026401	HMS00401	Yes[1]
83	Nopp140	FBgn0037137	HMS00564	Yes[1]
84	Not1	FBgn0085436	HMS00526	Yes[1]
85	nudC	FBgn0021768	HMS00258	Yes[1]

86	Nup358	FBgn0039302	HMS00803	Yes[1]
87	Ostgamma	FBgn0032015	HMS01113	Yes[1]
88	Patr-1	FBgn0266053	HMS01144	Yes[1]
89	pcm	FBgn0020261	HMS01169	Yes[1]
90	pdm3	FBgn0261588	HMS01468	Yes[1]
91	pk	FBgn0003090	HMS00408	Yes[1]
92	Pop2	FBgn0036239	HM05235	Yes[1]
93	Prosalpha5	FBgn0016697	HMS00095	Yes[1]
94	Prosbeta5	FBgn0029134	HMS00119	Yes[1]
95	Prosbeta6	FBgn0002284	HMS00110	Yes[1]
96	Rab5	FBgn0014010	HMS00147	Yes[1]
97	Rala	FBgn0015286	HMS01365	Yes[1]
98	Ref1	FBgn0010774	HMS01301	Yes[1]
99	rept	FBgn0040075	HMS00410	Yes[1]
100	Rho1	FBgn0014020	HMS00375	Yes[1]
101	ric8a	FBgn0028292	HM05121	Yes[1]
102	rpk	FBgn0022981	HMS01973	Yes[1]
103	RpL22	FBgn0015288	HMS00143	Yes[1]
104	RpLP0	FBgn0000100	JF01335	Yes[1]
105	RpS13	FBgn0010265	HMS00135	Yes[1]
106	RpS28b	FBgn0030136	HMS00635	Yes[1]
107	Rs1	FBgn0021995	HMS00354	Yes[1]
108	SA	FBgn0020616	HMS00272	Yes[1]
109	scrib	FBgn0263289	HMS01993	Yes[1]
110	Sec15	FBgn0266674	JF02649	Yes[1]
111	SelD	FBgn0261270	JF03230	Yes[1]
112	shrb	FBgn0086656	HMS01767	Yes[1]
113	skd	FBgn0003415	HMS01305	Yes[1]
114	SmF	FBgn0000426	JF02276	Yes[1]
115	Sobp	FBgn0033654	HMS00275	Yes[1]
116	sqh	FBgn0003514	HMS00830	Yes[1]
117	Ssl1	FBgn0037202	JF03401	Yes[1]
118	sty	FBgn0014388	HMS01599	Yes[1]
119	Su(var)2-10	FBgn0003612	HMS00705	Yes[1]
120	Su(var)2-HP2	FBgn0026427	HMS01699	Yes[1]
121	Su(var)3-9	FBgn0263755	HMS00704	Yes[1]
122	Syx5	FBgn0011708	JF03330	Yes[1]
123	Tctp	FBgn0037874	HMS00701	Yes[1]
124	TH1	FBgn0010416	HMS00283	Yes[1]
125	Tlk	FBgn0086899	HMS00943	Yes[1]
126	tum	FBgn0086356	HMS01417	Yes[1]
127	Uba1	FBgn0023143	JF01977	Yes[1]
128	unc-104	FBgn0267002	HM05162	Yes[1]
129	Usp8	FBgn0038862	HMS01941	Yes[1]
130	VhaAC45	FBgn0262515	HMS01717	Yes[1]
131	Vps2	FBgn0039402	HMS01911	Yes[1]

132	woc	FBgn0010328	JF02398	Yes[1]
133	wol	FBgn0261020	HMS01354	Yes[1]
134	Akt1	FBgn0010379	HMS00007	Yes
135	Atx2	FBgn0041188	HMS01392	Yes
136	aurB	FBgn0024227	JF03107	Yes
137	beta-PheRS	FBgn0039175	HMS01345	Yes
138	br	FBgn0283451	HMS00042	Yes
139	Caf1-180	FBgn0030054	HMS00480	Yes
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141	Cas	FBgn0022213	JF02972	Yes
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143	CG11985	FBgn0040534	HMS00097	Yes
144	CG16941	FBgn0266917	HMS00157	Yes
145	CG6066	FBgn0039488	HMS00995	Yes
146	chb	FBgn0021760	HMS01146	Yes
147	coro	FBgn0265935	HMS02007	Yes
148	CtBP	FBgn0020496	HMS00677	Yes
149	DCTN1-p150	FBgn0001108	JF02803	Yes
150	dia	FBgn0011202	HMS00308	Yes
151	dom	FBgn0020306	HMS00142	Yes
152	egg	FBgn0086908	HMS00112	Yes
153	eIF-4E	FBgn0015218	HMS00969	Yes
154	eIF3-S10	FBgn0037249	HMS01342	Yes
155	fs(1)h	FBgn0004656	HMS02723	Yes
156	Gs1	FBgn0001142	HMS02002	Yes
157	Hrb27C	FBgn0004838	HMS00597	Yes
158	hrg	FBgn0015949	HMS00252	Yes
159	M6	FBgn0037092	HMS01645	Yes
160	mars	FBgn0033845	HMS00878	Yes
161	mr	FBgn0002791	HMS02023	Yes
162	Mrtf	FBgn0052296	HMJ02106	Yes
163	msn	FBgn0010909	HMJ02084	Yes
164	msps	FBgn0027948	HMS01906	Yes
165	Nedd8	FBgn0032725	HMS00818	Yes
166	Nox	FBgn0085428	HMS00691	Yes
167	Ntf-2	FBgn0031145	JF03048	Yes
168	PCNA	FBgn0005655	HMS00634	Yes
169	piwi	FBgn0004872	HMS00185	Yes
170	Rm62	FBgn0003261	HMS00144	Yes
171	sgg	FBgn0003371	HMS01751	Yes
172	sim	FBgn0004666	HMS00491	Yes
173	sktl	FBgn0016984	JF02796	Yes
174	SmD3	FBgn0023167	HMS00361	Yes
175	SmE	FBgn0261790	HMS00074	Yes
176	Snr1	FBgn0011715	HMS00363	Yes
177	Stat92E	FBgn0016917	HMS00035	Yes

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180	tra	FBgn0003741	JF03132	Yes
181	tsr	FBgn0011726	HMS00534	Yes
182	tsu	FBgn0033378	HM05166	Yes
183	U2af38	FBgn0017457	JF02444	Yes
184	wds	FBgn0040066	HMS00746	Yes
185	Sep1	FBgn0011710	JF02789	No
186	Sep2	FBgn0014029	JF02838	No
187	5PtaseI	FBgn0259178	JF03299	No
188	7B2	FBgn0041707	JF02917	No
189	Abp1	FBgn0036372	HMS01735	No
190	Ace	FBgn0000024	JF01978	No
191	achi	FBgn0033749	HMS01127	No
192	Ack	FBgn0028484	HMS00892	No
193	Act79B	FBgn0000045	HMS00303	No
194	Adf1	FBgn0000054	JF03095	No
195	AdoR	FBgn0039747	JF02687	No
196	Aef1	FBgn0005694	JF02233	No
197	Akh	FBgn0004552	HMS00477	No
198	Alp4	FBgn0016123	HMS00537	No
199	alph	FBgn0086361	HMS02040	No
200	alphaCOP	FBgn0025725	HMS01272	No
201	alphaTub84B	FBgn0003884	JF01373	No
202	aly	FBgn0004372	HMS01614	No
203	amn	FBgn0086782	JF01814	No
204	amon	FBgn0023179	HM05071	No
205	Amph	FBgn0027356	HMS01933	No
206	Antp	FBgn0260642	JF02754	No
207	AnxB10	FBgn0000084	HMS01720	No
208	aop	FBgn0000097	HMS01256	No
209	aos	FBgn0004569	JF03020	No
210	AP-1-2beta	FBgn0010380	JF02963	No
211	AP-1sigma	FBgn0039132	HMS02143	No
212	Apc	FBgn0015589	HMS00188	No
213	Aplip1	FBgn0040281	JF02049	No
214	ara	FBgn0015904	JF02402	No
215	Arf79F	FBgn0010348	JF01809	No
216	ArfGAP3	FBgn0037182	JF01649	No
217	Arp5	FBgn0038576	HMS00809	No
218	Arpc3B	FBgn0065032	JF02679	No
219	arr	FBgn0000119	JF01261	No
220	Arr2	FBgn0000121	JF02392	No
221	Ars2	FBgn0033062	HMS00626	No
222	Art4	FBgn0037770	JF01307	No
223	Asciz	FBgn0035407	JF02336	No

224	asl	FBgn0261004	HMS01453	No
225	AsnS	FBgn0270926	HMS01481	No
226	AstA	FBgn0015591	JF01905	No
227	AstA-R1	FBgn0266429	JF02578	No
228	Asx	FBgn0261823	JF01708	No
229	Atac2	FBgn0032691	HMS00678	No
230	Atf3	FBgn0028550	JF02303	No
231	Atg12	FBgn0036255	HMS01153	No
232	Atg18a	FBgn0035850	HMS01193	No
233	Atg18b	FBgn0032935	HMS01194	No
234	atl	FBgn0039213	HMS01627	No
235	ato	FBgn0010433	HMS01278	No
236	Atpalpha	FBgn0002921	HMS00703	No
237	az2	FBgn0025185	JF02128	No
238	b	FBgn0000153	JF02661	No
239	B-H1	FBgn0011758	HM04018	No
240	baf	FBgn0031977	HMS00195	No
241	ball	FBgn0027889	JF01308	No
242	bap	FBgn0004862	JF02404	No
243	Bap170	FBgn0042085	JF02080	No
244	bbx	FBgn0024251	JF02113	No
245	beat-Vb	FBgn0038092	JF03186	No
246	Bgb	FBgn0013753	JF02111	No
247	bi	FBgn0000179	JF02976	No
248	bif	FBgn0014133	JF03009	No
249	Bka	FBgn0010520	JF01415	No
250	bmm	FBgn0036449	JF01946	No
251	boca	FBgn0004132	JF02863	No
252	bocks	FBgn0037719	HMS01817	No
253	brat	FBgn0010300	HMS01121	No
254	brp	FBgn0259246	JF01932	No
255	brv1	FBgn0036874	JF01061	No
256	bsf	FBgn0032679	HMS01022	No
257	bt	FBgn0005666	JF01107	No
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259	btl	FBgn0005592	HMS02038	No
260	btv	FBgn0023096	JF03010	No
261	Burs	FBgn0038901	JF02260	No
262	Bx	FBgn0265598	JF03390	No
263	by	FBgn0000244	HMS01743	No
264	C15	FBgn0004863	HMS01431	No
265	Ca-alpha1T	FBgn0264386	HMS01948	No
266	Ca-beta	FBgn0259822	JF03254	No
267	Cad88C	FBgn0038247	JF02443	No
268	Cad99C	FBgn0039709	HMS01451	No
269	CalpB	FBgn0025866	JF01983	No

270	Cals	FBgn0039928	JF01857	No
271	Calx	FBgn0013995	JF02937	No
272	CaMKI	FBgn0016126	JF02268	No
273	CaMKII	FBgn0264607	JF03336	No
274	CanA-14F	FBgn0267912	HMS01880	No
275	Cap-D2	FBgn0039680	JF01279	No
276	Capa	FBgn0039722	JF02981	No
277	CapaR	FBgn0037100	JF02577	No
278	capu	FBgn0000256	HMS00712	No
279	Cat	FBgn0000261	HMS00990	No
280	cato	FBgn0024249	JF02090	No
281	Cbl	FBgn0020224	JF02650	No
282	CCAP-R	FBgn0039396	JF01338	No
283	CdGAPr	FBgn0032821	HMS01730	No
284	Cdk12	FBgn0037093	HMS00155	No
285	Cdk2	FBgn0004107	HMS00174	No
286	Cdk5	FBgn0013762	JF02667	No
287	cer	FBgn0034443	JF01914	No
288	cg	FBgn0000289	HMS01145	No
289	CG10077	FBgn0035720	HMS00380	No
290	CG10321	FBgn0034643	JF02328	No
291	CG10344	FBgn0034729	HMS00197	No
292	CG10672	FBgn0035588	HMS00753	No
293	CG10814	FBgn0033830	HM05043	No
294	CG10864	FBgn0038621	JF01917	No
295	CG10933	FBgn0034264	HMS01708	No
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672	GstT4	FBgn0030484	HMS01607	No
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717	kel	FBgn0001301	JF01768	No
718	Khc	FBgn0001308	HMS01519	No
719	Kif3C	FBgn0039925	HMS02134	No
720	KLHL18	FBgn0037978	HMS01820	No
721	Klp10A	FBgn0030268	HMS00920	No
722	Klp3A	FBgn0011606	HMS02192	No
723	Klp64D	FBgn0004380	HMS02193	No
724	kon	FBgn0032683	JF01159	No
725	KP78b	FBgn0026063	HMS01981	No
726	kra	FBgn0250753	JF02556	No
727	ksr	FBgn0015402	HMS00730	No
728	Ku80	FBgn0041627	JF02790	No

729	kug	FBgn0261574	HMS02136	No
730	kune	FBgn0033032	HMS01755	No
731	l(1)G0289	FBgn0028331	HMS00700	No
732	l(1)sc	FBgn0002561	JF02399	No
733	l(2)gd1	FBgn0261983	JF02619	No
734	l(3)mbn	FBgn0002440	HMS00757	No
735	lab	FBgn0002522	JF02317	No
736	Lam	FBgn0002525	JF01389	No
737	lama	FBgn0016031	JF01294	No
738	Lap1	FBgn0033984	JF02362	No
739	Lar	FBgn0000464	HMS02186	No
740	lds	FBgn0002542	HMS01389	No
741	lft	FBgn0032230	JF03183	No
742	Lgr4	FBgn0085440	JF03070	No
743	Lhr	FBgn0034217	HMS01684	No
744	lic	FBgn0261524	JF01433	No
745	lid	FBgn0031759	HM05155	No
746	Lis-1	FBgn0015754	HMS01457	No
747	Lmpt	FBgn0261565	JF03218	No
748	lola	FBgn0005630	JF02254	No
749	LpR2	FBgn0051092	JF01627	No
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751	lqf	FBgn0028582	JF02672	No
752	Lrr47	FBgn0010398	HM05170	No
753	Lrrk	FBgn0038816	HMS01937	No
754	Lrt	FBgn0034540	HM05103	No
755	LS2	FBgn0034834	JF03339	No
756	Lsd-2	FBgn0030608	HMS00629	No
757	lsn	FBgn0260940	HMS01747	No
758	Lst8	FBgn0264691	HMS01350	No
759	lt	FBgn0002566	HMS00190	No
760	ltl	FBgn0268063	HM05205	No
761	luna	FBgn0040765	JF02430	No
762	lute	FBgn0262871	HMS01929	No
763	lwr	FBgn0010602	HMS01648	No
764	Mad	FBgn0011648	JF01263	No
765	mael	FBgn0016034	HMS00102	No
766	Magi	FBgn0034590	HMS00291	No
767	mahj	FBgn0034641	HMS01260	No
768	Map60	FBgn0010342	HMS00457	No
769	MBD-like	FBgn0027950	HMS01683	No
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771	Mbs	FBgn0005536	HMS00521	No
772	me31B	FBgn0004419	HMS00539	No
773	Med	FBgn0011655	JF02218	No
774	MED19	FBgn0036761	HMS00588	No

775	MED21	FBgn0040020	HMS01211	No
776	MED23	FBgn0034795	HMS01135	No
777	MED24	FBgn0035851	HMS01097	No
778	MED26	FBgn0039923	HM05058	No
779	MED7	FBgn0051390	HMS01140	No
780	MED9	FBgn0260401	HMS00542	No
781	Mef2	FBgn0011656	HMS01691	No
782	Men	FBgn0002719	HMS01700	No
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784	mew	FBgn0004456	JF02694	No
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787	Mhc	FBgn0264695	HMS01471	No
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793	Mlp84B	FBgn0014863	JF01128	No
794	Mmp1	FBgn0035049	JF01336	No
795	mnb	FBgn0259168	HMS01545	No
796	mol	FBgn0086711	JF03365	No
797	morgue	FBgn0027609	JF01507	No
798	Mp	FBgn0260660	JF02929	No
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801	Msh6	FBgn0036486	HMS01479	No
802	mspo	FBgn0020269	JF03397	No
803	MsR2	FBgn0264002	JF01850	No
804	mth	FBgn0023000	JF02645	No
805	Mtk	FBgn0014865	HM05032	No
806	mtm	FBgn0025742	HMS01806	No
807	mtRNApol	FBgn0261938	HMS01474	No
808	mud	FBgn0002873	HMS01458	No
809	mus201	FBgn0002887	JF01500	No
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815	nAChRalpha7	FBgn0086778	JF02570	No
816	NaCP60E	FBgn0085434	JF02037	No
817	Nak	FBgn0015772	HMS01793	No
818	ND-13A	FBgn0031684	HM05062	No
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823	nerfin-2	FBgn0041105	HM05037	No
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826	Neurochondrin	FBgn0037447	HMS02151	No
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831	NitFhit	FBgn0024945	HMS01875	No
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836	nompC	FBgn0016920	JF01067	No
837	nos	FBgn0002962	HMS00930	No
838	Nos	FBgn0011676	JF03220	No
839	Not10	FBgn0260444	HMS00751	No
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842	Nplp3	FBgn0042201	JF03188	No
843	Nplp4	FBgn0040717	JF03221	No
844	Nrt	FBgn0004108	JF03170	No
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846	nrv3	FBgn0032946	JF03367	No
847	Nrx-1	FBgn0038975	HMS00403	No
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852	Nup160	FBgn0262647	HMS00385	No
853	Nup44A	FBgn0033247	HMS01825	No
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855	O-fut1	FBgn0033901	JF02052	No
856	Oaz	FBgn0261613	JF01943	No
857	Obp50a	FBgn0050067	HMS00993	No
858	oc	FBgn0004102	HMS01314	No
859	Octbeta2R	FBgn0038063	HMS01151	No
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861	onecut	FBgn0028996	HMS01438	No
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863	Or23a	FBgn0026395	JF02885	No
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873	pad	FBgn0038418	JF02235	No
874	pain	FBgn0060296	JF01065	No
875	para	FBgn0264255	HMS00868	No
876	Parg	FBgn0023216	JF01703	No
877	park	FBgn0041100	HMS01651	No
878	Pask	FBgn0034950	JF03260	No
879	Patsas	FBgn0029137	JF01773	No
880	Pc	FBgn0003042	HMS00016	No
881	Pcl	FBgn0003044	HMS00896	No
882	Pcmt	FBgn0086768	HMS00768	No
883	pcx	FBgn0003048	HM05038	No
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898	Picot	FBgn0024315	JF01940	No
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902	PK1-R	FBgn0038201	JF02690	No
903	PK2-R2	FBgn0038139	JF03209	No
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905	Pkcdelta	FBgn0259680	JF02991	No
906	Pkg21D	FBgn0000442	JF02766	No
907	Pld	FBgn0033075	HMS00529	No
908	ple	FBgn0005626	JF01813	No
909	PlexA	FBgn0025741	HM05221	No
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914	pncr015:3L	FBgn0063083	HM05152	No
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917	polybromo	FBgn0039227	HMS00531	No
918	Poxm	FBgn0003129	JF02321	No
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921	Pp2C1	FBgn0022768	HMS01887	No
922	Pph13	FBgn0023489	JF02200	No
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925	ppk24	FBgn0039839	JF02031	No
926	ppk30	FBgn0039677	JF01828	No
927	ppk31	FBgn0051065	JF02433	No
928	ppk5	FBgn0053289	JF01834	No
929	ppk8	FBgn0052792	JF01832	No
930	pps	FBgn0082831	HMS01741	No
931	Ppt1	FBgn0030057	JF01972	No
932	PRL-1	FBgn0024734	HMS01826	No
933	Proc-R	FBgn0029723	JF03350	No
934	Prosalpha6	FBgn0250843	HMS00120	No
935	Prosap	FBgn0040752	HMS02177	No
936	Prosbeta1	FBgn0010590	HMS00139	No
937	Prp3	FBgn0036915	JF02798	No
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943	PTP-ER	FBgn0016641	HMS00350	No
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946	Ptpmeg2	FBgn0028341	HMS00731	No
947	put	FBgn0003169	HMS01944	No
948	Pvr	FBgn0032006	HMS01662	No
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954	Rab23	FBgn0037364	JF02859	No
955	Rab26	FBgn0086913	JF01684	No
956	Rab3	FBgn0005586	HMS01131	No
957	Rab3-GAP	FBgn0027505	JF01601	No
958	Rab30	FBgn0031882	JF01593	No

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974	reb	FBgn0033667	HMS01444	No
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977	Rel	FBgn0014018	HMS00070	No
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988	Ric	FBgn0265605	JF02670	No
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997	RpS27	FBgn0039300	HMS01581	No
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1046	Snap24	FBgn0266720	JF03146	No
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1073	Synj	FBgn0034691	HMS01368	No
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1078	Tab2	FBgn0086358	JF03353	No
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1081	tara	FBgn0040071	JF01421	No
1082	Task7	FBgn0037690	JF02568	No
1083	Taz	FBgn0026619	JF01564	No
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1090	tim	FBgn0014396	HMS02031	No
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1093	Tl	FBgn0262473	JF01491	No
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1096	Tollo	FBgn0029114	HM05005	No

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1104	Tre1	FBgn0046687	HMS00433	No
1105	Trl	FBgn0013263	HMS02188	No
1106	trn	FBgn0010452	HM05011	No
1107	Trpgamma	FBgn0032593	JF01244	No
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1114	Ubi-p5E	FBgn0086558	HMS01881	No
1115	Ublcp1	FBgn0027526	JF03179	No
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1117	Ulp1	FBgn0027603	JF01409	No
1118	Unc-115b	FBgn0260463	HMS02005	No
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1124	Utx	FBgn0260749	HMS00575	No
1125	uzip	FBgn0004055	JF03237	No
1126	vap	FBgn0003969	JF03327	No
1127	veli	FBgn0039269	JF03269	No
1128	velo	FBgn0035713	HMS00383	No
1129	vg	FBgn0003975	JF03404	No
1130	Vha100-1	FBgn0028671	JF02059	No
1131	Vha16-1	FBgn0262736	HMS02171	No
1132	Vha16-5	FBgn0032294	JF01821	No
1133	Vha44	FBgn0262511	HMS00821	No
1134	VhaSFD	FBgn0027779	HMS02144	No
1135	vis	FBgn0033748	HMS01480	No
1136	Vps4	FBgn0283469	HM04061	No
1137	Vps52	FBgn0031710	HMS01713	No
1138	Vrp1	FBgn0243516	HMS01593	No
1139	Vti1a	FBgn0260862	HMS01727	No
1140	vvl	FBgn0086680	JF02126	No
1141	w	FBgn0003996	JF02387	No
1142	wal	FBgn0010516	HMS01263	No

1143	wg	FBgn0004009	HMS00844	No
1144	Wnt4	FBgn0010453	JF03378	No
1145	wrapper	FBgn0025878	JF03240	No
1146	wry	FBgn0051665	JF03393	No
1147	wun2	FBgn0041087	HMS00372	No
1148	X11L	FBgn0026313	JF02449	No
1149	XNP	FBgn0039338	HMS00683	No
1150	Xrp1	FBgn0261113	HMS00053	No
1151	yki	FBgn0034970	HMS00041	No
1152	YT521-B	FBgn0027616	HMS01302	No
1153	Zasp52	FBgn0265991	JF01133	No
1154	zen2	FBgn0004054	HMS01124	No
1155	zormin	FBgn0052311	HMS01014	No
1156	zyd	FBgn0265767	JF01872	No

[1] no previous female sterile alleles reported

Cataloging biodiversity of cactophilic *Drosophila.*

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Information required for students of evolutionary biology interested in cataloging biodiversity includes accurate field annotation of natural populations to be subjected to comparative, experimental or observational analyses. Knowledge of the natural history of populations and species, as well as reliable field data on the locations of source populations is essential for new and experienced workers alike. Here, I describe collecting records for *Drosophila* species from 1985 to 2009, mostly from collecting trips to the deserts and arid lands of northwestern Mexico and the southwestern United States.

Most of these records have collection numbers that were part of William B. Heed's laboratory stock lists at the University of Arizona, but in more recent years after his retirement and then death in 2007, other collection labels were used. Some of these species are available at the San Diego Stock Center, some from the Ambrose Monell Cryo Collection (AMCC) at the American Museum of Natural History (https://figshare.com/articles/new_fileset/913592), and many of these stocks are still in culture or available frozen in ethanol from the author. All site locations are easily identified on good road maps or Google Earth. This report also acknowledges all colleagues, collaborators, and students who helped in the field - without them, these collections would not have been possible.

Colleagues/collaborators.

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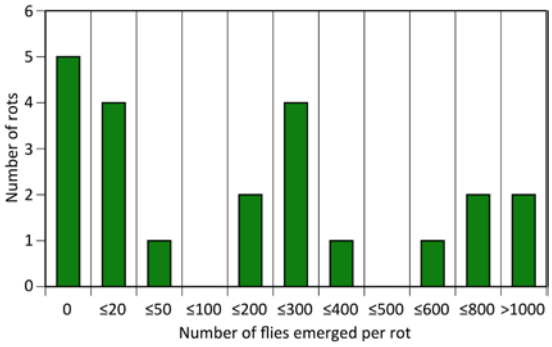
Luke Rochette, University of Arkansas
Gil Smith, University of St. Andrews
Pupulio S. N. A. Ssekimpi, University of Arizona
Melissa Stennett, University of Arkansas
David Stiers, University of Arkansas

1. A895 San Matias Pass, BC, Rte. 3, ca 32 miles after right turn from Rte 5 (before Valle Trinidad) May 24, 1985, w/Chris Nicholson. South facing slope covered in yellow-spined *Ferocactus cylindraceus*.

Ferocactus rot – no flies emerged



2. A896 Punta Prieta, BC, May 25-29, 1985, w/Chris Nicholson.

- 868 ♀ + 522 ♂ *D. mojavensis* aspirated from field agria rots, *Stenocereus gummosus*
- 8753 *D. mojavensis* ex-22 agria rots

3. A900 Santa Rosa Mountains, AZ, May 1985. w/WB Heed, WJ Etges, P. Ssekimpi
1 organ pipe rot, *Stenocereus thurberi*, produced 200-300 adult *D. mojavensis*

4. Los Gatos, CA, October 3, 1985, Marc Fey's backyard (former orchard)
ex-fermenting pears (12)
379 *D. mel/sim*
22 *D. pseudoobscura*

5. A907 Punta Onah, Sonora, October 1985, w/WB Heed, Stuart Barker, Andy & Karen Beckenbach
2 agria rots returned to the lab produced:

- 3246 *D. mojavensis*
- 200 *D. arizonae*
- 2 *D. nigrospiracula*

6. A911 Santa Rosa Mountains, AZ, January 25, 1986, w/WJE, WB Heed, T. Markow

- 74 ♀ + 81 ♂ *D. mojavensis* ex-organ pipe rots (3)

7. 11411 E. Sundance Dr., Tucson, AZ, February 1986.
ex-*Ferocactus wislizeni* rot in Etges front yard

- 79 *D. longicornis*
- 6 *D. hamatofila*
- 2 *D. nigrospiracula*

8. Organ Pipe National Monument, AZ, Ajo Mtn. loop drive, March 8, 1986. WJE
ex-organ pipe cactus rots (2)

- 659 ♀ + 415 ♂ *D. mojavensis*
- 2 *D. nigrospiracula*

9. Baja California trip, April 17-29, 1985, w/Phil Ganter and Ed Waltz.
A915 Rancho Santa Catarina, April 19, 1985

- 1 *Ferocactus gracilis* rot - no flies
- 3 *Myrtillocactus cochal* rots - no flies

A916 Punta Prieta, BC

- 103 ♀ + 130 ♂ *D. mojavensis* aspirated from agria rots
- 3685 *D. mojavensis* ex-11 agria rots (another 11 rots produced no flies)

A917 South of Vizcaino, BC on road up to Sierra San Francisco

- 5 ♀ + 32 ♂ aspirated from agria rots
- 1675 *D. mojavensis* ex-4 agria rots (another 6 rots produced no flies)
- 14 *D. mojavensis* ex-3 organ pipe rots (1 very large)

ex-2 *Opuntia* rots (on rocky hillside)

- 61 *D. aldrichi* (Beckenbach et al. 2008)
- 10 *D. mojavensis*
- 3 *D. melanogaster*

A918 South of Bahia de Concepcion, BCS, south of road to San Isidro

- 207 *D. mojavensis* ex-3 agria rots

A919 near San Lucas, BCS
1 cardon, *Pachycereus pringlei*, rot - no flies emerged

A920 San Lucas, BCS

- 217 *D. mojavensis* ex-2 of 5 agria rots

A921 km 90, south of El Rosario, BC

- 28 *D. mojavensis* ex-3 *Myrtillocactus cochal* rots

10. A922 Palm Canyon, Kofa Mtns., AZ with Tom Starmer and Bob Krebs (all baited)

- 19 ♀ + 18 ♂ *D. pseudoobscura*
- 47 ♀ + 38 ♂ *D. nigrospiracula*
- 2 ♀ + 2 ♂ *D. mettleri*
- 3 ♀ *D. repleta group?*
- 1 ♀ *D. mojavensis* (immature)

Ferocactus cylindraceus rot #1 - no flies emerged
F. cylindraceus rot #2

- 2 ♀ + 2 ♂ *D. nigrospiracula*
- 1 ♂ *D. pseudoobscura*

11. A923 Punta Onah, Sonora, March 7-8, 1987. w/WJE, Betsy Etges, Henar Alonso-Pimentel, and Cathlyn Klassen

- 135 ♀ + 146 ♂ *D. mojavensis* aspirated from agria rots
- 88 *D. nigrospiracula* aspirated from a cardon and a saguaro rot
- 53 *D. mettleri, ibid*

1 organ pipe rot from Noche Buena Rd. produced 8 *D. mojavensis*

12. A924 Santa Rosa Mtns., AZ, March 26, 1987.

- ~100 *D. mojavensis* aspirated from 2 organ pipe rots
- 3 ♀ + 1 ♂ *D. mettleri, ibid*

13. Trip to Tuxtla Gutierrez, Chiapas, April 6-13, 1987, w/Phil Ganter and Andre LeChance.
TG-2, north from Ocozocuautla, off road to Mal Paso, Rancho El Lider

- 124 *D. aldrichi* ex-6 *Nopalia* rots and fruits
- 144 *D.* ??. yellow testes, *D. mojavensis*-like, *D. longicornis*?
- 69 *Gitona americana*
- ~30 *D. simulans* (from *Nopalia* fruits)

TG-3 (A929), ~25 km down road to Cupia, many *S. pruinosus* as fence posts? Ejido Col. Libre.

- 1356 *D. arizonae* ex-4 *S. pruinosus* rots
- 1 *D. aldrichi* *ibid*

- 19 *D. repleta*?, smaller, darker, less pattern, orange testes
- 1 *D. nigrospiracula*-like
- 6 *D. wassermani*

SW-1, near screw worm plant
- 56 *D. arizonae* ex-*S. stellatus*(?) or *pruinosus*
- 8 ♀ + 3 ♂ *D. arizonae* aspirated
- 1 *mel/sim* aspirated

14. A925 Tucson, AZ, August 1987, w/Jim Fogleman
Off Speedway Blvd, east past town - aspirated from saguaro
- 36 *D. nigrospiracula*
- 9 *D. mettleri*

15. A926 Tucson, AZ, near Ft. Lowell and Wentworth, ibid.
- 235 *D. nigrospiracula* ex-saguaro rot #1
- 1 *D. longicornis* *ibid*

- 39 *D. nigrospiracula* ex-saguaro rot #2
- 1 *D. mettleri* *ibid*

16. Punta Prieta, Dec. 27, 1987-Jan. 2, 1988, and Punta Onah, Jan. 4-7, 1988, w/WJE, Tom Tobin, Liz Pennisi, George Ferguson, and Karen ? (TAM's graduate student).
PP88 (A931)
- 67 ♀ + 51 ♂ *D. mojavensis* aspirated from agria rots
- 330 ♀ + 386 ♂ *D. mojavensis* ex-7 agria rots

PO88 (drove from Punta Prieta, BC to Mexicali, Caborca, to Punta Onah, Sonora)
- 79 ♀ + 79 ♂ *D. mojavensis* aspirated from agria rots
- 1168 ♀ + 1103 ♂ *D. mojavensis* ex-10 agria rots

17. PP89 Punta Prieta, BC, March 21-23, 1989, w/WJE, Richard Halliburton and Greg Huckins.
- 541 ♀ + 259 ♂ *D. mojavensis* aspirated from agria rots
- 5239 *D. mojavensis* ex-15 agria rots
- 1 *D. arizonae* (?) *ibid*

18. PO91(A946) Punta Onah, Sonora, March 14-15, 1991, collected by WB Heed. All flies aspirated from rots.
- 224 ♀ + 278 ♂ *D. mojavensis*
- 4 *D. nigrospiracula*
- 3 *D. pachea*
- 23 *D. longicornis*-like

19. PP91 Punta Prieta, BC, March 16-20, 1991. Tracy Dunn, Phil Ganter, and Henar Alonso-Pimentel (WJE had to cancel at the last minute). Flies were aspirated and baited. Weather was rainy and cold.
- 176 ♀ + 99 ♂ *D. mojavensis* (+ 1913 *D. mojavensis* ex-8 agria rots)
- 1 ♀ + 2 ♂ *D. pseudoobscura*
- 2 ♀ *D. nigrospiracula*

20. PO92 Punta Onah, Sonora, May 15-18, 1992, w/WJE and Greg Huckins.

- 217 ♀ + 176 ♂ *D. mojavensis* aspirated from rots
- 4210 *D. mojavensis* ex-4 agria rots
  2 organ pipe rots produced no flies

21. Cerro Colorado and Punta Onah, Sonora, March 21-25, 1994, with WJE, Greg Huckins, Seth Isenberg, and Brian Newby.

CC94 Cerro Colorado

- 23 ♀ + 21 ♂ aspirated from 2 organ pipe rots
- 92 ♀ + 89 ♂ ex-organ pipe rot

PO94 Punta Onah

- 682 ♀ + 571 ♂ *D. mojavensis* aspirated from agria rots (1 ♂ tan body)
- 1 ♀ + 1 ♂ *D. melanopalpa* or *hamatofila*
- 1 ♀ + 2 ♂ *D. nigrospiracula*
- 2 ♂ *D. pachea*
- 1 ♀ *D. mettleri*

22. PSR94 Punta Santa Rosalillita (~20 miles NW of Rosarito, BC), April 4, 1994, collected by Pat O'Grady. All baited.

- 12 ♀ + 18 ♂ *D. mojavensis*

23. MSB94 Mission San Borja, BC, April 6-7, 1994, collected by Pat O'Grady. All baited.

- 17 ♀ + 26 ♂ *D. mojavensis*

24. MSF94 Mission San Fernando, BC, (76 miles south of El Rosario), April 8, 1994, collected by Pat O'Grady. All baited.

- undetermined # *D. mojavensis*

25. AB95 Anza-Borrego Desert trip, north side of Vallecito Mtns., March 16-25, 1995, w/WJE, Brian Newby, Melissa Stennett, and Peter Etges. baited.

- 21 ♀ + 25 ♂ *D. mojavensis*
- 41 *D. melanogaster*
- 87 *D. pseudoobscura*
- 7 *D. immigrans*
- 30 *D. hydei*
- 11 *D. hamatofila*

26. Baja California collecting trip, March 19-27, 1996, baited, w/Bill Etges, Conrad Istock, Chris Babcock. Species Identification: WB Heed, Pat O'Grady.

A975 Bahia Concepcion, BCS

- 15 *D. mojavensis*

A976 Santiago, BCS

- 43 *D. mojavensis* (+ 1 ♀ + 1 ♂ ex-agria rot)
- 6 *D. mel/sim*
- 4 *D. mainlandi*
- 3 *D. aldrichi*
- 1 *D. spenceri* ♂
- 1 *D. arizonae* ♂
- 1 *Gitona americana*

A977 Todos Santos, BCS
- 31 *D. mojavensis*
- 26 *D. mel/sim*
- 1 *Gitona bivisualis*

A978 Punta Agua Verde, BCS
- 11 *D. mojavensis*
- 6 *D. mainlandi*
- 4 *D. nigrospiracula*
- 3 *G. americana*
- 2 *D. aldrichi*
- 1 *D. mel/sim* ♂

A979 Punta Prieta, BC
- 65 *D. mojavensis* (+ 255 ♀ + 243 ♂ ex-4 agria rots)
- 19 *D. pseudoobscura*
- 5 *D. nigrospiracula*
- 1 *D. mel/sim*

A980 Observatory Road (San Pedro de Martir)
- 15 *D. pseudoobscura*
- 14 *D. tenebrosa*?
- 3 *D. busckii*
- 1 *D. hydei*
- 1 *D. mel/sim*
- 1 *D. hamatofila*?

A981 San Ignacio (near the date palm springs, during breakfast at La Pinta)
- 12 *D. pseudoobscura*
- 11 *D. mel/sim*

A982 East of Ciudad Insurgentes, BCS, 2 agria rots produced no flies

A983 South of San Agustin, BCS, 1 roadside agria rot
- 205 ♀ + 223 ♂ *D. mojavensis* ex-agria rot

A984 ~ 30 km South of Santa Rosalia, BCS, on road to San Jose de Magdelena
- 359 ♀ + 309 ♂ *D. mojavensis* ex-2 agria rots

A985 North of El Cien, north of Cabo San Lucas
1 agria rot produced no flies

27. Sonora/Sinaloa Collecting trip, April 15-23, 1996, w/Miguel Armella, Celeste Durando, Bill Etges, Greg Huckins, Francisco Molina.

A988 El Cardonal, Sonora (29° 29.27’ N, 111° 39.09’ W)
- 2 vials full *D. pachea* (ex-senita rot)

A989 Ejido Puerto Arturo, Sonora (28° 25.53’ N, 111° 25.04’ W) (baited and aspirated)
- 328 *D. mojavensis* (+ 407 ex-organ pipe rot)

- 3 *D. arizonae*
- 150 *D. nigrospiracula* (netted from cardon rot)
- 5 *D. mettleri (ibid)*
- 5 *D. pachea (ibid)*
- 1 *D. mel/sim (ibid)*

A990 Las Bocas, Sonora (26° 40.07’ N, 109° 19.81’ W) (baited and aspirated)

- 2359 *D. mojavensis* (+81 ex-organ pipe rot)
- 7 *D. arizonae*
- 957 *D. aldrichi*
- 15 *D. mettleri*
- 3 *D. mel/sim*

Cactus rots collected: O.P. rot # 1, no flies; O.P. rot # 2, 5 vials of flies, prob. *D. mojavensis*; cina rot, no flies; *Opuntia* rots (2 jars) about 30 flies, prob. *D. aldrichi*.

A991 El Fuerte, Sinaloa (26° 22.90’ N, 108° 42.91’ W) (baited and aspirated)

- 185 *D. mojavensis* (+49 ♀ + 35 ♂ ex-organ pipe rot)
- 7 *D. arizonae*
- 2 *D. aldrichi*
- 3 *D. simulans*
- 1 *D. subsigmoides*

(Note: many more flies were collected, but didn't survive for unknown reasons).
Cactus rots collected: O.P. rots # 1 and # 2, no flies; cina rot, no flies; *Opuntia* rots (2 jars), 10 flies, prob. *D. aldrichi*.

A992 27 km East of Navojoa, Sonora (27° 06.66’ N, 109° 10.22’ W) (no baits used)
Cactus rots collected: *Opuntia* rots (3 jars), 8 flies, prob. *D. aldrichi*.

A993 Rancho El Diamante, Sonora, km 82 on Rte 16, East of Hermosillo (28° 41.62’ N, 110° 18.13’ W) (baited and aspirated)

- 1550 *D. mojavensis* (+ 34 ex-organ pipe rot)
- 1 *D. arizonae*
- 39 *D. mettleri*
- 6 *D. pachea*
- 1 *D. nigrospiracula*
- 1 *D. nigrohydei*

Cactus rots collected: Organ pipe rot # 1, 2 1/2 vials of flies; O.P. rot # 2, no flies; cina rots # 1 and # 2, no flies.

A994 Punta Onah, Sonora (baited and aspirated)

- 1406 *D. mojavensis* (+296 ♀ + 232 ♂ ex-2 agria rots)
- 3 *D. pachea*
- 1 *D. mel/sim*
- 1 *Scaptomyza* spp.

27. Anza-Borrego SP and Eastern Mojave trip, November 13-21, 1996, w/WJE, Greg Huckins, Pat O’Grady. all baited.

A996: Mountain Palm Springs campground, Anza-Borrego State Park 11/13-11/14, few barrels

- 4 *D. hydei*
- 2 ♀ *D. pseudoobscura*

- 15♀ + 11♂ *D. mel/sim*

Vallecito, Anza-Borrego St. Park, very stormy, no flies, 11/14-11/15
Joshua Tree NP, Cottonwood campground, nothing. 11/16

A997 Providence Mtns. State Recreation Area, below visitor center near caves, elevation 1230 m. 11/17-19.

- 62♀ + 45♂ *D. mojavensis*
- 3 *D. mettleri*
- 4♀ *D. pseudoobscura*

A998: W. of Havasu City, N. of Whipple Mtns along power line road. 11/19-20

- 5♀ + 5♂ *D. mojavensis*
- 1 *Gitona americana*
- 2 *D. pseudoobscura*
- 2 *D. mettleri*
- 1 *D. nigrospiracula*

A999: Kofa Mts., AZ, off rt. 95 going W., King of Arizona Mine, Rte 21, barrels present, 11/20-21

- 203 *D. nigrospiracula*
- 87 *D. mettleri*
- 1 *D. hamatofila*
- 1♀ *D. pseudoobscura*

28. Western Mexico trip, March 16-24, 1997, to Tomatlan, Chamela, Jalisco, and Playa Azul, Infiernillo, Michoacan. w/WJE, Miguel Armella, Greg Huckins, Sandra Perez.

A1008 7 km west of Tomatlan, Jalisco

- 8 ♀ +10 ♂ *D. navojoa*
- 3 *mel/sim*
- 8 *D. ananassae* (yellow flies, not dark abdominal banding, keys out to Hirtodrosophila)
- 2 *Scaptodrosophila latifasciaeformis* (small yellow flies, abdominal bands fuller than *mel/sim*, fully extend to lateral margin as blocks)

A1009 Estacion de Biologia Chamela, Jalisco

- 34 ♀ +29 ♂ *D. navojoa*
- 31 *D. aldrichi*
- 8 *D. wassermani*
- 49 *mel/sim*
- 69 *D. eremophila*
- 5 *D. meridiana* (light body, cloudy crossveins)
- 3 *D. paranaensis* (light body, clear crossveins)
- 9 *D. spenceri* (dark body, clear crossveins)
- 12 *D. prosaltans* or *D. sturtevanti* (“very dark” flies - not *repleta*)
- 1 *D. hydei*
- 1 *D. ananassae*
- 2 *S. latifasciaeformis*
- 1 *D. cardini* (cardini-NOT)
- 2 diptera, not *Drosophila*

A1010 Playa Azul, Michoacan (baits set out overnight in coconut and banana plantations). 3/22/97.

- 170 *mel/sim*
- 7 *D. aldrichi*
- 9 *D. ananassae*
- 3 *D. cardini* (yellow flies, slightly clouded cross-veins, bright eyes, distinct abdominal bands)
- 1 large yellow fly, keys out to testacea group, distinctive abdominal bands.
- 3 *D. nebulosa*
- 12 *D. hydei*
- 1 ♂ *D. canapalpa*
- 3 *D. spenceri*
- 13 *D. prosaltans* or *D. sturtevanti*
- 24 *S. latifasciaeformis*
- 1 *D. meridiana rioensis*
- 1 *D. paranaensis*

A1011 near road to Infernillo, km 192 on HW 37, March 23, 1997.

- 24 *D. wassermani*
- 3 D. *mel/sim*
- 3 *D. eremophila*
- 2 *D. hydei*
- 1 *D. ananassae*
- 2 *Scaptodrosophila latifasciaeformis*

29. Tehuacan, Puebla, Oaxaca, and Metztitlan, Hidalgo Trip, August 3-15, 1997, w/Miguel Armella, Bill Etges, Greg Huckins. baited.

A1013 Zapotitlan des Salinas, Jardin Botanico and 6 km on road to Los Reyes Metzontla

- 18 *D. melanogaster*
- 6 *D. ananassae*
- 9 *D. aldrichi*
- 1 *D. cardini*
- 32 ♀ + 34 ♂ *D. hexastigma*
- 153 *D. pegasa*
- 1 *D. mulleri*
- 48 *D. eremophila*
- 7 *D. nannoptera* + 34 ex-*Myrtillocactus geometrizans*
- 1 *D. bifurca*
- 1 *D. pseudoobscura*
- 12 *D. hydei*
- 3 *D. saltans* group
- 1 big repleta, dark complete bands
- 55 ?? repleta group species
- 17 *Gitona americana*
- 17 *S. latifasciaeformis*

A1014 Oaxaca, pine forest on road to Tuxtapec.

- 6 *D. leonis* aspirated + 22 ex-*Opuntia*

A1015 Valley Site near Metztitlan.

- 50 *D. melanogaster*

- 134 *D. aldrichi* + 24 ex-*Opuntia*
- 7 *D. pseudoobscura*
- 72 *D. pegasa*
- 15 *D. hydei*
- 8 *D. hexastigma*
- 17 *D. longicornis* (all ex-*Opuntia*)
- 23 *D. arizonae*
- 2 *D. paranaensis*
- 1 *D. immigrans*
- 1 black repleta
- 1 big repleta, dull abdominal bands
- 5 muscids
- 523 ?? repletas
- 3 *Gitona americana*
- 2 *Rhinoleucophenga obesa*
- 1 *S. latifasciaeformis*

A1016 Upper Site, 1.5 km along road to Ganaderia Las Vaquerias, Metztitlan

- 32 *D. melanogaster*
- 6 *D. aldrichi* + 33 ex- 2 *M. geometrizans* rots
- 41 *D. arizonae*
- 32 *D. hexastigma*
- 8 *D. pseudoobscura*
- 2 *D. hydei*
- 1 *D. nannoptera* + 21 ex- 3 *Stenocereus dumortieri* rots
- 2 *D. bifurca*
- 288 ?? repletas

30. Valle de Infiernillo, Michoacan and Cañon Zopilote, Guerrero, January 1-16, 1998, w/Miguel Armella, Bill Etges, Greg Huckins, and Pat O'Grady.

A1017 Infiernillo: along side-road 5 km from town and at km marker 12.

- 1303 *D. wassermani* (baited)
  + aspirated 77 from *Stenocereus fricii* rot (rot #1)
  120 from *S. fricii* rot (rot #17)
  185 from *S. quevedonis* rot (rot # 14)
  119 from *S. marginatus* rot (rot # 4)
  + 8 ex- *S. quevedonis* rots (rots #11, 13, 17)
- 1 *D. nannoptera*
- 2041 *D. eremophila* (+2 aspirated from *S. quevedonis* rot; rot # 14)
- 473 *D. aldrichi*
- 9 *D. paranaensis*
- 2 *D. hydei*
- 98 *D. spenceri* +1 adult emerged from *S. quevedonis* rot #11
- 168 *D. anceps*
- 2544 *D. ananassae*
- 5 *D. busckii*
- 123 *D. nebulosa*
- 152 *D. mel/sim* +1 female ex- *S. quevedonis* rot #17
- 23 *D. cardini*

- 2 *D. gibberosa*
- 3 *D. sturtevanti*
- 1 *D. gigas*?
- 5 *D. funebris* +1 adult ex- *S. fricii* rot #15)
- 4 *D. saltans* group.
- 8 *S. latifasciaeformis*
- 6 *Gitona americana*

A1018 Cañon del Zopilote near Chilpancingo, Guerrero, 2 sites: at km 197 and one at km 176.

- 652 *D. aldrichi* (baited)
  + aspirated 36 from *P. weberi* rot (rot #6)
  104 ex- *Pachycereus weberi* rot (rot #6)
  13 ex- *Escontria chiotilla* (rot #7)
- 45 *D. wassermani* (baited)
  + aspirated 4 from *P. weberi* rot (rot #3)
  30 ex-*E. chiotilla* (rot #7)
- 156 *D. nannoptera* (baited)
  + aspirated 4 from *P. weberi* rots (rots # 3, 6)
  3 ex-*P. weberi* rots (rots # 5,6)
  40 ex- *E. chiotilla* (rot #7)
- 809 *D. eremophila* +206 aspirated from *P. weberi* rots
- 1041 *D. spenceri*
  + aspirated 13 from *P. weberi* rots
  3 ex- *P. weberi* rot (rots # 6)
- 4 *D. anceps* + 52 ex-*P. weberi* rots (rots # 5, 6)
  9 ex- *E. chiotilla* (rot #7)
- 7 *D. paranaensis*
- 1 *D. leonis*
- 1 *D. nigricruria*
- 1 *D. fasciola* group sp.
- 76 *D. hydei*
- 521 *D. mel/sim*
- 24 *S. latifasciaeformis*
- 29 *D. cardini*
- 51 *D. busckii*
- 14 *Gitona americana*
- 12 *D. nebulosa*
- 5 *D. subsigmoides* ?
- 1 *D. sturtevanti*
- 1 *D. pseudooobscura* (sex combs confirmed)
- 1 *D. tripunctata*?
- 5 *quinaria* group ?
- 5 big brown drosophilds - *D. gigas*?
- 1 *D. parthenogenetica*

31. Cuitcatlán, Puebla, Oaxaca City, and Acatlan, Puebla, July 3-11, 1998, w/Miguel Armella, Bill Etges, Eric Larsen, Barbara Gautschi, Luke Rochette, and Amaury Díaz Solís.

Cuitcatlán, Puebla, July 3-5, 1998.

- 2150 *D. melanogaster*
- 7 *D. aldrichi*

- 2 *D. nannoptera* + 140 ex- 2 *Escontria chiotilla* rots
- 2 *D. cardini*

2 other *Escontria* and 1 *Stenocereus pruinosus* rots produced no flies

Oaxaca City, 29 km south on road to Mitla, July 7-8, 1998.

- 102 *D. melanogaster*
- 4 *D. hydei*
- 2 *D. cardini*
- 38 *D. ritae*
- 2 *D. pegasa*
- 5 *D. hexastigma*

2 *Opuntia* and 1 *Stenocereus* rots produced no flies

Acatlán, July 9-11, 1998.

- 428 *D. melanogaster*
- 81 *D. nannoptera* +10 aspirated from *E. chiotilla* + 4 ex- 3 *E. chiotilla* rots
- *5* *D. aldrichi* + 1 aspirated from *E. chiotilla*
- 5 *D. nigrohydei*
- 2 *D. leonis*
- 1 *D. wassermani*
- 1 *D. pegasa*
- 5 *D. cardini*
- 3 *D. nebulosa*
- 1 *D. quinaria*

32. Metztitlán, Hidalgo, and El Infiernillo, Michoacan, March 15-29, 1999, w/Miguel Armella, Bill Etges, Art Gibson (Infiernillo) and students of Miguel.

Meztitlán, Road to Ganaderia Vaquerias, Marzo 16-19, 1999.

- 273 *D. hexastigma*
- 60 *D. longicornis* complex
- 12 *D. melanogaster*
- 6 *D. arizonae*
- 6 *D. pseudobscura*
- 4 *D. hydei*
- 2 *D. anceps*
- 1 *D. bifurca*
- 1 *D. pegasa*
- 11 *Gitona americana*

Rots: 3 *Stenocereus dumortieri*, 3 *M. geometrizans*, 2 *Opuntia* pads produced no flies

Metztitlán valley

- 137 *D. hexastigma*
- 91 *D. longicornis*
- 6 *D. melanogaster*
- 1 *D. aldrichi*
- 2 *D. pegasa*
- 2 *D. arizonae* (+2 ex-*Myrtillocactus geometrizans*)
- 2 *D. eremophila*
- 2 *D. nannoptera*
- 1 *D. longicornis* ??

- 3 *Gitona americana*

El Infiernillo, 5 km site, Marzo 21-25, 1999

- 3363 *D. wassermani*
- 67 *D. eremophila*
- 1 *D. aldrichi*
- 3 *D. mel/sim*
- 1 *Gitona americana*

El Infiernillo, 12 km site.

- 350 *D. wassermani* (+ 5 ex-*S. fricii*)
- 35 *D. eremophila*
- 19 *D. mel/sim*
- 8 *D. paranaensis*
- 3 *D. aldrichi*
- 1 *D. nebulosa*
- 1 *D. spenceri*

Rots: 5 *S. fricii*, 1 *Backebergia militaris*, and 2 *S. quevedonis* rots produced no flies

33. Near Zapotitlan, Valle de Tehuacan, Puebla, July 16-28, 1999, w/Miguel Armella, Miguel's nephew Jaime, Bill Etges, and Isaac Jones.

Zapotitlan de Salinas, July 18-25, ca. 5 km along road across the river, east of town.

- 1632 *D. melanogaster*‡
- 604 *D. eremophila* +13 aspirated from *M. geometrizans* rot
- 50 *D. hexastigma*
- 35 *D. longicornis*
- 26 *D. aldrichi*
- 19 *D. hydei*
- 13 *D. nigricruria*
- 4 *D. paranaensis*
- 3 *D. pegasa*
- 2 *D. ritae*
- 1 *D. bifurca*
- 1 *D. leonis*
- 4 *D. pseudoobscura*
- 15 *D. cardini*
- 1 *D. nannoptera* aspirated from *M. geometrizans* rot
- 15 *D. nebulosa*
- 13 *D. saltans* group
- 8 *D. anassasae*
- 1 *D. carbonaria* ?
- 6 *Gitona americana*
- 1 *D. ?? repleta* female, heavy clouding of crossveins, abdominal banding like *D. hydei*.

‡ Does not include the thousands of *D. melanogaster* released in the field.

Rots: 5 *M. geometrizans*, 1 *Stenocereus stellatus*, 2 *Pachycereus hollianus*, 1 *Neobuxbaumia tzetzo*, and 6 *Opuntia* pads produced no flies

34. San Francisco del Rincon, Guanajuato and Ixtlan del Rio, Nayarit, January 5-17, 2000, w/Miguel Armella, Bill Etges, Robin Gray, Raphael and Alejandro.

Stateline; South of San Francisco del Rincon on old road to Guadalajara (Jalisco 24), January 8-12.

- 1600 *D. longicornis* complex
- 325 *D. huckinsi sp. nov.* (Etges et al. 2001)
- 29 *D. huichole* (all males) sp. nov.
- 28 *D. eremophila*
- 1 *D. ritae*
- 4 *D. pseudoobscura*
- 1 *D. melanogaster*

ca. 4 km south of San Francisco del Rincon on road to Manuel Doblado, January 8-12.

- 1742 *D. longicornis* complex + 9 ex- 2 *Opuntia* rots
- 356 *D. huckinsi* sp. nov.
- 20 *D. huichole* (all males) sp. nov.
- 8 *D. eremophila*
- 1 *D. ritae*
- 1 *D. aldrichi*

Ca. 2-3 km west of town on old highway, next to a squash plantation, Ixtlan del Rio, Nayarit, January 12-14.

- 700 *D. longicornis* complex
- 632 *D. huckinsi* sp. nov.
- 437 *D. aldrichi*
- 150 *D. eremophila*
- 36 *D. hydei*
- 25 *D. melanogaster*
- 7 *D. ritae*
- 4 *D. busckii*
- 1 *D. paraenensis*
- 1 *D. leonis*
- 1 *D. pseudoobscura* (4-5 teeth in sex combs)
- 1 *D. repleta* ? (light bar tergite)
- 1 D. very black, *-virilis*-like
- 1 D. *nannoptera*-like

35. Los Tuxtlas Biological Station, Veracruz, March 21-24, 2000, Bill Etges, all baited. (Unfortunately, I did not have the time to identify all species returned to the lab)

- 945 *D. "big yellow"* (several species)
- 190 *D. cardini*
- 339 *D. sturtevanti*-like (brown)
- 214 *D. sturtevanti*-like (black)
- 5 *D. ellisoni*
- 14 *D. fulvimacula*
- 104 *D. mel/sim*
- 32 *S. latifasciaeformis*
- 31 *D. yellow body/dark legs*
- 1 *D. green leg bands*
- 1 *D. repleta*, big black, hunch back

36. Joluxtla, Oaxaca and San Luis Potosí, SLP, August 1-12, 2000, w/Miguel Armella, Bill Etges, Carolyn Lewis, and Adolfo Alvarado.

Joluxtla, Oaxaca on the rancho owned by Jaime Torres and family, Aug. 3-6.

- 523 *D. hexastigma*
- 315 *D. nannoptera* + ca 25 adults aspirated from *Escontria* rot
- 55 *D. longicornis* complex
- 95 *D. eremophila*
- 14 *D. racemova*
- 4 *D. hydei*
- 1 *D. ritae*
- 54 *D. pseudoobscura*
- 40 *D. melanogaster*
- 8 *D. cardini*
- 1 *D. willistoni*-like

Rots: 4 *E. chiotilla*, 1 *S. stellatus* produced 2 *D. pseudoobscura*, rest *D. mel/sim*

Punta El Tecolote, km 54 on Rt. 49 from SLP to Zacatecas, Aug. 7-10.

- 169 *D. bifurca*
- 162 *D. ritae*
- 97 *D. racemova*
- 95 D. *eremophila*
- 21 *D. longicornis* complex
- 6 D. *hamatofila*
- 15 *D. huichole*
- 2 *D. melanogaster*
- 2 *D. pseudoobscura* – like (both females)
- 1 *D. saltans* group
- 1 D. very black,-virilis-like

Rots: 1 *Opuntia robusta* produced 2 *D. repleta* group adults, rest *D. mel/sim*

La Parada, km 33 on Rt. 49 from SLP to Zacatecas, Aug. 9-10.

- 5 *D. bifurca*
- 9 *D. hexastigma*
- 21 *D. longicornis* complex
- 4 D. *hamatofila*
- 1 *D. melanogaster*
- 3 *D. pseudoobscura* (4-5 bristles on sex combs, both tarsi)
- 1 *D. hydei*
- 2 large yellow dipterans

37. Punta Prieta, Baja California and Punta Onah, Sonora, 2001, March 17-24, 2001, w/Miguel Armella, Bill Etges, and David Stiers

PP01 Punta Prieta, Baja California

- 15 ♀ + 20 ♂ *D. mojavensis* + 12 ex-agria rot (dry)
- 24 *D. pseudoobscura*
- 1 *D. nigrospiracula*

PO01 Punta Onah, Sonora

- 501 *D. mojavensis*
- 70 *D. arizonae*
- 774 *D. hydei*
- 143 *D. mettleri*

- 57 *D. nigrospiracula*
- 8 *D. pseudoobscura*
- 4 *D. pachea*
- 7 *D. mel/sim*

38. Oaxaca Pacific coast and Zapotitlan des Salinas, Puebla, January 2-12, 2002, w/Miguel Armella, Bill Etges, Pat O'Grady, Deodoro Oliveira, Harry Wistrand. all baited.

Penotepa National, Oaxaca

- 3 *D. melanogaster*
- 1 *D. aldrichi*
- 2 *D. malerkotliana*

Bahias de Huatulco, Oaxaca

- 3 *D. melanogaster*
- 34 *D. aldrichi*
- 35 *D. eremophila*
- 2 *D. spenceri*
- 18 *D. wassermani*
- 1♀ *D. acanthoptera*
- 1 *D. saltans group*
- 2 *D. malerkotliana*
- 1 *D. aldrichi??*

Zapotitlan des Salinas, Puebla (6 km on road to Los Reyes Metzontla)

- 56 *D. melanogaster*
- 23 *D. nannoptera*
- 9 *D. aldrichi*
- 10 *D. hydei*
- 131 *D. eremophila*
- 245 *D. longicornis*
- 288 *D. hexastigma*
- 42 *D. huckinsi*
- 9 *D. pegasa*
- 7 *D. spenceri*
- 15 *D. ritae*
- 2 *D. busckii*
- 16 *D. pseudoobscura*
- 1 *D. cardini* group
- 1 *D. brooksi* ?
- 3 *Gitona americana*

39. Valle San Quintin, Baja California, January 3-12, 2003, w/Bill Etges, Josh Mutic, Harry Wistrand

SQ03 Hillside agria patch, 2 km east main highway across from the road to the Old Mill Motel, (30°30'40.84"N, 115°53'30.58"W) and at km 36 south of Arroyo Hondo (30°10'14.32"N., 115°47'26.12"W)

- 3630 *D. melanogaster*
- 544 *D. mojavensis*
- 711 *D. hydei*
- 6 *D. longicornis* ?
- 65 *D. pseudoobscura*

- 131 *D. busckii*
- 2 *D. immigrans*
- 2 *D. floricola*
- 1 *S. latifasciaeformis*
- 2 *Gitona americana*

40. Punta Onah, Sonora, November 12-14, 2003, Collectors: Bill Etges and Luciano Jaureguy

PO03 Heed Camp, Punta Onah (baited).

- 819 ♀ + 1187 ♂ *D. mojavensis** + 36 ♀ + 26 ♂ aspirated from agria rots
- 198 *D. arizonae*
- 325 *D. nigrospiracula*
- 97 *D. mettleri*
- 53 *D. pseudoobscura*
- 107 *D. melanogaster*
- 24 *D. hydei*
- 1 *D. spenceri* male (?) dead
- 1 *D. pachea*
- 1 *D. cardini group*
- 1 *Gitona americana*

* 1 male was a yellow body mutant, all flies very polymorphic for body coloration, brown versus black.

41. Santa Catalina Island, December 22-23, 2004, collector: Brian Counterman.

SC05 Little Harbor (baited).

- 252 *D. mainlandi*
- 50 *D. mojavensis* + 63 ex-*Opuntia*
- 7 *D. wheeleri* + 8 ex-*Opuntia*
- 4 *D. mettleri* + 1 ex-*Opuntia*
- 22 *D. pseudoobscura*
- 17 *D. simulans*

42. Big Bend National Park, Texas, March 12 -20, 2004, Collector: Bill Etges. baited.

Chisos Basin (3/14-3/15).

- 207 *D. longicornis*
- 134 *D. pseudoobscura*
- 8 ♂ *D. desertorum*
- 2 *D. hydei*

Cottonwood Campground (3/18).

- 34 *D. pseudoobscura*
- 27 *D. longicornis*
- 1 *D. hydei*
- 1 *D. melanogaster*

Boquillas Canyon Road (3/19-3/20)

- 146 *D. pegasa*
- 91 *D. longicornis* and *D. hamatofila*
- 21 *D. melanogaster*
- 1 *D. hydei*

Trail SE of Rio Grande Village campground

- 15 *Notogramma purpuratum* Cole (Diptera: Otitidae) ex-*Echinocactus horizonthalonius* (Turk's head cactus)

43. Big Bend National Park, Texas, March 18 -26, 2005, Collector: WJ Etges. baited.
Where are the *D. desertorum* females? (Etges and Heed 2005)

Chisos Basin Campground (3/20-3/23).

- 625 *D. longicornis* (est. number)*
- 512 *D. hamatofila* (est. number)
- 463 *D. pseudoobscura* [+]
- 9 ♂ *D. desertorum*
- 9 *D. ritae*
- 1 *D. hydei*

Chisos Basin Hillside (3/20-3/23).

- 1201 *D. longicornis* (est. number)
- 605 *D. hamatofila* (est. number)
- 300 *D. pseudoobscura*
- 76 *D. desertorum* (mostly males)

Rio Grande Village Campground (3/23-3/25). A mesquite forest next to the rio.

- 290 *D. longicornis* (est. number)
- 145 *D. hamatofila* (est. number)
- 981 *D. pseudoobscura*
- 9 *D. hydei*
- 1 *D. pegasa*
- 2 *D. repleta* dark abdominal bands (1 female laid many eggs, none hatched)
- 46 *D. mel/sim*
- 15 *D. carbonaria*
- 2 *D. cardini* group
- 2 *D. funebris*
- 1 *D. macrospina limpiensis* (?)
- 23 *Notogramma* sp.

est. number – *D. longicornis* and *D. hamatofila* were initially sorted into one group, and then separated. Some had died, so I estimated the original numbers using the ratio of *long/ham* in the survivors.

[+] I did not check for the presence of *D. azteca*.

44. Punta Onah, Sonora, November 14 -18, 2007, w/Bill Etges, Allen Gibbs, Jackson Jennings, and Michael Brewer

PO07 Heed Camp, Punta Onah (baited).

- 472 *D. mojavensis* (includes many aspirated from agria rots)
- 825 *D. arizonae* (ibid)
- 937 *D. nigrospiracula/mettleri* *
- 28 *D. pseudoobscura*
- 33 *D. melanogaster/simulans*
- 37 *D. hydei*
- 15 *D. pachea*

- 2 *D. spenceri* male (?)
- 3 *D. wheeleri*
- 11 *D. "light repleta"*
- 2 *D. carbonaria* (?)
- 1 *Gitona americana*
- 5 *Zaprionus indianus*

* didn't sort all, probably also includes *D. ritae*.

45. Punta Prieta and San Quintin, Baja California, January 13 -17, 2008, w/Bill Etges, Allen Gibbs, Julie Havens

PP08 Punta Prieta (baited).

- 456 *D. mojavensis*
- 45 *D. nigrospiracula*
- 106 *D. pseudoobscura*
- 2 *D. hydei*

SQ08 San Quintin, same hillside as 2003 (baited).

- 17 *D. mojavensis* (+ 355 ex-3 agria rots)
- 11 *D. arizonae*
- 190 *D. hydei*
- 60 *D. pseudoobscura* (+2 ex-agria)
- 328 *D. melanogaster/simulans* (+ 9 ex-agria)
- 2 *D. repleta* dark

46. Brazil collecting trip, August 5-14, 2008, w/C. de Oliveira, F. de Faria Franco, and A. Esguícero. Most flies baited, some reared from cactus rots, no other data provided (Franco 2009).

N36 Petrolina, Pernambuco (9.1º S, 40.6º W)
D. gouveai, *D. serido*, *D. borborema*

N47 Juazeiro, Bahia (9.5º S, 40.5º W)
D. gouveai, *D. serido*, *D. borborema*

N37 Morro da Bahiarrinha, Bahia (9.9º S, 40.3º W)
D. gouveai, *D. serido*, *D. borborema*

N39 Morro do Chapéu, Bahia (11.6º S, 41.2º W)
D. serido, *D. seriema*

N40 10 km S de Morro do Chapéu, Bahia (11.65º S, 41.29º W)
D. borborema, *D. seriema*, *D. serido*

N42 Cach. Ferro Doido, Bahia (11.63º S, 41.00º W)
D. serido, *D. seriema*

N43 Irecê, Bahia (11.22º S, 41.95º W)
D. serido

N44 Xique-Xique, Bahia (10.90º S, 42.54º W)
D. gouveai

N45 Mucugê, Bahia (13.0º S, 41.4º W)
D. serido, D. seriema

47. Sonora Trip, March 3 -10, 2009, w/Bill Etges, Alfredo Ruiz, Deodoro Oliveira, and Gil Smith. baited

CH09 El Choyudo, Sonora (east of the road to the playa)

- 315 *D. mojavensis*
- 32 *D. arizonae*
- 841 *D. hydei*
- 1 *D. mettleri*
- 32 *D. mel/sim*

LB09 Las Bocas, Sonora

- 1264 *D. mojavensis* + 9 ex- 3 cina, *S. alamosensis*, rots
- 446 *D. arizonae* + 6 ex-3 cina rots
- 16 *D. navojoa*
- 211 *D. aldrichi*
- 12 *D. pachea*
- 118 *D. hydei*
- 70 *D. spenceri*
- 39 *D. huckinsi*
- 24 *D. longicornis (?*)*
- 1 *D. nigricruria*
- 5 *D. eremophila*
- 815 *D. mel/sim*
- 1 *D. busckii*
- 3 *Zaprionus indianus*
- 3 neriids (*Odontoloxozus longicornis*?)
- 23 small black dipterans (drosophilid-like)

* probably also includes *D. ritae*.

Alamos, Sonora

- 1 *D. hydei*
- 42 *D. mel/sim*

PO09 Heed Camp, Punta Onah

- 641 *D. mojavensis* + some aspirated + 1070 ex-agria rot
- 11 *D. arizonae* + 4 ex-agria rot
- 70 *D. nigrospiracula*
- 7 *D. pseudoobscura*
- 29 *D. melanogaster/simulans*
- 30 *D. hydei*
- 6 *D. pachea*
- 1 *D. aldrichi*
- 2 small dark dipterans
- 4 large dipterans with wing spots ex-agria rot

Conclusions

Despite habitat loss and increasing human population pressures in the arid lands of northwestern Mexico and elsewhere, there remains a diverse fauna of drosophilids easily obtained from nature. Cataloging this biodiversity is challenging - both the diverse numbers of drosophilid and cactus species throughout the New World demands familiarization with fly identification and preparation of male genitalia (Vilela, 1983) as well as field cactus identification (Gibson and Horak, 1978; Gibson, 1982; Gibson and Nobel, 1986; Gibson *et al*., 1986; Gibson, 1991; Zappi, 1994; Turner *et al*., 1995; Manfrin and Sene, 2006). Certainly there remain species difficult to identify as evident from the many questionable or unidentified species listed above. The often described numbers of *D. repleta* group species as "ca. > 100 species" (Oliveira *et al*., 2012) has increased as we know there are undescribed, cryptic species present (Beckenbach *et al*., 2008; Heed and Castrezana, 2008), and more new species uncovered through continuing field studies (Stensmyr *et al*., 2008; Acurio and Rafael, 2010; Acurio *et al*., 2013).

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New collection of drosophilids from Font Groga site (Barcelona, Spain).

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On 4th October 2016, a new sample of drosophilids was obtained from Font Groga (Barcelona). This is a well-described site, characterized by a vegetation composed of pinewoods (*Pinus pinea*) with some ilexes (*Quercus ilex*) and Mediterranean brushwood (Araúz *et al*., 2009). Flies were trapped from 16:30 to 19:40 pm using 12 baits containing fermenting bananas placed along a trail. Individuals were classified according to species and sex (Table 1).

It is worth observing that *melanogaster* group (*D. melanogaster* and *D. simulans*) is the dominant, as was also reported in the 2015 collection (Rosselló *et al*., 2016). *D. subobscura*, the following species in abundance, scarcely reached 12%. The sex imbalance in this species is also interesting, being females more common than males. In comparison with previous collections, the invasive species *D. suzukii* presented a drastic reduction in percentage: 8.97% in 2015 (Rosselló *et al*., 2016), 20.35% in 2014 (Esteve and Mestres, 2015), 7.98% in 2013 (Pineda *et al*., 2014), and 9.20% in 2012 (Canals *et al*., 2013). Furthermore, only females of this species were trapped. The remaining species sampled presented percentages under 1%. With

this drosophild distribution the values of *H'* (Shannon diversity index) and *J* (Shannon uniformity index) were 0.474 and 0.294, respectively. For these indexes, the trend to decrease detected in the last two years continues (Rosselló *et al.*, 2016; Esteve and Mestres, 2015).

Table 1. Classification of flies according to species and sex (Font Groga site, Barcelona).

Species	Number	Percentage
D. subobscura (♂)	5	0.91
D. subobscura (♀)	61	11.11
D. simulans (♂)	230	41.89
D. melanogaster (♂)	1	0.18
D. melano / *simulans* (♀)	240	43.72
D. suzukii (♀)	7	1.28
D. phalerata (♂)	1	0.18
D. phalerata (♀)	1	0.18
D. hydei (♂)	1	0.18
Scaptomyza sp.	2	0.36
Total	549	100

References: Araúz, P.A., F. Mestres, C. Pegueroles, C. Arenas, G. Tzannidakis, C.B. Krimbas, and L. Serra 2009, J. Zool. Syst. Evol. Res. 47: 25-34; Canals, J., J. Balanyà, and F. Mestres 2013, Dros. Inf. Serv. 96: 185-186; Esteve, C., and F. Mestres 2015, Dros. Inf. Serv. 98: 20; Pineda, L., C. Esteve, M. Pascual, and F. Mestres 2014, Dros. Inf. Serv. 97: 37; Rosselló, M., R. Madrenas, V. Ojeda, and F. Mestres 2016, Dros. Inf. Serv. 99: 18-19.

Flubendiamide inflicts tissue damage and alters detoxification status in non-target dipteran insect, *Drosophila melanogaster*.

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Abstract

This study aims to assess the safety of a lepidopteran insecticide, Flubendiamide, in a non-target dipteran model insect, *Drosophila melanogaster*, at tissue/cellular and enzyme/protein levels. Enhanced blue coloration through Trypan blue dye exclusion test suggests greater tissue damage. Furthermore, dose-dependent increase ($p < 0.05$) in the cytochrome P450 1A1 enzyme activity suggests activation of the Phase-I detoxifying mechanism. Thus, this study confirms Flubendiamide-induced toxic stress in *Drosophila* that might be replicated in other non-target organisms. Keywords: Cytochrome P450, *Drosophila*, Flubendiamide, Tissue damage.

Introduction

Flubendiamide ($C_{23}H_{22}F_7IN_2O_4S$, CAS No: 272451-65-7), a lepidopteran insecticide, is widely used in agriculture and has been suggested to be chemically safe for non-target insects like *Drosophila melanogaster* (Tonishi *et al*., 2005). Approximately 60 µg/mL Flubendiamide has been recommended for use in case of cotton by Fluoride Action Network Pesticide Project (2007), whereas proposals of US EPA (2010) for soya bean and grain are up to 60 and 103 µg/mL. The recommended Indian field doses in case of paddy and cotton are 50 µg/mL and 100 µg/mL (Government of India, Ministry of Agriculture, 2009). But recent studies revealed that Flubendiamide at very low concentration (far below the agricultural doses) may elicit severe effects on stress gene expression, neurophysiology, and external morphology of dipteran non-target *D. melanogaster* (Sarkar *et al*., 2015a, 2015b). Several workers recognized *Drosophila* as a remarkable model organism for pesticide-induced toxicity monitoring studies (Aurosman Pappus *et al*., 2017; Dutta *et al*., 2017; Rajak *et al*., 2017).

Cytochrome P450 belongs to a group of heme-containing, mono-oxygenase enzymes, which are present in eukaryotes in large numbers. There are 85 functional P450 genes present in *Drosophila melanogaster* (Tijet *et al*., 2001), whereas humans have 57 (Lewis, 2004). Several researchers suggested Cytochrome P450 enzymes to be involved in metabolism of several endogenous steroids as well as xenobiotic compounds (Chung *et al*., 2009). P450s are reported to metabolize numerous pharmaceutical drugs (Nebert and Russell, 2002), pesticides (Joussen *et al*., 2007), and plant toxins (Mao *et al*., 2006). Chung *et al*. (2009) suggested that CYP450 is found in brain, midgut, hindgut, Malpighian (renal) tubules, fat body, gonads, and so forth of *D. melanogaster* at the time of detoxification. Thus, the present study aims to observe the tissue damage through the dye exclusion test and to monitor changes in activity of cytochrome P450 mono-oxygenase enzymes in *D. melanogaster* larvae to identify the effect of the chemical on the organism as well as the response of the organism (detoxification ability) towards the chemical-induced stress at sub-lethal concentrations.

Materials and Methods

Drosophila *strain*

Drosophila melanogaster Oregon 'R' were reared at 25±1ºC and 65% relative humidity on Standard *Drosophila* Medium (SDM) containing agar-agar, corn meal, sucrose, and yeast in 360 mL distilled water (Dutta *et al*., 2014). As preservative and fungicide, 1 mL Propionic acid and 5 mg Nipagin were used.

Dye exclusion assay

The degree of tissue damage was observed in 3rd instar larvae through Trypan blue dye exclusion test following Krebs and Feder (1997) with some modifications. First instar larvae of *D. melanogaster* were subjected to chronic exposure to Flubendiamide (0.25, 0.5, 2, and 3 µg/mL) at different treatment concentrations. Treated (0.25 – 3 µg/mL) as well as control larvae were dissected cautiously to expose insect gut on a grooved slide using Poel's Salt Solution (PSS), and were carefully washed in phosphate buffer saline (PBS) for three times. 0.2 mg Trypan blue (Himedia, India) solution in 1ml PBS was used to stain tissues for 30 minutes with continuous shaking at room temperature (24±1°C) and again washed with phosphate buffer saline (PBS) three times. Immediate observation revealed tissue damage indicated by blue coloration under binocular microscope (Model: Magnus MS-24). The experiment was performed in triplicate sets.

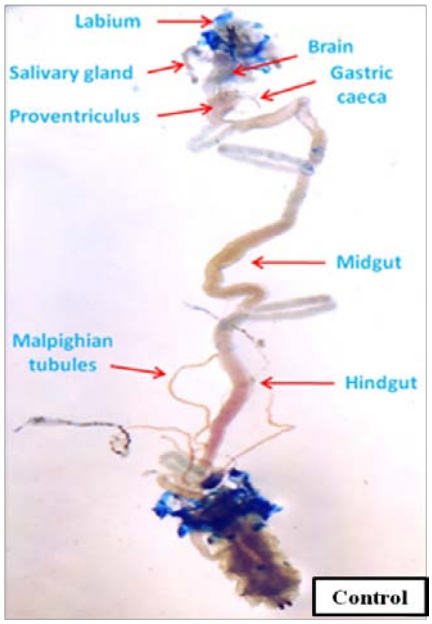
Activity of larval Cytochrome P450 (*CYP450*) *in* Drosophila (*EROD assay*)

EROD assay was carried out to observe the activity of Cytochrome P450 (CYP450 1A1) according to the method described by Klotz *et al*. (1984) with some modifications. Healthy third instar Flubendiamide-treated (0.25 – 3 µg/mL) larvae were collected from food and rinsed in Poel's salt solution (PSS). 10% homogenates were prepared with sodium pyrophosphate buffer (pH 7.4) and centrifuged at 14000 RCF for 20 min. Then, supernatants collected containing crude cytosolic (post-mitochondrial fraction) protein was used for the assay. The entire process was carried out at 4°C. Resorufin (RR) standard curve was prepared using Tris-Cl buffer for unknown samples referencing and protein was estimated following Lowry *et al*. (1951).

The typical reaction mixture contains 0.1 M Tris-Cl buffer (pH 7.8) including 0.1 M NaCl, 2 µM 7-ethoxyresorufin, and 20 µg microsomal protein to compose a total volume of 1 mL. 0.5 mM NADPH was added to initiate the reaction. Activity of 7-ethoxyresorufin o-deethylase (EROD) was recorded after 2 min at 25-30°C in UV-Vis Spectrophotometer (Model: Shimadzu UV-1800) at 572 nm. The experiment was done in triplicate sets with ten repetitions.

Statistical analysis

Non parametric Kruskal-Wallis test, followed by mean comparison Steel-Dwass-Critchlow-Fligner (*Post hoc test*) test according to the methods of Zar (1999) was performed using statistical software XLSTAT 2010, to calculate the exposure dependent differences in larval CYP450 level of different treatment concentrations in comparison to control counterparts. $p < 0.05$ was considered as the level of significance.



Results

Effect of Flubendiamide in larval tissue damage (*Trypan blue dye exclusion Assay*)

Figure 2 has revealed differential patterns of coloration in 3rd instar larval body of *D. melanogaster* exposed to different concentrations of Flubendiamide (0.25 – 3 µg/mL) after Trypan blue dye exclusion assay. Following Krebs and Feder (1997), scoring procedure was adopted based on an average composite index per larva with the help of the photographs (Table 1). From the scores, it appeared that all the treatment concentrations (0.25 – 3 µg/mL) manifested a significant ($p < 0.05$) increase in blue color which is reflected in greater Trypan blue score in comparison to control counterpart (Figure 1). Maximum tissue damage due to Flubendiamide exposure was observed after 3 µg/mL treatment (Figure 2). The uniform blue color noticed at the anterior and posterior skin regions of control as well as treated larvae might be due to the injury caused during the time of dissection. This color was ignored during scoring.

Figure 1. Trypan blue staining pattern in control 3rd instar larvae of *Drosophila melanogaster*. This figure is a representation of the different parts of larvae given special attention during observation.

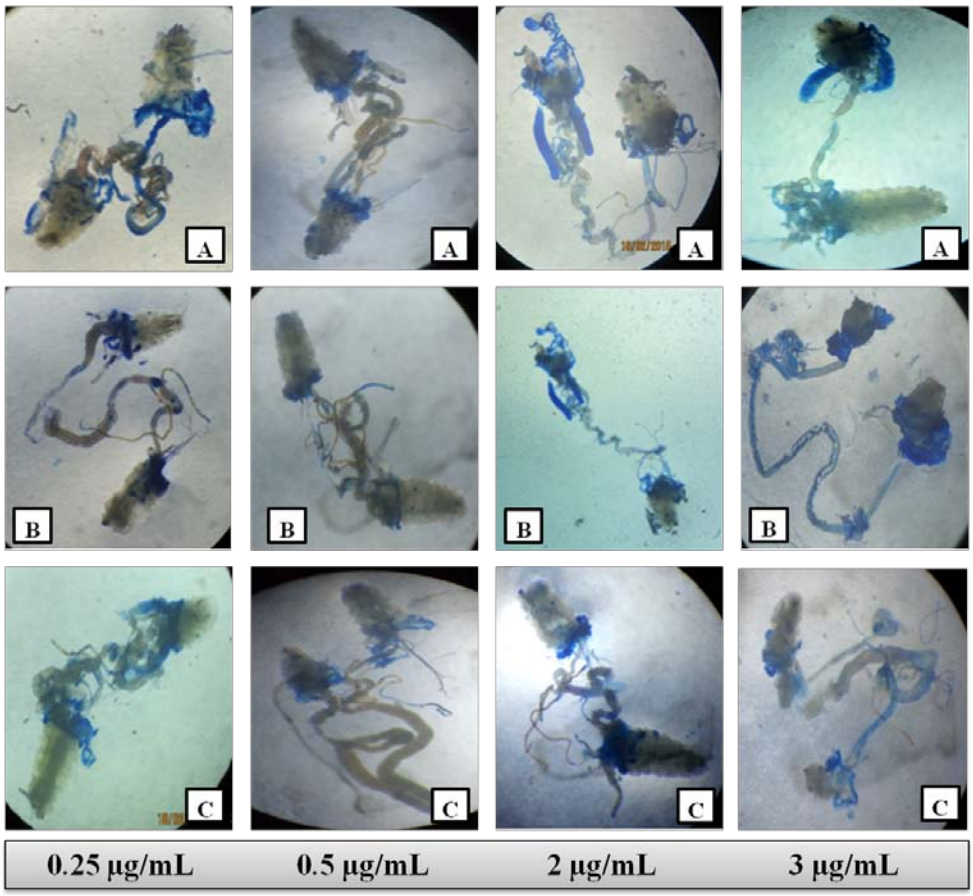


Figure 2. Trypan blue staining pattern in different tissues of third instar larvae of *Drosophila melanogaster* treated with 0.25 – 3 µg/mL Flubendiamide. Experiments were carried out in triplicate sets and scoring was done depending on development and intensity of blue color in different body parts.

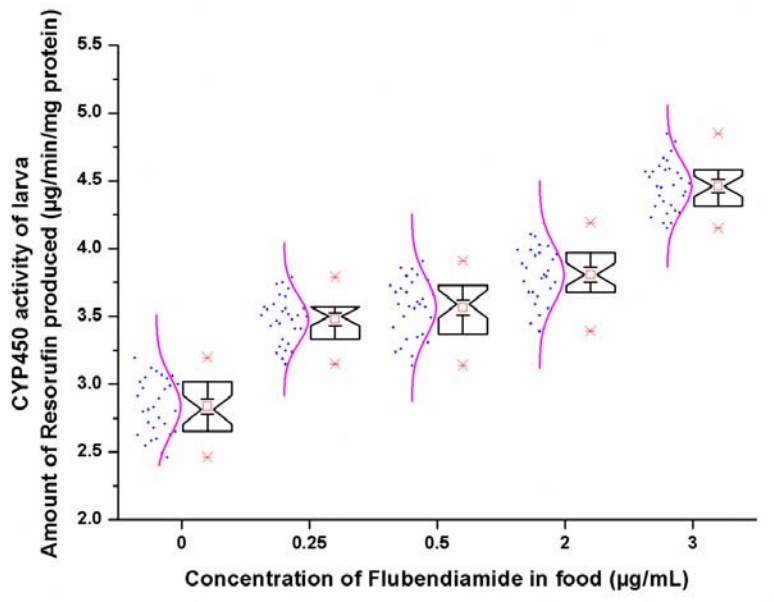


Figure 3. Variation in CYP450 activity calculated through amount of resorufin formed (µg/min/mg protein) in *D. melanogaster* larvae after treatment with Flubendiamide. The data are expressed in notch box plot, where blue dots stand for real data, pink curve denotes normal curve, and notch box represents middle 50% data (26% - 75%, *i.e.*, 2nd and 3rd quartile data). Moreover, small red boxes within the notch boxes indicate mean values, vertical lines within notch boxes symbolize standard errors (SE), notches of the notch boxes signify median values, whereas lower and upper cross of notch boxes are 1st (1% - 25%) and 4th (76% - 99%) quartile data, respectively.

Table 1. Quantification of Trypan Blue staining, an indicator of tissue damage, observed in 3rd instar larvae of *D. melanogaster* exposed in different concentrations (0.25 – 3 µg/mL) of Flubendiamide.

Conc. Of Flubendi-amide (µg/mL)	Group	B	SG	P	GC	FG	MG	HG	MT	PS	Score	Mean	S.E.
Control	A	0	0.5	0	0	1	0.5	0	0	0	2	2.33	0.3
	B	0	1	0	0	0.5	1	0	0	0	3		
	C	0	0.5	0.5	0	0	1	0	0	0	2		
0.25	A	1	1	0.5	1	0	3	3	0	0.5	10	10.5*	0.2
	B	1	2	2	1	0.5	1	1	1	1	10.5		
	C	2	2	1	1	1	1	1	2	0	11		
0.5	A	2	2	0.5	1	1	1	1	2	1	11.5	10.7*	0.3
	B	1	1	1	1	0.5	1	2	2	1	10		
	C	2	1	1	1	1	2	1	1	1	1		
2	A	1	3	1	1	1	2	1	1	1	14	12.8*	0.5
	B	2	3	1	1	1	2	1	1	1	13		
	C	1	2	1	1	1	2	0.5	2	1	11.5		
3	A	3	3	2	1	1	2	2	1	0.5	15.5	13.2*	0.4
	B	2	2	1	0.5	2	2	1	0.5	0	11		
	C	1	1	1	2	1	2	1	2	2	13		

B- brain; SG- salivary gland; P- proventriculus; GC- gastric caeca; FG- foregut; MG- midgut; HG- hindgut; MT- Mapighian tubule and PS- posterior skin.
The numerical '0' = no color, '0.5'= very pale blue color, '1'=pale blue color, '2'=moderate blue color, '3'= dark blue color. In the present study scoring pattern was adopted from Krebs and Feder (1997) with some modifications. The experiments were carried out in triplicate sets and each set consisted of 20 larvae. '*' represents significant difference from control group ($p<0.05$).

Effect of Flubendiamide in larval Cytochrome P450 assay (*EROD*)

Cytochrome P450 (CYP6 family) activity was measured by the formation of Resorufin (µg)/ min/ mg protein. This present study revealed that all the Flubendiamide-treated 3rd instar *D. melanogaster* larvae were significantly different ($p < 0.05$) in Cytochrome P450 activity from control counterpart (2.84±0.04). Treatment with (0.25 – 3 µg/mL) test chemical revealed a significant ($p < 0.05$) increase (22.63%, 25.79%, 34.28%, and 62.54%) in Resorufin production or enzyme activity as compared with control (Figure 3).

Discussion

Trypan blue is a vital dye which can easily infiltrate through the cell membrane of dead cells or tissues, whereas live cells with intact cell membrane resist the dye from penetration. When cells or tissues were exposed to Trypan blue, then dead or dying cells were unable to eliminate the dye and dead cells or tissues reveal distinct blue color. On the contrary, live cells do not absorb any dye and remain unchanged. In the present study, all the treated larvae (0.25 – 3 µg/mL) were observed with blue coloration in their brain, salivary gland, proventriculus, gastric caeca, foregut, midgut, hindgut, and Mapighian tubule as compared with control counterparts (Figure 1 and Figure 2). Furthermore, quantification of Trypan Blue staining (according to Krebs and Feder, 1997), an indicator of tissue damage, revealed a significant ($p < 0.05$) dose dependent increase in treated *Drosophila* larvae exposed in different concentrations (0.25 – 3 µg/mL) of test chemical (Table 1). Flubendiamide and/or metabolites of Flubendiamide might cause toxic stress on larvae that result in tissue or cellular damage. Thus larvae exposed to Flubendiamide reveal an increase in dead cell number as observed through trypan blue dye exclusion test, thereby confirming the Flubendiamide-induced stress. Dose-dependent greater tissue damage might be due to reduced number of viable cells as suggested by Gupta *et al*. (2005a) in *Drosophila* exposed to an organophosphate pesticide, Nuvan. Several workers reported tissue damage in response to chemical stress (Siddique *et al*., 2013; Dutta *et al*., 2017; Rajak *et al*., 2017). As the test chemical might cause cellular or tissue damage, initiation of detoxification was observed to overcome the stress.

As suggested by Mukhopadhyay and Chottopadhyay (2014), all organisms have their own detoxifying systems for overcoming toxic stress. Normally, detoxifying system is composed of Phase I, Phase II, and Phase III pathways (Rajak *et al*., 2017). In phase I pathway, toxic substances convert into polar and water soluble compounds facilitated following pathways (Phase II and Phase III) act upon and are excreted from body through bile or kidney (Benson and Di Giulio, 1992). Cytochrome P450 is very well known for its Phase I detoxifying mechanism against xenobiotic compound including pesticides (Joussen *et al*., 2007; Chung *et al*., 2009). Hence, insects exposed to chronic sub-lethal Flubendiamide (0.25 – 3 µg/mL) might be under stress that activates Cytochrome P450 detoxifying system (Phase I). In the present study, treated larvae of *D. melanogaster* show a concentration dependent significant ($p < 0.05$) increase of Cytochrome P450 enzyme activity in comparison to control, thereby confirming a greater Flubendiamide-induced stress (Figure 3). Similar increase in Cytochrome P450 activation was also found in *Drosophila melanogaster* in response to toxic stress of NaF (Dutta *et al*., 2017). As Flubendiamide is a fluoride containing chemical (Tonishi *et al*., 2005), thus, free fluoride ion released from test chemical might be responsible for toxic stress (oxidative stress) (Guo *et al*., 2003). Thus, this study confirms Flubendiamide-induced toxic stress in *Drosophila melanogaster* and might show similar hazardous effects to other related non-target organisms.

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Appl-GAL4 driven transcription in adult heads.

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Drosophila melanogaster is one of the most important invertebrate model organisms to study the aging process. The proper application of genetic tools that depend on the GAL4/UAS bipartite gene expression system to overexpress or downregulate genes of interest in aging research requires GAL4 drivers that provide well-characterized expression levels in specific adult tissues. It was previously reported that Appl-GAL4 driven expression of UAS-Atg8a lines increased maximal and average lifespan, while the widely used pan-neuronal elav-GAL4 driver did not provide similar effect (Simonsen, 2008), suggesting that Appl-GAL4 provides stable adult expression. To assess the expression level and expression pattern of Appl-GAL4 in the adult nervous system we decided to characterize UAS transcription driven by the P{w[+m*]=Appl-GAL4.G1a}1, y[1] w[*] line (BDSC stock no. 32040) that is advertised to express GAL4 in the nervous system.

In order to determine UAS transgene expression levels driven by Appl-GAL4 during aging we collected freshly eclosed *Appl-GAL4/w; UAS-GFP/+* progeny of *Appl-GAL4* females and *w; UAS-GFP* males and maintained them at 25°C by passing them to fresh vials every 2-3 days. We prepared RNA samples from heads of 1 day, 3 days, 1 week, 2 weeks, 3 weeks, 4 weeks, and 6 weeks old female flies (10 heads per sample, three biological replicates per age group) using QIAzol Lysis Reagent (Qiagen). We generated first strand cDNA from 0.5 µg RNA per sample using TaqMan Reverse Transcription Reagents (Thermo Scientific) with random hexamer primers, then measured transgene expression levels in a PikoReal Real-Time PCR system (Thermo Scientific) using GoTaq qPCR Master Mix (Promega) with primers specific for pUAST (Fw: CTG TGG TGT GAC ATA ATT GGA CAA, Rev: TGC TCC CAT TCA TCA GTT CC A, designed for the SV40 polyA/terminator region in the pUAST vector) and with primers for the rp49 housekeeping gene that was used for normalization. qPCRs were performed in duplicates and transcript levels were determined by setting Ct values against cDNA template calibration curves. We found that the transcript level of UAS-GFP significantly decreased over time (Spearman's correlation coefficient $\rho = -0.60553$, $P = 0.00363$). A gradual reduction in Appl-GAL4 driven UAS-GFP expression was most pronounced after 2 weeks of age, with heads of 4 and 6 weeks old flies having transcript levels below 60% of that of freshly eclosed adults (Figure 1).

Next, we investigated the expression pattern of Appl-GAL4 in the adult nervous system by visualizing GFP expression in dissected brains of 1 week old *Appl-GAL4/w; UAS-GFP/+*females under a Nikon Eclipse

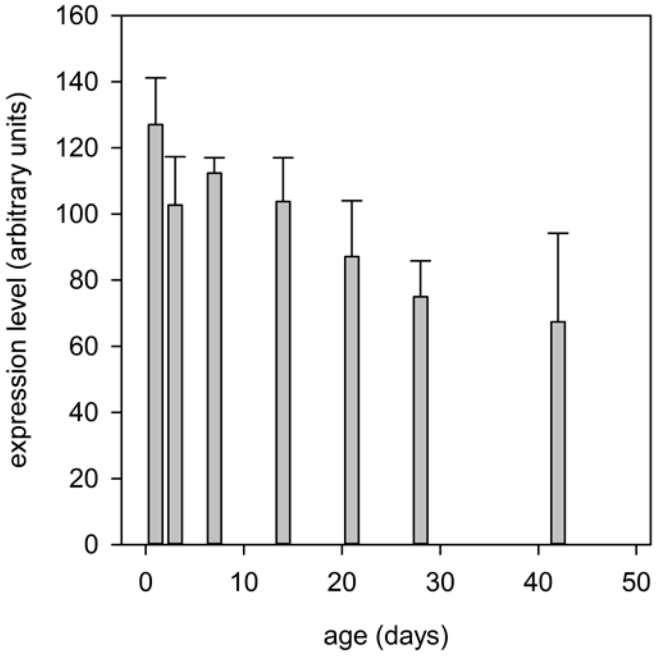

Figure 1. Expression level of Appl-GAL4 driven UAS-GFP in heads of aging adults. Bars show the average of three biological replicates; error bars represent the standard error of mean.

80i fluorescent microscope (Figure 2). We found that the adult neuronal expression driven by Appl-GAL4 is not uniform. Although low levels of GFP could be detected in most parts of the brain, high level expression was detected only in the mushroom body.

In conclusion, we found that Appl-GAL4 driven UAS expression is not uniform in the adult brain and its level decreases during aging – both issues should be taken into consideration in aging studies.

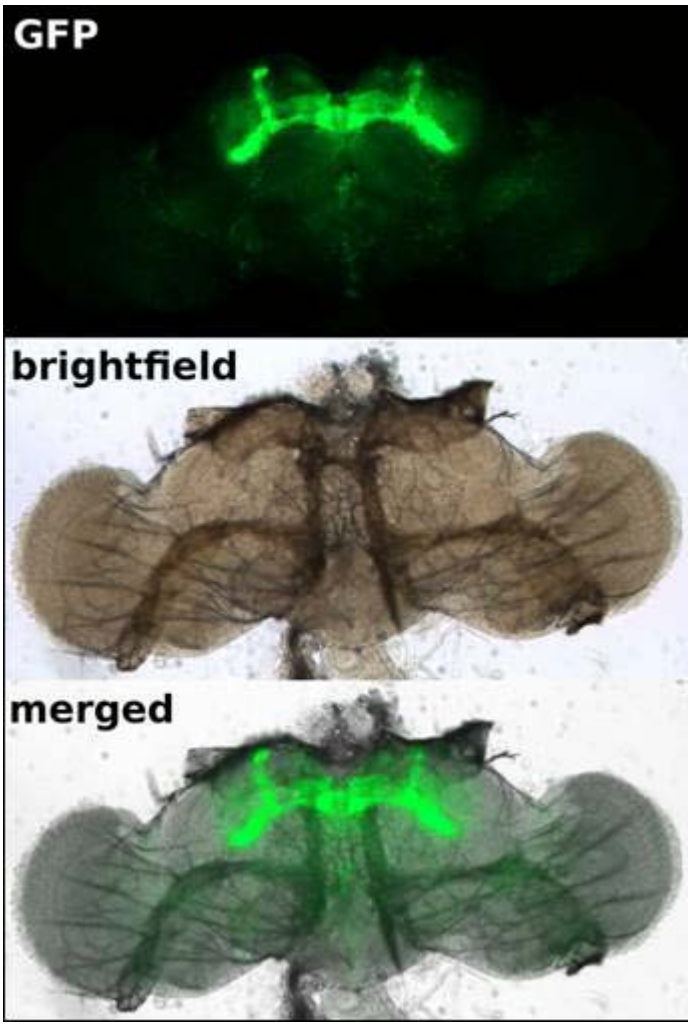

Figure 2. Appl-GAL4 driven expression of UAS-GFP in the adult brain.

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Co-amplification of Hymenoptera parasitoids' sequences during DNA Barcoding assessments of mycophagous drosophilids.

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Introduction

Drosophilidae have been the target of several studies in many areas of biological knowledge, mainly due to the performance of *Drosophila melanogaster* Meigen, 1830:85 as an effective model organism (Jennings, 2011). However, some basic aspects of the biology of the family remain unknown. One of these topics concerns the frequency of parasitism by Hymenoptera species (Fernandes *et al.*, 2009). In fact, few studies described the association of drosophilids with Hymenoptera parasitoids (Wertheim *et al.*, 2000; Marchiori *et al.*, 2003; Guimarães *et al.*, 2004; Yorozuya, 2006; Fernandes *et al.*, 2009). Fernandes *et al.* (2009), for example, found *Ganaspis* sp. (Figitidae, Eucoilinae) parasitizing an unidentified species of frugivorous drosophilids, that was hypothesized to be *Zaprianus indianus* Gupta, 1970 due to the length of the pupae. In addition, Wertheim *et al.* (2000) identified 673 individuals of hymenopteran drosophilids' parasitoids associated with 9,305 specimens of Drosophilidae in mushroom emergence evaluations.

Trying to enhance studies with mycophagous drosophilids, Machado *et al.* (2017) tested and proved the effectiveness of DNA Barcoding as an important way to identify and discover species in this poorly explored Drosophilidae group. In that study, the authors analyzed cytochrome oxidase subunit I (COI) sequences of 218 mycophagous drosophilids collected at 20 sites in the Brazilian Amazonian, Atlantic Forest, and Pampa Biomes, and revealed a high number of potential new species, some of which have already been described (Junges *et al.*, 2016). However, an unexpected result obtained by Machado *et al.* (2017) refers to the co-extraction and co-amplification of Hymenoptera parasitoids' DNA sequences. So, the aim of the present study is to report the presence of Hymenoptera parasitoids associated to at least two individuals of the obligatory mycophagous species *Mycodrosophila projectans* (Sturtevant, 1916).

Materials and Methods

Adult flies were first collected from macroscopic fungi fruiting bodies using an entomological aspirator (Machado *et al.*, 2014) and then fixed and stored in absolute alcohol. The two individuals whose DNA Barcoding sequences indicated the presence of Hymenoptera parasitoids were collected in Floresta Nacional de Caxiuanã, in Melgaço city (coordinates -51.457129, -1.737762), and in Horto Botânico UFPel, in Pelotas city (coordinates -52.431944, -31.816111), both located in the Brazilian Amazonian and Pampa biomes, respectively. The flies were identified as *M. projectans* based in male genitalia (Wheeler and Takada, 1963).

Total DNA was extracted using phenol-chloroform protocol (Sassi *et al.*, 2005), and COI fragments were amplified using primers HCO1490 (Folmer *et al.*, 1994) and C1N2329 (Simon *et al.*, 1994) or LCO2198 (Folmer *et al.*, 1994). The PCR products were purified and directly sequenced using an ABI 3730XLs automatic sequencer with the same primers used in PCR. The electropherograms were assembled using the Staden Package Gap 4 program (Staden, 1996), and each contig was checked for adequate sequence quality. To confirm species DNA identity, Nucleotide Blast (Altschul *et al.*, 1990) searches were performed using Gap4 consensus sequences as queries. Identity inferences were based on the returned hit with maximum score value, whose E-value and percentage of identity was taken as an indication of identification accuracy. The

obtained result was further confirmed through a search performed in the Bold system (Ratnasingham and Hebert, 2007), whose system is interconnected with GenBank. The sequences were deposited in Genbank under the numbers MF615211 and MF615212.

Results and Discussion

Due to the low number of Hymenoptera parasitoids' DNA sequences deposited in GenBank and Bold systems, accurate identification of the parasitoid species was not possible. In this sense, despite the divergence of 13% between both parasitoid sequences, Blast tool recovered for them a maximum identity of 87% to the same *Hymenoptera* sp. (GenBank access number JF271365.1) from Papua New Guinea, which is a parasitoid of caterpillars (Lepidoptera) (Hrcek *et al.*, 2011). Given that COI is the animal molecular marker most frequently used (Pentinsaari *et al.*, 2016), this result highlights the poor knowledge related to Diptera parasitoids taken as a whole.

Moreover, our results also suggest an apparent low infection rate among wild caught mycophagous drosophilids, given that only two cases of infection were detected among more than 200 analyzed specimens. Considering that some other infections may not have been adequately detected by our indirect approach, this infection rate is in agreement with other studies addressing this subject in mycophagous drosophilids. In this sense, Wertheim *et al.* (2000) reported for this group a Hymenoptera infection rate of 8% in Dutch woodland, and Yorozuya (2006) found for them a constant annual parasitism rate ranging between 6.7% and 9.8% in Japan. Nevertheless, this last author observed some seasonal variations in these values.

Interestingly, Fernandes *et al.* (2009) found a similar infection rate [8.3% - by *Ganaspis* sp. (Figitidae, Eucoilinae)] among frugivorous drosophilids colonizing fruits of *Coffea arabica* in Brazil. This suggests that the low infection rate is probably a characteristic of the family, independent of the resource explored, although some species could be more prone to parasitoid infection than others. In this sense, among the 54 different species which had their COI sequences characterized by Machado *et al.* (2017), the two cases of infection were detected for *M. projectans*, in populations distant by more than 3,000 kilometers. Thus, this report calls attention to the need for more studies in this unexplored group, whose diversity is largely misunderstood and whose impact in drosophilids dynamics must be further evaluated.

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First report of *D. polychaeta* Patterson and Stone from India.

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Abstract

Drosophila flies were collected from different localities of Dharwad district of Karnataka state, India. The present report is on collection, identification, and description of *D. polychaeta* Patterson and Stone for the first time. The species was found at vegetable fields of Navalgund taluk in Dharwad district. A few morphological characters of the species are discussed.

Introduction

Drosophila is used as a model organism in many fields of biology. Taxonomy and biodiversity of this genus play a vital role in analyzing the faunal composition in an ecosystem. At present in the family of Drosophilidae there are 4217 species described, which consist of 77 genera. In genus *Drosophila* there are 1178 species among which 751 species belong to subgenus *Drosophila*, whereas 335 species belong to subgenus *Sophophora* (Bachli, 2014). Taxonomic studies on Drosophilidae were reviewed by Gupta on the Indian subcontinent during 1974. Kandpal and Singh (2010a) reported a total of 319 species of Drosophilids from India, of which 148 species belong to genus *Drosophila*. In South India there are only 50 species (Hegde *et al*., 2001). Most of the species reported from India mainly belonged to *melanogaster* species group of subgenus *Sophophora* (Bock and Wheeler, 1972; Hegde *et al.*, 2001), or else belonged to *immigrans* and *repleta* subgroups of subgenus *Drosophila* and many other genera, such as *Zaprionus* of family Drosophilidae (Fartyal *et al*., 2014). In South India most of the faunal studies were concentrated in and around Mysore and Western Ghats (Hegde *et al*., 2001). Srinath and Shivanna (2012, 2013, and 2014a) did a complete survey of *Drosophila* fauna of Dharwad district. Two rare species belonging to the subgroup of *Polychaeta* under subgenus *Drosophila*, *D. daruma* Okada and *D. latifshahi* Gupta and Ray-Chaudhari, were recorded from Dharwad district (Srinath and Shivanna, 2012, 2014b). The frequent survey is required to keep the data updated regarding the richness of species and diversity; hence the survey was continued and new rare species were recorded.

Materials and Methods

Drosophila were collected from different vegetable fields in Navalgund taluk of Dharwad district using Bottle trapping and net sweeping methods (Hegde *et al*., 2001). The collected flies were brought to the laboratory and were separated according to their respective sexes. The males were directly used for identification whereas the females were cultured in separate vials consisting of wheat cream agar medium. Later the males were used for identification of species (Hsu, 1949; Bock and Wheeler, 1972; Markow and O' Grady, 2006).

Results

The morphological characters of *D. polychaeta* collected from Dharwad district, Karnataka, India are given below.

Length of the imago: male: 2.7 to 2.9 mm (Figure 1a); female: 3.2 to 3.5 mm (Figure 1b).

Head: Arista with 5 branches above and 3 below.

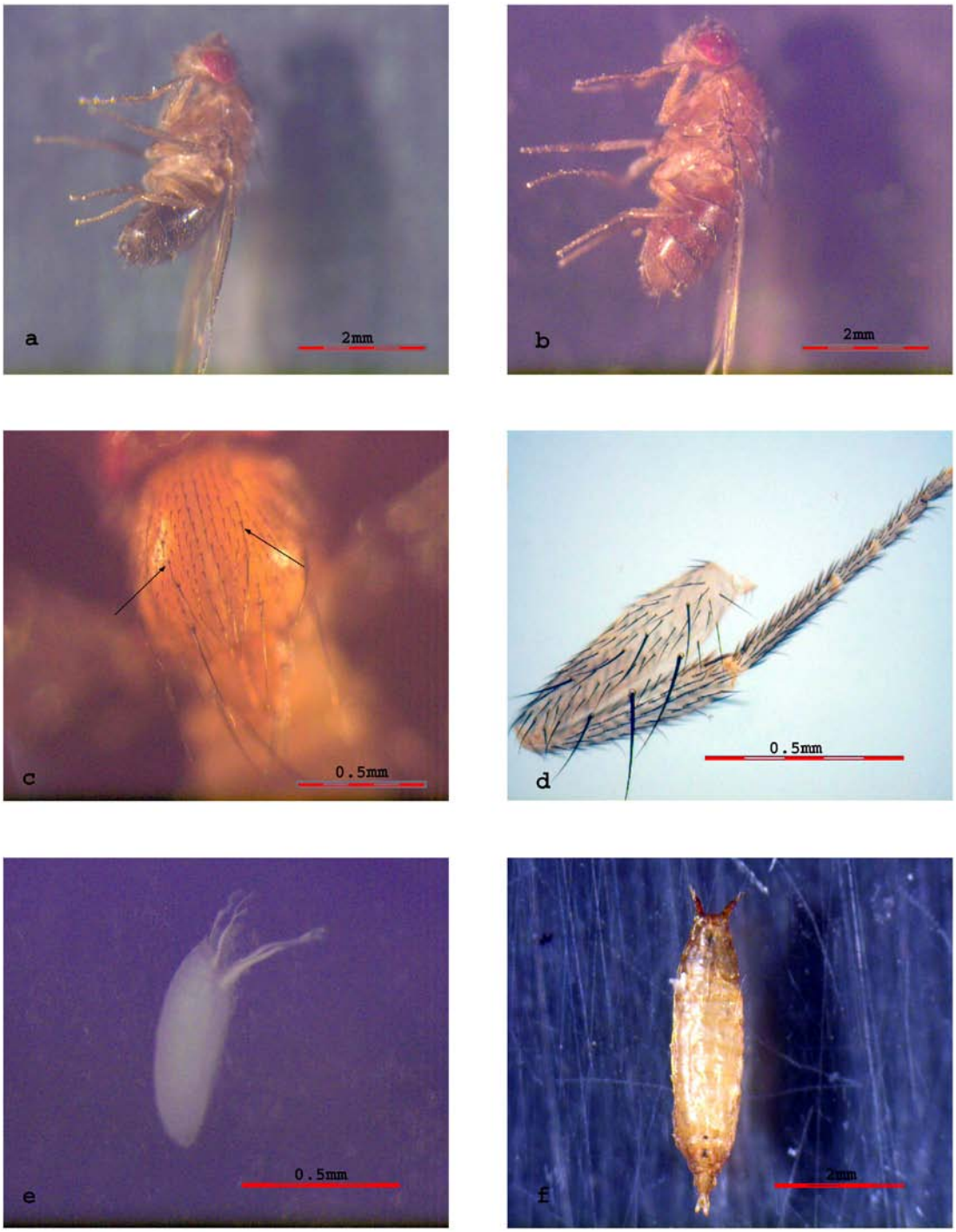

Figure 1. *D. polychaeta* lateral view of male, 1a; female, 1b; thorax, 1c; foreleg of male, 1d; egg, 1e; pupa, 1f. Arrow indicates the presence of extra pair of dorso-central setulae.

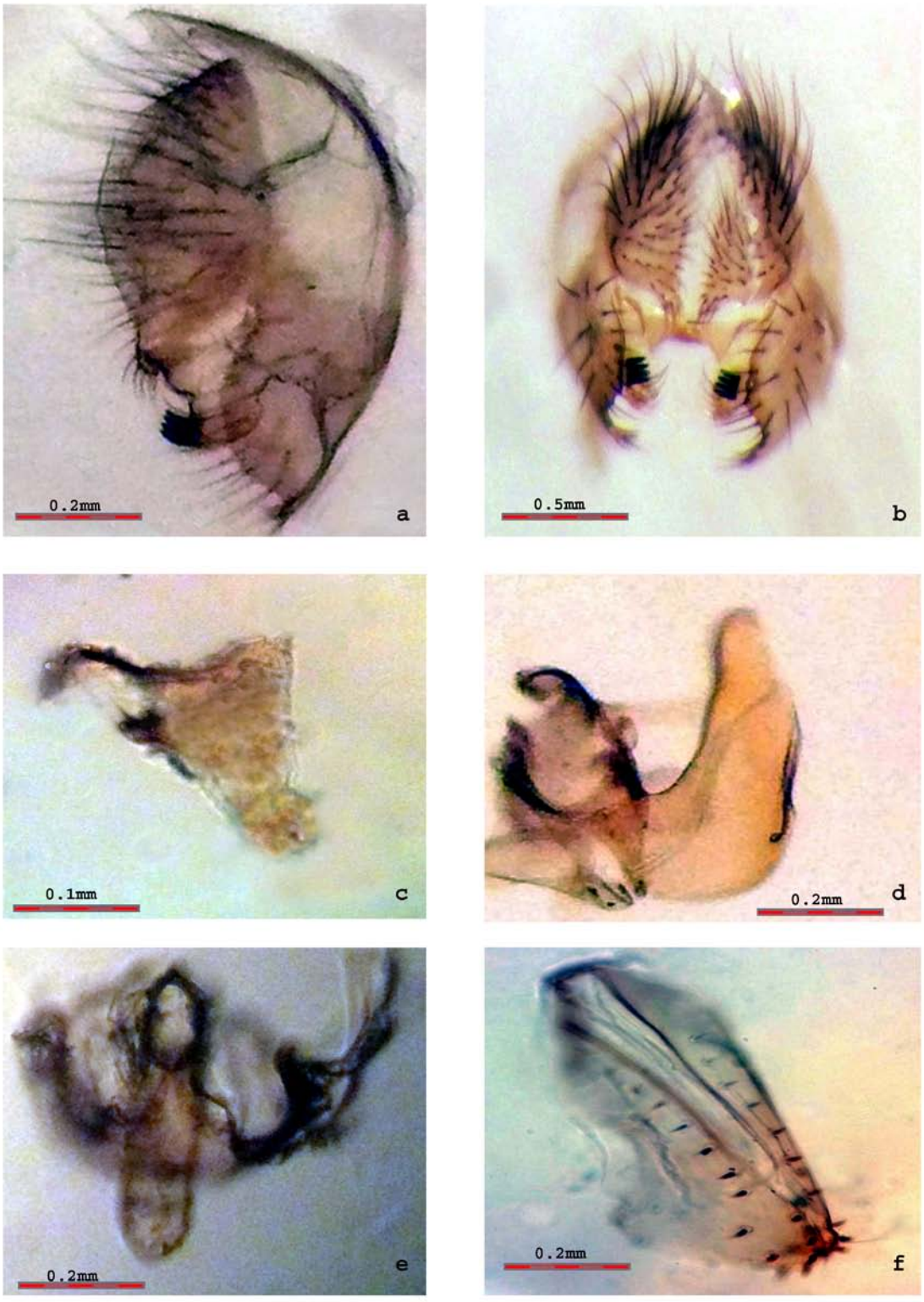

Figure 2. *D. polychaeta* Periphallic organ lateral view, 2a; whole view, 2b; Decasternum, 2c; Phallic organ lateral view, 2d; Phallic organ ventral view, 2e; egg guide, 2f.

Antenna: Dark yellowish color; carina uniformly shaped and sulcate; cheek broad. At the base of cheek 3 rows of bristles were present; 1st row with 7 bristles, 2nd row with 8 bristles, and 3rd row with 5 bristles.

Anterior orbital bristle proclinate and larger than middle orbital bristle and almost equal to posterior orbital bristle; middle and posterior orbital bristle reclinate; post vertical bristles present; vertical bristles present and reclinate; eyes are red in color and oval in shape with females having broader eyes and looks quadrate in shape; ocellar triangle present and pointed anteriorly.

Thorax: Yellowish in color; a dark median stripe present internally; acrostichal hairs are in 7 irregular rows; dorsocentrally an extra pair is present (Figure 1c); anterior dorsocentral is smaller than posterior dorsocentral; pre-scutellar setulae absent; anterior scutellar bristle longer than posterior scutellar; posterior scutellar crossed. Sterno – index is 0.6 in females and 0.82 in males.

Legs: Sex-combs absent in tarsal region of forelegs; femur consists of three prominent bristles (Figure 1d).

Egg with four filaments and pupa with 2 distinct anterior spiracles and two elongated and partially fused posterior spiracles (Figures 1e and f).

Wing: Wings hyaline and its venation clear; subcostal break prominent; halteres yellowish. Wing indices calculated according to Okada (1956).

	C-index	4V-index	4C-index	5X-index
Male	1.99	2.24	1.38	1.60
Female	2.05	2.30	1.40	1.56

Abdomen: First abdominal tergite is yellowish at anterior and dark at posterior, whereas other abdominal tergites are tannish at anterior and completely dark at the posterior. Sternites brownish; on ventral view female looks lighter in color except the sternite, whereas the males are orange because of color of testis.

Periphallic organ: Genital arch brown, broad, and pubescent; about 24 to 25 bristles present on lower half. Toe pointed and heel absent; surstylus looks attached to the anal plate and upper region is broad and pubescent in nature, whereas lower region consists of 4-5 primary teeth with about 2 to 3 bristles on the distal margin and 5 to 6 on caudoventral region. Anal plate attached to genital arch at the center and has a convex shape when viewed from lateral side and has a pointed end; it consists of approximately 49 to 50 bristles; the pointed end has approximately 6 to 7 bristles (Figures 2a, 2b). Decasternum triangular in shape (Figure 2c).

Phallic organ: Aedeagus pointed at distal end and broad at proximal end. Aedeagal apodeme small. Anterior paramere without sensilla; posterior paramere absent. Novasternum slipper shaped (Figure 2 d, e).

Egg guide: Lobe of ovipositor pale yellow with 21 teeth surrounding the internal margin (Figure 2f).

Specimens examined: 2 ♂, 2♀, INDIA: Karnataka, Navalgund; Dharwad. Coll: Shweta K.

Distribution: Neotropics, Micronesia, Hawaii, North America, Europe, China, India (n. loc.): Navalgund.

Discussion

The coloration and the presence of an extra-pair of dorsocentral bristles confirm the species belonging to the *polychaeta* species group (Okada, 1956; Toda and Peng, 1989; Watabe *et al*., 1990; Markow and O' Grady, 2006). The present species differs from earlier description made by Hsu (1949) in the shape of genital

plates. Hsu (1949) mentions that the anal plate is fused with genital arch at the upper half and the number of teeth present on the primary clasper region is 5-6 and they are not similar in size. Whereas the present species has anal plate fused with genital arch at the center and lower side and the number of teeth present on the primary clasper is 4-5 and they are equal in their size (Figure 2b). The collected *D. polychaeta* is very much similar to the characters mentioned by Watabe *et al*. (1990). Species belonging to *polychaeta* species group are rarely reported by *Drosophila* taxonomists across India. *D. daruma* was first reported by Vaidya and Godbole (1976) from Poona and surrounding regions. Similarly, *D. latifshahi* was reported by Gupta and Raychaudhuri (1970) from Chakia forest in North India, but they categorised this species under subgenus *Scaptodrosophila*. Later Toda and Peng (1989) reclassified this species under *polychaeta* species group. These species were reported for the first time from South India by Srinath and Shivanna (2012, 2014) from Dharwad and surrounding areas. *D. polychaeta* is reported for the first time from the Indian subcontinent. Hence this species is the third addition to the present list of species under *polychaeta* species group reported from India.

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Interaction of chlorophyllin with radiation-induced autosomal recessive lethals.

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Abstract

Chlorophyllin (SCC), sodium copper chlorophyll, presents protective action against damage induced by different physical and chemical agents. In *Drosophila melanogaster*, this effect has been reported for somatic cells. However, on germ cells for sex chromosomes, the inhibitory effect was not found. We are interested in the lethal induced effect on the second chromosome of this species. Canton-S males were given a 24-hour pre-treatment with and without 69 mM of SCC and later exposed or not to 40 Gy of gamma radiation. Those males were screened with the Cy L /Pm technique for detection of recessive lethal genes. Results showed the SCC pre-treatment did not produce significant changes in frequency for recessive lethal genes in the second chromosome, due to 40 Gy gamma radiation. In order to evaluate the effects of chlorophyllin on damage done by radiation, we considered the presence of autosomal lethals and semi-lethals. We observed that, even with radiation, the frequency of semi-lethals did decrease when chlorophyllin is applied but not significantly. For lethals, either with or without radiation, the frequency slightly increased. Keywords: chlorophyllin, radiation, lethal genes, *Drosophila*

Introduction

For almost three decades, research towards evaluating the modifying action of sodium copper chlorophyll (SCC) on ionizing radiation mutagenicity has been conducted at Instituto Nacional de Investigaciones Nucleares (ININ) *Drosophila* laboratory. SCC is a compound with strong protective action against damage induced by different physical and chemical agents, and it has been evaluated on different biological systems. We are interested in the effect it has on several *Drosophila melanogaster* tests. Graf *et al.* (1984) evaluated its effect through the mutation and somatic recombination test. Zimmering *et al.* (1990) found that, when applied at a concentration of 69 mM as a pre-treatment, the frequency of gamma radiation induced mutation and chromosomal break would significantly decrease. The same concentration for a 24-hour pre-treatment during larval stage was able to prevent damage from gamma radiation (Pimentel *et al.*, 1999).

Based on the fact that X-rays or gamma rays induce somatic recombination with a quadratic dose-response relationship or a two-strike phenomenon (Merriam and Fyffe, 1972) and that, theoretically, this should be valid for all somatic cells, with the results of this research, SCC is suggested to inhibit, on a lower grade, events related to ruptures in comparison with the ones related to mutations (Pimentel *et al.*, 2000).

Regarding SCC's action against damage induced by direct chemical agents, it was found to protect against damage induced by chromium trioxide (Olvera *et al.*, 1997) only during the first two days; however, the effect is reversed and mutation frequency increases (Cruces *et al.*, 2003). The same result was returned against damaged induced by N-ethyl-N-nitrosourea (ENU) (Pimentel *et al*., 2003) and damage induced by dimethylhydrazine; after that time it behaved as a damage promoter (Guerrero, 2004). In addition, it is important to point out that there are very few tests regarding CCS's inhibition or promotion of induced genetic damage on germ cells. Of course, the significance of damage induced on this kind of cells is greater, since it can be transferred through generations. In this regard, studies have been made to detect SCC's effect on tests with and without radiation by sex-linked recessive lethals screening (Pimentel and collaborators, unpublished data), who did not see any protective effect from SCC against radiation. With this background, we set out to achieve determining SCC's effect against gamma radiation keeping in mind the damage it causes to *D. melanogaster*'s second chromosome.

Materials and Methods

Two strains of *D. melanogaster* were used, the Canton-S that acted as treated strain and the Cy L /Pm; H /Sb as the marker one.

Groups of Canton-S males from 3 to 5 days of age were previously treated for 24 hours as follows: two control groups, one fed with 5% sucrose and another one with a 69 mM chlorophyllin solution and two experimental groups that received the same pre-treatment but were then subject to gamma radiation with a 40 Gy dose provided at the Institute's Gamma Cell.

Both the chlorophyllin and the sucrose were administered orally. The study was done by several stages since the work overload impedes the simultaneous analysis on all four treatments. After treatment, males were crossed individually, according to the technique described by (Wallace, 1956) for this species' second chromosome recessive lethals detection, with 2-3 virgin females of strain Cy L /Pm; H /Sb 2-5 days old. From this cross, a Cy L /+ male was extracted from each culture and back-crossed with 2-3 virgin females of mother strain Cy L /Pm; H /Sb.

At time when the offspring of this back-cross emerged, five virgin females were selected per culture, since with these crosses there are also Cy L /Pm and +/+ individuals, and five Cy L /+ males, which were interbred. This series of crosses allows us to track down a chromosome II of each treated male, represented on cross like +/+.

When the offspring of this last crossbreed was born, each culture's counts were done. Since these are Mendelian crosses, it is expected for the progeny to be two kinds of individuals: Cy L /+ (curved wings and partially reduced eyes) and +/+ (wild ones) with a 2:1 proportion. Cultures with this proportion are considered normal, meaning those whose chromosome did not suffer any damage, since deviations from this proportion indicate the presence of damaged genes.

When +/+ individuals do not show, there is a lethal-gene carried in the chromosome and if there are +/+ flies in a frequency about 10% of individuals, with wild +/+ phenotype and genotype, then it carries a semi-lethal gene.

All crosses were conducted at 25 ± 1° C and 60% relative humidity. The supplied medium was the common use one at the lab, made from corn flour, sugar, beer yeast, and agar. Data obtained was screened with an X^2 test to identify differences.

Results

A total of 949 second chromosomes were analyzed, 232 of them were treated only with sucrose, 217 with chlorophyllin, 213 with sucrose and radiation, and 287 with chlorophyllin and radiation; as shown on Table 1, which also indicates the number and frequency of each category of genes: normal, semi-lethal, and lethal ones, calculated from each culture's counts. If we consider the observed frequencies on the treatment with sucrose as a basis, we see that in three out of four possible comparisons –that we are interested in– chlorophyllin itself decreases the frequency of semi-lethals. On the other hand, the comparison that did not decrease was the frequency of lethals when treated without radiation; in this case it slightly increased.

Nevertheless, our goal is to know how significant these differences are, but a direct observation on the relative frequency of the different categories shows that differences are so small that they do not require a statistical analysis because of their similarity.

Discussion

Chlorophyllin's inhibition or promotion effect against radiation varies according to the system used. Among the different systems using *Drosophila*, the one carried out by Zimmering *et al.* (1990) stands out. They obtained evidence of action as radio-protective substance and Pimentel *et al.* (1999) confirm its persistence as radio-protective agent as well as indicate evidence of action as inhibition or promotion agent (Pimentel *et al.*, 2000).

Meanwhile, Olvera *et al.* (1997) consider it an anti-mutagen agent. It is also considered a genetic-damage promotor or inhibitor agent when its effect has been seen against chemical agents such as CrO_3 (Cruces *et al.*, 2003) or against 1,2-dimethylhydrazine (Guerrero, 2004). All of the previous cases dealt with somatic effects since, fundamentally, the assays were carried out via the mutation and somatic recombination test on *Drosophila*'s wing. When dealing with germ cells, the regular test is the sex-linked recessive lethals screening and, in this case, Pimentel and collaborators (on unpublished data) informed us about not having found a promotion or inhibition effect of SCC against radiation when performing this test.

The fact that no inhibition effect was observed on the sex-linked recessive lethals screening Pimentel and collaborators (unpublished) led us to analyze the effect of SCC on damage produced by gamma radiation via this species' second chromosome recessive lethals test.

Table 1. Relative frequency of normal, semi-lethal and lethal genes obtained through sucrose, SCC with and without 40 Gy of gamma radiation treatments.

	Sucrose	Chlorophyllin	Sucrose +40 Gy	Chlorophyllin + 40 Gy
Normal	212; 91.3%	209; 95%	188; 88.3%	256; 89.2%
Semi-lethal	17; 7.3%	6; 2.7%	15; 7%	17; 5.9%
Lethal	3; 1.3%	5; 2.3%	10; 4.7%	14; 4.9%
n	232	220	213	287

The results shown on Table 1 indicate on the semi-lethal gene category when they are a product exclusively of the sucrose or SCC pre-treatment, the effect of the latest is to inhibit the appearance of semi-lethals. In this case, there is a significant difference $P \leq 0.01$. However, when comparing both pre-treatments plus the radiation, this effect did not show up.

Nevertheless, in the case of SCC treatment alone, the frequency of observed lethal frequency did not decrease; it actually increased slightly. However, when besides the SCC there was exposure to radiation, a slight increase was obtained on the frequency of this kind of genes. On the remaining comparisons, to see the effect of chlorophyllin there are no possible significant differences.

The fact that there was no inhibition effect seen on the previously obtained sex-linked recessive lethals test matches with the effect observed by us on the second chromosome, as a result of this assay and with the one observed on the third chromosome on somatic cells. All the above show a similar action of SCC among sex and autosomal chromosomes against gamma radiation effect.

In virtue of the above data, we can conclude that chlorophyllin's action as pre-treatment against gamma radiation has no response that benefits a protective or inhibiting action as it also happens on sex chromosomes, which indicates chlorophyllin does not work on sex nor autosomal chromosomes against gamma radiation.

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What happens when exotic species arrive in a new area? The case of drosophilids in the Brazilian Savanna.

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Abstract

Drosophilids have been widely used as models in scientific research, including the area of biological invasions. In the past two decades, three exotic species of these flies have arrived in the Neotropics. *Zaprionus indianus* was first detected in 1999, and after two years it was widely distributed throughout the Neotropics. Currently, it dominates drosophilid assemblages under specific environmental conditions. *Drosophila suzukii* and *D. nasuta* were recorded in southern Brazil in 2013 and 2015, respectively, and have rapidly expanded their distribution northward. In this paper, we describe the temporal variations in these two recently introduced species in the Brazilian Savanna, a two million-km^2 biome located in the center of South America, where we have been regularly collecting drosophilids since 1998. *Drosophila nasuta* and *D. suzukii* were first detected in the Brazilian Savanna in December 2013. Two years after their arrival, *D. nasuta* seems to have succeeded in colonizing forests, reaching peak abundance in the rainy season, whereas *D. suzukii* remains rare. We conclude that these exotic species differ in their ability to establish viable populations in natural environments and recommend monitoring them to understand the dynamics of the early stages of biological invasion.

Introduction

The world is becoming biotically homogenized, with cosmopolitan taxa replacing geographically restricted species (McKinney and Lockwood, 1999). In Brazil, *ca.* 300 species of neotropical drosophilids coexist with 12 species from other biogeographical domains (Tidon *et al*., 2016). These exotic species have probably been arriving since at least the 16th century, transported by ships and airplanes. However, in the late 20th century, the establishment and spread of *Zaprionus indianus* Gupta (Vilela, 1999) caught the attention of the scientific community. In addition to economically impacting fig plantations, it provided an informative model system for understanding the ecological and evolutionary processes of invasions (Gilbert *et al*., 2016).

Drosophila suzukii Matsumura and *D. nasuta* Lamb have arrived in the Neotropics in the 21st century. The former is a widespread species known as the spotted-wing *Drosophila* due to the prominent dark spot on the wings of males (Walsh *et al*., 2011). The female has a long and narrow ovipositor with many teeth that is used to infest soft-skinned fruit crops, causing enormous economic damage on commercial plantations (Walsh *et al*., op cit.). *Drosophila suzukii* is endemic to southeastern Asia and has spread to western regions; it was found in Hawaii in the early 1980s and more recently in North America and Europe, where it has reached invasive pest status (Asplen *et al*., 2015). In Brazil, it was first detected in the southern region in February 2013 (Deprá *et al*., 2014). *Drosophila nasuta* is a tropical species characterized by a brownish stripe on the pleurae, an iridescent silvery-whitish frons, and a row of cuneiform setae on the first femur (Vilela and Goñi, 2015). It probably originated in southeastern Asia (Kitagawa *et al*., 1982) and spread by passive transportation to Africa, the Oriental region, the Pacific Islands, and western North America (David and Tsacas, 1981; Brake and Bächli, 2008). In Brazil, it was first recorded in the southeastern region in March 2015 (Vilela and Goñi, op cit.).

The Brazilian Savanna, locally known as Cerrado, is the second-largest South American biome. Its vegetation is highly variable on the well-drained interfluves, while gallery forests follow the watercourses (Oliveira and Marquis, 2002). The predominant vegetation type, called *cerrado sensu stricto*, harbors a unique array of drought- and fire-adapted plant species. The climate of the region is seasonal with an average annual rainfall of 1500 mm concentrated between November and March (Eiten, 1972), and mean temperatures range from 22°C to 27°C. The drosophilid communities in the Brazilian Savanna have been intensively studied since 1998 and currently include 122 Neotropical and eleven exotic species (Roque *et al*., 2016).

Here, we provide the first record of *Drosophila nasuta* in the Brazilian Savanna and describe temporal fluctuations in *D. suzukii* and *D. nasuta* populations at two sites in this biome. Therefore, this study contributes to the understanding of exotic species establishment in new areas.

Materials and Methods

The flies were collected in two areas near Brasília, the capital of Brazil. The first area was the IBGE Ecological Reserve (IBGE) (15°56'S; 47°53'W), where ten sites were sampled bimonthly. The collections occurred from October 2013 to April 2016 (from five sites in gallery forests) and from October 2013 to February 2016 (from five sites in *cerrado sensu stricto*). At each site, nine retention traps (Roque *et al.*, 2011) were distributed in three non-spatially autocorrelated sample units (SUs), corresponding to a 1,345-trap sampling effort. The second sampled area was a gallery forest located approximately 60 km from IBGE at the *Campus Planaltina* of the *Instituto Federal de Brasília* (PLA) (15°38'S; 47°41'W). This forest was sampled monthly from September 2015 to April 2016 using five retention traps (Roque *et al*., op cit.) in each collection expedition, resulting in a total 40-trap sampling effort. The traps were baited with bananas fermented with dried baker's yeast (*Saccharomyces cerevisiae* Hansen) and left in the field for four consecutive days.

Captured flies were stored in 70% ethanol and identified using taxonomic keys and descriptions. Based on their biogeographical origin, all specimens were classified into four categories: *D. suzukii*, *D. nasuta*, other exotic species (EXO), and native Neotropical species (NEO). Voucher specimens were deposited in the Collection of the *Laboratório de Biologia Evolutiva* of the *Universidade de Brasília* and the *Laboratório de Biologia Animal* of the *Instituto Federal de Brasília* (*Campus Planaltina*).

Table 1. Abundance of drosophilids in gallery forest and *cerrado sensu stricto* of the IBGE Ecological Reserve between October 2013 and April 2016. NEO: Neotropical species, EXO: other exotic species. Gray columns indicate the core rainy season.

	2013		2014					
	Oct	Dec	Feb	Apr	Jun	Aug	Oct	Dec
Forest IBGE								
NEO	26	654	11080	4379	323	110	32	123
EXO	25	441	1613	146	34	1948	326	411
D. suzukii	0	0	0	0	0	0	0	0
D. nasuta	0	1	1	0	0	0	0	0
Total forest	51	1096	12694	4525	357	2058	358	534
Cerrado *sensu stricto* IBGE								
NEO	27	375	1847	318	246	80	54	397
EXO	14	10685	2133	114	118	156	59	3053
D. suzukii	0	3	0	1	0	0	0	5
D. nasuta	0	0	0	3	0	0	0	0
Total cerrado	41	11063	3980	436	364	236	113	3455

(Table 1 continues), Last column represents totals for each species in each region.

	2015						2016		Total
	Feb	Apr	Jun	Aug	Oct	Dec	Feb	Apr*	For each species 2013- 2016
Forest IBGE									
NEO	2488	17567	1363	205	53	80	2455	348	41286
EXO	164	367	123	1581	258	940	298	31	8706
D. suzukii	0	10	1	0	0	0	0	0	11
D. nasuta	0	6	0	0	0	228	260	8	504
Total forest	2652	17950	1487	1786	311	1248	3013	387	50507
Cerrado *sensu stricto* IBGE									
NEO	311	2454	397	198	40	78	567	-	7389
EXO	2337	3093	105	329	47	4415	172	-	26830
D. suzukii	14	12	0	0	0	6	0	-	41
D. nasuta	0	2	0	0	0	4	6	-	15
Total cerrado	2662	5561	502	Aug	87	4503	745	-	34275

Results and Discussion

Among the 86,658 drosophilids collected, 52 specimens were identified as *Drosophila suzukii* (Table 1). The male collected at IBGE in December 2013 by Paula *et al*. (2014) was the first record of this species in the Brazilian Savanna, but in reviewing the collections made by these authors, we identified two female

spotted-wing *Drosophila*. Therefore, ten months after the first record in South America (Deprá *et al*., 2014), we found *D. suzukii* in the Brazilian Savanna. Since then, periodic collections have caught only a few individuals, primarily during the rainy season and in savanna vegetation.

Benito *et al*. (2016), based on the thermal requirements of the spotted-wing *Drosophila*, suggested that it prefers a moderate climate. In fact, the temperate southern region of the country is where invasive populations are spreading and causing fruit production losses. According to scenarios modeled by these authors, most of the Brazilian Savanna is not favorable for the establishment of this species except the area surveyed in the present study. It is worth noting that *D.suzukii* females survived an average of 20 or more days at 94% relative humidity (RH) in the laboratory, whereas below 20% RH they could not reproduce (Toshen *et al*., 2016). As rains in this tropical biome are heavily concentrated between November and March, the stressful dry seasons probably cause local extinctions of *D. suzukii*. On the other hand, if Southern immigrants transported by the fruit trade arrive during the rainy season, these propagules will be able to establish new populations. Therefore, it is too early to say whether *D. suzukii* will establish in central Brazil. Continued monitoring of drosophilids in preserved and converted areas will contribute to answering this question.

We primarily captured *Drosophila nasuta* in forests and throughout the rainy season (Tables 1 and 2). The first five specimens were collected in IBGE in December 2013 and early 2014, but we were not able to identify them until after the publication of the paper by Vilela and Goñi (2015). These authors recorded *D. nasuta* in southeast Brazil (São Paulo city) in March 2015 and provided an accurate species description that allowed us to identify it among our samples. The first peak in the abundance of *D. nasuta* in the Brazilian Savanna was observed in both study areas in December 2015.

Table 2. Abundance of drosophilids in a gallery forest of the *Campus Planaltina* of the *Instituto Federal de Brasília* between September 2015 and April 2016. NEO: Neotropical species, EXO: other exotic species. Gray columns indicate the core rainy season.

	2015				2016				
	Sep	Oct	Nov*	Dec	Jan	Feb	Mar	Apr	Total
NEO	11	7	0	24	10	127	167	75	421
EXO	48	2	0	639	68	285	131	84	1257
D. nasuta	0	0	0	8	73	66	33	18	198
Total	59	9	0	671	151	478	331	177	1876

* In November 2015, the traps were placed in the field, but no drosophilids were captured.

Drosophila suzukii and *D. nasuta* seem to differ in their ability to establish viable populations in the natural environments of the Brazilian Savanna landscape. Two years after it was first recorded, *D. nasuta* succeeded in colonizing forests; in both areas surveyed (IBGE and PLA), it corresponded to approximately 11% of the drosophilids collected in the rainy season. In contrast, the spotted-wing *Drosophila* has never reached a relative abundance of 1% in a single collection and so can be considered a rare species in these communities. These results strongly contrast with those obtained for *Zaprionus indianus*. When first recorded in the Brazilian Savanna, the African fig fly corresponded to 7% of the drosophilids collected (February 1999), and a year later (February 2000), its relative abundance had reached almost 90% of the drosophilids in savanna-type vegetation (Tidon *et al*., 2003).

It is challenging to predict the fate of an introduced propagule in a new area, and we are aware that extensive monitoring is required to estimate the spread and population growth of *Drosophila suzukii* and *D. nasuta*. For example, the exotic species *D. ananassae* Doleschall and *D. busckii* Coquillett have been recorded in Brazil for decades but remain rare in most collections (Tidon, 2006; Roque *et al*., 2013), whereas *D. simulans* Sturtevant and *Zaprionus indianus* respond rapidly to favorable environmental cues, undergoing extensive population growth and dominating drosophilid assemblages under specific circumstances (Döge *et al*., 2015). Therefore, studies documenting the establishment of recently introduced species, as presented here

for *D. suzukii* and *D. nasuta*, are fundamental to understanding the dynamics of biological invasions.

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A burst of spontaneous mutations in isofemale lines of *Drosophila melanogaster* from Senegal.

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Introduction

With more than a century of use *Drosophila melanogaster* has proven to be a good model for genetic studies (Green, 2010). In addition to the ease of handling the species in laboratory, it is particularly interesting because of its ability to produce spontaneous mutants (Lindsley and Zimm, 1992). These mutants have played a fundamental role in understanding genetic mechanisms and most of them are now characterized at the molecular level and the function of the affected genes is known. Therefore, recovering classical mutant phenotypes is today of little value as they are not going to improve our knowledge much. However, recovering these phenotypes might be relevant for indicating genetic instability in natural populations. Indeed a large proportion of natural mutations is due to mobile element activity, and this mutagenic activity is associated with stress (Capy *et al*., 2000) or possibly with the admixture of divergent populations as in the P-M system (Kidwell *et al*., 1977).

From the biogeographical distribution of species closely related to it, *D. melanogaster* probably

originated in Africa and then invaded the world as a human commensal (Lachaise *et al*., 1988). Genetic divergence is now observed between the cosmopolitan populations outside of Africa and their African counterparts (Begun and Aquadro, 1993; Kauer *et al*., 2002; Baudry *et al*., 2004). Genetic conflicts are thus possible when secondary contacts occur between African and cosmopolitan populations. In this context it is particularly interesting to observe the presence of several mutants in isofemale lines from Senegal in Africa. Several phenotypes affecting wings or eyes have been observed in these lines. Two of them we isolated and characterize here.

Materials and Methods

Isofemale lines were established from flies collected at Salemata (SM) in Senegal in 2008. Since then some lines have gone extinct, but 17 SM lines are still in culture. Two other stocks have been used for outcrossing: vg^{null}/ *Cy*O (from Institut Jacques Monod, Paris) and *y cn bw sp* (from Kyoto Drosophila Genetic Resource Center). The vg^{null} allele is a full deletion of the coding sequence of the *vestigial* gene generated by excision of the P element of the vg^{21} allele (Bernard *et al*., 2003). Crosses were made in vials at room temperature with usual fly medium. 5-10 virgin females were outcrossed to males of the appropriate other stock.

Results

Wingless mutants were isolated from the line SM15. The phenotype was typical of *vestigial* (*vg*) and apparently similar to vg^{1}. Virgin females were crossed to males of vg^{null}/*Cy*O. Two phenotypes were observed in the progeny: [*vg*] and [*Cy*] indicating that the mutation found in SM15 line affects the *vestigial* gene and is recessive. The new allele is denoted vg^{SM15}.

Mutants with bright red eyes were isolated from the line SM23. The mutant was crossed to wildtype flies from another SM line. The progeny were completely wildtype, indicating that the mutant was recessive. The mutant was also crossed with a *y cn bw sp* stock that was available at the laboratory. The progeny all had the [*cn*] phenotype indicating that the mutation found in the line SM23 affected the *cinnabar* gene. We denoted the new allele cn^{SM23}.

Discussion

We isolated two mutants and identified the affected genes as *vestigial* and *cinnabar* through crossing. These mutants have very obvious phenotype and are viable and fertile. They are then easily detected and consequently many similar alleles are already known. Indeed, FlyBase lists 390 classical alleles for *vestigial* and 225 for *cinnabar*. Other phenotypes, mostly involving position of wings, were observed and are currently under isolation. More careful observation of these lines is necessary to reveal more discrete phenotypes.

These mutations with large phenotypic effect are expected to be at very low frequency in natural populations due to strong selection against them. It is therefore unlikely that so many mutations were sampled by chance in isofemale lines. Thus, the presence of large numbers of mutants in the Salemata lines suggests a genetic basis at the origin of the mutational process. This genetic basis is probably the insertion of mobile elements at different loci. Mutagenic activity by mobile elements can increase under environmental stress (Capy *et al*., 2000) or when a genomic conflict arises by the admixture of two diverging populations. Senegal is at the north-western edge of the Afrotropical realm and is an important step on the commercial route with Europe. It is consequently very likely that this area is a contact zone between native African populations and cosmopolitan ones that differ in their mobile element composition. Under these conditions, numerous insertional mutations would be present in the field and so be collected. However, it is also possible that the mutation process has also been exacerbated in the laboratory by increasing homozygosity in the isofemale lines.

The identification of mutated genes is the first step necessary before characterization of the mutations at the molecular level. Further studies are thus necessary to identify the true nature of these mutations and to validate a possible genomic conflict following population admixture.

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Phenology or resources limit *Drosophila* local biodiversity in a southern Asian continental subtropical forest.

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Introduction

Biodiversity is typically greater in the tropics and semitropics than in temperate areas. The question then is, how is this greater biodiversity achieved? High diversity would result if there were more resources in low latitudes than in high with a small number of species using each resource. High diversity would also result from greater spatial or temporal (*i.e.*, phenological) variability in species identities. This mechanism also suggests that local species numbers will be small. The proportional sampling model, however, predicts high local diversity (Cornell and Lawton, 1992). This is because the proportional model holds that interspecific competition is relatively unimportant. The number of species coexisting locally is then a simple proportion of the number of species in the regional pool.

As an initial step towards examining this question in *Drosophila*, we sampled flies in a species rich semitropical forest in continental southern China.

This genus is species rich with 2874 species world-wide (Wheeler, 1981, 1986) and provides a very diverse but phylogenetically restricted fauna. The genus is better known, particularly in the tropics, than most dipteran taxa. We looked at continental eastern Asia because there are no major barriers to north-south movement of faunas at this longitude. The existing faunas are, therefore, unlikely to be affected by restricted post-glacial colonization. Major barriers do exist at other longitudes. The Himalayas and Caucuses, together with deserts, limit exchange in western Asia. The subtropical African faunas are separated from Europe by the double barrier of the Sahara desert and the Mediterranean sea. And in the Americas the relatively recent rise of the Isthmus of Panama is a similar complicating factor. Diverse drosophilid faunas are, however, found in tropical mainland Asia. In an area of only 1130 ha, our main study site in southern China has over 115 drosophilid species. Of these, 63 are currently in the genus *Drosophila* (Toda and Peng, 1989; Peng *et al.*, 1990a, 1990b). This contrasts markedly with 54 *Drosophila* species recorded for the entire British Isles. We, therefore, concentrated our study on the *Drosophila* faunas of two locations near Guangzhou, Guangdong Province, China. Local biodiversity was remarkably low. This completely contradicts the proportional sampling model. We are now investigating the contribution of spatial, temporal, and resource variability in species richness to the high biodiversity of continental southern Asia.

Methods

Dinghu Shan (DHS) (23°08' N 112°35' E) is a mountainous area of remnant forest 86 km west of Guangzhou City in the southern subtropical biogeographic zone of China. The mountain summits are 150-500 m with the highest peak at 1000.3 m. The topography is rugged and steep-sloped (Wu *et al.*, 1982). The underlying rocks are Devonian sandstones or slates and the soils are generally acidic and humus-rich (He *et al.*, 1982). The area has a subtropical monsoon climate (Huang and Shen, 1982) with tropical storms and typhoons bringing heavy rain from April to October (Huang and Fan, 1982). Annual precipitation reaches 1962.9 mm and annual insolation 10.48 Kcal cm^{-2} (Huang and Shen, 1982). The dominant vegetation type is subtropical monsoon evergreen broadleaf forest composed mainly of *Castaneopsis*, but also including *Caryota* and *Calamus* palms, screwpine *Pandanus*, and treeferns. Plant diversity is very high with 1489 species (Wang *et al.*, 1982).

We made limited additional collections on the campus of the Guangdong Institute of Entomology (GEI). This is within the urban area of Guangzhou city at 23°05" N 113°17' E. It contains research and domestic buildings, gardens, ponds, and trees. It is at the south west corner of the 117 ha Zhongshang University campus. This campus is well wooded and park-like.

Few of the tree species at DHS were fruiting, so we collected *Drosophila* by putting out fruit baits. Small pieces of banana or of pineapple were put into 1 × 3" glass vials. Twenty-four baits, 12 of each fruit, were placed in pairs at two sites and 26, 13 of each, at two other sites in unfrequented montane evergreen broadleaved forest 200-400m away from Qing Yun Si temple at DHS. These 4 sites were at least 100 m away from each other. At a fifth site 10 tubes of each bait type were exposed among scrubby undergrowth and young trees. We exposed the baits for 6 days and then collected them. A second series of baits was then set out in the same way as the same locations and these in turn were collected 6 days later. All the samples were, therefore, made within 12 days in the last 2 weeks of July.

The same procedure was followed at one site on the GEI campus. On the first occasion we set out 25 banana and 10 pineapple baits and on the second 20 of each.

After collection the baits were maintained at the GEI in an outdoor insectary (at 29 ± 2° and 80-100% RH). They received natural light but were shaded from direct sun. We identified the adults that emerged to species and recorded the numbers of each taxon.

Results

A total of 224 baits were successfully recovered. Many of the missing baits, particularly the banana baits, exposed at DHS had been removed by ants. The proportions of baits of the two types recovered from each site varied greatly (Table 1). Of the recovered baits, 15 produced no adults. We have omitted these baits, and those lost, from our analysis.

Table 1. Mean number of flies of each species per bait (and standard deviation) for each bait type at each of the two locations. The number of successful, recovered baits is given in brackets after the bait type.

Site	Bait Type	*Drosophila albomicans*	*Drosophila bochi*	*Drosophila kikkawai*	*Drosophila lini*	*Drosophila melanogaster*	*Drosophila montium*	*Drosophila punjabi*	*Drosophila suzukii*	*Drosophila takahashi*	*Drosophila tani*
Dinghu Shan	banana (59)	53.85 (47.52)	0.05 (0.22)	0.02 (0.13)	0.17 (0.70)		0.66 (2.05)	0.05 (0.39)	0.02 (0.13)	5.97 (17.39)	0.73 (1.54)
	pineapple (88)	21.10 (17.86)	0.01 (0.11)		0.14 (0.57)		0.23 (0.62)			1.36 (3.17)	1.17 (2.44)
Guangzhou	banana (40)	8.05 (12.39)	0.25 (1.28)			18.30 (21.29)	0.15 (0.66)	0.05 (0.32)	0.03 (0.16)	1.60 (2.66)	
	pineapple (22)	4.45 (6.91)	0.05 (0.21)			40.05 (41.94)			0.18 (0.85)	2.32 (3.14)	

We reared out 7194 adult flies from 10 *Drosophila* species taxa. There were 5454 *D. albomicans*, 15 *D. bochi*, just one *D. kikkawai*, 22 *D. lini* (lini-complex), 1613 *D. melanogaster*, 65 *D. montium* (montium-complex), 5 *D. punjabi*, 6 *D. suzukii*, 587 *D. takahashi*, and 146 *D. tani*.

We identified 9 species from DHS baits and 7 from those at GEI (Table 1). We reared two species, *D. lini* and *D. tani*, from DHS that were not found at GEI. *Drosophila melanogaster* emerged only from GEI baits and not at all from those at DHS. It is of interest that we reared *D. suzukii* from fruit baits exposed on the ground despite the frequent claim that this species specializes on ripe rather than decaying fruit. We also reared a very few *Asobara*-like braconids from DHS. From GEI there were a few *Leptopilina*-like eucoilids and a single braconid (not like *Asobara*). At both sites there were occasional individuals of at least 2 beetle species and a species of large, long legged acalypterate fly.

The *Drosophila* fauna we collected was dominated by very few species, *D. albomicans*, *D. takahashi* and, in GEI, *D. melanogaster* (omitting the infrequent *D. kikkawai*, *D. punjabi*, and *D. suzukii*) (Figure 1). There were significant faunistic differences between DHS and GEI ($F^{1}_{195} = 40.20$, $p < 0.001$) by permutated nested Anderson manova using Jaccard similarity. There were also significant differences between bait types within sites and locations ($F^{7}_{195} = 2.67$, $p < 0.001$). However, there were no significant differences in faunas between sites within locations ($F^{5}_{195} = 1.37$, $p < 0.079$) (see also Figure 1).

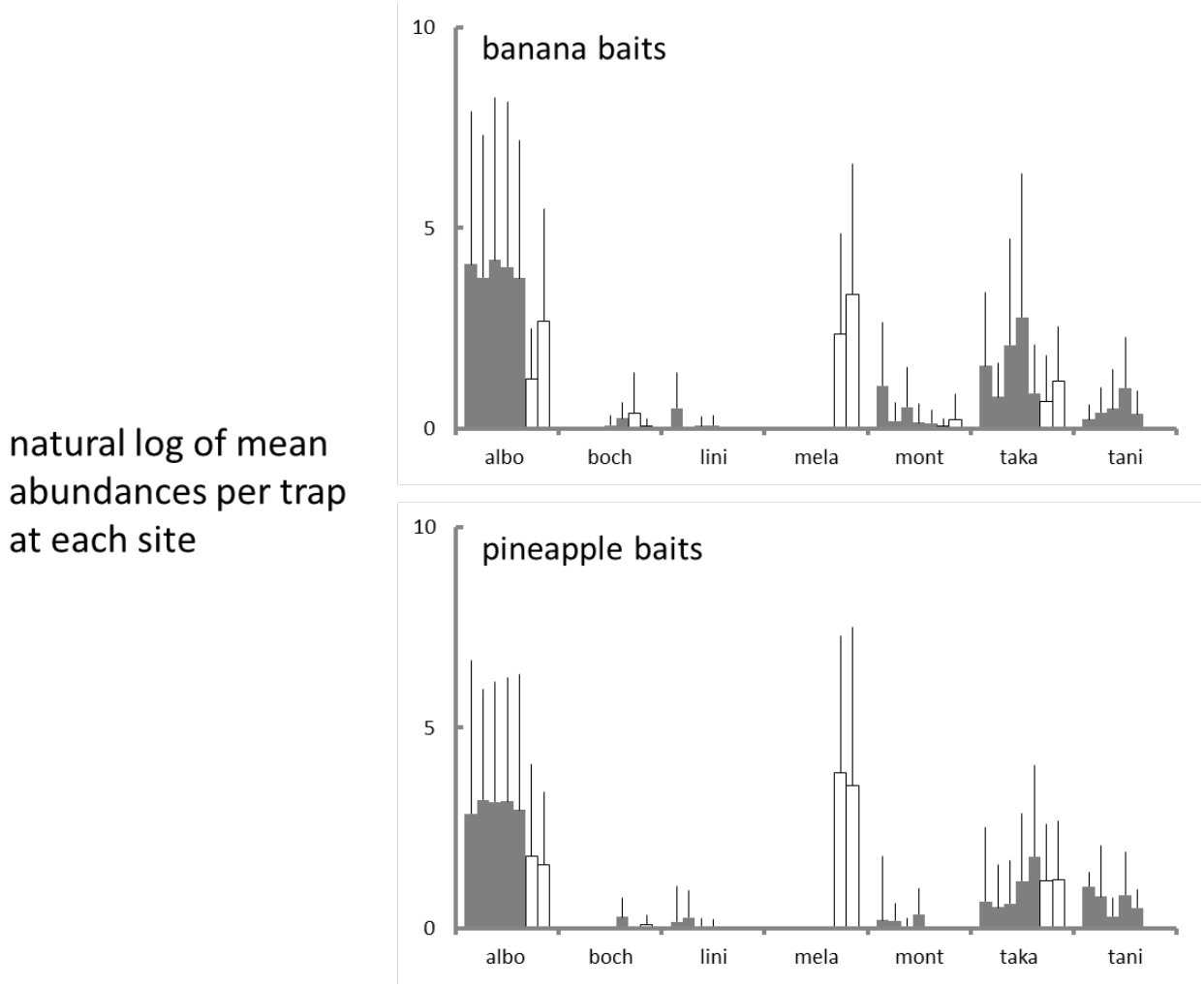

Figure 1. Average numbers of adults (as natural logs of abundance) at 5 sites in DHS (gray) and 2 in GEI (white) emerging from the 2 different bait types. The error bars are the natural log of 1 standard deviation of the corresponding mean.

Discussion

The small number of species from DHS and GEI contradicts the proportional sampling model (Cornell and Lawton, 1992), because more would be expected under this model given the 63 *Drosophila* species recoded from the area. That we only reared 10 species, only 7 in any numbers, is not the result of using banana and pineapple as baits. It is true that neither is present in DHS. However, banana has been present in eastern Asia for millennia and so is likely to have accumulated a *Drosophila* fauna. Banana, furthermore, is attractive to many *Drosophila* species, particularly those breeding on fruit, even species that never breed on banana naturally. Therefore, the banana baits probably attracted all the fruit breeding *Drosophila* species active in the trapping area during late July. The similar number of species reared from pineapple suggests that this fruit is also a broadly attractive bait.

We would have reared such a small number of species despite a recorded fauna of at least 63 species if the majority of the 63 were not fruit breeders. This is not the case as the faunal list for DHS is derived from static retention trapping using fruit bait (Peng *et al.*, 1990b). Most of the *Drosophila* listed usually or often breed on fruit.

Other mechanisms must, therefore, produce the high species diversity of DHS and of semitropical continental east Asia generally. One mechanism is a high turnover in species present with distance, *i.e.*, spatial variation in species. There were differences in the species found in DHS and in GEI, particularly the occurrence of *D. melanogaster* at GEI. However, there were no significant differences between sites within DHS or within GEI suggesting that spatial variation in species, if it occurs, does so on a scale greater than 100-500 m, the distances between traps. Another possible mechanism is variation in species between different breeding resources. The operation of this mechanism is suggested by the significant differences in the faunas reported from banana and pineapple in both DHS and GEI. Collections of *Drosophila* species from a wider range of fruits is needed to substantiate this mechanism. However, the differences in faunas between banana and pineapple are not very great. A further mechanism is variation in species over time as a result of different species phenologies. There are, indeed, considerable differences in phenology among the species occurring in DHS (Peng *et al.*, 1990a). This mechanism thus probably contributes greatly to the high total diversity of *Drosophila* species in semitropical east Asia and is a prime target for future research. The local diversity, however, is limited to a few species by phenology as our study occurred within 2 weeks and resources as we used only 2 bait types.

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Instigating an "identity crisis" to investigate how a *Hox* gene acts in fly legs.

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The three pairs of legs in *Drosophila melanogaster* have starkly different bristle patterns (Hannah-Alava, 1958). The first leg in particular is easily recognizable by the presence of a unique row of bristles on its basitarsus. This "sex comb" gets its name from (1) its presence only in males, and (2) its resemblance to a hair comb: its bristles are thick, dark, blunt, curved, and aligned in a single file. Hence, the bristles of the sex comb are commonly called "teeth."

The foreleg (in both sexes) also bears a series of evenly-spaced rows of bristles on the anterior face of the tibia and basitarsus (Shroff *et al.*, 2007). These transverse rows ("t-rows") are aligned perpendicular to the proximal-distal axis of the leg, whereas the sex comb runs parallel to it. The t-rows are used for grooming during resting (Szebenyi, 1969; Vandervorst and Ghysen, 1980), while the sex comb is used for grasping during mating (Hurtado-Gonzales *et al.*, 2015). Curiously, the comb arises as an ordinary t-row, but then rotates ~90˚ during the pupal period to assume its final position on the adult leg (Held *et al.*, 2004; Atallah *et al.*, 2009).

How do cells on the foreleg choose fates that differ from those of the simpler midleg, which constitutes an evolutionary ground state (Casares and Mann, 2001)? The foreleg veers away from a midleg fate by expressing the *Hox* "selector" gene *Sex combs reduced* (*Scr*) in precisely those parts of the leg that form the sex combs and t-rows (Barmina and Kopp, 2007; Devi *et al.*, 2012). If *Scr* is disabled, then foreleg cells fail to make either structure and instead act as if they belong to a midleg (Held, 2010; Shroff *et al.*, 2007; Struhl, 1982).

Historically, there have been two schools of thought about how such selector genes function (Castelli-Gair and Akam, 1995; Foronda *et al.*, 2009). The "hierarchical" school postulates that they trigger downstream (effector) genes indirectly via a chain of command, like a cascade of falling dominoes (Doe, 2017; Tsubota *et al.*, 2008). The "micromanager" school asserts that they assign cell states directly in combination with other regulatory factors, like the digits of an area code (Akam, 1998; García-Bellido, 1975).

Operationally, it should be possible to test these models by silencing *Scr* at a late stage. The key question is: do sex comb cells need their *Scr* gene during the final stages of bristle differentiation in order to form a proper row of teeth? The hierarchical model implies that *Scr* should be dispensable since it would have triggered the next gene in the cascade long ago, whereas the micromanager model implies that *Scr* should be needed throughout differentiation.

An experiment of this kind was performed by Tanaka *et al.* (2011). They used the TARGET system (see below) to disable *Scr* in nascent bristle cells and found ~50% fewer teeth but no change in comb rotation or tooth morphology. Their results imply that *Scr* is dispensable for bristle differentiation, though it evidently does play a role in setting tooth number. However, similar experiments by Atallah *et al.* (2014) argue that two other regulatory genes—*Distal-less* (*Dll*) and *dachshund* (*dac*)—are needed for comb rotation and tooth morphology, as well as for tooth number (Randsholt and Santamaria, 2008).

The discrepancy between the role of *Scr* on the one hand and *Dll* and *dac* on the other could be due to a difference in their respective modes of action, but it could be partly illusory, given how the experiments were done. Atallah *et al.* used a different *Gal4* driver from Tanaka *et al.* (see below). In order to rule out artifacts that might be due to this uncontrolled variable, we repeated the Tanaka *et al.* analysis of *Scr* by using the *Gal4* driver employed by Atallah *et al.*

Materials and Methods

Like both Tanaka *et al.* and Atallah *et al.*, we used the "TARGET" (temporal and regional gene expression targeting) procedure (McGuire *et al.*, 2004), which relies on the yeast transgenes *Gal4*, *UAS*, and

Gal80^{ts}. *Gal4* encodes a transcription factor that binds the upstream activating sequence *UAS* (Leung and Waddell, 2004). When *Gal4* is inserted in the *cis*-regulatory region of a given gene—call it *gene A*—then *Gal4* will be expressed at the same time and place as *gene A*, and a desired gene—call it *gene B*—can be turned ON congruently if artificially linked to *UAS*. Tanaka *et al.* used *neuralized*(*neur*)-*Gal4*, but Atallah *et al.* used *scabrous*(*sca*)-*Gal4*.

The distinction between these *Gal4* drivers may seem trivial, but it might explain the difference in outcomes. Both *sca* and *neur* are expressed in bristle precursor cells (Yeh *et al.*, 2000), but *sca* is also expressed in the preceding pool of epidermal cells—the "proneural cluster"—whence precursors are chosen (Renaud and Simpson, 2001; Troost *et al.*, 2015). Thus, we used the same *UAS* as Tanaka *et al.* but the same driver as Atallah *et al.—i.e.*, *sca-Gal4:UAS-dsScrRNAi*. Double-stranded (*ds*) *Scr* RNAi (interfering RNA) stifles transcription of native *Scr*, thus allowing us to assess the effect of disabling *Scr* at a slightly earlier stage than Tanaka *et al.*

The timing of *Gal4* expression is manipulable by the *Gal80^{ts}* component of the system. *Gal80^{ts}* is a temperature-sensitive (*ts*) allele of the yeast's *Gal80* gene, and its transgene is expressed constitutively due to its *tubulin* (*tub*) promoter. At permissive temperature (18°C), Gal80^{ts} protein blocks *Gal4* from activating the gene linked to *UAS*, but at restrictive temperature (30°C) the Gal80^{ts} protein itself becomes nonfunctional. Hence, whatever gene is linked to *UAS* can be artificially turned ON at any desired stage of development by merely shifting developing individuals of the appropriate genotype from an 18°C incubator to a 30°C incubator.

Herein lies another potential problem with how to interpret the data of Tanaka *et al.* They shifted their *tub-Gal80^{ts}*:*neur-Gal4:UAS-ScrRNAi* individuals to 30°C as wandering larvae just before puparium formation (PF), but the ~16 h that appears to be required for full *Gal80^{ts}* inactivation (Pavlopoulos and Akam, 2011) might delay *Scr* knockdown when *neur* turns ON in bristle precursors. Indeed, Atallah *et al.* found that *tub-Gal80^{ts}*:*rotund-Gal4:UAS-[dac or Dll]* larvae had to be shifted ~24 h before PF to attain a maximal effect on sex comb development, though *rotund* (unlike *sca*) is ON throughout the larval period, so this comparison may be moot. Detectable expression of *sca* in the sex comb area begins ~15 h after PF (Atallah *et al.*, 2009).

Given the confounding uncertainties concerning the kinetics of the interacting processes, we decided to document sex-comb effects over a wide span of shift times before (BPF) and after (APF) PF. One obvious virtue of using these post-embryonic shifts (*vs*. *Gal4:UAS* sans *Gal80^{ts}*) is that they bypass any possible complications from *sca* expression during the embryonic period (Mlodzik *et al.*, 1990). Another benefit is that they provide a time line for future investigations.

We crossed *sca-Gal4/CyO; tub-Gal80^{ts}* females with *UAS-ScrRNAi* (on chromosome 3) males and raised the offspring on Ward's *Drosophila* Instant Medium plus live yeast at 18°C.

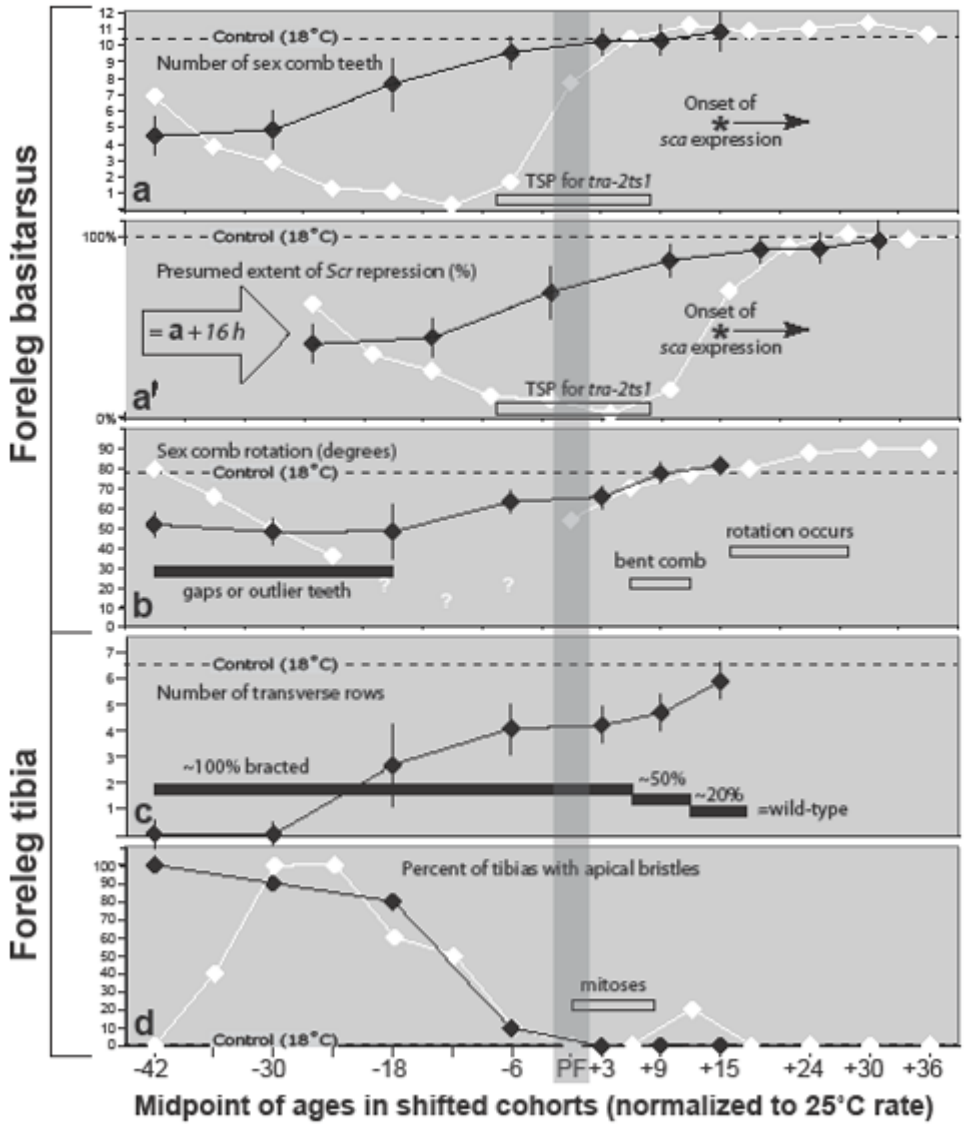
As a control, we let F_1 offspring complete development at 18°C. For pre-PF shifts we transferred bottles of 3rd-instar F_1 larvae from 18°C to 30°C and collected F_1 pupae every 12 h. For post-PF shifts we maintained the bottles at 18°C, harvested F_1 pupae at 12 h intervals, placed them in humidified petri dishes, and kept them at 18°C until shifting to 30°C. All ages were normalized to 25°C time (Ashburner, 1989). Thus, 12-h (real time) cohorts at 18°C are reported here as 6-h (adjusted) cohorts @25°C due to development being two times slower at 18°C (Held, 1990), while 12-h (real time) cohorts at 30°C are reported as such (development rate≈ rate at 25°C).

In addition to this loss-of-function (LOF) strategy we also conducted a gain-of-function (GOF) experiment where we turned *Scr* ON in *sca*-expressing proneural cells. To that end we crossed *sca-Gal4/CyO; tub-Gal80^{ts}* females with *UAS-ScrWT* (on chromosome 2) males (*WT* denotes the wild-type allele) and raised offspring as before. The F_1 offspring we examined were those with non-Curly wings—namely, *sca-Gal4/+; tub-Gal80^{ts}/UAS-ScrRNAi* males for the LOF analysis (abbreviated *sca>ScrRNAi*) and *sca-Gal4/UAS-ScrWT; tub-Gal80^{ts}/+* males for the GOF analysis (abbreviated *sca>ScrWT*). Curly-winged siblings served as internal controls.

Finally, we crossed *neur-Gal4*, *Kg/TM3*, *Sb* (#6393) males with *UAS-ScrRNAi* (#50662) females to construct *neur>ScrRNAi* F_1 flies (like the ones studied by Tanaka *et al.* but without any *Gal80^{ts}*) so that we could compare the effects of knocking out *Scr* in proneural clusters (*sca-Gal4*) *vs*. disabling it in bristle

precursors alone (*neur-Gal4*). We tried a similar strategy to construct *neur>ScrWT* individuals but were unsuccessful due to poor infertility of the parents.

Nutrition was monitored to avoid overcrowding. Adults were preserved in 70% ethanol. Legs were mounted in Faure's medium (Lee and Gerhart, 1973) between cover glasses and photographed at 200× or 400× magnification with a Nikon compound microscope. Legs were also photographed under a dissecting microscope before mounting, so that the extent of comb rotation could be measured precisely at a consistent viewing angle before any twisting of the legs that often occurs as a result of being sandwiched between cover slips.



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LOF Analysis: Results and Discussion

Figure 1 summarizes the phenotypes of *Scr*-LOF males. All of these phenotypes constitute partial homeoses from T1 to T2 segmental identity. Like Tanaka *et al.* (2011) we saw ~50% fewer sex comb teeth (Figure 1a) in cohorts shifted before PF, but, unlike them, we observed effects on sex comb rotation (Figures 1b and 2a). This difference is not surprising because the motive force for rotation is generated by nearby cells rather than by the bristle cells themselves (Atallah *et al.*, 2009; Atallah *et al.*, 2014; Malagon and Larsen,

2015). An effect on rotation makes sense for our *sca-Gal4* driver, which is expressed not only in bristle cells (like Tanaka *et al.*'s *neur-Gal4* driver), but also in surrounding epidermal cells within the proneural cluster.

Figure 1. *Scr*-LOF phenotypes caused by transferring cohorts of *sca>ScrRNAi* larvae or pupae from 18°C to 30°C in order to repress *Scr* in proneural clusters. In each panel, data are plotted (y axis) against developmental age at the time of the shift (x axis; normalized to 25°C) relative to puparium formation (PF). Horizontal dashed lines indicate means for control flies raised only at 18°C. Black diamonds denote midpoints of ages when shifted, with vertical lines marking standard deviations. Each point is the mean of 20 legs (= left and right legs of 10 males), unless stated otherwise. Solid bars designate temperature-sensitive periods (TSPs). For comparison, data (white diamonds) are included from a previous *Scr*-LOF experiment (Held, 2010) where pulses—instead of shifts—were used to repress *Scr* throughout the tarsus and distal tibia (via *Dll-Gal4*), and a few other relevant TSPs (open rectangles) are also plotted. **a**. Number of teeth (stout bristles) in the comb. *Asterisk:* earliest time when *sca* expression is detected in the comb area (Atallah *et al.*, 2009). *Open rectangle:* TSP when bristle number is finalized in *transformer2*^{ts1} (*tra2*^{ts1}) sex combs (Belote and Baker, 1982). For *neur>ScrRNAi* flies sans *Gal80*^{ts}, (data not plotted) we saw an average of 4.7 sex comb teeth (s.d. = 0.8, N = 10). **a'**. Data from **a** are re-plotted here, assuming a ~16 h lag until *Gal80*^{ts} is totally disabled after being shifted to 30°C (Atallah *et al.*, 2014; Pavlopoulos and Akam, 2011), though McGuire *et al.* (2004) report a shorter (~ 6 h) lag. Note that the "valley" of *Dll>ScrRNAi* pulses (white diamonds) now coincides with the *tra2*^{ts1} TSP, and the upper "tail" of *sca>ScrRNAi* shifts (black diamonds) now extends beyond the *sca* onset. **b**. Angle of the sex comb relative to transverse axis (perpendicular to proximal-distal leg axis), such that 0° indicates no rotation. N ≈ 20 except for the last three time points, where N = 7, 3 and 5, respectively. Combs of *neur>ScrRNAi* males (data not plotted) rotated normally (~80°; N = 10), as reported previously by Tanaka *et al.* (2011), and 3/10 had gaps or outliers. *Black bar:* TSP for misaligned combs that have a gap or ectopic teeth outside the comb. *Open rectangles:* TSP for bent-comb anomaly (Held, 2010), and period during which the comb rotates in wild-type pupae (Held *et al.*, 2004; Atallah *et al.*, 2009). *N.B.:* Bent-comb TSP was plotted incorrectly in Figure 2b of Held (2010); it is correct here. **c**. Number of t-rows (defined as ≥2 bristle sockets touching transversely) along the proximal-distal axis. Basitarsal t-rows reappeared with a similar time course (data not shown). For *neur>ScrRNAi* males (data not plotted) there was an average of 4.3 t-rows on the tibia (s.d. = 0.9; N = 10) and 4.7 t-rows on the basitarsus (s.d. = 1.8; N = 6), and all tibial t-row bristles were uniformly brown and bracted (as is true for *sca>ScrRNAi*). *Black bars:* Gradual loss of bracts from t-row bristles with later shifts, ultimately reaching the wild-type level where all t-row bristles, except those at the lateral edges, lack bracts. **d**. Extent to which foreleg tibias resemble midleg tibias by the presence of a thick, blunt apical bristle at the distal end of the segment (ventral face). Apical bristles were scored as present even though most were less than half the normal length. Similar effects were observed for *neur>ScrRNAi* males (data not plotted): 70% of male legs (N = 10) had a short apical bristle, while the remainder had none. *Open rectangle:* Time when the precursor of the apical bristle undergoes its two differentiative mitoses (Nottebohm *et al.*, 1994).

In our previous *Dll>ScrRNAi* analysis (Held, 2010) we charted the temperature-sensitive period (TSP) for *Scr*'s role in assigning the number of teeth. That TSP lasts ~2 days @25°C. We used shifts here instead of pulses, so the number would be expected to gradually approach the wild-type level as shifts overlap less and less with this TSP at later times. If we take into account the ~16 h lag of *Gal80*^{ts} inactivation following shifts to 30°C (Pavlopoulos and Akam, 2011), then the "true" TSP would span ~26 h BPF to ~22 h APF (Figure 1a'), instead of ~42 h BPF to ~6 h APF (Figure 1a). With this adjustment, the midpoint of the *Scr*-LOF TSP coincides with a comparable TSP (8 h BPF to 8 h APF) for the sex-determining gene *transformer2* (Belote and Baker, 1982). Given that *sca* turns ON at ~15 h APF (Atallah *et al.*, 2009), enough time should be left in the *Scr*-LOF TSP (~7 h) to permit a partial (~50%) blockage of tooth initiation or maintenance. Based on direct observations of pupal legs (A. Kopp, personal communication), the number of teeth is set by ~15-16 h APF, just before the comb starts to rotate.

This same logic applies to comb rotation, which begins around the time of *sca* activation (Atallah *et al.*, 2009). If *sca>ScrRNAi* is blocking *Scr* at the tail end of its tooth-number TSP, then we might expect disruptions in bristle alignment, since bristle cells merge into a single file around this time (Atallah *et al.*, 2009). Indeed, 8/20, 8/20, and 15/20 legs showed a gap or outlier teeth (Figure 2a) for shifts at 36-48 h, 24-36 h, and 12-24 h BPF, respectively (Figures 1b and 2a). Presumably, the misaligned cells possessed enough Scr to become bristle cells within the proneural cluster, but then suffered a loss of Scr function before they could join together in a chain via homophilic adhesion. Cell adhesion is apparently also impaired on the foreleg tibia because *Scr*-LOF bristle cells fail to form t-rows during this same period (Figures 1c and 2c).

One oddity of the wild-type foreleg is its bracts. Bracts are tiny triangular structures that are induced by bristle cells via the EGFR pathway (del Álamo *et al.*, 2002; Held, 2002b). Most tibial t-rows lack bracts, whereas all basitarsal t-rows possess them (Schubiger *et al.*, 2012). Suppression of *Scr* by *Dll>ScrRNAi* (Held, 2010) or *sca>ScrRNAi* causes virtually all bristles in the t-row area of the foreleg tibia to acquire bracts (Figure 1c), so *Scr* is clearly required for the bractless state of tibial t-rows, and it turns out to be sufficient as well, because overexpressing *Scr* via *Dll>ScrWT* (Held, 2010) or *sca>ScrWT* (see GOF Analysis) deletes bracts from all six legs. Another oddity of tibial *vs*. basitarsal t-rows in wild-type flies is that tibial bristles are yellow, while basitarsal ones are brownish like most other leg bristles (except sex comb teeth, which are black). Among the *Scr*-LOF flies, all t-row bristles were brown, regardless of shift time—indicating the necessity of *Scr* for yellow coloration and suggesting that the TSP for color assignment lies outside the range of ages examined (*i.e*., beyond 15 h APF).

The contemporaneous need for *Scr* gene function when tooth identity, comb rotation, and cell alignment occur is consistent with analogous findings of a need for *Ubx* when midleg-specific traits—sternopleural bristles and the tibial apical bristle—are suppressed during the final stages of bristle differentiation on the hindleg (Rozowski and Akam, 2002). Both of these *Hox* genes appear to be behaving more like intrusive micromanagers than as aloof executives.

Our suppression of *Scr* activity in foreleg proneural cells is evidently causing them to adopt a midleg identity at the very time that they are executing their foreleg-specific instructions. In order to see whether midleg-specific traits can be evoked at such a late stage, we examined foreleg tibias for the presence of the apical macrochaete that is normally only found on midlegs (Hanna-Alava, 1958). Indeed, 20/20, 18/20, and 16/20 forelegs showed an enlarged, thick, blunt bristle at the apical site for shifts at 36-48 h, 24-36 h, and 12-24 h BPF (Figure 1d). However, these bristles never attained more than half the length of a midleg apical bristle (Figure 3), and side-by-side duplicate bristles were common (6/20, 3/20, and 4/20, respectively)—implying that several cells in the proneural cluster start to become apical bristles but then fail to compete by lateral inhibition (Castro *et al.*, 2005) so as to yield a single "victor" whose rival cell regresses back to an epidermal state.

Mechanistically, our conclusion about *Hox* genes acting as micromanagers implies that Hox proteins bind the *cis*-enhancers of effector genes when bristle identity is being implemented (Pavlopoulos and Akam, 2011). If so, then the targeted *cis*-regulatory regions would resemble a rugby scrum where transcription factors from all levels of the control hierarchy area converge simultaneously. Other suspected regulators inside this "huddle", aside from Scr, include Dsx-M, Dac, and Dll (Atallah *et al.*, 2014; Kopp, 2011; Tanaka *et al.*, 2011).

The main exception to this rule of combinatorial action, as Tanaka *et al.* have noted, is bristle shape. Once *Scr* licenses the decision of a bristle precursor to be (or not to be) a sex comb tooth, it evidently delegates the implementation of the tooth's unique shape to subsidiary genes, since no intermediate morphologies were found in the present *Scr*-LOF/GOF study nor in our previous one (Held, 2010), regardless of the time of the shift or pulse. Indeed, the TSP for altering bristle shape—as ascertained using *transformer2*—appears to occur just after the TSP for tooth number (Belote and Baker, 1982). Although sex-determining genes like *tra2* and *dsx* may act directly in assigning bristle shapes (Tanaka *et al.*, 2011; *cf*., Atallah *et al.*, 2014), *Scr* apparently does not.

GOF Analysis: Results and Discussion

We employed the same *UAS-ScrWT* construct (#7302) from the Bloomington Stock Center (*WT* = wild-type allele) that we had previously used with a *Dll-Gal4* (*Distal-less-Gal4*) driver (Held, 2010). In that case, *Dll-Gal4* drove *ScrWT* expression throughout the epidermis of the distal tibia and tarsus, starting at either 20 h or 12 h BPF (when we shifted flies to 30°C). Under those conditions *Scr*-GOF caused ectopic sex combs and t-rows on all six legs, plus yellow bristle pigmentation and pervasive loss of bracts. Curiously, the extra t-rows occurred not only on the anterior surface of midlegs and hindlegs—as would be expected from *Scr*'s role on forelegs—but also on the posterior surface of midlegs (Figure 4d). Some extra t-rows were also found on the posterior side of the foreleg, but to a much more limited extent, as discussed below.

Milder versions of those same *Dll>ScrWT* effects were observed here with *sca>ScrWT*, except for yellowish pigmentation, which might require earlier or longer expression of *ScrWT* than available with *sca-Gal4*. Because our *Scr*-GOF effects were so subtle, we focused on flies that had maximal phenotypes—namely, those from the earliest age we shifted (36-48 h BPF). No experimental (non-Curly) flies survived to eclosion, so we had to dissect pharate adults out of their pupal cases to study them. The anatomical features of those flies will now be described.

Ectopic teeth were only found on forelegs (not on midlegs or hindlegs as in *Dll>ScrWT*): either one (17/20 legs) or two (3/20 legs) isolated teeth were present on the 2nd tarsal segment, and 15/20 forelegs also had a tooth on the 3rd (3/20 cases) or 4th (9/20 cases) tarsal segment or on both of them (3/20 cases) at locations homologous to the sex comb site on the basitarsus. This tendency for extra teeth to arise at these tarsal locations is attributable to regulatory genes other than *Scr* (Barmina and Kopp, 2007; Randsholt and Santamaria, 2008; Tanaka *et al.*, 2011).

The foreleg tibias of control flies (raised at 18°C) contained an average of 6.6 t-rows on their anterior face (s.d. = 0.5; N = 20) like wild-type flies. In contrast, the t-row area on foreleg tibias of shifted (*sca>ScrWT*) flies expanded proximally to reach twice that number in some cases (max. = 13; mean = 9.8; s.d. = 1.7; N = 20; Figure 5a). Strangely, we found a similar, albeit weaker, effect on the posterior side of the hindleg tibia (Figure 5c), which canonically has a single row (Figure 5d), but in *sca>ScrWT* flies had an average of 3.0 rows (s.d. = 1.1; N = 20). The extra rows had as few as two bristles each but arose at intervals typical of foreleg t-rows.

The hindleg is governed by *Ubx*, so *Scr* and *Ubx* could be cooperating synergistically there. This notion finds support in the dramatic effects of *Dll>ScrWT* on hindleg tibias (Figure 8a), where the number of posterior t-row bristles skyrockets four-fold or six-fold from a mean of 8 (wild-type) to a mean of 34 (shift = 20 h BPF) or 47 (shift = 12 h BPF). For some reason the excess t-rows are more irregular on hindlegs than on forelegs (Figure 5e).

Ectopic t-rows that are caused by *sca>ScrWT* (on all six legs) usually consist of only a few adjacent bristles each, instead of the much broader t-rows seen in *Dll>ScrWT* flies (Figure 4). On *sca>ScrWT* forelegs, such minimal—or "incipient"—t-rows were detectable on the posterior face of the tibia and basitarsus, while on hindlegs, they were visible on the anterior face (Figure 6). Comparable ectopic t-rows have been reported for midleg and hindleg basitarsi when *ScrWT* is expressed via a *rotund(rn)-Gal4* driver (Shroff *et al.*, 2007), but it is unclear whether the t-rows in that case arose on both faces of *rn>ScrWT* midlegs or only on the anterior side (T. Orenic, personal communication).

Midlegs offer an ideal opportunity to test the idea that *Scr* fosters t-row formation both anteriorly and posteriorly, because they manifest no t-rows whatsoever in wild-type flies (Figure 7) and hence provide a "blank slate" baseline. Hence, we tallied extra bristles between longitudinal rows ("l-rows") 7 and 8 (anterior face) and between l-rows 1 and 2 (posterior face)—recording only those bristles whose sockets were (1) transversely aligned with a socket of an l-row bristle and (2) physically touching that socket. In a sample of 20 midleg basitarsi, there was a mean of 4.25 thusly defined t-row bristles on the anterior side (s.d. = 2.07) and a mean of 4.60 such bristles on the posterior side (s.d. = 1.43). These averages do not differ significantly ($p > 0.05$; t-test), and thus are consistent with the symmetry hypothesis. Interestingly, *Ubx*-GOF can also induce t-rows on midlegs like *Scr*-GOF (Shroff *et al.*, 2007), but we do not yet know whether it induces them symmetrically on both sides of the leg (T. Orenic, personal communication).

This ability of *sca>ScrWT* to evoke partial t-rows equally on both faces of the midleg suggests that *Scr* function is not being inhibited by *engrailed* (*en*)—a selector gene for the posterior compartment in all legs (*cf*., Held, 2002a). On the other hand, the relative inability of *Dll>ScrWT* to elicit more than a few t-rows on the posterior face of the foreleg (Figure 4b) implies that *en* is preventing *Scr* from inducing t-rows there. This paradox prompted us to go back and comprehensively measure the extent to which extra t-rows are induced on the anterior (A) *vs*. posterior (P) faces of forelegs, midlegs, and hindlegs by *Dll>ScrWT* (vis-à-vis *sca>ScrWT*).

Figure 2. Effect of *sca>ScrRNAi* (*Scr*-LOF) on the foreleg basitarsus (**a**, **b**) and tibia (**c**, **d**). All images are from right legs, oriented with proximal-distal from top to bottom (transverse axis runs perpendicularly) and ventral to the right, at the same scale (bar = 100 microns). **a**. Basitarsus (anterior view) from a *sca>ScrRNAi* male shifted to 30°C at ~42 h BPF (cohort: 36-48 h BPF). There are six teeth, five of which occupy a single file (lower arrowhead) oriented at 52° to the transverse axis. The sixth tooth (upper arrowhead) is an outlier that might have migrated on its own, but it could instead have arisen *in situ* since it belongs to the most distal t-row—a common anomaly in artificially selected strains (N. Malagon, personal communication). Anteroventral bristles (right half of this image), which would normally form t-rows, are fewer and less aligned, with no bristle sockets in contact. The reduction in tooth number and absence of t-rows reflect homeosis. **b**. Basitarsus (anteroventral view) from a control *sca>ScrRNAi* male raised entirely at 18°C (wild-type phenotype). The sex comb has 12 teeth, all of which occupy a single file, and the comb has rotated to ~80°. Seven t-rows are visible in the upper part of the segment, where the sockets of adjacent bristles are nearly all in contact. The dark triangular structures above the bristle sockets are bracts. **c**. Tibia (anterior view) from a *sca>ScrRNAi* male shifted to 30°C at ~42 h BPF (cohort: 36-48 h BPF). The macrochaete (lower left) is the pre-apical bristle (pAB), whose thickness is typical of a midleg. The apical bristle is out of focus on the opposite side of the segment. Note the absence of t-rows, the ubiquity of bracts (except on pAB), and the sparseness of bristles compared to t-row area in **d**. **d**. Tibia (anterior view) from a control *sca>ScrRNAi* male raised entirely at 18°C (wild-type phenotype). T-rows of lighter colored bristles decorate the anteroventral face. The central bristles within each t-row lack bracts; the same is true for the solitary t-row at the distal tip of the hindleg tibia (Figure 5d). The pAB (out of focus) is thinner than the pAB in **b**—another indication, albeit subtler than the apical bristle (Figure 3b), that the *Scr*-LOF leg in **b** has undergone a partial T1-to-T2 homeosis.

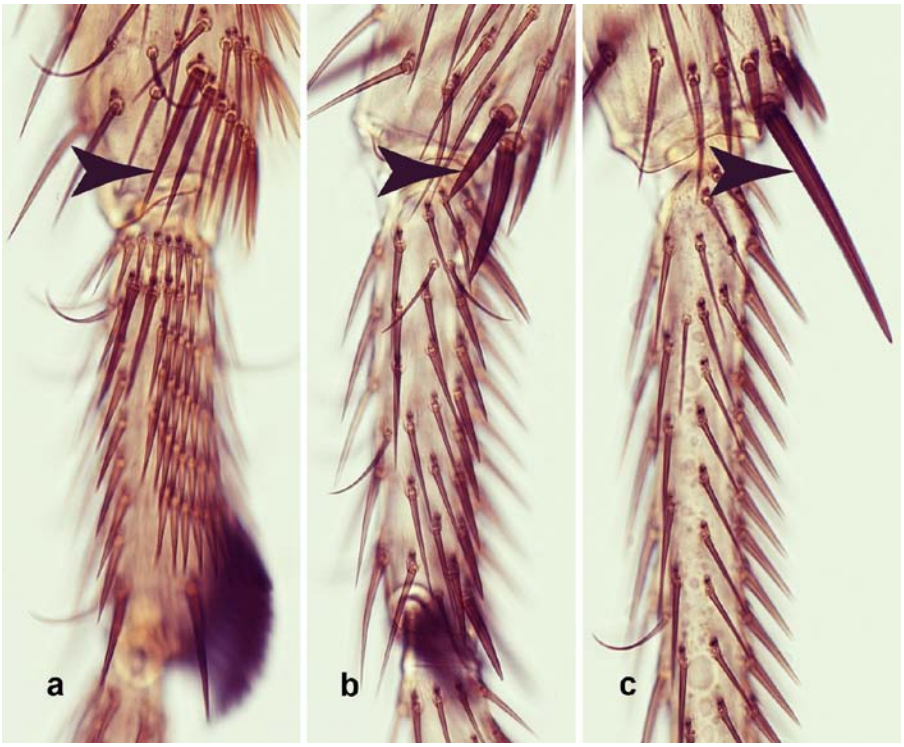

Figure 3. Homeotic production of one or more midleg-like apical bristles on the *sca>ScrRNAi* (*Scr*-LOF) foreleg tibia. All images are at the same scale. **a**. Distal tip of the left foreleg tibia and basitarsus (ventral view) from a control *sca>ScrRNAi* male raised at 18°C (wild-type phenotype). Arrowhead indicates the distalmost t-row, whose two most lateral bristles are characteristically larger and darker than other t-row bristles. **b**. Distal tip of the left foreleg tibia and basitarsus (posterior view) from a *sca>ScrRNAi* male shifted to 30°C at ~42 h BPF (cohort: 36-48 h BPF). T-rows are absent. In place of the last t-row are two bristles (arrowhead), both of which resemble a midleg apical bristle in thickness and pigmentation, but neither attains full length. These bristles could be transformed versions of the dark bristles denoted in **a** (based on unpublished data supplied by M. Rozowski, personal communication), though they reside more distally (see text for an alternative idea). **c**. Distal tip of the right midleg tibia and proximal three-fourths of the basitarsus (posterior view) from a control *sca>ScrRNAi* male raised entirely at 18°C. (The original image was flipped left-right here for ease of comparison.) Arrowhead points to the apical macrochaete. It is crowned by an arc of stout, dark spur bristles (Hannah-Alava, 1958) that are aligned like a foreleg t-row but lacking socket-to-socket contact.

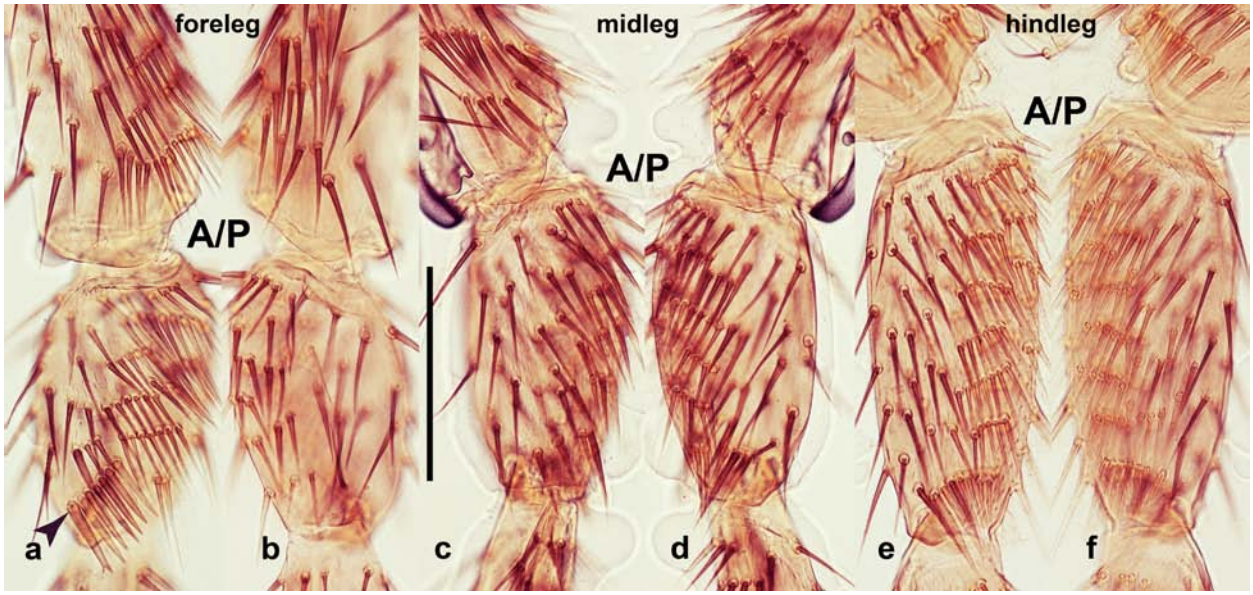

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Figure 4 (previous page). Extra t-rows induced by *Dll>ScrWT* (*Scr*-GOF) on a foreleg (**a**, **b**), a midleg (**c**, **d**), and a hindleg (**e**, **f**). These specimens are from a previous study (Held, 2010), and they are depicted here to facilitate comparison with the similar—but milder—effects of *sca>ScrWT*. They are representative examples of phenotypes seen when *Dll-Gal4/UAS-ScrWT; tub-Gal80ts/+* larvae are shifted to 30°C at 12 h BPF. All photos are at the same magnification, but images for the (left) midleg and hindleg were flipped left-right so as to match the (right) foreleg. Bar length = 100 microns. Leg segments are shorter and wider than the wild type (*cf*., Figure 2b), and bristles are yellower and bractless. Segments are viewed from anterior (A) or posterior (P). **a**, **b**. "Mug shots" of A (**a**) and P (**b**) surfaces of the distal tibia and basitarsus from a foreleg. Note the partly rotated sex comb (arrowhead) and the single outlier tooth on the A side, and the sparsity of ectopic t-rows on the P side of the tibia and basitarsus. **c**, **d**. A (**c**) and P (**d**) surfaces of the distal tibia and basitarsus from a midleg. Note the roughly equal numbers of ectopic t-rows on both faces. (Brown arc at edge is a bubble.) **e**, **f**. A (**e**) and P (**f**) surfaces of the distal tibia and basitarsus from a hindleg. The number of t-rows is roughly symmetric. The t-rows are wide, but they fail to encircle the circumference. Why? Probably because *Scr* is repressed by Dpp (T. Orenic, personal communication). Dpp diffuses from the dorsal midline (Held, 2002a), and if the Dpp gradient has a fixed slope, then its inhibition of *Scr* might explain why the wild-type t-row area is triangular on the (conical) tibia, but rectangular on the (cylindrical) basitarsus.

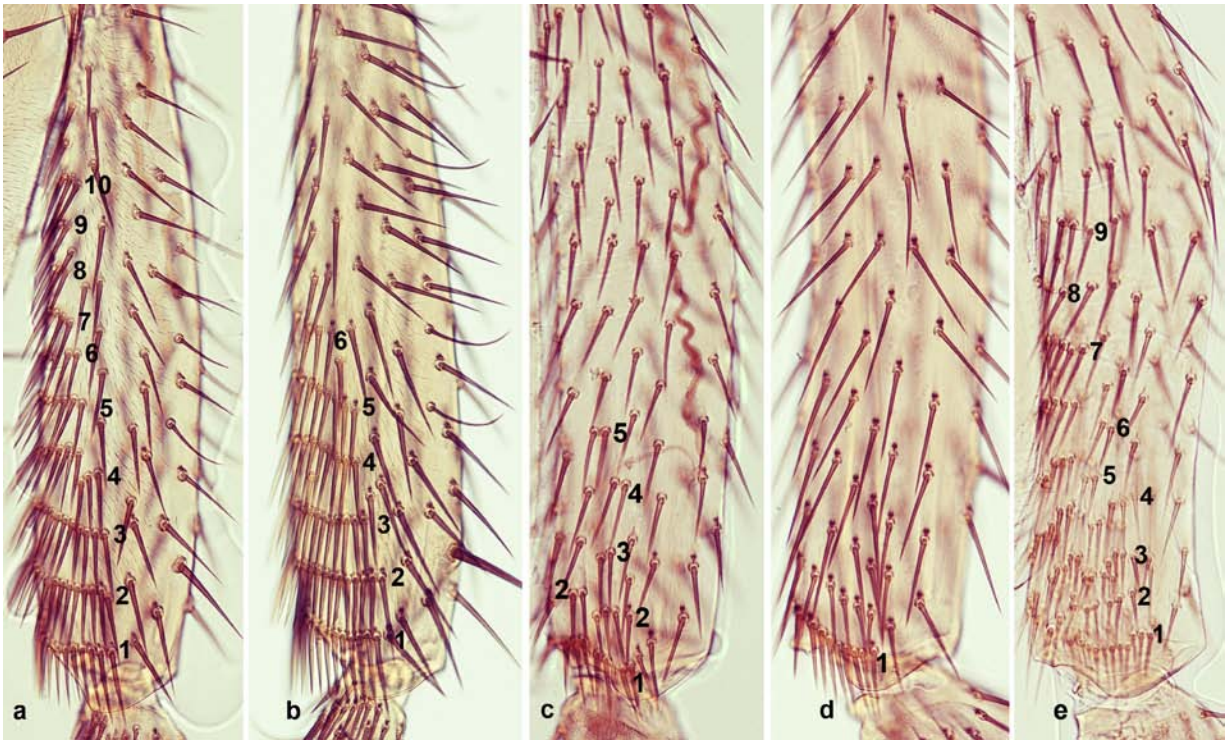

Figure 5. Effects of *Scr*-GOF on t-rows of the foreleg and hindleg tibias. All images are at the same magnification. **a**. Left foreleg tibia (anterior view, ventral to the left) from a *sca>ScrWT* male shifted to 30°C at ~42 h BPF (cohort: 36-48 h BPF). T-rows are numbered from the distal end. Four extra t-rows (#7-10) are visible relative to the control tibia (**b**), presumably due to the extra dosage of Scr in the t-row region (*cf.*, Shroff *et al.*, 2007). The maximum we saw in any specimen was a total of 13 t-rows. Extra rows also develop on foreleg tibias of *Dll>ScrWT* flies (Held, 2010). **b**. Left foreleg tibia (anterior view) from a

Figure 5 (continued). control *sca>ScrWT* male raised entirely at 18°C (wild-type phenotype). Control tibias typically have 6 (as here) or 7 t-rows. Most tibial t-row bristles lack bracts (except at lateral termini)—unlike basitarsal t-row bristles (Figure 2b)—and they are yellower. In contrast, *Sca*-GOF tibial t-row bristles (**a**) are browner, like the surrounding non-t-row bristles. **c**. Right hindleg tibia (posterior view, ventral to the left) from a *sca>ScrWT* male shifted to 30°C at ~42 h BPF (cohort: 36-48 h BPF). T-rows are numbered from the distal end. Four extra, albeit tiny, t-rows (#2-5) are visible (an extreme case) relative to the control tibia (**d**), presumably due to the extra dosage of Scr in the t-row region (*cf*., Shroff *et al.*, 2007). The second t-row has a gap, and rows #3-5 contain only two bristles each. Most bristles lack bracts, unlike the control tibia (**d**). **d**. Right hindleg tibia (posterior view, ventral to the left) from a control *sca>ScrWT* male raised entirely at 18°C (wild-type phenotype). Control tibias have only one t-row (as shown here) containing an average of 8.1 bristles (N = 20). The central bristles of that row consistently lack bracts—like the t-rows on the foreleg tibia (**b**). **e**. Right hindleg tibia (posterior view, ventral to the left) from a *Dll>ScrWT* male shifted to 30°C at 12 h BPF. This profusion of t-rows may stem from a synergy between endogenous *Ubx* and ectopic *Scr* (see text). Numbering of t-rows is approximate because of their many irregularities. The yellow color of bristles in the distal half of the tibia (**e**) is attributable to a longer exposure to *Scr* in the *Dll* region than that afforded by *sca-Gal4* (**c**), but bracts are missing in both cases.

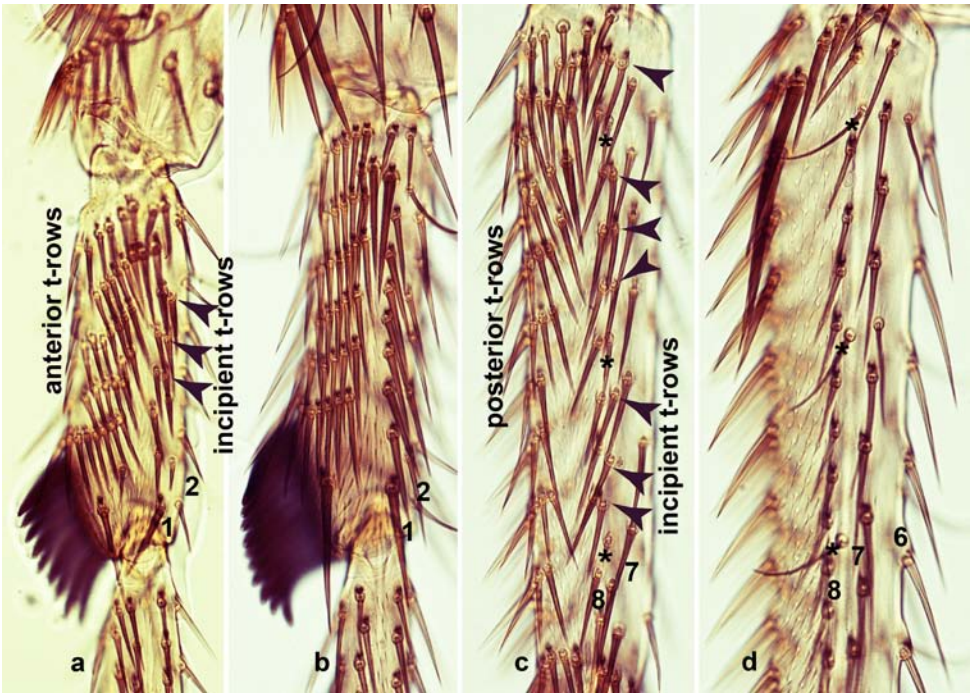

Figure 6. Induction of incipient ectopic t-rows on foreleg and hindleg basitarsi of *sca>ScrWT* (*Scr*-GOF) flies. All photos are at the same magnification. **a**. Right foreleg basitarsus (ventral view) from a *sca>ScrWT* male shifted to 30°C at ~42 h BPF (cohort: 36-48 h BPF). Arrowheads indicate 3 extra bristles between l-rows 1 and 2 (numbered below), which meet the criteria of t-rows (≥2 bristle sockets touching transversely). Evocation of t-rows by *Scr*-GOF on the posterior side challenges the traditional view that *Scr*'s presumed role is to serve as an anterior-specific t-row selector gene (Shroff *et al.*, 2007). **b**. Right foreleg basitarsus (ventral view) from a control *sca>ScrWT* male raised entirely at 18°C (wild-type phenotype). Note the lack of bristles in the narrow corridor between l-rows 1 and 2, except for one chemosensory (curved) bristle near the proximal end. **c**. Left hindleg basitarsus (ventral view) from a *sca>ScrWT* male shifted to 30°C at ~42 h BPF (cohort: 36-48 h BPF). Arrowheads indicate 7 extra bristles between l-rows 7 and 8 (numbered below), which meet the criteria of t-rows. Asterisks label the shafts of

Figure 6 (continued). chemosensory bristles that are recognizable by socket shape, shaft curvature, and thin shaft at base (Held, 2002a). In wild-type legs (**d**) such bristles lack a bract, but *Scr*-GOF deletes many bracts, thus precluding identification here based on that feature alone. Curiously, two of the asterisked bristles appear to be forming incipient t-rows, even though such bristles don't normally abut l-row bristles in wild-type flies (**d**). **d**. Left hindleg basitarsus (ventral view) from a control *sca>ScrWT* male raised at 18°C. Note the absence of bristles in the corridor between l-rows 7 and 8, except for three chemosensory bristles (asterisks) near row 8.

Figure 7. Induction of incipient ectopic t-rows on midleg basitarsi of *sca>ScrWT* (*Scr*-GOF) flies. This slight T2-to-T1 homeosis is trivial compared with the massive eruption of t-rows on *Dll>ScrWT* midlegs (Figure 4)—attributable to a shorter duration of *ScrWT* action from *sca-Gal4*. **a**, **b**. "Mug shots" of right foreleg basitarsus from a *sca>ScrWT* male shifted to 30°C at ~42 h BPF (cohort: 36-48 h BPF), as seen from the anterior (**a**) or posterior (**b**). **a**. Arrowheads indicate five extra bristles between l-rows 7 and 8 (numbered below), which meet the criteria of t-rows (≥2 bristle sockets touching transversely). Most of the extra bristles abut row-8 bristles, but rarely (*e.g.*, lowest arrowhead) they abut a bristle in row 7. **b**. Arrowheads denote four extra bristles between rows 1 and 2 (numbered below), which meet the criteria of t-rows. Three of the extra bristles (first, second, and fourth counting from proximal end) are also aligned with (though not touching) a bristle in row 2. Many basitarsal bristles are missing bracts—another effect of *Scr*-GOF (see text). **c**, **d**. Right foreleg basitarsus from a control *sca>ScrWT* male raised at 18°C (wild-type phenotype). There are no adventitious bristles between l-rows. Curved (bractless) bristles are chemosensory (Held, 2002a).

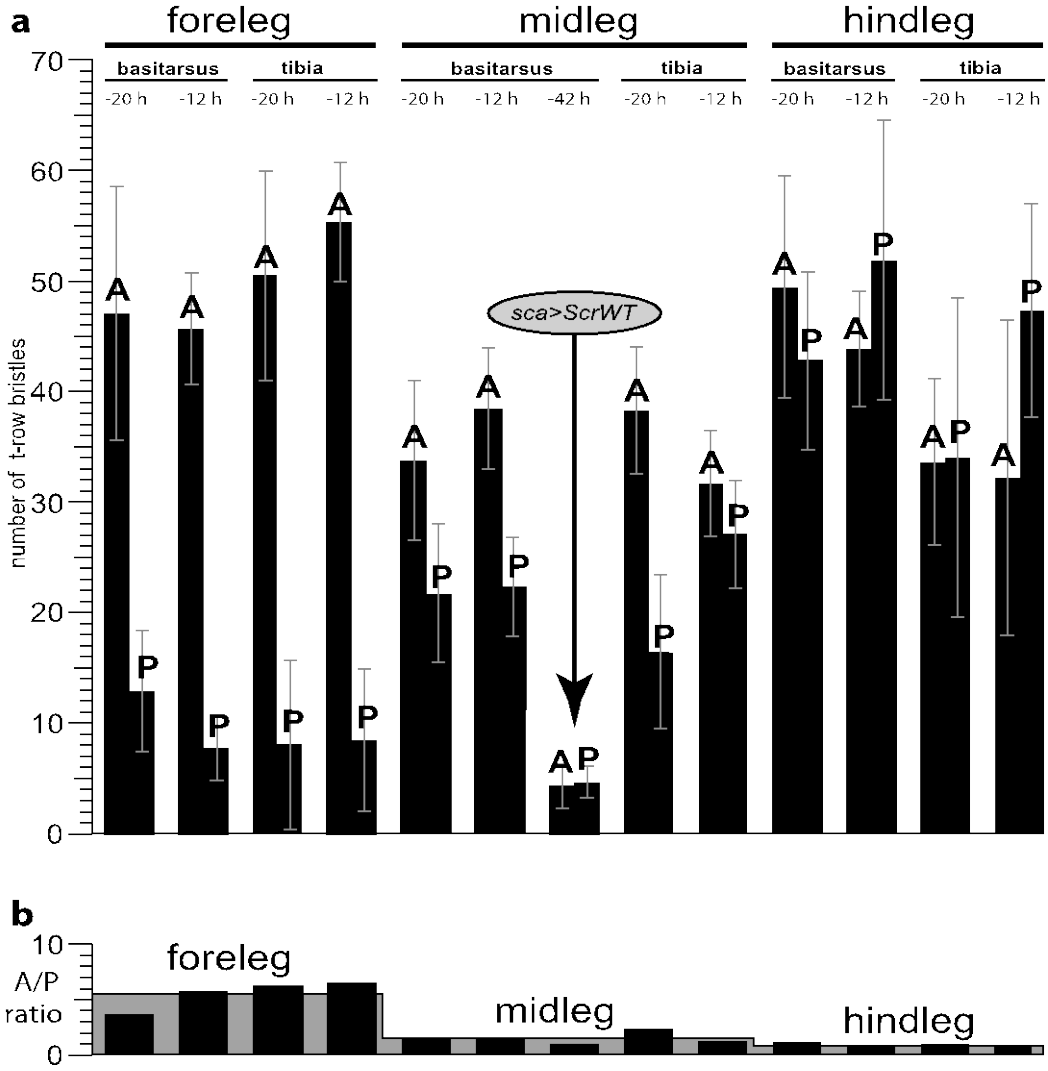

Figure 8. Quantitative analysis of *sca>ScrWT* (present study) *vs*. *Dll>ScrWT* (Held, 2010) with regard to the number of t-row bristles on anterior (A) and posterior (P) faces of forelegs, midlegs, and hindlegs. **a**. Average numbers of t-row bristles (error bars = standard deviations) plotted on the y axis for A and P faces of basitarsi and tibias from *Dll>ScrWT* flies (N = 8 legs/histogram bar) shifted to 30°C at 20 or 12 h BPF, plus a center pair of histograms for *sca>ScrWT* flies (N = 20 legs/histogram bar) shifted to 30°C at ~42 h BPF (cohort: 36-48 h BPF). Mean number of t-row bristles in controls (≅ wild -type) is 42.3 for foreleg basitarsus (N = 10), 64.3 for foreleg tibia (N = 10), and 8.6 for hindleg tibia (N = 10), which contains only a single row. Note the 4- or 6-fold increase in bristle number for the P side of hindleg tibia (20 or 12 h BPF, respectively) above the control level (8.6). **b**. A/P ratios (y axis) computed from the average numbers of t-row bristles on A *vs*. P faces of basitarsi and tibias plotted in histograms above (**a**). The averages of these ratios for forelegs, midlegs, and hindlegs are plotted as gray rectangles.

Figure 9. *Scr*-GOF (*sca>ScrWT*) pharate adult male (**a**) compared to a control (*Curly*-balancer) male sibling (**b**), both of which were shifted from 18°C to 30°C at ~42 h BPF (cohort: 36-48 h BPF). **a**. Most head and thoracic macrochaetes are missing, though tiny bristles do remain at a few sites. Thoracic microchaetes are relatively unaffected. Abdominal bristles are appreciably smaller. **b**. This control fly had partly eclosed from its pupal case when it died, so its body is longer than that of the *sca>ScrWT* fly, its forehead is bulging, and its wings are partly unfurled.

The results of that retrospective analysis are plotted as histograms in Figure 8. The data confirm our preliminary suspicion that forelegs are somehow able to suppress t-row formation (induced by *Dll>ScrWT*) on their P surface more strongly than are midlegs: an average of only ~10 t-row bristles were typically elicited on the P side of basitarsi or tibias of forelegs, whereas twice that number was induced on the P side of basitarsi or tibias of midlegs. Likewise, the A/P ratio of t-row bristles averaged 5.6 for foreleg (basitarsi and tibias), but only 1.7 for midlegs (*vs*. 1.0 for *sca>ScrWT* in the present study) and 0.92 for hindlegs, respectively.

To solve this mystery it might help to recall a peculiar fact about midlegs: they express *Ubx* in their P half (Brower, 1987; Stern, 1998)—like hindlegs but more weakly (Held, 2002a). If (1) *Scr* does interact with *Ubx* as argued above, and (2) *en* can inhibit *Scr* on the P side of forelegs and midlegs, then the quantitative trends for *Dll>ScrWT* (Figure 8) are explicable as follows: (1) the *Dll-Gal4* driver can't raise *Scr* levels enough in the foreleg to overcome *en* inhibition, but (2) it is able—in combination with *Ubx*'s low background level—to double the number of t-row bristles on the P side of the midleg relative to the foreleg. Even with that *Ubx* boost, however, *Dll>ScrWT* can't surmount *en* inhibition all the way to an A/P ratio of 1.0—earning an A/P ratio of only 1.7 instead. So how does *sca>ScrWT* manage to attain a 1.0 ratio on the midleg while *Dll>ScrWT* falls short? Perhaps it fosters a higher level of *Scr* expression at later stages than *Dll>ScrWT*. These conjectures should be testable by (1) expressing *ScrWT* and *UbxWT* coordinately or (2) inducing *Ubx*-LOF or *en*-LOF clones in an *Scr*-GOF background.

Most of the extra t-row bristles that we found on *sca>ScrWT* midleg basitarsi abutted bristles within l-row 8 or row 1, rather than rows 7 or 2 (Figure 7). This affiliation of incipient t-rows with the ventral-most l-rows (8 and 1) suggests that t-rows on the foreleg and hindleg might normally develop similarly by adding bristle cells starting ventrally and spreading dorsally until they span the whole t-row area, instead of the other way around (*i.e*., dorsal to ventral).

Our finding that ectopic *Scr* can impose foreleg-specific traits (t-rows) on midlegs and hindlegs at a late (proneural cluster) stage agrees with the demonstrated ability of ectopic *Ubx* to induce hindleg traits (thin preapical bristle and stout tarsal bristle) in midlegs during bristle differentiation (Rozowski and Akam, 2002). In each case, bristle precursors can evidently be swayed by *Hox* influence to "change their minds" (leg identity) at the very last minute.

Expressing *ScrWT* via *Dll-Gal4* suppressed bracts on all six legs (Held, 2010), but expressing *ScrWT* by *sca-Gal4* deleted them to a variable extent on different leg segments. On *sca>ScrWT* forelegs, 57.9% (s.d. = 5.6; N = 10) of tibial bristles outside the t-row area lack bracts (*vs*. 7.4%; s.d. = 1.0; N = 10 in wild-type), whereas only 29.3% (s.d. = 6.4; N = 10) of bristles within the basitarsal t-row area lack them (*vs*. 0% in wild-type). The midleg tibia is missing bracts to a greater extent (70.4%; s.d. = 6.9; N = 10). We have conjectured

that high levels of Scr may be causing the bractless condition in the tibial t-rows of wild-type flies (Held, 2010), though it is hard to ascertain from published photos to what extent *Scr* is expressed more strongly on the tibia than on the basitarsus (Barmina and Kopp, 2007; Devi *et al.*, 2012; Randsholt and Santamaria, 2008; Shroff *et al.*, 2007; Tanaka *et al.*, 2011). For species that lack sex combs, such a bias (*i.e.*, higher on tibia) is indeed quite noticeable (A. Kopp, personal communication).

Many of these abnormalities were anticipated from our *Dll>ScrWT* study (Held, 2010), but one effect was a surprise (because our *Gal4* driver had been confined to legs): expression of *ScrWT* via *sca-Gal4* suppressed macrochaetes on the head and thorax (Figure 9). Macrochaetes are huge bristles that occupy constant positions from one fly to the next (Held, 2002a). Conceivably, *Scr* might impact them more than the smaller microchaetes, because their longer growth period (via polytenization) would prolong exposure to this "alien" transcription factor. Likewise, apical macrochaetes were missing from all *sca>ScrWT* midlegs (N = 20), and the big coxal bristles were shortened, but genuine signs of T2-to-T1 homeosis were also discernable: spur bristles at the apical site (Hannah-Alava, 1958) elongated to resemble tapered bristles of the foreleg's t-rows.

Conclusions

Hox genes are notorious for establishing "area codes" along the body axis in bilaterians (Held, 2017), and such broad "brush strokes" were probably their first major role in this clade. However, they also appear to have been recruited at various times for sundry "touch-up" chores (*cf.*, Stern, 1998 and 2003), and fly legs offer us a window into how such micro-managerial tasks are handled. Forelegs and hindlegs evolved peculiar rows of bristles (t-rows and sex comb) to serve adaptive needs aside from sensation—namely, grooming and grasping—and *Scr* and *Ubx* were somehow co-opted to implement the modular "subroutines" that govern those rows.

Whereas *Hox* genes encode segmental identity digitally as ON-OFF switches in embryos, *Scr* and *Ubx* seem to govern the later creation of t-rows and combs by an analog mode of action. The higher the dose of *Scr*, for example, the more t-rows arise on the foreleg tibia (Figure 5a), and *Scr* and *Ubx* appear to interact synergistically on the hindleg tibia (Figure 8a). Furthermore, the dosage of *Scr* may be instrumental in enforcing the bractless and yellowish state of t-row bristles on the tibia (*vs.*, the tarsus), though we still have no idea whether either of those tibia-specific traits are useful to the fly … or merely accidental side-effects of some obscure adaptive process.

Ultimately we would like to know how the t-rows and sex combs arose evolutionarily, how they are assembled developmentally, and how they are encoded genetically (Kopp, 2011). Answers to those questions might shed light on the genomic programming of bilaterian anatomy. Ever since Hanna-Alava mapped their intricacies in 1958, fly legs have offered a microcosm in which to explore deeper questions about patterning, and they continue to taunt us, despite all of the embarrassing situations we've contrived in an effort to pry from them their deepest secrets.

Acknowledgments: Fly stocks were obtained from Jeff Thomas (double-balancer strain, *y w; Sp/Cyo; Dr/TM3, Sb*, used to build our *sca-Gal4/CyO; Gal80^{ts}* stock) and from the Bloomington Stock Center: *sca-Gal4/CyO* (#42741), *neur-Gal4, Kg/TM3, Sb* (#6393), *UAS-dsScrRNAi* (B-50662), *UAS-ScrWT* (#7302), and *w; Sco/CyO; tub-Gal80^{ts}* (#7018). Critiques of the manuscript were provided by Markus Friedrich, Artyom Kopp, Nicolas Malagon, Marion Rozowski, Marc Srour, Kohtaro Tanaka, and Teresa Orenic, who helped resolve our A/P mystery. Zack Fitzgerald assisted in collecting virgins. This paper is dedicated to the memory of the Gerold Schubiger (1936-2012), who pioneered the use of fly legs as a model system 50 years ago (Beira and Paro, 2016; Schubiger, 1968). After receiving a diagnosis of the illness that would end his life, he contacted one of us (L.I.H.) to consult in the writing of his final paper on this topic—a masterpiece of Swiss precision (as usual). That paper, published in the year of his death (Schubiger *et al.*, 2012), inventoried all of the peculiar differences among the three pairs of legs.

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Enhancer of *dumpy-vortex* [*e*(*dp*^{v})] also enhances *dumpy-oblique* (*dp*^{ov1}).

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The complex *dumpy* gene (Wilkin *et al*., 2000) is an important component of the extracellular matrix of epithelial cells throughout development. *Dumpy* mutants fall into three classes, those that affect wing shape (oblique mutants), those that affect tendon cell attachment to the adult dorsal thoracic cuticle (vortex mutants), and those that affect viability (lethal mutants). Single mutants can affect one, two, or all three of these phenotypes. Following Grace *et al*. (1980), we have developed a scoring system for the oblique phenotype (Carmon *et al*., 2010). Wild type wings are scored as zero, whereas dumpy mutant wings are scored from one to five with five indicating the most severe truncation and/or distorted wing shape.

As part of our ongoing study of dumpy's interactions with other genes, we have examined the extent of the interaction between dumpy mutants and a gene discovered about 100 years ago by Calvin Bridges (Bridges and Mohr, 1919) originally called "enhancer of dumpy vortex" and now designated as *e*(*dp*^{v}) in flybase. We made double mutant combinations of *e*(*dp*^{v}) and the canonical dumpy oblique mutant, *dpy*^{ov1}, which shows an intermediate expression of the oblique phenotype but variable expression of dumpy vortex. Our results, using the scoring system described above are shown in the table below:

1.	dp^{ov1}/dp^{ov1} ; $e(dp^{v})^{+}/e(dp^{v})^{+}$	females (79) 1.9	males (65) 2.3
2.	dp^{ov1}/dp^{ov1} ; $e(dp^{v})/e(dp^{v})^{+}$ -	females (23) 1.9	males (21) 2.6
3.	dp^{ov1}/dp^{ov1} ; $e(dp^{v})/e(dp^{v})$ -	females (53) 3.4	males (74) 3.4

Clearly, $e(dp^{v})$ also enhances the dumpy oblique phenotype as well, indicating the interaction is recessive for both mutants and taking place in both the developing wing disc and the tendon cells of the thoracic flight muscles. To date, the $e(dp^{v})$ gene, which maps at 40.4 on the third chromosome, has not been annotated nor has the lesion producing the dumpy interaction been identified.

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Temperature shock effects on *dumpy* wing expression.

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Introduction

The *dumpy* locus is a complex gene and developmental system. Through alteration of its' primary product, a large extracellular protein, three major mutant phenotypic effects have been noted. These are a truncated wing termed *oblique* (dp^{o}), a rearrangement of thoracic bristle pattern termed *vortex* (dp^{v}), and lethality (dp^{l}). Different *dumpy* alleles can exhibit a single one of these phenotypes or a combination, *e.g.*, both *oblique* and *vortex* (dp^{ov}). Phenotypic expression of *dumpy* can be modified by a number of genetic factors, among them are single second site enhancer or suppressor genes, *e.g.*, $en^{(dpv)}$, the accumulative effects of polygenic modifiers, and position effect. Not yet examined are the effects of environmental factors such as temperature shock during pupal development on the phenotypic expression of *dumpy* mutants in the adult fly. This approach was used in the 1960's in a number of studies on the genetics and development of the posterior crossvein (Thompson, 1967). In these studies both enhancement and suppression of the mutant phenotype, missing portions of the posterior crossvein, was dependent on the time during pupal development when heat shock was applied, age response, and the length of the heat shock treatment, dose response. This study shows that the wing phenotype of *dumpy-oblique* mutants can be altered by high temperature shock in a fashion similar to that observed with *crossveinless-like* mutants.

Materials and Methods

The four different *dumpy* mutations, dp^{ov1}, dp^{ov7b}, dp^{ov56a}, and dp^{ovA12}, used in this study are a part of the *dumpy* stocks maintained in the laboratory of Dr. Ross J. MacIntyre of Cornell University. Stocks were maintained on a standard medium at room temperature, 23°C. Experimental cultures were handled as described by Thompson (1967).

White prepupae were collected and placed on the inside walls of plastic shell vials (25 × 95 mm), plugged with moist cotton and allowed to continue development at 23°C. Whiteness of the prepupae indicates that it was collected in less than one hour after the onset of puparium formation, and the time of collection is

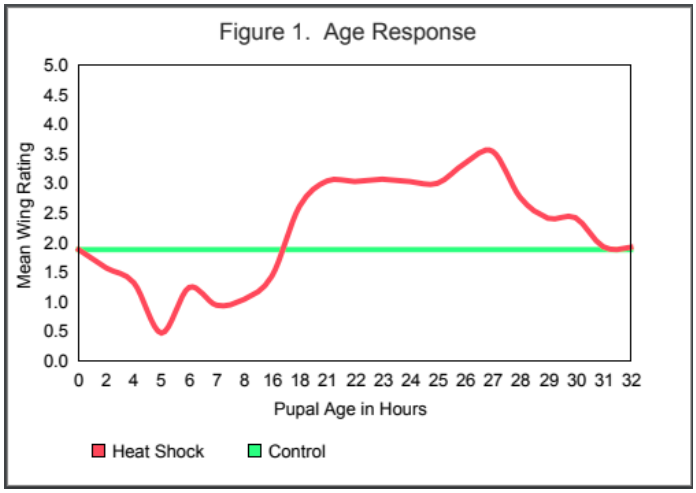
taken as time zero in pupal development. Temperature shocks were carried out in a constant temperature water bath set at 40.5°C by insertion of the collection vial into the water bath up to 15 mm of the plugged opening so that all collected pupae were treated but not submerged. After the pupae were subjected to treatment, the vials were removed from the water bath and pupae were allowed to complete development at 23°C until eclosion. In like manner, control samples were collected but were untreated and completed all development at 23°C.

Following eclosion, the treated and control flies were rated for wing length and appearance using the rating scale of 0 (wild-type) to 5 (extreme reduction in wing length and size) as described by Carmon *et al*. (2010). The average ratings of treated or control flies are presented as mean wing rating (mwr).

Three types of temperature shock experiments were performed. (1) Age Response: Pupae of the dp^{ov1} stock were collected over a range of developmental time from 2 through 32 hours and subjected to temperature shock for 20 minutes at 40.5°C. This stock was selected because of its moderate level of wing expression and because historically this was the first *dumpy* mutant described, originally as *truncate*, by Morgan and his colleagues at Columbia University in the famous "Fly Room" (Altenburg and Muller, 1920). The purpose of this type of experiment was first to determine if *dumpy* is temperature sensitive, secondly to determine if there are peak periods of sensitivity, and lastly to characterize the type of response(s). (2) Dose Response: Pupae of the dp^{ov1} stock were collected and at a peak response developmental age subjected to varying duration of temperature shock to determine the length of treatment for maximum response. (3) Comparative Response: Pupae of each of the *dumpy* stocks listed above were collected and treated at the peak response developmental ages for 20 minutes at 40.5°C. The purpose of this study was to determine whether the response to temperature shock is similar in all *dumpy oblique* mutations or if each reacts differently.

Results and Discussion

(1) Age Response: Figure 1 shows the combined female and male results of the age response study with dp^{ov1}. In this figure, the horizontal line represents the control level of expression with a mean wing rating of 1.88, and the curved line represents the heat shock responses. In addition to showing that *dumpy* is temperature sensitive, it clearly shows that dp^{ov1} has two major responses to temperature shock, an early suppression of the mutant effect and a later enhancement of the mutant effect. The early suppression occurs during the stage of development that Waddington (1940) characterized as the prepupal stage. The peak suppression response occurs at 5 hours of development with a mean wing rating of 0.47. The prepupal phase ends with the transition from prepupae to the pupal stage at 17 hours of development. This directly corresponds to Waddington's (1940) description of the prepupal to pupal transition point in development and is characterized with the eversion of the head of the developing fly. Enhancement begins with the start of the pupal stage at 18 hours of development and ends at 31 hours of pupal development. The peak enhancement response occurred at 26.5 hours of pupal development with a mean wing rating of 3.42.



(2) Dose Response: Figure 2 illustrates the Dose Response effect with dp^{ov1} pupae at 26.5 hours of development. In this figure, the curved line represents the expression (mwr) at varying lengths of heat shock. The response to varying treatment length shows a subthreshold response prior to 10 minutes and that treatment lengths from 10 to 20 minutes are linear in response after which time it plateaus. Thus, we concluded that 20 minutes is the optimum treatment time for maximum response. Dose response at the early suppression age of 5 hours also had a maximum response at 20 minutes of treatment.

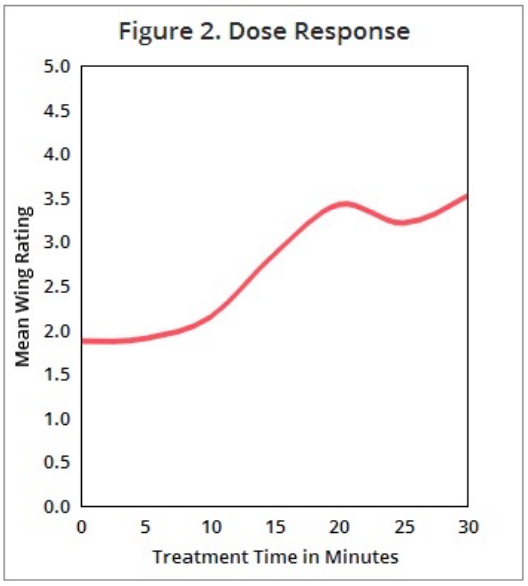


Table 1. Comparison of mean wing rating in dumpy-oblique mutants with Control (no treatment) and Heat Shock at 5 and 26.5 hours of pupal development. Values in parentheses are number of flies rated.

dumpy allele		Control (mwr)	5 Hour (mwr)	26.5 hour (mwr)
ov1	female	1.85 (255)	0.52 (126)	3.32 (244)
	male	1.90 (228)	0.42 (108)	3.55 (183)
ov7b	female	1.05 (64)	0.44 (34)	1.82 (66)
	male	0.60 (58)	0.10 (20)	1.52 (50)
ov56a	female	1.75 (149)	0.89 (35)	2.49 (126)
	male	1.45 (128)	0.60 (25)	2.06 (111)
ovA12	female	4.70 (146)	3.63 (52)	4.74 (77)
	male	4.74 (115)	3.67 (56)	4.46 (65)

(3) Comparative Response: Table 1 shows that all of the *dumpy-oblique* mutants tested have similar responses at both early (suppression) and late (enhancement) developmental ages as we have illustrated with dp^{ov1}. The exception to this conclusion being that 26.5 hour treatments with *dpovA12* do not show enhancement, but the phenotype of this mutant is so extreme, average mwr of 4.72, that it would be difficult to determine an enhancement effect with our method of scoring wing phenotype. This mutant does follow the pattern of suppression at 5 hours of development. Dose response studies at 5 hours of development with this extreme mutant follow the same pattern as seen with dp^{ov1} except that at high doses of heat shock, above 25 minutes, there is a reduction in viability.

This pattern of early suppression and later enhancement was also observed with the *crossveinless-like* studies of the 1960's (Thompson, 1967) with minor differences in timing. Whether this pattern applies to other wing mutations is unknown with the exception of an initial attempt to induce early suppression with *vestigial* which did not respond to 20 minute heat shock. It may be that moderate wing morphology mutations, for example *miniature*, and venation mutations such as *cubitus interruptus* and *abrupt* could be better candidates to respond to heat shock than the more extreme morphology changes seen with *vestigial*.

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Evaluation of the neuroprotective potential of aged garlic extract and grape flour in *Drosophila melanogaster*.

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Abstract

Oxidative stress is considered the main cause of aging and diverse neurodegenerative diseases, such as Alzheimer's, Parkinson's, and Huntington's. To decrease the number of these diseases, the antioxidant effects of many compounds have been explored. In this research, lines of *Drosophila melanogaster* were exposed to

Aged Garlic Extract and Grape Flour and the Rapid iterative negative geotaxis (RING) test was used to evaluate their aging. Additionally, the Lethal Dose 50 test was performed to evaluate the toxicity of the extract to flies. There was a decrease in the motor performance of the tested organisms from the 14 days, but a tendency to stabilize the aging process of the flies exposed to the medium with the aged garlic extract in the 5× concentration from 21 days. At the end of the experiment, the number of individuals at 28 days was higher in medium containing the highest concentrations of garlic extract and grape in relation to the control. In the LD50 test, it was not observed a significant number of deaths so that the lethal dose of the garlic extract could not be stipulated. Key-words: Antioxidants, aging, RING test, oxidative stress.

Introduction

In the last decades, the population's life expectancy has been increasing and the frequency of neurodegenerative diseases is also expected to increase. In the United States of America, the number of elders with Alzheimer's disease is expected to double in the next decade (Thies and Bleiler, 2013). Although age is a determinant factor for the development of some diseases, the influence of other factors has been estimated. There is growing evidence that aging can be accelerated through oxidative damage to cells. Free radicals (reactive oxygen species and reactive nitrogen species) may break DNA strands, damage desoxyribose or modify purine and pyrimidine bases (Lobo *et al.,* 2010).

With the recognition of the oxidation process of DNA, proteins and lipids as a central mechanism in the development of neuropathies and aging, antioxidant functional foods that might complement the defense mechanism of the body have been exploited to fight them (REF). Many compounds are indicated as antioxidants, and phenolics have received special attention. Aged Garlic Extract (AGE) is an important antioxidant, but was studied only in cell cultures or in mouse lines (Ray, Chauhan, and Lahiri, 2011; Kohda *et al.,* 2013; Morihara, 2016).

For the production of AGE, garlic is stored in ethanol solution for about two years. This long term extraction confers its aged characteristic, altering its antioxidant properties through molecular changes. During this process, the production of cystein (S-allylcysteine, SAC) is intensified, which confers a higher antioxidant activity in relation to *in natura* garlic (Ide and Lau, 1997; Imai *et al.,* 1994; Borek, 2001). Among the role of AGE in protecting against aging-related cognitive impairment, the inhibition of lipid peroxidation, and Low Density Lipoprotein (LDL) cholesterol oxidation (Ide and Lau, 1997) can be highlighted. In this way, it can decrease the risks of degenerative diseases such as Alzheimer's. The AGE's antioxidant effect has already been tested *in vitro* in mouse and *in vivo* cells in transgenic mice with familial Alzheimer's disease and demonstrated important neuroprotective activity (Chauhan and Sandoval, 2007; Ray *et al*., 2011).

Another important functional food is grape. These have high antioxidant content, with phenolic compounds as resveratrol and anthocyanins (Xia *et al.,* 2010). In this way, Grape Flour (GF) has the main functional antioxidant properties of fresh grapes. A previous study with GF added to *Drosophila* culture medium influenced the longevity of flies. Female longevity increased 127% and male longevity increased 50% (Fernandes, 2006), an indication that functional foods might have an important role on fighting against aging.

Many of the common age-related behavioral changes in humans are also observed in *Drosophila*, including motor decline, olfactory, memory, and circadian rhythmic ability (Jones and Grotewiel, 2011). *Drosophila* has been used as a study model in the neurotoxicology of cisplatin and acrylamide (Podratz *et al*., 2011; Prasad and Mudalihara, 2012). Therefore, *Drosophila* might be an important model organisms in aging studies.

In the present study, *Drosophila* were maintained on culture media enriched with phenolic compounds (AGE and GF), and their motor activity was evaluated weekly. Flies submitted to the culture medium enriched with AGE and Grape Flour in higher concentrations had their populations conserved, such as better motor performances with the aging process.

Material and Methods

In this experiment, a Canton-S *Drosophila melanogaster* line was used. Flies were raised on standard corn culture medium and virgin adults were transferred and maintained on enriched medium with Grape Flour or Aged Garlic Extract (powdered) in two concentrations, 1,200 mg/kg of medium and 3,000 mg/kg (corresponding to two and five times the dose recommended for daily human consumption, from now on called the 2× and 5× treatments). In addition, raw garlic powder at concentration of 600 mg/kg was used for preliminary tests. Virgin flies maintained on corn medium as negative controls. Five replicates of each treatment (AGE and GF) were established.

To evaluate aging of flies, the Rapid iterative negative geotaxis (RING) test, proposed by Gargano *et al*. (2005), was used. In this test, the motor ability of flies is measured as the high flies ascend after being tapped, and was inspected weekly (7, 14, 21, and 28 days after hatching). Following the protocol of Nichols *et al*. (2012), about twenty flies were inserted into each of the falcon tubes of the device. For each trial, two photographs were taken and one was used for analysis.

The evaluation of the lethal dose of AGE through LD50 test was performed in groups with about 80 flies reared in standard culture medium, 7-10 days after hatching. Flies were exposed to different concentrations of AGE diluted in 5% sucrose solution and applied 100 µL to pieces of 2×2 cm filter paper. The survival of the flies was evaluated by the number of individuals at the end of 24 hours and after 48 hours of exposure to the extract.

According to previous published works, the initial concentration in the LD50 test would be 600 mg/kg (solution); flies were maintained in vials with 10× that concentration, 150× and 300× the indicated concentration, besides the control group (without addition of age). In total, 321 flies were used in this test, performed in cylindrical glassware with added pink dye to the solution for better visualization. 24 hours after the start of the test, the filter papers were changed to avoid the lack of solution, maintaining the same concentrations, and fly survival was again registered 24 hs later.

In the RING test, differences in fly heights were evaluated by Univariate Analysis of Variance (ANOVA), using treatments as a predictor variable. As the result was statistically significant, Tukey's multiple comparisons test was performed in order to verify which comparisons were significant. In all tests, $p < 0.05$ was used. All analyses were performed in the Statistica 10 program (StatSoft).

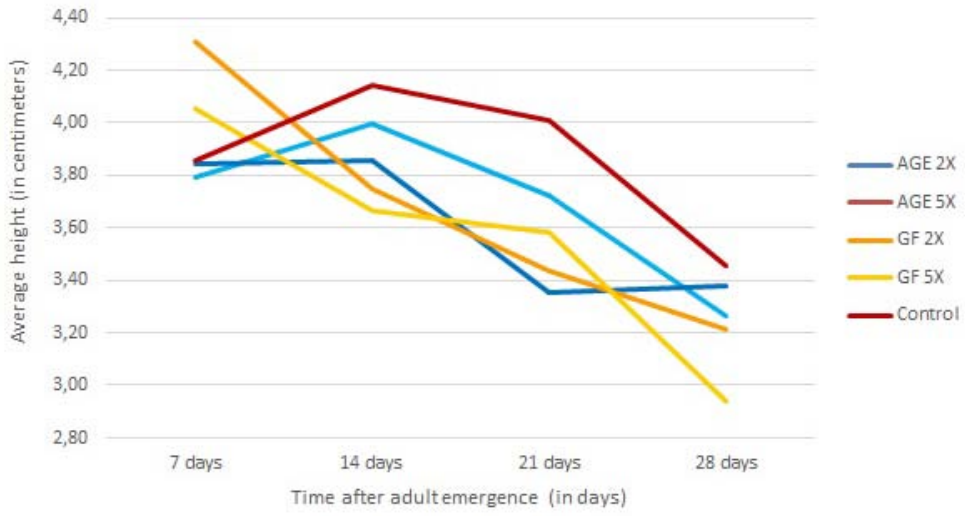

Figure 1. Average heights in RING Test analysis of the different treatments in *Drosophila melanogaster*. Flies were maintained on media enriched with AGE - aged garlic extract, or GF - grape flower, or non-enriched (control).

Results and Discussion

About 6800 observations were listed with RING test between the control groups and treatments. In general, there was a decrease in fly motor performance after 14 days from hatching (Figure 1). This effect was

expected due to the consequences of aging, as Gargano (2005) demonstrated in his protocol and recorded in other studies (Jones, 2010; Simon *et al.*, 2006; Grotewiel *et al*., 2005). An increase in average heights at the beginning of the experiment was not expected, although similar behavior was seen in other works, such as in Piazza (2009).

It was possible to trace a motor development line due to aging in each of the treatments used in *Drosophila*. In the animals exposed to the medium with the 5× garlic extract concentration, there is a tendency to stabilize the heights from the 21 day period, showing an apparent and possible relation with its neuroprotective potential.

A comparison of the heights between the two treatments and the control group was performed separately (Table 1).

Table 1. Univariate analysis of variance (ANOVA) between the control group and the treatments. AGE - Aged Garlic Extract; GF - Grape Flour.

	7 days		14 days		21 days		28 days	
	F test	p	F test	p	F test	p	F test	p
AGE	1.90	ns	1.11	ns	5.48	**	0.35	ns
GF	3.41	*	4.92	**	5.03	**	1.46	ns

* $p < 0.05$ and ** $p < 0.01$ and ns: non-significant.

There was no difference between treatments when compared to their concentrations (2× to 5×). Differences found were restricted to the relationship between the control group and one of the treatments.

Flies maintained on 5× AGE medium presented lower locomotor activity at 21 days, which was not maintained at 28 days. However, the treatments that presented lower performance in the averages of the heights were those that presented a better survival rate at the end of the experiment (5× Grape Flour and 5× AGE concentrations).

Flies maintained on 2× GF medium presented higher locomotor activity at 7 days after hatching, and lower activity at 14 and 21 days. Regarding the 5× GF treatment, at 14 days the height was also lower than the control.

After the tests, a qualitative analysis of the flies survival was carried out (Table 2). Both 5× AGE and 5× GF presented higher survival rates after 28 days.

Table 2. Population survival rate (%) after seven to 28 days after hatching. AGE - aged garlic extract; GF -grape flour.

	7 days	*14 days*	*21 days*	*28 days*
Control	100	68.36	28.77	11.81
2x AGE	100	64.83	37.93	16.23
5x AGE	100	81.36	47.31	36.16
2x GF	100	47.90	41.08	26.43
5x GF	100	67.91	53.56	38.15
Control	100	68.36	28.77	11.81

The decay rate of the populations was slower from 21 to 28 days, at which point the control presented higher contrast due to the onset of aging. These groups stand out from the others, which can be attributed to the higher concentration of antioxidant compounds present in the culture medium. The 2× AGE treatment presented survival rates similar to the control, which might be explained by a dose-dependent effect.

Borek (2001) reports the power of garlic extract in increasing the longevity of mice studied. In our research, we did indeed find evidence that AGE treatment had similar effects, increasing the survival rate of the exposed flies in relation to the control, at least in high concentrations.

The longevity of the *Drosophila* lineages in this study may be associated with the neuroprotective potential of AGE, as well as may be related to the tendency of stabilization of the heights of the treated group in higher concentration. Thus, the AGE study becomes suggestive about the use of antioxidants from the diet to improve quality of life.

In the LD50 test, it was not observed a significant number of deaths in order to estimate the lethal dose of AGE. The 300× AGE solution presented only 6% deaths after 48 hs. This is strong evidence for AGE safety and an indication that their antioxidant compounds might not act as pro-oxidants in high concentrations, as found for ascorbic acid (Carr and Frei, 1999).

In our tests, we found some unforeseen side-effects. AGE, odorless in regular concentrations (2× and 5×), presented a strong and characteristic odor in solutions with higher concentrations (150 and 300×). Studies with *Drosophila* show that these are influenced by odors, which act as stimulants, and in this way may increase the activity of these flies or even provoke environmental disapproval (Budick, 2006). This attitude of aversion could be observed in the preliminary tests with culture medium added in garlic powder *in natura*, which for having more prominent odor did not succeed in egg laying and establishment of the lineage. This type of behavior is not observed in other model study organisms due to the influence of body weight and different olfactory sensitivities.

It was also observed that in the LD50 trials, glasses containing the highest concentrations of AGE presented higher amounts of residues. This may be due to a possible increase in the metabolic activity of the flies, which had slightly altered behaviors regarding agitation and excretion. This alteration in metabolism, somehow, may have masked the antioxidant action of garlic extract, due to its increase being related to the anticipation of aging.

One weakness of the present study design is that we could not determine the daily dose of antioxidant compounds ingested by the flies, as there was no control of how much of the extract the flies were fed from the culture medium. Future studies that directly administer food compounds to the individuals might overcome this limitation.

Conclusion

The data obtained in this research show that there was an increase in the life time of the flies treated with AGE and GF. Nutraceuticals may increase the longevity of individuals, as can be seen in Soh *et al*. (2012) and also in studies with trans-resveratrol (Fernandes, 2006) and D-chiro-inositol (HADA, 2012). The difference in relation to the survival rate may be associated with the antioxidant potential of the treatments, which presents dose-dependent effects. We found no evidence of toxicity of AGE on *Drosophila*, even when flies were submitted to high doses. We suggest that further research should be done at higher concentrations of AGE, and might use *Drosophila* lines that are more susceptible to oxidative stress.

Acknowledgments: The authors would particularly like to thank the professors Daniela de Toni and Norma Silva, from Federal University of Santa Catarina (UFSC), who kindly donated the *Drosophila melanogaster* Canton-S lines used in this research. The authors also thank Mr. Yukihiro Kodera, senior researcher from Drug Discovery Laboratory (Wakunaga Pharmaceutical Co., Ltd., Hiroshima, Japan) for donation of the Aged Garlic Extract.

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First record of *Drosophila incompta* Wheeler 1962, *flavopilosa* group, in the Brazilian state of Minas Gerais.

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Drosophila incompta belongs to the *flavopilosa* group, which comprises 17 species (Bächli, 2017), distinguished mainly by their internal male genitalia morphology (Wheeler *et al*., 1962). This group presents a remarkable characteristic, being ecologically specialized and totally dependent on flowers of *Cestrum sp* (Solanaceae) for both feeding and reproduction (Brncic, 1966). In this sense, these species developed several adaptations to their host, including their small to medium size, the yellowish color (cryptic to *Cestrum* flowers), and the presence of strong spines on the outer region of the female genitalia (Brncic, 1983; Ludwig *et al*., 2002).

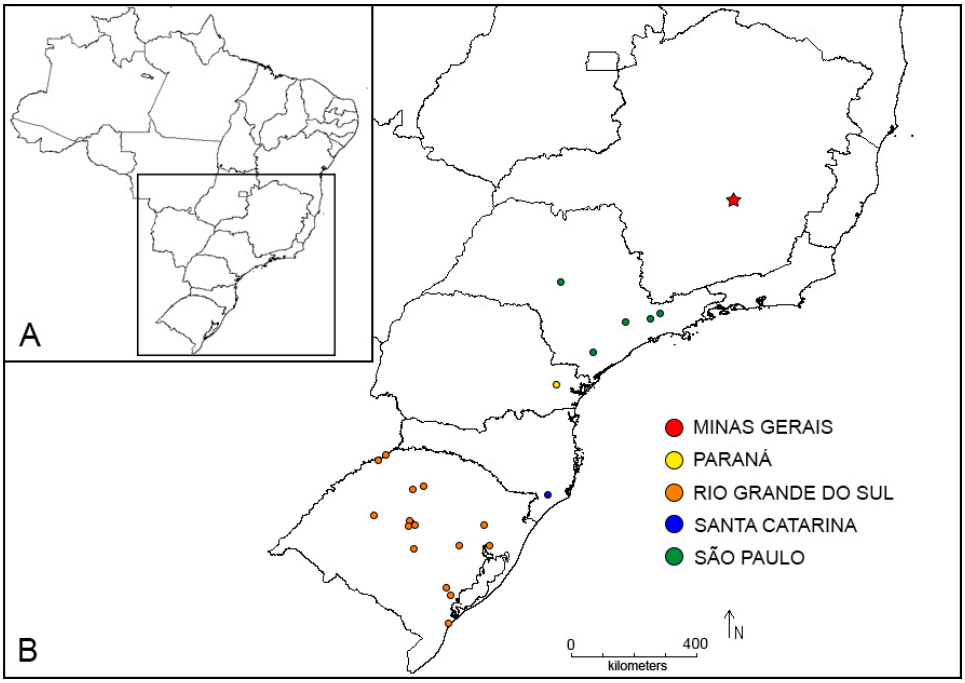

Figure 1. Known geographical distribution of *D. incompta* in Brazil, according to Bächli (2017). Orange, blue, yellow, and green circles correspond to sample locations in the states of Rio Grande do Sul, Santa Catarina, Paraná, and São Paulo, respectively. The red star corresponds to the first occurrence location of *D. incompta* in the state of Minas Gerais.

Due to their restricted ecology, the members of the *flavopilosa* group are geographically restricted to areas with *Cestrum* sp., which seems to be highly abundant in the Neotropics (Robe *et al*., 2013). Even so, most species are specialized to a small array of *Cestrum* species (Santos and Vilela, 2005) and are widely endemic to a restricted area (Robe *et al*., 2013). *Drosophila incompta* appears to have the widest distribution among the members of this group, being found from Mexico to northern Argentina (Bächli, 2017). Nonetheless, across this area, the distribution of the species is predominantly scattered, with registers encountered only for Antilles (1 record), Colombia (2 records), and Panama (2 records), besides Mexico (2 records), Brazil (22 records,

Figure 1), and Argentina (1 record) (Bächli, 2017). Despite this, Robe *et al*. (2013) emphasize this may reflect a bias of the sampling efforts. In this article, we report the first record of *D. incompta* in the state of Minas Gerais.

The flowers of *Cestrum* were collected in the municipality of Belo Horizonte (19°47′S, 43°57′21″W), taken to the laboratory and kept until the hatch of adult flies. These were captured using an entomological aspirator (Machado *et al*., 2014) and immediately fixed in absolute ethanol. The flies were separated by sex through their external morphology and further identified by the internal male genitalia morphology, as described by Wheeler *et al*. (1962). A total of 61 flies were collected, from which 27 were male. All male individuals were identified as *D. incompta*, according to their internal genitalia morphology patterns.

This report is congruent with the predictions based on Environmental Niche Modeling strategies performed by Robe *et al*. (2013), according to which the potential distribution of *D. incompta* would extend from the southern region of Brazil, in which it can be found in sympatry and even syntopy with *D. cestri*, *D. corderoi*, and *D. flavopilosa*, to the central region of the country. Nevertheless, although this report extends the known distribution range of *D. incompta* in Brazil, the wide area of unsuitable habitats that was projected to follow to the north of this area (Robe *et al*., 2013) remains to be further assessed.

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Effects of three common orange flavored drinks on survival and phenotype of *Drosophila melanogaster.*

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Abstract

In this study, *Drosophila melanogaster* flies were exposed to three local and common orange flavored drinks (Nutri-C, Sari-C, and Eve). The *Drosophila melanogaster* flies were fed on a Banana-Garri medium containing the test substances in varying concentrations (1, 2, 5, 10, 25, 50, and 100%). The flies were bred in the media in the ratio of a male to three females, left to mate and lay eggs for six days. The number of deaths of parent flies and phenotypic defects in F_1 flies were noted. It was observed that the drinks caused the death of some parent flies and that females were more affected than males. Phenotypic defects of the wings and abdomen were also observed in F_1 flies. Food products containing chemicals should be adequately tested before release into the market. Further research should be carried out to determine the mode of action of these substances on *D. melanogaster* and on mammalian test systems. Keywords: *Drosophila melanogaster*, F_1 flies, fruit drinks, survival, phenotypic defect

Introduction

Environmental toxins pose a constant challenge to the survival of living organisms. These toxins enter the body by physical contact, inhalation, or ingestion and can originate from a wide range of sources (Misra *et*

al., 2011). There are more than 80,000 chemicals in commercial use today, and approximately 2,000 new chemicals are introduced yearly (Rand, 2010). These chemicals comprise food additives, drugs, narcotics, antibiotics, pesticides, cosmetics, contraceptives, air pollutants, water pollutants, and others.

Genetic manipulability and ease of detecting phenotypes made *Drosophila* the model of choice for mutagenesis screens of the 1980's and 1990's (Rand, 2010). *Drosophila* is a genus of small flies, belonging to the family Drosophilidae whose members are often called 'Fruit flies'. The genus is diverse phylogenetically, geographically, and ecologically (Dilon *et al*., 2009). Many species are easily reared in the laboratory. One species of *Drosophila* in particular, *Drosophila melanogaster*, has been used a lot in genetic research and is a common model organism in developmental biology. *Drosophila melanogaster* has an abundance of molecular and genetic tools and is a leading model system for investigating metazoan biology (Dilon *et al*., 2009). *D. melanogaster* is uniquely useful in genetic toxicology and mutation research due to extensive knowledge of its genetics and biology together with genetic homology to mammals. Many hundreds of thousands of offspring can be generated in a relatively short period of time (2 weeks at 25°C), easy to maintain, and the complex spectrum of somatic and heritable alterations can be detected under the microscope with low power magnification (Deepa *et al*., 2012).

Orange flavored drinks are a common part of the Nigerian diet and are consumed especially by children because of their sweetness and color. There has been no study done to investigate the toxicity of these drinks. This study aims to investigate the effects of three common orange flavored drinks in the Nigerian market on morphology and survival of *Drosophila melanogaster*.

Materials and Methods

Flies were collected from specific sites in the University of Lagos, Akoka campus. These were separated into different sexes after etherization based on morphological differences as described by Parvathi *et al*., 2009. The culture medium was the Banana-Garri medium containing Banana (250 g), agar (10 g), propionic acid (5 ml), garri (50 g), and distilled water (1 L) (Williams and Akpabio, 1993). The three orange flavored drinks were sourced from a local market in the Lagos metropolis. 10 ml of different concentrations (1, 2, 5, 10, 25, 50, and 100%) of these drinks (Nutri-C, Sari-C, and Eve) were added to 50 ml of the culture medium and poured into a vial. The drinks were applied to eggs, larvae, and adults by means of nutrition, adding it to the culture medium. The control culture was the Banana-Garri medium without the addition of the drinks.

Flies in the ratio of 1 male to 3 females were bred on the culture media containing different concentrations of the test substances and reared at room temperature (27ºC). These were left to mate and lay eggs for six days. The parent flies which were alive were then removed after six days. The F_1 offspring were observed and phenotypic changes recorded using a stereomicroscope. This observation was done until no adult eclosion was observed in media.

Results

Survival of parent flies

The number of deaths recorded is presented in Table 1. Some parent flies died but this was not in a linear relationship with concentration. No parent fly died in the control as shown in Table 1. It was observed that 28.57% of parent flies, 33.33% of female parent flies, and 14.29% of male parent flies died in Nutri-C- and Sari-C-containing medium. 35% of parent flies, 40% of female parent flies, and 20% of male flies died in Eve-containing medium. It was obvious that more females died than did males.

Phenotypic changes

Abnormal phenotypes were observed in F_1 progeny exposed to the test media as shown in Table 2. These changes in phenotype were not observed in F_1 flies exposed to the control medium. Nutri-C had the least incidence of phenotypic effects on the phenotype of F_1 flies while more incidences were observed in F_1 flies exposed to Sari-C and Eve.

Table 1. Survival of parent flies.

Media	Parent Flies	Total Dead (%)	Dead (female) (%)	Dead (male) (%)
Nutri-C	28 (21 females, 7 males)	28.57 (8/28)	33.33 (7/21)	14.29 (1/7)
Sari-C	28 (21 females, 7 males)	28.57 (8/28)	33.33 (7/21)	14.29 (1/7)
Eve	20 (15 females, 5 females)	35 (7/20)	40 (6/15)	20 (1/5)
Control	4 (3 females, 1 male)	0	0	0

Table 2. Abnormal phenotypes in F_1 flies.

	Media		
	Nutri-C	Sari-C	Eve
Number of flies	2	9	5
Defect	Curly Wings	Folded wings	Short wings & smashed abdomen
		Rough and short wings	Short bent wings
		Curly wings	Abnormal wing
		Smashed abdomen	Rough & raised wings
		Splattered wings & curved abdomen	Short wings
		Short wings	

Discussion

It was observed that the media containing high concentrations (50, 100%) of Eve did not solidify. This suggests the possibility of chemicals at high concentration that could degrade the Banana-Garri medium. It also indicates that the Banana-Garri medium can be modified and thus provides the basis for further research into the effects of such modifications on several aspects of the biology of *Drosophila melanogaster*.

Parent flies were fed on media containing different concentrations of the three test substances. These parent flies died more in the media containing Eve (35%) than in media containing Nutri-C (28.57%) and Sari-C (28.57%). This is important as it raises issues on the frequent and continuous consumption of these drinks. These results indicate that these substances (Eve, Nutri-C, and Sari-C) particularly at high concentrations are toxic to parent flies of *Drosophila melanogaster*. Such strong toxic effects observed at the highest concentration of exposure were also observed by Tripathy *et al*. (1996), Ding and Wang (2009), and Uysal *et al*. (2013).

The female fly was found to be more susceptible to the toxic effects of the chemicals than males. This may be due to sexual variation and differences in feeding activity, which is at higher levels for female adults. Wong *et al*. (2009) found that females fed more than males over a 30-minute period, because they spent a greater proportion of time with the proboscis extended (2.8-fold more on average) than males. This higher intake by female flies is presumably related to their nutrient usage in egg production. A similar result was obtained by Gayathri and Krishnamurthy (1980) who found that the viability of females is more affected, indicating that female *D. melanogaster* are more susceptible to toxic effects of 1-amino-2-naphthol-4-sulphonic acid. Such toxic effect on adult females than adult males was also reported by Obeidat (2008) following exposure of *D. melanogaster* to Jordanian *Bacillus thuringiensis* isolates.

These substances also effected several morphological changes as indicated. Pre-adult stages of *D. melanogaster* are particularly susceptible as this is the time of active growth and development. Nutri-C showed the least effect on morphology. This was not the case for Sari-C and Eve exposed flies, which showed more abnormal phenotypes. Wing and abdominal defects were the morphological defects observed in F_1 flies. Haq *et al*. (2012) observed morphological changes to wings, abdomen, and color when *D. melanogaster* larvae were fed with lead acetate. Wing alterations were also observed when *D. melanogaster* was exposed to ethidium bromide (Ouchi *et al*., 2011). Some flies exposed to Eve were also seen to have orange colored abdomen probably indicating indigestion.

These substances thus have the ability to affect development in pre-adult stages and induce detrimental changes to the eclosed adult. The toxicological effect of Eve may be due to its much higher concentration and its composition of dyes (Sunset yellow) and artificial sweeteners (Aspartame). Sayed *et al*. (2012) demonstrated the mutagenic action of sunset yellow also showing an increase of morphological abnormalities in spermatozoids of mice.

The toxicological effect of Nutri-C and Sari-C may also be due to its composition of aspartame, tartrazine, colorings, and acesulfame-K in Sari-C. Gomes *et al*. (2013) found that tartrazine yellow dye has anti-proliferative activity action and potential to cause cellular aberrations using the *Allium cepa* test.

Conclusion

This study demonstrated signs of toxicity of the orange flavored drinks on *D. melanogaster*. A reduction in survival of parent flies as well as morphological changes in F_1 progenies was observed. At the very least, this has shown that these substances can affect some aspects of the biology of fruit flies. Further research should be carried out to determine the mode of action of these substances on *D. melanogaster* and on mammalian test systems.

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How nutritive conditions determine life-history traits in *Drosophila melanogaster*?

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Drosophila melanogaster uses various fruits and vegetables in different manners: as a food, egg-laying sites, or for reproduction (Shorrocks, 1972). Since flies are often exposed to different quality, quantity, and availability of nutritional resources, adjustment to new nutritional environment induces adaptive plastic responses, which include changes in morphological, physiological, life-history, and behavioral traits (Djawdan

et al., 1998; Bross *et al*., 2005; Broughton *et al*., 2005; Carsten *et al*., 2005; Partridge *et al*., 2005; Burger *et al*., 2007; Sisodia and Singh, 2012; Reddiex *et al*., 2013; Trajković *et al*., 2013, 2017a, 2017b; Abed-Vieillard *et al*., 2014; Rodrigues *et al*., 2015; Kristensen *et al*., 2016). Adaptation of individuals to different environmental conditions by developmental plasticity could be manifested, among other things, through the larval developmental time and viability (Kolss *et al*., 2009).

Our previous research confirmed that long term rearing of fruit flies on different diets (standard corn-meal substrates, as well as tomato, banana, carrot and apple diets) resulted in significant differences in developmental time, eclosion dynamic, and viability (Trajković *et al*., 2017a). Namely, flies maintained on carrot diet for more than 300 generations had the fastest developmental time, while flies reared on apple diet expressed the slowest development (Trajković *et al*., 2017a). In this respect, the purpose of this research was to explore and quantify potential changes in certain life-history traits, when flies grown on carrot diet were transferred to apple diet and *vice versa*.

In this experiment, we used *D. melanogaster* flies which were reared for more than 300 generations on carrot (C) and apple (A) diets. Media were prepared according to recipes published by Kekić and Pavković-Lučić (2003). Over the years, flies were maintained in 250 ml glass bottles (about 100 individuals *per* bottle), in optimal laboratory conditions (temperature of ~ 25^{o}C, relative humidity of 60%, 300 lux of illumination, and 12 h: 12 h light: dark cycle).

For experimental purposes, flies maintained on carrot diet (C flies), which had the fastest developmental time, were transferred to the apple diet (C-to-A flies), and flies maintained on apple diet (A flies), which were previously characterized by the slowest developmental time (Trajković *et al*., 2017a), were transferred to the carrot diet (A-to-C flies) (Figure 1). After that, three life history traits were scored: developmental time, dynamics of eclosion, and viability.

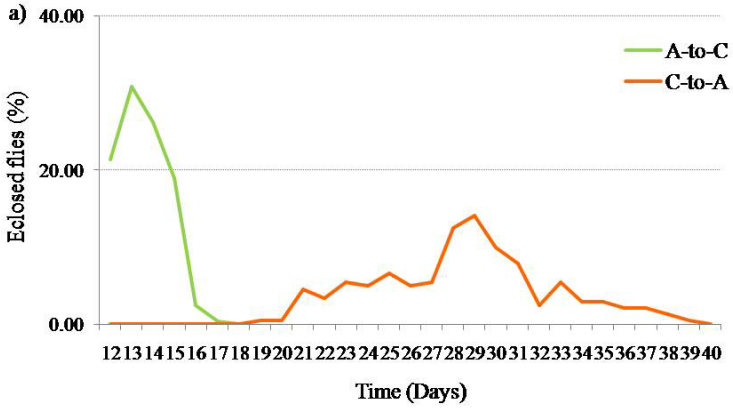
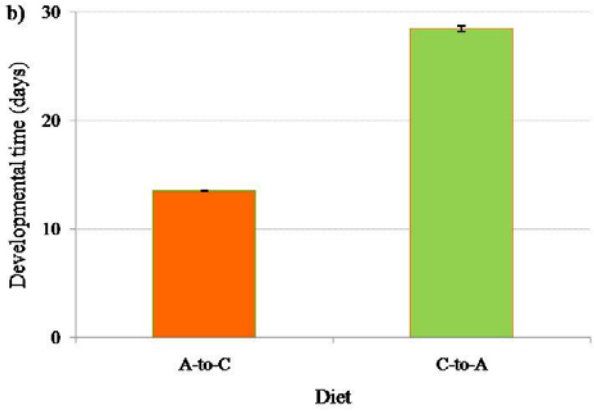
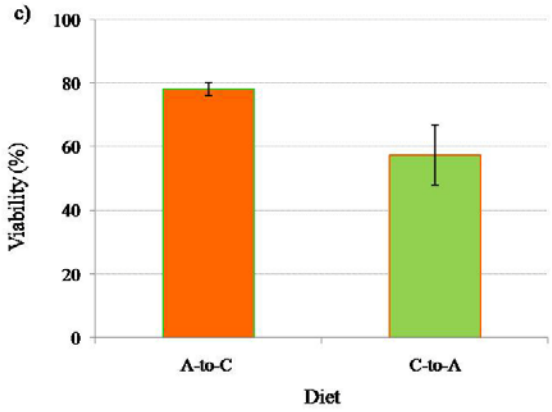


Figure 1. The scheme of experimental design.





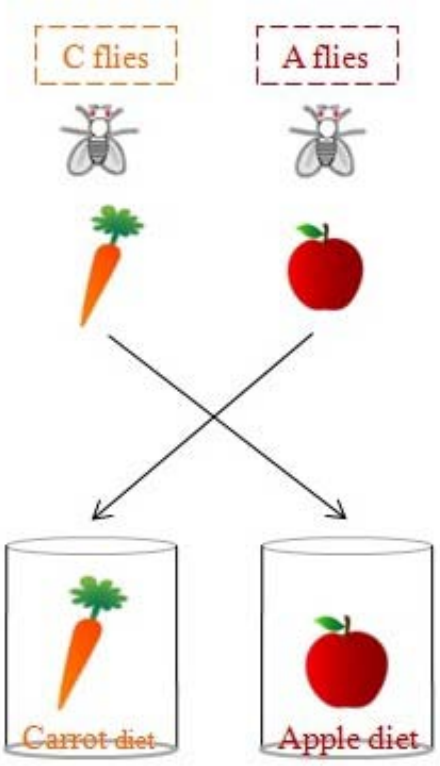


Figure 1 (continued).

Thirty to fifty fertilized females of C and A strains were transferred to the egg laying vials filled with their "native" substrates. Further, sixty eggs were collected and transferred to the new vials in the manner shown in Figure 1, and 5-7 such replicates were obtained *per* both combinations. Life-history traits were determined according to Trajković *et al*. (2017a). Dynamics of eclosion were presented as percentage of flies emerged *per* day, while developmental time was calculated as the average time weighted by the number of adults emerged. Egg-to-adult viability was expressed as the ratio of emerged flies and eggs placed in the vial.

Dynamic of eclosion, mean developmental time, and egg-to-adult viability are presented in Figure 2.

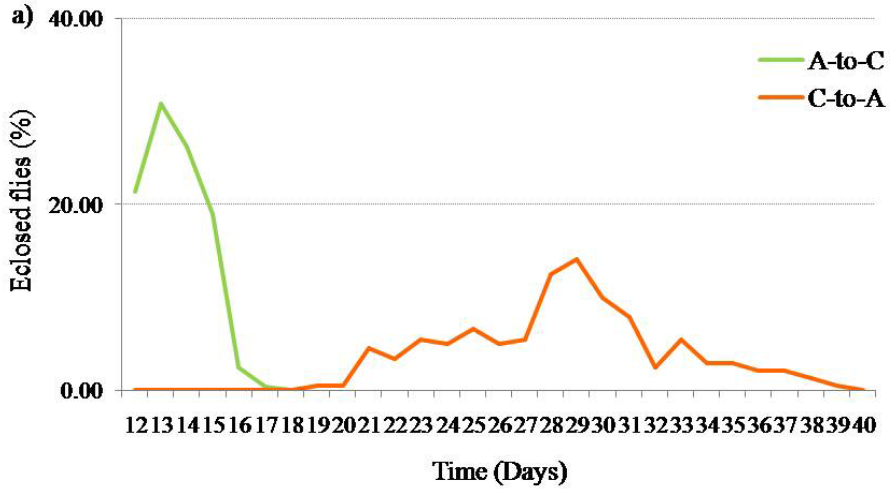
A-to-C flies hatched from the 12th to 17th day, and the largest number of eclosed flies was recorded on day 13 (Figure 2a). Emergence of C-to-A flies started at day 19 and lasted until day 39 (Figure 2a). The largest number of emerged C-to-A flies was recorded at 29th day (Figure 2a).

A-to-C flies have significantly shorter development (mean developmental time: 13.51 ± 0.06 days; Figure 2b) in comparison with C-to-A flies (mean developmental time: 28.44 ± 0.27 days; Figure 2b) ($F = 192.211$, $df = 1$, $p < 0.001$). Also, development of A-to-C flies lasted considerably shorter compared with development of A flies ($F = 74.323$, $df = 1$, $p < 0.001$). On the other hand, developmental time of C-to-A flies was significantly prolonged in comparison with C flies ($F = 235.829$, $df = 1$, $p < 0.001$).

After transferring eggs from the apple to the carrot diet, egg-to-adult viability significantly increased, from 53.71% (Trajković *et al*., 2017a) up to 78.10% ($F = 36.568$, $df = 1$, $p < 0.001$) (Figure 2c). In the reverse situation, when eggs from the carrot diet were transferred to the apple diet, egg-to-adult viability decreased from 82.22% (Trajković *et al*., 2017a) to 57.38% ($F = 32.941$, $df = 1$, $p < 0.001$) (Figure 2c). Further, A-to-C flies manifested significantly higher egg-to-adult viability than C-to-A flies ($F = 13.173$, $df = 1$, $p < 0.01$).

Under natural conditions, it is very important for *D. melanogaster* to adjust to the diverse environmental variations (including nutritional), which is mostly achieved by metabolic and physiological adaptations. Numerous studies pointed out that *Drosophila* life-history traits depend on both quality and amount of nutritive resources (Rodrigues *et al*., 2015; Abed-Vieillard and Cortot, 2016; Kolss *et al*., 2009; Kristensen *et al*., 2011; Lee *et al*., 2008; Schwarz *et al*., 2014).

To our knowledge, only one experimental evolutionary study on *D. melanogaster* confirmed the existence of adaptations to malnutrition. Namely, flies reared on poor larval nutrition showed a higher egg-to-adult viability and faster development on poor in comparison to the standard medium (Kolss *et al*., 2009). In our study, dynamics of eclosion, developmental time, and egg-to-adult viability were considerably dependent on the diet type. Previously, chemical analysis of diets used for growing flies in our laboratory revealed differences in protein/carbohydrate (P: C) ratio (Trajković *et al*., 2017a). Apple diet contains very low amounts of proteins, and flies maintained for many years on this diet type exhibited the slowest developmental time and the lowest viability (Trajković *et al*., 2017a). When those flies were transferred to standard (Trajković *et al*., 2017a) or, as in this research, to carrot diets, which contain higher protein amounts, they developed faster and expressed higher viability. In the opposite situation, when C flies which exhibited the fastest development and higher viability were transferred to the apple diet, their development was prolonged and viability reduced.



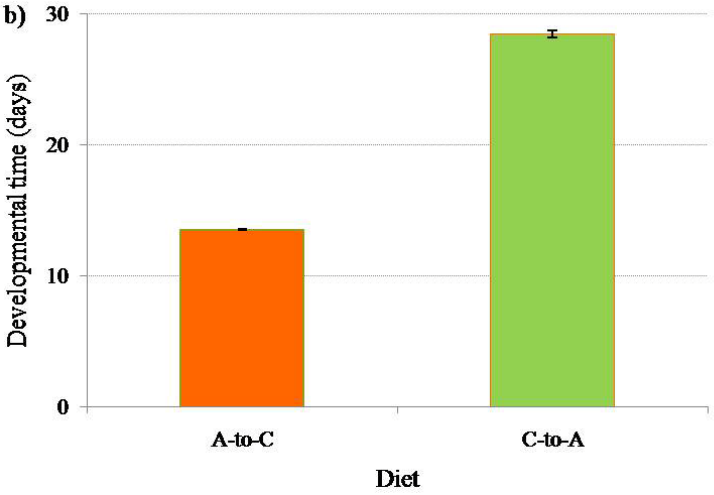


Figure 2. Dynamics of eclosion (a), mean developmental time (b), and mean egg-to-adult viability (c) of *D. melanogaster* A-to-C and C-to-A flies.

Results of this research confirmed the existence of developmental plasticity when *D. melanogaster* flies were exposed to different nutritional environments, and that developmental time is not deeply channeled. Furthermore, presence of developmental plasticity gives flies the possibility to adjust to highly variable environmental conditions.

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White eye mutation in *Drosophila melanogaster* does not affect fitness – a support for a neutral theory of molecular evolution.

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Abstract

White eye mutation in *Drosophila melanogaster* resulted in significant reduction in pre-adult development time. However, this reduction in pre-adult development time was accompanied by non-significant reduction in adult dry weight, life-time oviposition, and longevity lending a fortuitous support to the 'neutral theory of molecular evolution'. Key words: white eye, life history, oviposition, longevity, lipid content

Introduction

Mutations are an important source of heritable variation. They are acted upon by evolutionary forces such as natural selection and genetic drift in a population. New mutations could arise from DNA replication and repair infidelity, spontaneous point mutations, transposable elements, and a variety of other sources (reviewed in Mackay, 2010).

White eye, the first mutant phenotype identified in *Drosophila* by Morgan in 1910, is due to a mutation in an ABC transporter gene (Sullivan *et al.*, 1974; Mackenzie *et al.*, 1999). White functions with products of either scarlet or brown genes as paired heterodimers for transport of pigment precursors, tryptophan and guanine, respectively, into the eye of the fly (Ewart and Howells, 1998). The red pigments- drosopterins, and the brown pigments- ommochromes, are synthesized from guanine and tryptophan, respectively (Summers *et al.*, 1982). The repercussion of inefficient transport of pigment precursors is correlated to defective vision in the mutant flies at different wavelengths of light (Cosens and Briscoe, 1972), partially attributable to the inability to screen stray light due to the lack of optical insulation provided by the pigments (Hengstenberg and Götz, 1967). This also results in 'dazzling' the flies because of over-flow in daylight conditions (Krstic *et al.*, 2013). The white eye flies are positively phototactic but may completely lack optomotor responses (Kalmus, 1943) and have abnormal electroretinograms (Wu and Wong, 1977). Due to the low levels of expression of White, molecular studies are often difficult to conduct and hence characterizing expression in tissues other than the eye is problematic, though its expression in the CNS has been established and expected to express in the PNS too (Krstic *et al.*, 2013).

Furthermore, the white gene has been implicated in a plethora of complex processes such as mating behavior in males, transport of biogenic amines involved in memory formation (Sitaraman *et al.*, 2008), and

cGMP transport in the malpighian tubules (Evans *et al.*, 2008). Varying transport of biogenic amine precursors by White and its ligands, leads to concomitant diminished accumulation and differential release of the neuromodulators histamine, dopamine, and serotonin in mutants of *white*, *scarlet*, and *brown* genes (Borycz *et al.*, 2005, 2008). The effects of reduced serotonin and dopamine have been correlated with the differences in memory performance of the white eye mutants (Sitaraman *et al.*, 2008).

Studies have demonstrated differences in behavior due to varied neuronal action on exposure to volatile general anesthetics (Campbell and Nash, 2001), and recorded reduction in spatial learning (Diegelmann *et al.*, 2006), olfactory learning (Anaka *et al.*, 2008), and suppression of phototactic personality (Kain *et al.*, 2012) amongst white mutants and wild type. White mutants have been observed to court both decapitated males and females in the dark unselectively, or form chains in daylight conditions when placed in groups of males (Zhang and Odenwald, 1995; Hing and Carlson, 1996). It has also been reported that both the mis-expression of *mini-white* or mis-localization of the same and the lack of extra-retinal White can cause alteration in the sexual behavior of male flies (Zhang and Odenwald, 1995; Anaka *et al.*, 2008; Krstic *et al.*, 2013). Further, it is also argued that deficit of White function heightens the sexual arousal of males in general, indirectly leading to male-male courtship (Krstic *et al.*, 2013).

Pre-reproductive and reproductive activities are costly as they make high energetic demands. This is typically manifested as increased death rate of non-virgin female flies as opposed to virgin female flies (Partridge *et al.*, 1986; Partridge and Fowler, 1990), or the high mortality of frequent maters compared to less frequently mating females (Fowler and Partridge, 1989), due to large amount of seminal fluid molecules transferred by males (Chapman *et al.*, 1995). Male *D. melanogaster* flies, too, invest heavily in mating (Cordts and Partridge, 1996). Significant behavior and growth related trade-offs have been seen as a consequence of increased male reproductive effort in *Poecilia reticulate* (Jordan and Brooks, 2010). Cost of reproduction significantly affects life history evolution including key traits such as future survival and age-specific oviposition schedules in animals that reproduce sexually (Partridge and Farquhar, 1983; Partridge *et al.*, 1985). In addition to energetic costs of reproduction, courtship itself carries a cost. Courtship harassment by males is shown to affect the fitness of females in *Drosophila melanogaster* (Partridge and Fowler, 1990). Taken together, the white eye mutants are expected to impose higher cost due to heightened arousal and thus be less fit compared to their wild type relatives.

In this paper we have evaluated the fitness and fitness related traits of white eye mutant and discussed their implication to understanding the evolutionary concept of 'the neutral theory of molecular evolution' (Kimura, 1968, 1983, 1991) that suggests no affect/effect of mutations on fitness of the organism bearing them. We have assessed the effect of white eye mutation on important life history traits namely, pre-adult development time, egg to adult viability, adult weight, lipid content in adult fly, adult longevity, and life time egg production. The results from this study clearly are in support of the 'neutral theory of molecular evolution'.

Materials and Methods

Drosophila melanogaster is well known for its extensive use in research due to its numerous advantages as a model organism. It is a holometabolous insect with life cycle inclusive of four discrete stages-egg, larva, pupa, and adult (imago) form. Wild type (JB) and white eye (WE) populations were used in this study. Ancestry of the wild populations used in this study can be traced back to the IV population (Ives, 1970). They are maintained on a 21 day egg-to-egg discrete generation cycle under standard laboratory conditions of 25±1°C temperature, 24:0 LD cycles and 75±5% relative humidity (Mishra *et al.*, 2017; Chandrashekara and Shakarad, 2011). Adults in cages are given yeast supplement 3 days prior to (on day 18) the egg collection (day 21) for starting the subsequent generation. A fresh uncontaminated food plate is then provided and flies are allowed to lay eggs on it for 18 hours. Eggs from this plate are transferred to 6 mL media vials at approximate densities of 40-60 eggs. Forty such vials were maintained per line. All adults emerging from the 40 vials were transferred to pre-labelled Plexiglass cages on day 12 and provided with fresh food plates every alternate day until day 18. The white eye mutant was a natural mutant isolated from the wild (JB) population and bred to build a complete population by standard back-cross procedure. The white eye mutant population was also maintained on a 21 day egg-to-egg discrete generation cycle. The population maintenance was

identical to the wild type JB populations. The breeding population sizes in both the wild and mutant populations, was 1600-1800 each. The wild JB populations had been through 311 generations, while the white population had been through 40 generations of maintenance in the laboratory at the time of assaying their life history traits.

Fly food: Composition and Preparation

A fine paste of 205 g banana, 35 g jaggery, 25 g barley, and 36 g yeast (Prestige) ground in 180 mL water was added to 12.4 g agar dissolved in 1L water. The mixture was brought to a boil on low flame and cooled down to ~37°C using a cold waterbath. Following this, 2.4 g methyl paraben (Fisher Scientific) dissolved in 45 mL of absolute ethanol (Changshu Yangyuan Chemicals China) was added to the cooled media and mixed thoroughly. 2 mL and 6 mL of media were poured into 8 dram vials for oviposition and developmental time assays, respectively.

Egg to adult Development time Assay

Flies were provided with fresh uncontaminated food plate and allowed to lay eggs for 1 hour. The plates were taken out and eggs were harvested using moist, fine tip camel hair brush. Exactly 50 eggs were counted under stereo zoom microscope and dispensed into 6 mL food vials. 10 vials each were set up for the wild and mutant populations. The vials were incubated under SLC, on pupation the vials were inspected twice daily for eye spots in pupae. On observation of eyespots, vigil checks were conducted every four hours and emerging flies were collected into pre-labelled empty dry vials, sorted based on gender under light CO_2 anaesthesia and transferred to holding vials with fresh media till used in further experiments.

Mid-point of egg laying window was considered to be the 0^{th} hour and mid-points of two successive 4 hourly checks considered as the time of emergence. The 4 hourly checks were carried out until no fly emerged from any of the vials. Finally, the pupae on the walls and those on the bottom of the vials were scored to assess pupation rate.

Adult Dry weight and Lipid Content

Virgin males and females from both mutant and wild populations, from the development time assay were over-etherized using diethyl ether and transferred to pre-labelled, clean glass vials in batches of 10 flies each, and dried at 70°C in oven for 36 hours and weighed to obtain dry weights. The flies were then put into pre-labelled 1.5 mL centrifuge tube to which 1.3 mL diethyl ether (≥99.5 purity, Merck) was added and placed on a gel rocker set to 2000 rpm and rocked for 36 hours at room temperature with a change of ether every 12 hours. The flies were then transferred to new centrifuge tubes and given 1 wash with diethyl ether, dried at 40°C in oven for 1 hour and weighed again to obtain lipid free weights. Three replicate vials per fly type per gender were set up.

Longevity and Lifetime Oviposition Assay

Flies transferred to holding vials were segregated into 40 pairs (one male + one female) and transferred to 2 mL media vials. Flies were subsequently transferred to fresh media vials every 24 hours and eggs laid during the preceding 24 hours were counted under the microscope and recorded. The death of every fly was recorded to calculate longevity. The assay was terminated following the death of all the flies.

Statistical analyses

The egg to adult development time, pupation percentage, egg-to-adult viability, and average longevity data were subjected to two-sample t-test. The dry weight and lipid content data were subjected to mixed model analyses of variance (ANOVA), treating replicate vials as a random factor and fly type and gender as fixed factors crossed with vial. In all cases, the vial means were used as the units of analysis and, therefore, only fixed-factor effects and their interactions can be tested for significance (Prasad *et al.*, 2001). Further, the gender ratio data were analysed using 2×2 contingency χ^2 test. The difference in the adult survival probability curves was analysed by Kaplan-Meier log-rank test (Fisher and Van, 1993).

Results

Egg-To-Adult Development Time

There was a significant effect of population type on egg-to-adult development time (t_{18} = 3.27, p < 0.005). The white eye mutant flies emerged as adults (226.577 h) nearly 3 hours earlier compared to their wild type ancestors (229.53 h). The significant difference in the egg-to-adult development time was largely due the reduction in development time of the males (t_{18} = 3.410, p < 0.005) as compared to the females (t_{18} = 2.653, p < 0.05). The male flies from the mutant population emerged in approximately 9 days and 11 h (227.316 h) compared to 9 days and 15 h (230.794 h) of the ancestral wild populations, and the female mutant flies emerged in nearly 9 days and 10 h (225.963 h) as compared to 9 days and 12 h (228.348 h) of the wild population, respectively (Figure 1).

Figure 1. Mean (±se) of development time for wild and white eye populations.

Dry Weight and Lipid Content

The mutant and their ancestral wild relatives had non-significantly different dry weights ($F_{1,2}$ = 6.23, p = 0.129, Figure 2). The gender of the fly had significant effect on its dry weight ($F_{1,2}$ = 4635.843, p = 0.0002). Average dry weight of male flies from mutant and wild type populations were found to be 257.23 µg and 269.57 µg, whereas those of the females were 364.97 µg and 389.93 µg, respectively. The fly type × gender interaction was not significant ($F_{1,2}$ = 7.467, p = 0.112).

Lipid content of the flies was not affected by the fly type ($F_{1,2}$ = 7.332, p = 0.114, Figure 3). The gender of the flies had significant effect on its lipid content ($F_{1,2}$ = 280.957, p = 0.004). Fly type × gender ($F_{1,2}$ = 0.121, p = 0.761) interaction was not significant. Both male and female wild type flies had non-significantly higher lipid content with 50.17 µg and 67.2 µg than their WE mutant counterparts with 41.43 µg and 60.7 µg, respectively.

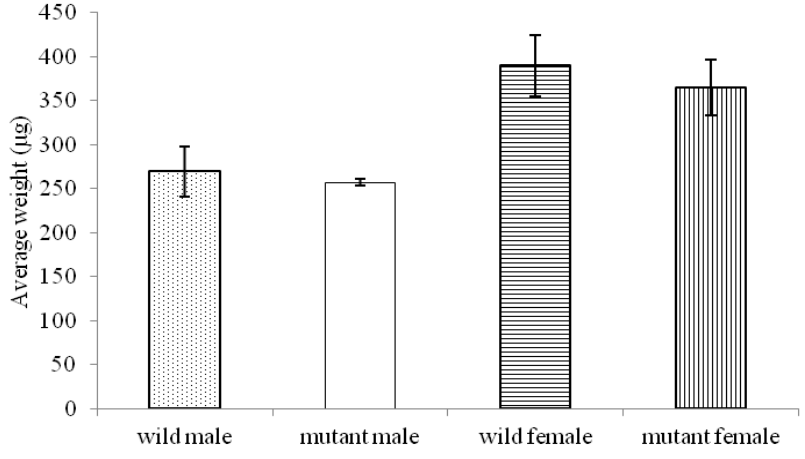

Figure 2. Mean (±se) dry weight of male and female flies from the wild and white eye populations.

Pupation, Viability and Gender Ratio

There was no significant effect of mutation on percentage pupae formed (t_{18} = 0.709, p > 0.05; Figure 4) as well as egg to adult viability (t_{18} = 0.795, p > 0.05; Figure 5). The mean pupation and viability for wild and WE flies was found to be 87% and 88.8%, and 84.2% and 86.6%, respectively. There was no significant effect of fly type on the sex ratio of the emerging flies (χ^2 = 0.101, p > 0.05, Figure 6).

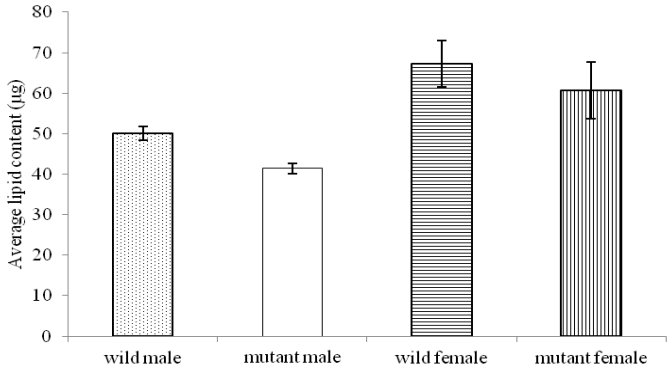

Figure 3. Mean (±se) lipid content in wild and white eye populations.

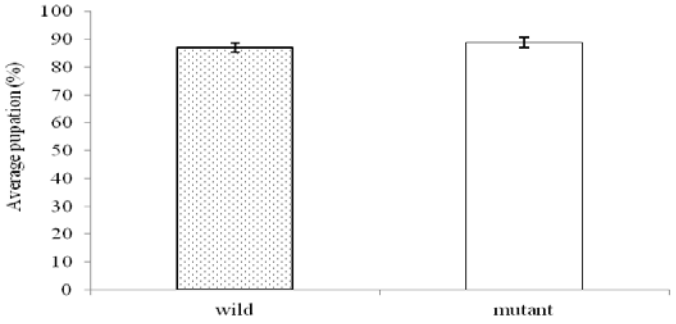

Figure 4. Mean (±se) percentage pupation for wild and white eye populations.

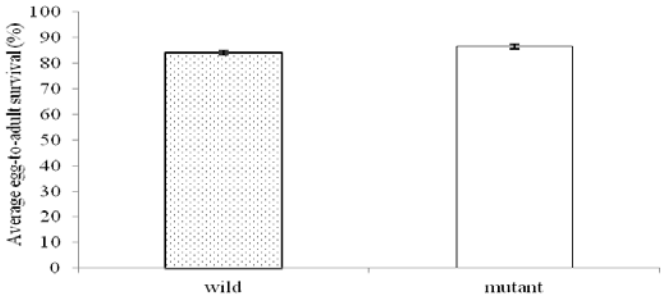

Figure 5. Mean (±se) of egg-to-adult viability of wild and white eye populations.

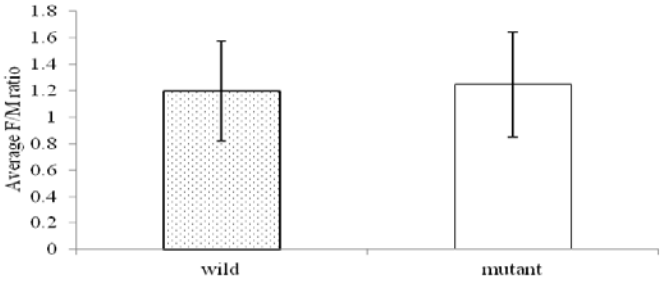

Figure 6. Mean (±se) of sex ratio of the wild and white eye populations.

Longevity and Survival Probability

Average longevities of the white eye and wild-type flies pooled across genders were not significantly different ($t_{74} = 0.529$, $p > 0.05$; Figure 7), even though mutant population lived ~ 2 days longer. Overall, there was a significant effect of gender on the average longevity ($t_{74} = 2.923$, $p < 0.01$). The average longevity of male flies pooled across population types was 44.57 d as against 36.34 d in females. Average lifespan of adult wild type males was 42.73 days as compared to 46.31 days of the mutants, whereas that of the wild-type and

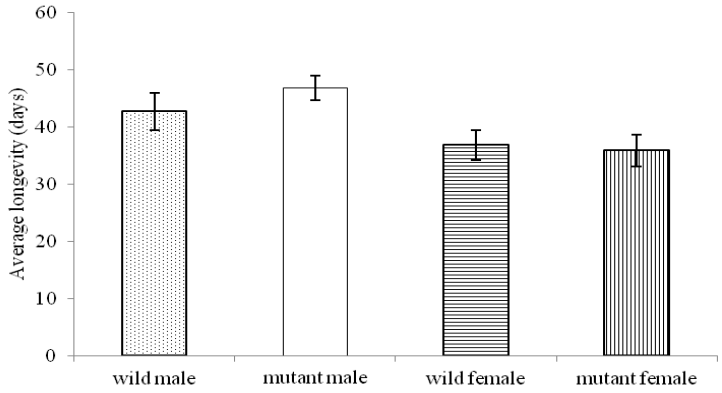

Figure 7. Mean (±se) longevity of wild and white eye populations.

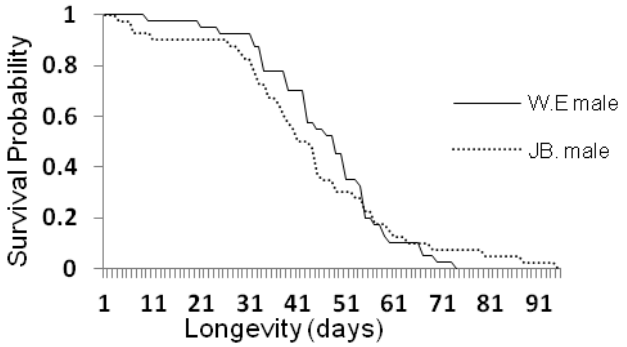

Figure 8. Kaplan-Meir survival curves for mutant and wild type males.

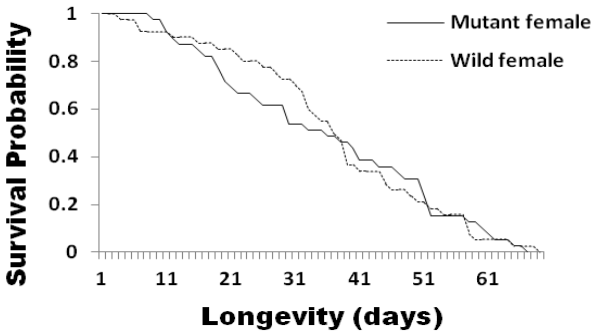

Figure 9. Kaplan-Meir survival curves for mutant (WE) and wild type female flies.

mutant females was 36.78 days and 35.92 days, respectively. The average is only a descriptive statistic that does not provide an insight into the biological process(es), thus necessitating comparison of the survival probabilities (Chandrashekara and Shakarad, 2011; Chandrashekara *et al.,* 2014). The survival probability of the white eye mutant and their ancestral wild type flies were significantly different for males ($\chi^2 = 24.19962$, $p < 0.001$; Figure 8) as well as females ($\chi^2 = 35.95932$, $p < 0.001$; Figure 9).

Lifetime Oviposition

Although an average WE mutant fly laid fewer eggs (400.05) compared to its wild relative (435.82), the differences were not statistically significant ($t_{37} = 1.074$, $p > 0.05$; Figure 10).

Discussion

Pre-adult development time is an important trait that has a direct impact on the life-history in all holometabolous insect species, especially those that thrive on ephemeral habitat, such as *Drosophila melanogaster*. The significant reduction in the white eye mutant development time compared to their wild type ancestors could be due to founder effect as single male fly was identified and expanded into a full population by crossing with 100 virgin female siblings. Unlike other studies that showed significant reduction in adult body size as a correlated response (Zwaan *et al.*, 1995; Nunney, 1996; Chippindale *et al.*, 1997; Prasad *et al.*, 2000, 2001; Handa *et al.*, 2014), the reduction in body size was only marginal in the present study. Further, the other associated traits, namely lipid content, longevity, viability, pupation, gender ratio, and

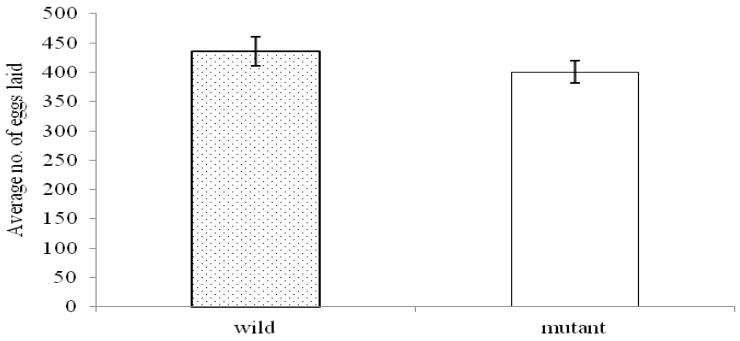

Figure 10. Mean (±se) life-time oviposition for female flies from wild and white eye populations.

oviposition did not show any significant difference between the white eye and wild-type populations. The non-significant decrease in the body size of the white eye flies was accompanied by non-significant decrease in the life-time oviposition, which in turn was accompanied by non-significant increase in the longevity. The relationship between fecundity and female size is shown to be close to 1 in many insect species (Honek, 1993). In addition, several studies have reported a trade-off between reproduction and longevity (Chippindale *et al*., 1993; Chapman and Partridge, 1996; Tu and Tatar, 2003). The reduction in life-time oviposition of white eye mutants in the present study could either be due to courtship harassement by males (Partridge and Fowler, 1990) or due to reduced courtship, a result of lowered sexual arousal exhibited by the white eye mutant males (Krstic *et al*., 2013). Partridge and Fowler (1990) also reported a reduction in longevity of females as a result of exposure to courting males. However, the non-significant increase in the longevity of the white eye mutants in our study suggests that the males are perhaps in a lowered arousal state (Krstic *et al*., 2013).

Reproductive life-span is an important fitness trait for all iteroparous organisms. Reproductive life-span is a composite variable trait influenced by the genotype for longevity and reproduction, both of which are highly influenced by the environment. The variability in any biological trait is due to mutations and shuffling of genome due to sexual reproduction. Our results show that white eye mutation does not significantly affect the fitness of the organisms, yet has the potential to alter the course of evolution through drift. This is the first study that provides experimental support to neutral theory of molecular evolution (Kimura, 1968, 1983, 1991).

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Genetic composition of seven microsatellite DNA loci of females and males from a natural population of *Drosophila mediopunctata* collected in a highland Araucaria forest fragment of Brazil.

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Introduction

As evolutionary processes primarily act shaping the genetic variability of a population, studies on genetic variability of natural populations are extremely important to understand the evolutionary history of a species. *Drosophila mediopunctata* belongs to the *tripunctata* group (Frota-Pessoa, 1954), which is endemic of the Neotropical region and includes 64 species that inhabit forest fragments (Vilela and Bächli, 2000). It is the second largest group of *Drosophila* in this region, and the largest of Neotropical forests (Klaczko, 2006). *Drosophila mediopunctata* is a strictly forest dwelling species that is not associated to human habitats, it has a wide geographic distribution and can be found in Brazil and El Salvador (Val *et al.*, 1981). It is very abundant in some areas, particularly in the south of its distribution or in high altitudes during the coldest months of the year (Saavedra *et al.*, 1995).

Laborda *et al.* (2009) described 134 microsatellite DNA loci for *D. mediopunctata*, and Cavasini *et al.* (2015) establish the chromosomal location of seventeen of these loci, one from each of the five major linkage groups previously published (Laborda *et al.*, 2012), and twelve new loci. So, a very important and well-described genetic marker is available for this species. Thus, the objective of this work was to perform a preliminary analysis of the genetic composition of seven microsatellite DNA loci of naturally collected females of *D. mediopunctata* and their offspring (from which we were able to infer the parental male composition), obtained from a highland Araucaria forest fragment in the South of Brazil.

Material and Methods

This work was performed in a fragment of highland Araucaria Forest phytophysiognomy (Mixed Ombrophylous Forest) of the Atlantic Forest biome, named Parque Municipal das Araucárias (25°23'36" S, 51°27'19" W), where *Araucaria angustifolia* (Coniferae: Araucariaceae) is the predominant vegetal species. This fragment is located in the third plateau of the State of Parana, Brazil, in the subregion named as Plateau of Guarapuava by Maack (1981). According to the climatic classification of Köeppen, this subregion has humid

and superhumid mesothermic climate, without dry seasons and with fresh summers. The mean temperatures in hottest months are below 22ºC. Frosts are severe and frequent, and the average temperature in the coldest month is 12.9ºC.

The collection was performed according to dos Santos *et al.* (2010), using fermented bananas and oranges on plates on the ground. The collected females of *D. mediopunctata* were put individually into vials containing standard *Drosophila* culture medium (banana/agar) and the offspring (when produced) were transferred to a new vial, thus obtaining isofemale lines. From each isofemale line, the parental female and 15 F1 females were separated and stored at -20°C in 1.5 mL microtubes containing 70% ethanol. DNA extractions were performed using Promega Kit, EDTA (pH = 8.0), Proteinase K (20 mg/mL), and isopropanol.

Seven microsatellite loci (SSR034, SSR057, SSR087, SSR095, SSR096, SSR099, and SSR118) were amplified through PCR using a touchdown program, varying the annealing temperature from 65°C to 48°C, lowering 1°C each cycle, as described by Laborda *et al.* (2009). The PCR products were resolved in 6% PAGE (Machado *et al.*, 2003) and stained using silver nitrate (Sanguinetti *et al.*, 1994). All gels were photographed using the L-Pix (Loccus) image system, and the alleles were numbered sequentially from the smallest to the largest. From our sample we were able to identify the genetic composition of the collected parental female and to identify the parental male composition through the F1 female. The presence of null alleles was detected as described in Machado *et al.* (2010).

Table 1. Genetic composition of seven microsatellite DNA loci of females and males of *Drosophila mediopunctata* obtained from the natural population of Parque Municipal das Araucárias, Guarapuava/PR. X = presence; - = absence

		Alleles									
Loci	Sex	null	1	2	3	4	5	6	7	8	9
SSR034	F	X	X	X	-	-	X	X	X	X	X
	M	-	X	X	X	X	-	X	X	X	X
SSR057	F	-	X	X	X	-	-	-	-	-	-
	M	-	X	X	X	X	-	-	-	-	-
SSR087	F	-	X	X	X	-	-	-	-	-	-
	M	-	X	X	X	-	-	-	-	-	-
SSR095	F	-	X	X	X	X	X	-	-	-	-
	M	-	X	X	X	X	X	X	-	-	-
SSR096	F	X	X	X	X	X	-	-	-	-	-
	M	-	X	X	X	X	-	-	-	-	-
SSR099	F	-	X	X	X	X	-	-	-	-	-
	M	-	X	X	X	-	-	-	-	-	-
SSR118	F	X	X	-	X	X	X	-	-	-	-
	M	-	X	X	X	X	X	X	-	-	-

Results and Discussion

The collections and establishment of isofemale lines from Parque Municipal das Araucarias resulted in 8 strains of *Drosophila mediopunctata*. From each of these 8 strains, the analysis of the parental female and their 15 F1 females generated the genetic composition of females and males presented in Table 1. The SSR034 was the locus with the highest number found of alleles (8 in both sexes), and SSR087 had the lowest number (3 in both sexes). Null alleles were found in three loci, SSR034, SSR096, and SSR118, out of seven analyzed. Laborda *et al.* (2009) studied the genetic variability of thirteen strains of *D. mediopunctata* and in general they detected higher number of alleles than our results. The number of alleles observed in their work was 9 for SSR034, 6 for SSR057, 5 for SSR087, 6 for SSR095, 9 for SSR096, 3 for SSR099, and 5 for SSR118. These differences in the number of alleles detected in these two samples should be due to the sample size of each work. We analyzed 8 females and their F1 females, which resulted in the inferred genetic

composition of the parental males. Laborda *et al.* (2009) performed their study using 30 flies from each strain. Therefore, it is expected that if we analyze more samples our number of alleles must increase.

This work was a preliminary study that will be important to generate data to evaluate the number of parental males that contributed to the formation of each isofemale line, which will be critical to understand genetic parameters of sperm competition in *Drosophila mediopunctata*. Sperm competition is one of several aspects that are relevant to understand reproductive characters in this species.

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The invasive *Drosophila suzukii* (Diptera: Drosophilidae) uses native plant species of the Brazilian savanna as hosts.

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Introduction

Drosophila suzukii (Matsumura), also known as spotted wing drosophila, is a native species from western Asia, characterized by damaging soft-skinned and unwounded fruits to perform oviposition (Acheampong, 2010). For this reason, this species is considered an important pest, especially in agricultural systems, bringing economic loss to diverse crops (*e.g.*, strawberry, Wollmann *et al.*, 2016). In recent decades, surveys of the occurrence of this species have shown its rapid expansion around the world (*i.e.*, Asia, Europe, South and North America). This wide geographic distribution of *D. suzukii* suggests high potential of colonization of different environments, as well as high thermal tolerance (Cini *et al.*, 2012). Furthermore, its short life cycle and high fecundity capacity (average production of 600 eggs throughout its life cycle) allow an explosive population growth in this species when under favorable conditions (Cini *et al.*, 2012).

In Brazil, records of this species are recent and occurred in the south and center-west of the country. Paula *et al.* (2014) were the first to find individuals of this species in the Cerrado region in 2013, a biome known for its high biodiversity, and threatened by the constant agricultural expansion (Lahsen *et al.*, 2016). However, little is known about the influence of this species on natural environments and its effect as an invasive species and fruit pest of native species. In this study, we document and discuss about *D. suzukii* as a fruit predator of three native species of Cerrado biome.

Materials and Methods

This study was conducted at IBGE Ecological Reserve (15° 56'S, 47° 53'W), a protected area located in the south of Federal District, Brazil, with more than 10,000 ha (Paula *et al.*, 2014). This site is embedded in

the biome Cerrado, a hotspot of biodiversity (Myers *et al.*, 2000). In our study site, vegetation type is cerrado *stricto sensu*, characterized by the presence of continuous ground layer of grasses, and a cover (ranging from 10-60%) of shrubs and wood trees. The climate is tropical (Köppen Aw), characterized by dry (May to September) and wet (October to April) seasons.

The samplings occurred from September to December (2016) in an experimental area consisting of different treatments of soil fertilization with sixteen 15 × 15 m plots separated by at least 10 m from each other. Since 1998, soil nutrients (N, P, N plus P and Ca) are applied in these plots, which are arranged in a randomized design (see Bustamante *et al.*, 2012). For this work, two transects (15 × 1 meters) were established in each plot. In each transect, the number of seed units per plant species was counted, a seed unit typically being a fruit, or head in the case of Asteraceae. Subsequently, for individuals with less than 100 units per transect all seed units were collected, while for plant species with larger number of seed units, a total of 100 seed units per individual were collected. In the laboratory, biweekly checks (five in total for each) were made for each paper bag to the collection of insects (adults) that emerged from the seed units. The insects were stored in eppendorf tubes with 70% alcohol for later identification by taxonomists.

Figure 1. Male of spotted wing drosophila (*D. suzukii*) collected in fruits of native plant species of Cerrado.

Results

We collected more than 800 insects (Coleoptera, Diptera, Hymenoptera, and Lepidoptera) from 32 plant morphospecies. Of a total of 92 Diptera individuals, *ca*. 68% belonged to the genus *Drosophila*. Males of the exotic *Drosophila suzukii* (Figure 1) were reared from three native plant species: *Miconia albicans* (Sw.), Triana (Melastomataceae), *M. fallax* DC, and *Blepharocalyx salicifolius* (Kunth) O. Berg (Myrtaceae). Considering only these three plant species, we collected in total 2498 seed units, out of which 55.4% were from *M. albicans*, 20.4% from *M. fallax*, and 24.0% from *B. salicifolius*. The incidence rate of *D. suzuki* males was similar in all three species (see Figure 2). Further taxonomic checks are needed to verify the incidence of females.

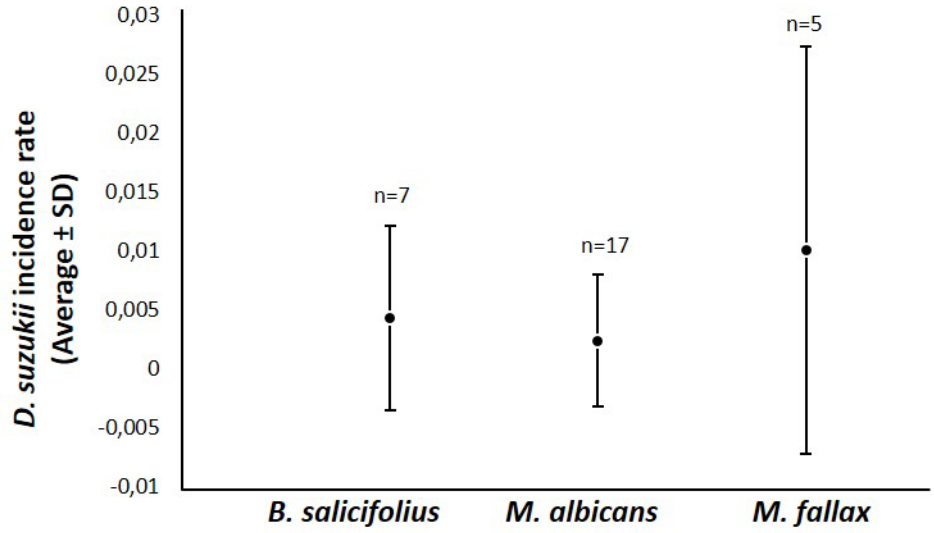

Figure 2. Incidence rate of *D. suzukii* in three plant species of cerrado.

Discussion

This study shows that the invasive *Drosophila suzukii* is able to use native species of Brazilian savannas as larval resources. As expected, the samplings occurred from the beginning of the rainy season, because it is a season where breeding sites are more available to drosophilid populations (Paula *et al.*, 2014). Annual precipitation is an important abiotic variable for the establishment of populations of *D. suzukii* (dos Santos *et al.*, 2017), and the southern region of Brazil is thought to have more favorable climatic conditions to the establishment success of *D. suzukii* than the study areas (Benito *et al.*, 2016). However, our findings indicate that this species is not occurring occasionally, but already established in the study region. As most crop pests can sustain their populations within agricultural regions, such establishment is likely a spill-over effect from fruit production areas in Cerrado region, and have the potential of threatening native plant populations.

Studies indicate that the greatest losses on fruit production through oviposition of *D. suzukii* occur in species of plants with small fruits (Werts and Green, 2014). Plants of the genus *Miconia* (Melastomataceae), characterized by their wide distribution in the cerrado biome, usually have small and fleshy fruits, which would explain the greater number of flies emerging from their fruits in this work. These results reinforce the need to consider the management of exotic pests not only in agricultural systems but also in natural ecosystems given the potential for invasion *of D. suzukii* and its negative effect on the reproductive success of native species. More surveys of the occurrence of this species in Cerrado biome should be done to better evaluate its current distribution in that region. Finally, the identification of spotted wing drosophila' parasitoids in Cerrado areas would be helpful for the creation of efficient management strategies including these potential biocontrol agents.

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Expression of *UAS-lacZ*$^{4\text{-}2\text{-}4b}$ under the control of *elav-Gal4* significantly reduces lifespan in *Drosophila melanogaster.*

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Introduction

The *UAS/Gal4* system created by Brand and Perrimon (1993) is used extensively in *Drosophila* for the expression of transgenes under specific conditions. This technique can utilize ribonucleic acid interference (RNAi) to post-transcriptionally silence endogenous genes (Fire *et al.*, 1998), in order to determine the phenotypic consequences of loss of function and deduce their functions and mechanisms (Perrimon *et al.*,

2010; Ni *et al*., 2011). Both the presence of *Gal4* and the activity of the responding transgene can influence the phenotypes in *Drosophila melanogaster*; therefore, negative control critical class individuals must be generated using the most benign transgene(s) possible.

While it has been commonly believed that *Gal4* transgenes produce few negative effects in *Drosophila melanogaster*, this is not always true. For example, *GMR-Gal4*^{12} expression can result in an apoptosis-dependent "rough-eye" phenotype (Kramer and Staveley, 2003). In addition, maternally-inherited *arm-Gal4* in combination with *UAS-lacZ*$^{4-1-2}$ reduces the lifespan of critical class males (Slade and Staveley, 2015). Finally, *UAS-GFP*, which has been often utilized as a benign transgene in negative controls, has been shown to reduce lifespan and to impair climbing ability over time when expressed in some neuronal tissues (Mawhinney and Staveley, 2011). As transgenes commonly believed to be benign have been shown to produce subtle negative effects, examination of the commonly-used control transgenes is warranted.

The *UAS-lacZ* transgene is one of the most common negative controls utilized in such experiments. Multiple insertions of this transgene exist, including *UAS-lacZ*$^{4-1-2}$ and *UAS-lacZ*$^{4-2-4b}$. Our research group has conducted a number of experiments using *UAS-lacZ*$^{4-1-2}$ as a negative control, which have shown that this transgene results in a longer median lifespan than RNAi constructs which inhibit endogenous genes such as the autophagy genes, *Autophagy-related 6* or *Atg6* and *Phosphotidylinositol 3 kinase 59F* or *Pi3K59F* (M'Angale and Staveley, 2016a), *CG2076/GHITM* (M'Angale and Staveley, 2016b), *Mitochondrial calcium uptake 1* or *Micu1* (M'Angale and Staveley, 2017a), and *Pdxk* (M'Angale and Staveley, 2017b). However, while several experiments have utilized *UAS-lacZ*$^{4-2-4b}$ as a negative control (Armstrong *et al*., 2002; Elfring *et al*., 1998), a comparison of the effects of this transgene with those of the *UAS-lacZ*$^{4-1-2}$ transgene seemed desirable. The aim of the present study is to determine if the directed expression of *UAS-lacZ*$^{4-2-4b}$ may result in differences in lifespan compared to the standard *UAS-lacZ*$^{4-1-2}$ control.

Materials and Methods

Drosophila stocks and media: *UAS-lacZ*$^{4-1-2}$, *UAS-lacZ*$^{4-2-4b}$, *arm-Gal4*, and *elav-Gal4* lines were obtained from the Bloomington Drosophila Stock Center at Indiana University. Stocks and crosses were maintained on a medium consisting of 65 g/L cornmeal, 15 g/L yeast, 5.5 g/L agar, and 50 ml/L fancy grade molasses. In order to inhibit the growth of mold, 5 mL of 0.1 g/mL methyl paraben in ethanol and 2.5 mL of propionic acid were added to the medium. Stocks were maintained at room temperature (22ºC).

Drosophila crosses: To produce critical class males, *arm-Gal4* or *elav-Gal4* females were crossed with either *UAS-lacZ*$^{4-1-2}$ or *UAS-lacZ*$^{4-2-4b}$ males. Crosses were performed at either 22ºC or 29ºC.

Longevity assays: Longevity assays comparing the lifespan of the critical class progeny of the crosses described above were conducted at 22ºC and 29ºC. Critical class male flies were collected from each genotype and were placed in vials containing no more than 25 flies to avoid overcrowding. Every two days following the initial isolation date, the vials were examined to determine the number of dead flies, and fresh medium was supplied every six days. GraphPad Prism 5.03 was used to create survival curves for the longevity experiment and to conduct the Mantel-Cox test of statistical significance.

Results and Discussion

The expression of *UAS-lacZ*$^{4-2-4b}$ under the direction of maternal *elav-Gal4* resulted in a shorter lifespan compared to that of *UAS-lacZ*$^{4-1-2}$ at both 22ºC and 29ºC, as determined by the Mantel-Cox test ($p < 0.0001$). The median lifespan of the *elav-Gal4/UAS-lacZ*$^{4-1-2}$ flies was 90 days at 22ºC and 56 days at 29ºC, while the *elav-Gal4/UAS-lacZ*$^{4-2-4b}$ flies lived only 72 days at 22ºC, and 40 days at 29ºC. There was no significant difference in the lifespan of flies expressing *UAS-lacZ*$^{4-1-2}$ versus *UAS-lacZ*$^{4-2-4b}$ under the control of *arm-Gal4* at either temperature. The *arm-Gal4/UAS-lacZ*$^{4-1-2}$ flies had a median lifespan of 62 days at 22ºC, and 44 days at 29ºC, while the *arm-Gal4/UAS-lacZ*$^{4-2-4b}$ flies survived for 60 days at 22ºC and 44 days at 29ºC.

From this study, it is evident that *UAS-lacZ*$^{4-1-2}$ and *UAS-lacZ*$^{4-2-4b}$ can have different effects on lifespan, depending on the tissues in which they are expressed. Low-level ubiquitous expression of these transgenes, directed by *arm-Gal4*, does not appear to affect lifespan, whereas expression in the neurons, directed by *elav-Gal4*, caused a reduced lifespan in the flies expressing *UAS-lacZ*$^{4-2-4b}$. From this, we can

conclude that while both *UAS-lacZ* transgenes can result in the same findings when expressed in certain conditions, caution should be exercised when using *UAS-lacZ*$^{4\text{-}2\text{-}4b}$ as a negative control, as the short lifespan that can result may lead researchers to make erroneous conclusions with regards to the effects of certain transgenes on survival. Future studies should investigate whether the differences in lifespan observed between *UAS-lacZ*$^{4\text{-}1\text{-}2}$ and *UAS-lacZ*$^{4\text{-}2\text{-}4b}$ arise when other common *Gal4* lines are used to direct their expression.

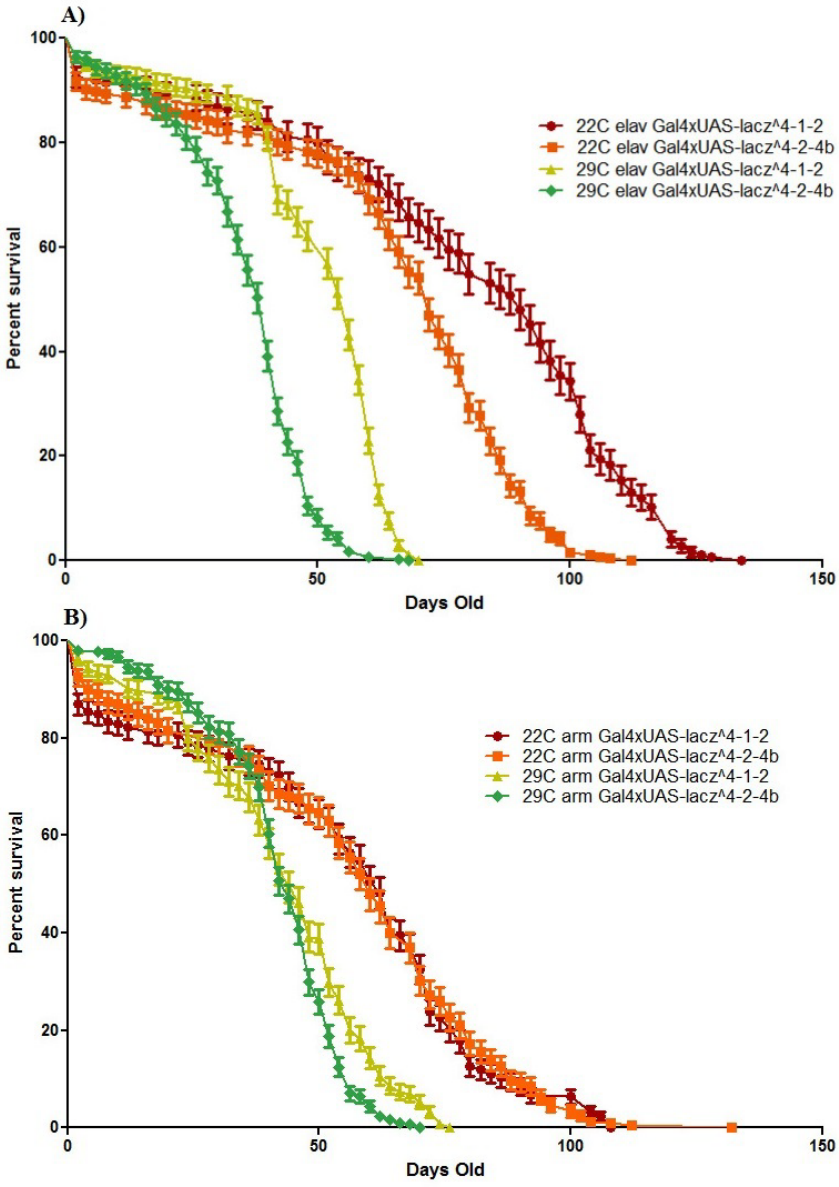

Figure 1. Survival of critical class males possessing either *UAS-lacZ*$^{4\text{-}1\text{-}2}$ or *UAS-lacZ*$^{4\text{-}2\text{-}4b}$ controlled by either A) maternal *elav-Gal4* or B) maternal *arm-Gal4* at 22ºC and 29ºC. Genotypes are *elav-Gal4/UAS-lacZ*$^{4\text{-}1\text{-}2}$ (n = 175 @ 22ºC; n = 301 @ 29ºC), *elav-Gal4/UAS-lacZ*$^{4\text{-}2\text{-}4b}$ (n = 266 @ 22ºC; n = 304 @ 29ºC), *arm-Gal4/UAS-lacZ*$^{4\text{-}1\text{-}2}$ (n = 253 @ 22ºC; n = 245 @ 29ºC), and *arm-Gal4/UAS-lacZ*$^{4\text{-}2\text{-}4b}$ (n = 238 @ 22ºC; n = 298 @ 29ºC). Error bars represent the standard error of the mean.

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Hydrogen peroxide, toluene, and dimethyl sulfoxide affect some development and reproductive parameters in *Drosophila melanogaster*.

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Abstract

Hydrogen peroxide (H_2O_2) is a compound used in numerous daily use products and exposure to them has been related to disturbing effects on human and animal reproduction and the increase of reactive oxygen species (ROS). Because high levels of Cyp450s enzymes have been correlated with an increase in ROS, we treated third instar larvae of flare (*flr*^{3}) and OR(R)-flare (OR(R)) strains (P_0), that have inducible and high levels of Cyp450s enzymes, respectively, to search the *in vivo* effects of hydrogen peroxide (H_2O_2, 20 mM) treatments on development (total and daily emergence, sexual proportion) and reproductive parameters (total fecundity, reproductive performance, and fertility percentage) in *D. melanogaster*. In addition, the emergence of the F_1 was tested to search for a putative hereditary effect. H_2O_2 did not alter the reproductive parameters but it modified development ones. Toluene (TOL, 10 mM), used as a positive control, affected significantly the development and reproductive traits with significant differences between the strains, confirming Cyp450s' participation in TOL's metabolism. DMSO, TOL's solvent, altered the sexual proportion and the total fecundity. No effect was observed on the F_1 in any of the traits studied. Our results agree with the concern that chronic exposure to these compounds could affect some development and reproductive processes in *D. melanogaster*. Keywords: oxidative stress, toxicity, cryoprotectant, chronic exposure.

Introduction

People and other living beings may subject themselves to an increased oxidation stress through exposure to chemicals that generate Reactive Oxygen Species (ROS) or increase free radicals; therefore, it is possible that constant exposure to such chemicals in high enough or chronic concentrations may cause disorders related to infertility (Agarwal *et al*., 2006). Hydrogen peroxide (H_2O_2) increases free radicals as hydroxyl radical ($^{\bullet}OH$) (Halliwell and Gutteridge, 2007) and is used as an active compound in numerous daily use products (Watt *et al*., 2004). The major source of human exposure to H_2O_2 is through the use of hair dyes. More than 60% of women and 5% to 10% of men dye their hair an average of 6-8 times a year (Benaiges, 2007) and H_2O_2 represents between 6% and 12% of the oxidant solution in the product. Heikkinen *et al.* (2015) suggested an association between the uses of hair dyes with breast cancer incidence. Oehninger *et al.* (1995) reported that H_2O_2 directly affects the human spermatic functions. Pandey *et al.* (2010) found that H_2O_2 acts as a signaling molecule and modulates diverse aspects of the oocyte physiology, such as meiotic cell cycle arrest. Cui *et al.* (2011) observed that H_2O_2 moderately increases the ROS content in porcine oocytes. In other contexts, humans are exposed to toluene (TOL) through products where it is used as a solvent. Exposure to TOL increases the generation of ROS (Kodavanti *et al*., 2015; Sarma *et al*., 2011) that may lead to neurodevelopmental disabilities (Grandjean and Landrigan, 2014; Win-Shwe *et al*., 2007). Long term exposure to TOL can induce apoptosis (Al-Ghamdi *et al*., 2004; El-Nabi Kamel *et al*., 2008) and exposed paint workers are at genotoxic risk (Priya *et al*., 2015); it has been suggested that its genotoxicity effects increase the risk of cancer (Kang *et al*., 2013). Bowen and Hannigan (2013) showed some negative effects of TOL in the reproductive performance of rats, while Webb *et al.* (2014) showed that similar effects are also observed in humans. Kawamoto *et al*. (1995) correlated the human cytochrome P450s (CYP450s), CYP1A1 and CYP2E1, with the metabolism of TOL, and Nakajima *et al*. (1997) described CYP2E1 as the main enzyme in producing benzyl alcohol, with other CYP450s enzymes participating in the production of *o*- and *p*-cresol, but Kim *et al*.

(2015) reported that they only found significant correlations between TOL´s metabolism and glutathione S-transferase (GSTM1 or GSTT1) enzymes. Singh *et al.* (2009) evaluated the effect of TOL in *Drosophila melanogaster* larvae, with inducible Cyp450s enzymes, as a response to oxidative stress. The solvent dimethyl sulfoxide (DMSO) is widely used in laboratories (Hallare *et al*., 2004; Turan *et al*., 2008) and direct exposure to it is limited. But in medicine, DMSO is also widely used as a vehicle for many pharmaceuticals (Santos *et al*., 2003), because it easily penetrates the skin, as an analgesic for muscle and joint pain (Ruso and Santarelli, 2016), and also is used as a chemically related nutritional supplement for the treatment of osteoarthritis (Brien *et al*., 2008). It has been reported to have radioprotective effect against the ROS produced by radiation (Cigarran *et al*., 2004; Jia *et al*., 2010), but it is concerning that it is also used as a cryoprotectant agent in embryo vitrification (Oikonomou *et al*., 2017). Taking in account that in *D. melanogaster* it had been demonstrated that Cyp450s' activity is analogous to the vertebrate liver and immune system (Yang *et al*., 2007), and there is a 80-90% global identity with mammals, for nucleotides and proteins' sequences of functional domains (Bhan and Nichols, 2011), we tested *in vivo* the capacity of H_2O_2, TOL, and DMSO to alter development and reproductive processes through chronic treatments in two *D. melanogaster* strains that differ in their Cyp450s levels: the flare (flr^3) strain with basal o inducible levels of Cyp450s, and the insecticide resistant Oregon-flare (OR(R)) strain with higher levels of these xenobiotic enzymes (Graf and van Schaik, 1992; Saner *et al*., 1996).

Methods

D. melanogaster

Eggs from flr^3 (flr^3/TM3, Bd^{Ser}) and OR(R) (*ORR*(1);*ORR*(2);flr^3/TM3, Bd^{Ser}) strains (full descriptions of the genetic characteristics of these strains may be found in Lindsley and Zimm, 1992) were collected 8 h separately by shaking the flies into bottles of 250 mL (Graf and van Schaik, 1992); the eggs collection bottles were kept undisturbed at 25°C and a relative humidity (RH) of 65 %. Three days later, the 72 ± 4 h, third instar larvae were collected through a fine-meshed stainless steel strainer and thoroughly washed free of yeast (Guzmán-Rincón and Graf, 1995).

Chemicals

H_2O_2 30% (w/w) stabilized solution (Sigma-Aldrich, Steinheim, Germany), CAS No. 7722-84-1; TOL 99.5% (J.T. Baker, México), CAS No. 108-88-3 (10 mM); DMSO 99.9% (Fluka Chemie, Switzerland), CAS No. 67-68-5 (0.3%); *Drosophila Instant Media* (DIM) (Carolina Biological Supply Co. Burlington N.C. USA).

Mortality assay with H_2O_2

Four independent experiments for each strain were carried out with three replicates for each of the following aqueous H_2O_2 concentrations: 5, 25, 50, 100, 150, and 200 millimolar (mM) (Courgeon *et al*., 1988). Third instar larvae were transferred to each vial and incubated at 25°C and 65% of RH until emergence of imagoes which, in turn, were recovered to quantify survival from 0-100% for each treatment. Finally, these data were plotted against the previously mentioned concentrations to calculate LC_{50} and LC_{20} with the correlation equation.

Chronic exposure

Third instar larvae from both strains were divided into four groups, each one consisting of five vials; two independent experiments were done. Each group was exposed to either: 0.5 g of DIM with 2 mL of milli-Q water (W) as negative control; H_2O_2 [20 mM] dissolved in W; TOL [10 mM] as positive control (Singh *et al*., 2009) dissolved in DMSO (0.3%); DMSO (0.3%) as solvent control (Nazir *et al*., 2003) dissolved in W. The concentration of H_2O_2 used corresponded to subtoxic LC_{20} and was chosen according to our results in the survival assay. All the vials remained approximately during 48 h in the incubator at 25°C and 65% of RH until emergence of imagoes.

Development Parameters

Total and daily emergence

The total emergence was calculated according to Arellano (2002), with some modifications:

$$Total\ emergence = \frac{F(X_i) + M(X_i)}{F(0) + M(0)}$$

$F(X_i) = Number\ of\ emerged\ females\ in\ the\ corresponding\ treatment.$

$M(X_i) = Number\ of\ emerged\ males\ in\ the\ corresponding\ treatment.$

$F(0) = Number\ of\ emerged\ females\ in\ the\ negative\ control.$

$M(0) = Number\ of\ emerged\ males\ in\ the\ negative\ control.$

This calculation was carried out in the exposed generation (P_0) to test for an acute effect and the descendants (F_1) to test for a possible hereditary effect in emergence values (Espinoza-Navarro *et al*., 2009). To obtain the daily emergence data, the number of imagoes from each group was daily counted between the 10th and 12th day after egg laying.

Sexual proportion

After emergence, imagoes from P_0 were segregated by sex and quantified. The sexual proportion (SP) for females (F) and males (M) corresponding to each treatment were calculated according to Arellano (2002):

$$SP = \frac{F\ (X_i)}{F\ (X_i) + M\ (X_i)}$$

$$SP = \frac{M\ (X_i)}{F\ (X_i) + M\ (X_i)}$$

Reproductive parameters

The reproductive parameters were calculated according to Gayathri and Krishnamurthy (1981) with some modifications:

Total fecundity

After emergence, virgin female and male flies (P_0) obtained from the incubation of chronically exposed larvae were segregated by sex and isolated in vials for 6 h. Five pairs of flies obtained from each treatment and strain were then transferred to individual plastic vials with media (Martínez-Castillo *et al*., (2012). For a period of ten days, the flies were daily transferred to new vials containing media to count the number of eggs they laid. The number of laid eggs was used in turn to determine the total fecundity (total laid eggs/ 10 days).

Reproductive performance and fertility percentage

The reproductive performance was calculated as the total number of hatched imagoes along 10 days. The fertility percentage was calculated based on the number of hatched flies in 10 days/ total laid eggs in 10 days × 100.

Statistical analyses

The development and reproductive parameters were individually analyzed by factorial ANOVA with *D. melanogaster* strain, sex, day, and treatment as factors, and also all the possible interactions between them. In the case of an ANOVA's difference ($p < 0.05$) the Fisher's Least Significant Difference (LSD) test was then used to determine whether or not there were differences ($p < 0.05$) between the factors. All the statistical analyses were performed using Minitab 17 software. Box plots were done with data where the box represents

50% with median as a horizontal line; the other 50% is represented by vertical lines and asterisk represents data out of range.

Results

Mortality assay with H_2O_2

Mortality assay's results (LC_{50}) were analyzed as a single data group, since only differences between the concentrations were observed (concentrations: $F = 37.54$, $p = 0.0$). Quadratic curves (*flr*^{3} strain: $r^2 = 0.8158$; OR(R) strain: $r^2 = 0.8638$) indicated that the LC_{50} of H_2O_2 was 38 mM for the *flr*^{3} strain and 48 mM for the OR(R) strain. Although the lineal slope of H_2O_2 yielded higher values in the OR(R) strain than in the *flr*^{3} strain, a *t*-test revealed that for this compound there was no difference between the strains. Table 1 shows sensitivity and lineal slope data obtained from the fitted curves. With these results we calculated the concentration that could yield 20% of mortality (LC_{20}) as a treatment for both strains, to evaluate the H_2O_2's effect on development and reproductive parameters.

Table 1. Values of sensivity, lineal slope, r^2 and equation of polinomic tendency for flies from flr^{3}and OR(R) strains treated with H_2O_2 (0, 5, 25, 50, 100, 150 and 200 mM).

Strain	Sensivity	Lineal Slope	r^2	Equation
flr^3	-0.003	0.9932	0.8158	$y = -0.003x^2 + 0.9932x + 16.241$
OR(R)	-0.003	1.0325	0.8638	$y = -0.003x^2 + 1.0325x + 8.0362$

Development parameters

Total emergence of P_0: ANOVA analyses of total emergence of flies (P_0) obtained from treated third instar larvae, indicated differences in treatments; the LSD test showed that the differences are in H_2O_2 *vs.* W and H_2O_2 *vs*. DMSO (Table 2 and Figure 1) with more dispersion and a lower median in the *flr*^{3} strain treated with H_2O_2, but not statistically different from OR(R) strain. Because, for this parameter, we did not obtain differences in DMSO *vs.* W or TOL *vs*. DMSO, we conclude that the total emergence was affected only by H_2O_2 and in a similar manner in both strains.

Table 2. Summary of the ANOVA and LSD results obtained from data's experiments.

		‡Factorial ANOVA			‡LSD Values				
		Strain factor (flr^3, OR(R)	Treatment factor (W, H_2O_2, TOL, DMSO)	Treatment-strain interaction	H_2O_2 vs. W	TOL vs. W	DMSO vs. W	H_2O_2 vs. TOL	H_2O_2 vs. DMSO
Development Parameters	Total emergence	ns	$p = 0.048$*	ns	$p = 0.012$	ns	ns	ns	$p = 0.018$
	Sexual proportion	ns	$p = 0.023$*	ns	$p = 0.011$	$p = 0.006$	$p = 0.006$	ns	ns
Reproductive Parameters	Total fecundity	ns	$p = 0.006$*	ns	ns	$p = 0.044$	$p = 0.004$	$p = 0.035$	$p = 0.003$
	Fertility percentage	ns	ns	$p = 0.036$*	ns	§.	ns	ns	ns

‡Statistically significant differences when $p \leq 0.05$. *See the corresponding LSD values in the second column on the right: §LSD values: TOL-flr^3 vs. W-flr^3 ($p = 0.018$); TOL-flr^3 vs. TOL-OR(R) ($p = 0.011$); TOL-flr^3 vs. DMSO-flr^3 ($p = 0.013$); TOL-flr^3 vs. DMSO-OR(R) ($p = 0.007$) after comparing between treatment and strain factors; ns = not significant values.

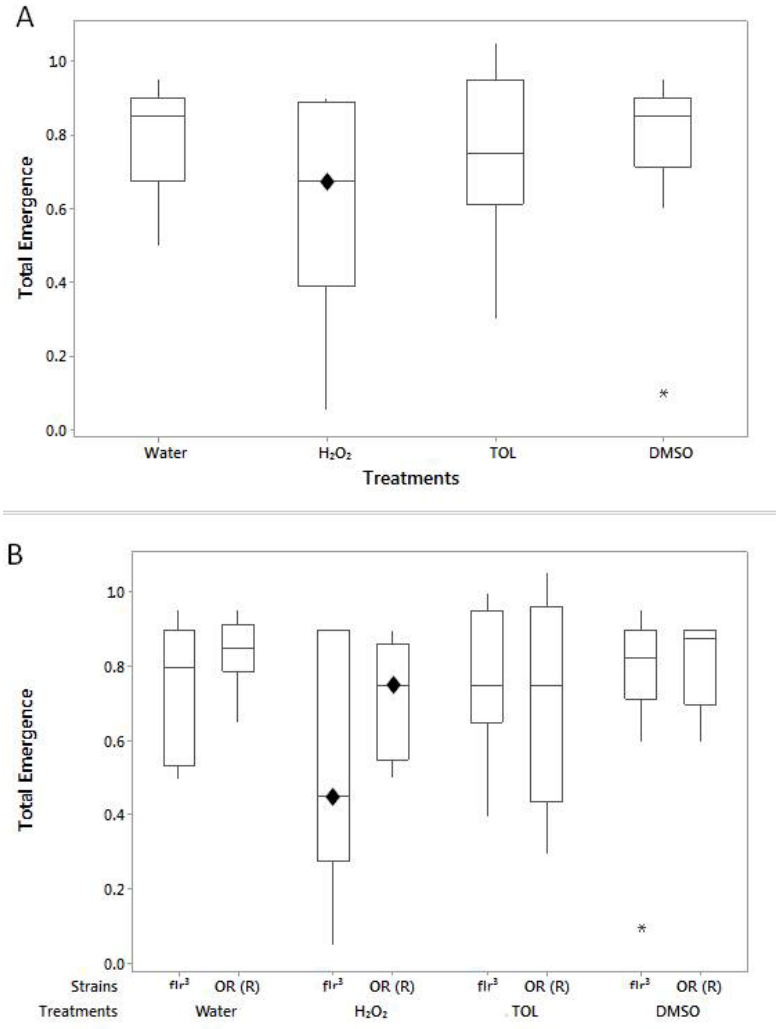

Figure 1. Medians and dispersions of (A) total emergence per treatment (Water, H_2O_2, 20 mM; TOL, 10 mM; DMSO, 0.03%) and (B) total emergence per treatment and strains (*flr*^{3}, OR(O)). ♦LSD test statistically significant differences ($p < 0.05$).

Total emergence of F_1: The comparison of the F_1´s hatches, all of them derived from progenitors (P_0) exposed to controls and treatments, showed similar medians and no statistical significant differences between them. This means that total emergence was affected only in P_0.

Daily emergence of P_0: Strain-treatment, strain-day, or strain-treatment-day interaction factors did not show differences in females, nor in males, but for males we found differences in day factor (Table 3 and Figure 2). Also, differences were found when comparing H_2O_2 *vs*. W treatments in males' emergence with a delay, and lower medians that are almost half of females' in both strains. Therefore, males' daily emergence was affected only by H_2O_2.

Table 3. Summary of the ANOVA and LSD statistically significant values obtained for the daily emergence parameter.

‡Factorial ANOVA								‡LSD
Strain		Treatment		Day		Strain-Treatment-Day		H_2O_2
(flr^3, OR(R))		(W, H_2O_2, DMSO, TOL)		(1-10)		interaction		vs. W
Females	n.s.	Females	n.s.	Females	$p = 0$	Females	n.s.	$p = 0.001$
Males	n.s.	Males	$p = 0.014$*	Males	$p = 0$	Males	n.s.	

‡Statistically significant differences when $p \leq 0.05$.*See the corresponding LSD value in the column on the right; n.s. = non-significant values.

Sexual proportion of P_0: In both strains all treatments yielded a sexual proportion that was statistically different from that of the W control, with lower medians in males than in females (Table 2 and Figure 3), but no differences between strains. Clearly, there is a biological component in males that makes them more susceptible than females, but it seems that the Cyp450s' different levels between the *flr*^{3} and OR(R) did not affect these results.

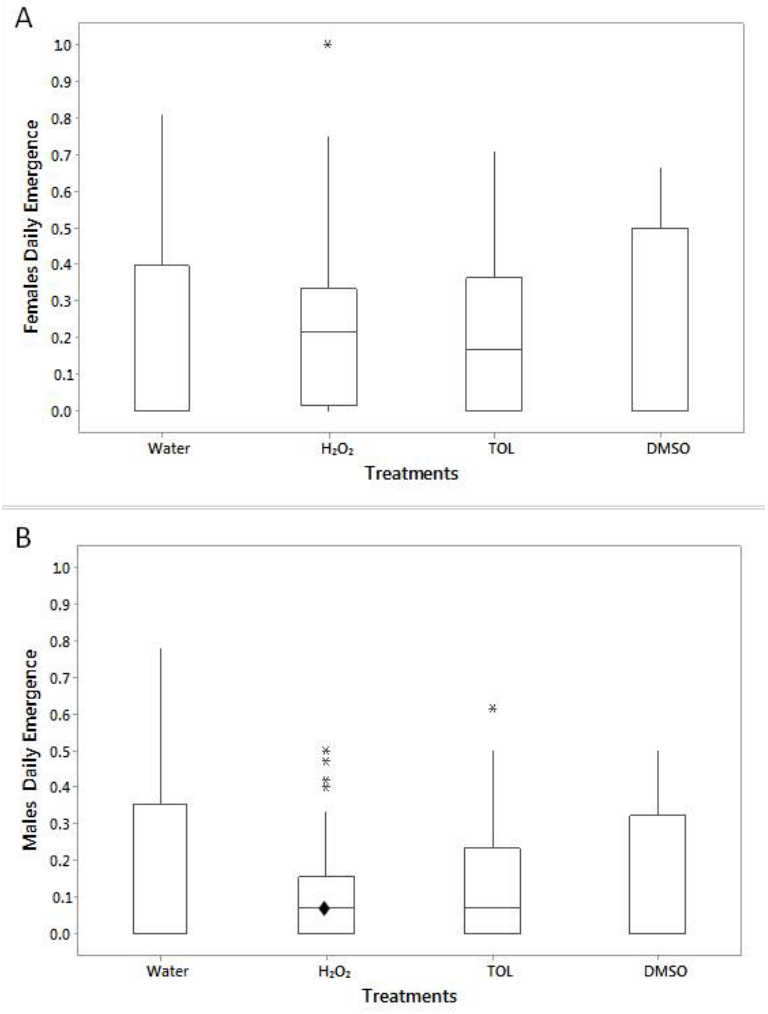

Figure 2. Medians and dispersions of (A) females and (B) males daily emergence per treatment (Water, H_2O_2, 20 mM; TOL, 10 mM; DMSO, 0.03%) and strain (*flr*^{3}, OR(O)). ♦LSD test statistically significant differences ($p < 0.05$).

Reproductive parameters

Total fecundity: As was expected, W produced the most homogeneous data of total fecundity, but they were not statistically different from H_2O_2's. TOL's data were the most dispersed and its median was similar to DMSO's. The analyses showed differences when comparing between the following treatments: H_2O_2 *vs*. DMSO, H_2O_2 *vs*. TOL, DMSO *vs*. W, and TOL *vs*. W (Table 2 and Figure 4), because total fecundity was lower in the DMSO and TOL treatments, remarkably with less dispersion in the first one. Then, these two compounds affected notably this parameter in the same manner to both strains.

Reproductive performance: The total number of hatched imagoes during 10 days did not show differences between strains, treatments, or interactions.

Fertility percentage: For the treatment-strain interaction ANOVA analyses, the fertility percentage was different ($p = 0.036$) with very low numbers in TOL *vs*. *flr*^{3}. When comparing between them we obtained LSD test differences between treatment-strain (Table 2 and Figure 5). Therefore, third instar larvae from the *flr*^{3} strain, with basal and inducible levels of Cyp450s, were more affected by TOL.

Discussion

H_2O_2

It is well-known that H_2O_2 produces the hydroxyl radical that has a very high oxidation potential that can decompose organic compounds in a very short time (Siu *et al*., 2009); it is also well documented that H_2O_2 is a free radical generator that can alter cell functions (Halliwell and Gutteridge, 2007) and it has been proved to exert toxic effects on *D. melanogaster* (Pomatto *et al*., 2017). On the other hand, it has been reported that it up-regulates developmental pathway, signaling and nucleobase metabolism genes (Landis *et al*., 2012). Here, the H_2O_2 altered the development parameters of both strains by delaying the total and daily emergence of imagoes from exposed third instar larvae. Furthermore, it affected the 1:1 sex proportion with a significant decrease in male individuals. Interestingly, Pomatto *et al*. (2017) demonstrated in *D. melanogaster* that female, but not male, adults adapt to H_2O_2 (10 and 100 μM) stress. Also, Pickering *et al*. (2013) demonstrated in *D. melanogaster* adults that pretreatment with 10–1000 μmol l^{-1} H_2O_2 yields a progressive

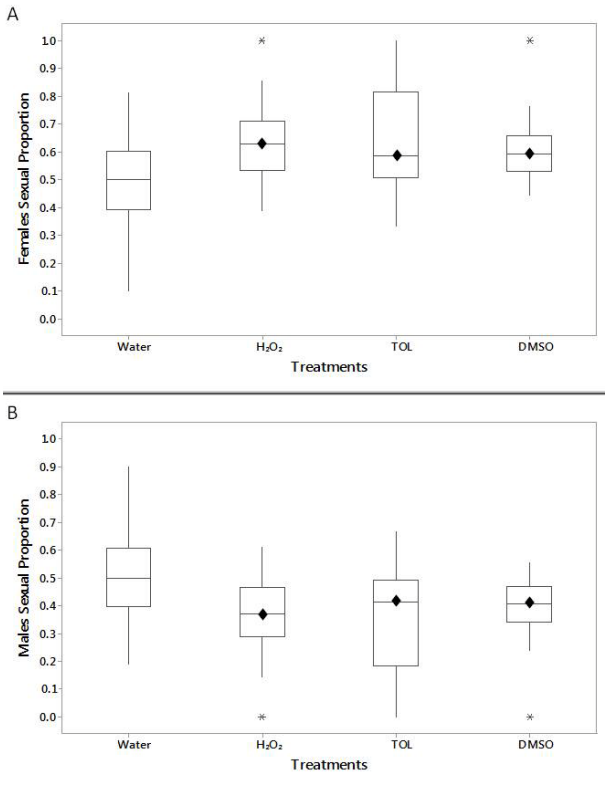

Figure 3. Medians and dispersions of sexual proportions of (A) females and (B) males per treatment (Water, H_2O_2, 20 mM; TOL, 10 mM; DMSO, 0.03%). ♦LSD test statistically significant differences ($p < 0.05$).

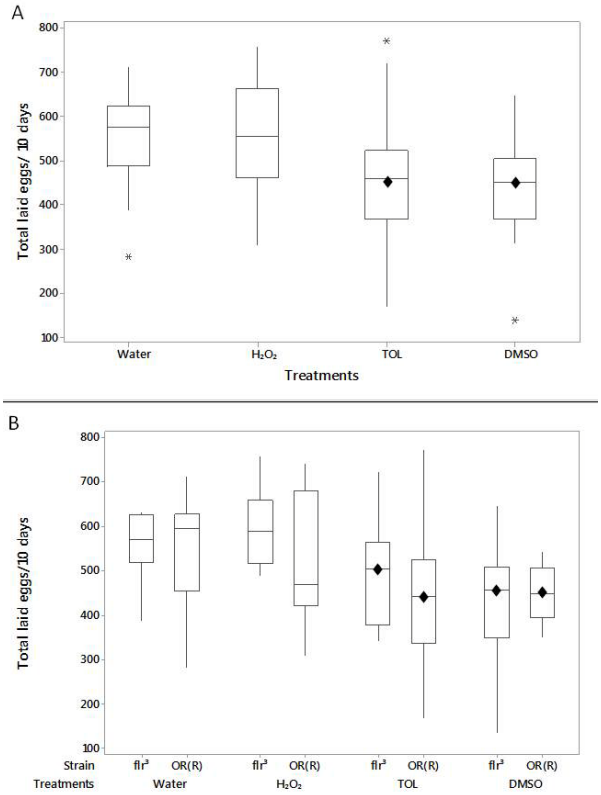

Figure 4. Medians and dispersions of (A) total fecundity (total laid eggs/10 days) per treatment (Water, H_2O_2, 20 mM; TOL, 10 mM; DMSO, 0.03%) and (B) total fecundity per treatment and strain (flr^3, OR(O)). ♦LSD test statistically significant differences ($p < 0.05$).

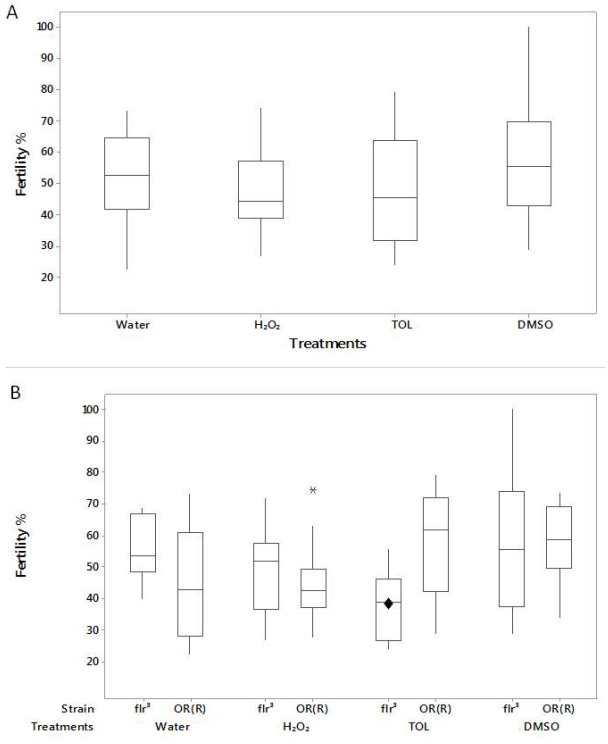

Figure 5. Medians and dispersions of (A) fertility percentage (No. flies in 10 days/No. of laid eggs in 10 days × 100) per treatment (Water, H_2O_2, 20 mM; TOL, 10 mM; DMSO, 0.03%) and (B) fertility percentage per treatment and strain (flr^3, OR(O)). ♦LSD test statistically significant differences ($p < 0.05$).

increase in the proteolytic capacity of females' lysates, but a decrease of proteolytic capacity from male fly lysates. We fed third instar larvae with a higher H_2O_2 concentration (20 mM), and we suggest that the difference in sex proportion, due to males' decrease in total and daily emergence, could be related to the less male's capacity to cope with oxidative stress as reported by Pomatto *et al*. (2017) and Pickering *et al*. (2013). So,

we infer that the observed effects in development parameters of treated larvae of both strains might be due to an oxidative stress and the different levels of Cyp450s between strains did not influences effects of H_2O_2. Surprisingly, H_2O_2 did not affect the reproductive parameters of adults obtained from treated larvae; it is well known that H_2O_2 treatment could induce the pro-survival program characterized by an alteration in insulin-like signaling, an increase in mitochondrial biogenesis and an increase in the de-acetylase activity of sirtuins (Stefanatos *et al*., 2012) or the clearances of damage tissue through apoptosis (Siu *et al*., 2009); then, putative cell defense mechanisms linked to this compound could explain the lack of detectable effect on the reproductive parameters only if damage in larvae's germ line cells could be repaired, or their damaged cells could be eliminated by apoptosis, during larval and metamorphosis stages.

TOL

We used TOL (10 mM) as a positive control, because Singh *et al.* (2009) reported in a strain with inducible Cyp450s, as the *flr*^{3} strain used here, that TOL (10-100 mM) affected the reproduction of *D. melanogaster,* by delaying emergence of imagoes up to 48 h, significantly decreasing the number of imagoes, and diminishing the reproductive performance in an inverse proportion to the concentration. They suggested that it was due to an alteration in development that could be the result of an increase in ROS that induced apoptosis (Singh *et al.*, 2009). Our experiments with both strains showed that exposure to TOL affected the development parameter: sexual proportion and the reproduction parameters: total fecundity and fertility percentage (Table 2), but contrary to Singh *et al*. (2009) it did not affect total emergence. Interestingly, TOL treatment was the only one where we got statistical differences in fertility percentage, between treatment-strain factors and between TOL and controls (LSD values in Table 2), confirming that alteration of fertility percentage by TOL occurred only in the strain with inducible Cyp450s. Because of the well described differences in Cyp450s expression levels between these strains (Saner *et al*., 1996; Vázquez-Gómez *et al*., 2010), we suggest that in the TOL treatment, the differences found in development parameter and reproductive parameters must be related to these different enzyme levels. Contrary to Kim *et al*. (2015), affirmations that TOL's metabolism is related only with GSTM1 or GSTT1 enzymes, our results contribute to assure the Cyp450s enzymes participation in TOL's metabolism (Kawamoto *et al*., 1995; Nakajima *et al*., 1997). We propose that the high levels of Cyp450s in the insecticide resistant OR(R) strain diminished cell damage, and TOL's biotransformation caused the differences observed when comparing its results with those of the *flr*^{3} strain.

DMSO

We used DMSO (0.3%) as a solvent control of TOL solution. Nazir *et al*. (2003) reported that a dietary concentration of 0.3% of DMSO is not harmful for the development of *D. melanogaster* and Singh *et al*. (2009) used in this model this concentration as a negative control. Also, Traut (1983) reported that a 2% solution does not induce aneuploidy in oocytes, making it a good solvent for mutagenicity screening in *Drosophila melanogaster*. Moreover, in the wing spot test we observed lower frequencies of mutant clones when DMSO was used as a solvent, presumably due to its scavenger properties (Dueñas-García *et al*., 2012). While DMSO is generally considered an antioxidant (Da Silva Duarte *et al*., 2004), under certain circumstances it causes oxidative stress (Sadowska-Bartosz *et al*., 2013). Surprisingly in this work, exposure to a DMSO concentration of 0.3% affected the 1:1 sexual proportion in both strains with a significant decrease in male individuals. This finding directly contradicts Singh *et al*. (2009) results and the observed decrease in total fecundity agrees with a cytotoxic effect reported by Nazir *et al.* (2003), although they used a higher concentration of 0.5%. Our results will be added to other adverse effects of DMSO demonstrated in human cells (Ruiz-Delgado *et al*., 2009; Shu *et al*., 2014) and other models (Choi *et al*., 2015; Herrid *et al*., 2016; Sadowska-Bartosz *et al*., 2013).

Conclusions

In conclusion, H_2O_2 significantly affected total emergence, daily emergence, and sexual proportion in both strains. TOL affected sexual proportion, total fecundity, and fertility percentage. Although TOL was dissolved in DMSO, the different Cyp450s levels between the two strains used, *flr*^{3} and OR(R), yielded

differences only for TOL treatment. This is important, because it supports the fact that the effects of this chemical on cells could depend on the expression levels of Cyp450s enzymes. Exposure to DMSO treatment affected in strains, sexual proportion, and total fecundity. We demonstrated that these three chemicals affect the studied parameters. Our results agree with the concern that chronic exposure to these compounds may affect human and animal development and reproductive processes (Pulver *et al.,* 2011; Misra *et al*., 2014).

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Thermotolerance of *Drosophila* hybrids: a new mode of adaptation in extreme climatic conditions.

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Abstract

Hybridization is not common in natural *Drosophila* populations. Trait expression and ecological performance of hybrids determine the consequences of hybridization such as degree and direction of gene flow or generation of phenotypic novelty. We investigated plastic effects on thermoresistance traits by growing parental species namely, *Drosophila jambulina* and *Drosophila punjabeinsis* and their hybrids, at four growth temperatures. Hybrids show increased variation in thermotolerance traits than their parental species. Hybrids show higher plasticity as well as cumulative fitness under variable environmental conditions. Acclimation effects were also significant in hybrids concluding adaptive potential. Results suggest that hybridization increases genetic variation that produces adaptation to new environments. We conclude that plasticity studies on hybrids and their progenitors are useful for testing basic predictions about evolution, as well as for understanding the evolutionary significance of hybrids. Key words: sibling species; *Drosophila jambulina*; *Drosophila punjabeinsis*; hybrid zone; genetic variation; fitness; acclimation

Introduction

Evolutionary significance of natural interspecific hybridization has gained much attention in recent years. In hybrids, trait expression and their ecological performance depends on the genetic control of the traits and the nature of genetic differences in their parental species. The outcome of natural hybridization varies in different taxonomic groups, but it has played a part in the evolution of large number of contemporary and

extinct organisms (Arnold, 1997). There are studies that have argued for an important role for hybridization in the evolution of species (*e.g*., Anderson and Stebbins 1954). Many attempts have been made to hybridize different species of *Drosophila*, but most of them were unsuccessful (Sturtevant, 1920), except in a few cases, *e.g*., *D. serrata* and *D. birchii* (Ayala, 1965). However, the majority of species do not hybridize in nature, but in laboratory they do so (Bock, 1984). Interspecific hybridization often leads to embryonic or adult lethality and reduced viability or fertility of hybrids (Sperlich, 1962; Llopart *et al.*, 2005) due to accumulation of genes that cause recessive incompatible epistatic interactions between the parental species (Dobzhansky- Muller model, Presgraves, 2003). Hybridization can also provide insights into plasticity as a mechanism for evolutionary change.

For ectothermic organisms, differences in thermal resistance match geographical distribution patterns of species, *i.e*., a high level of cold resistance is linked with higher latitudes while a reverse trend occurs for heat resistance (Hoffmann, Sorensen, and Loeschcke, 2003; Chown and Nicolson 2004). There are studies that abound on consequences of hybridization on speciation, formation of novel populations, fitness of hybrids and parents, genetic variation (Hatfield and Schluter, 1999; Schartl *et al*., 1995; Norris *et al*., 1999). Comparison of plasticity of hybrids and their parents will provide insights about persistence and adaptability of hybrids. Genetic variations for heat and cold resistance have been examined by comparing species and populations as well as on the basis of laboratory selection experiments (Gilchrist and Huey, 1999; Hoffmann, Sorensen, and Loeschcke, 2003). Thus, there are heritable genetic variations in thermoresistance traits, which confer adaptations to climatic conditions.

Consideration of closely related species and their hybrids is a way of examining genetic divergence on a longer time scale (Hercus and Hoffmann, 1999). Hybridization studies would be instrumental in describing how a species increases its ecological tolerance and expansion of geographical range. Analysis of variance in hybridizing species will provide necessary evidence for natural selection that helps a species in adapting to new environments. Further, plastic studies are essential in hybrids as their fitness relative to the parents may vary depending on the environment (Mercer *et al*., 2006; Darwish and Hutchings, 2009). Analysis of reaction norms is important for across- environment studies (Morris *et al*., 2011).

D. jambulina and *D. punjabeinsis* (Prashad and Paika, 1964) are Asian endemic *Drosophila* species. These two sibling species present an ideal situation for looking at the effect of plasticity in hybrids. In both species light allele is dominant over dark allele, but only the later one shows plasticity to some extent. In spite of huge work on hybridization and phenotypic plasticity, there are very few studies regarding phenotypic plasticity of hybrids in extreme conditions. This study addresses the influence of plasticity on hybrid fitness and adaptability. To study the potential effect of natural hybridization and phenotypic plasticity in the genus *Drosophila*, this study focuses on the conditions that involve natural hybridization between these two sibling species. We used morphometric and physiological approaches to quantify effects of hybrid plasticity in drosophilids from different sites. Comparative analysis was used to study what role hybrid phenotypic plasticity plays in adaptation to extreme conditions. Fitness analysis related to thermotolerance assays was performed to examine significance of hybridization and capability of evolutionary adaptation. We tried to score out whether plasticity for heat and cold traits differ between the two parental species and their hybrids. Specifically, we ask how plasticity and acclimation capacity generated due to effect of hybridization help in adaptation at a broader thermal range. Both the parent species have different thermal limits and occur in different biogeographical regions. Plasticity and acclimation capacity generated due to effect of hybridization help in adaptation in a broader thermal range.

Material and Methods

Collection and cultures

D. jambulina and *D. punjabeinsis* are sibling species inhabiting different biogeographical regions. The former one is a tropical species and the latter inhabits subtropical localities. *D. punjabeinsis* exhibits considerable variation in abdominal variation. Both these species are very similar and were confused with each other. Also, their hybrids were often misleading one who separates the mixed wild stocks. Both exhibit color dimorphism, having light allele dominant over darker one. The collections of sympatric populations of *D. jambulina* and *D. punjabeinsis* were made in a single trip during pre-winter months from six altitudinal

sites. From each site, about 200-300 flies were collected using net sweeping and bait trap methods. Isofemale lines from wild caught individuals were maintained at 25°C on standard cornmeal yeast agar medium.

Isofemale lines were distinguished among *D. jambulina*, *D. punjabeinsis*, and hybrids. To generate hybrid populations in the laboratory, crosses were undertaken by single pair mating using true breeding isofemale lines from both species in both directions. Morphological characters were used for confirmation of hybrid status. An equivalent number of parental lines of the two species were also set up to serve as controls. Climatic data for the sites of origin of populations were obtained from Indian Institute of Tropical Meteorology (IITM; www.tropmet.res.in).

Trait measurement

Thermotolerance of hybrids and their parents were measured in both control as well as acclimated groups. For thermotolerance traits and acclimation assessment, heat knockdown and chill coma tolerance were preferred, because these are ecologically relevant and confer repeatability. Effects due to age, sex, anesthesia, ambient room temperature, and thermal conditions of assay vials were controlled. Seven day old flies were aspirated and introduced to assay vials, which were pretreated at experimental temperatures for 6 hours so as to minimize effects due to thermal variations. For all traits, measurements were made in a thermocontrolled room at 21°C.

We analyzed plastic effects on thermoresistance traits by growing parental species and their hybrids at four growth temperatures (17 to 28°C). All experiments were performed on adult flies selected randomly from each iso-female line. For examining growth range (lower and upper developmental temperature), eggs of *D. punjabeinsis*, *D. jambulina*, and their F1 hybrids were transferred to 14, 15, and 16°C for lower and 28, 29, 30, and 31°C for the upper limits. Temperatures at which egg to adult development occurs at the both ends were considered as lower and upper developmental temperatures.

For acclimation effects, adult acclimation treatment by exposing adults at high and low temperatures for varying durations of time was undertaken. Experiments were carried out with females from isofemale lines of both the parental species and their hybrids. Acclimation temperatures were similar to those that the parental forms encounter in the field. Adults were exposed to high and low temperatures for 2, 4, 6, 8, and 10 days and were transferred to growth temperature for 24 hours before experimental analysis.

Fitness analysis was performed by mimicking natural habitat conditions of the two parental species. For estimating fitness, survival rate (w) was calculated as the proportion of group (*D. punjabeinsis*, *D. jambulina*, or hybrid) that survive after selection in two habitat conditions by dividing the number of surviving individuals by total number of individuals of that group. Secondly, fitness was computed by dividing each genotype survival rate by maximal survival rate. Finally, cumulative fitness was calculated.

Statistical analysis

Since the thermoresistance traits showed high repeatability across G1 and G2 generations, such data were pooled. For all the traits, isofemale line means (n = 20) along with s.e. or s.d. were used for illustrations and tabular data. For trait variability analysis, ANOVA helped in comparing F values and their percent variation contribution. Acclimation effects were compared through t-test (Zar, 1996). For estimating hybrid superiority, fitness as well as cumulative fitness was calculated. Statistical calculations and illustrations were made with the help of Statistica™ 5.0.

Results

D. punjabeinsis and *D. jambulina* are sibling species that are adapted to different biogeographical regions and are thermal specialists. Adults show only slight morphological differences and are hard to distinguish. For six localities, flies were identified as hybrids and pure species on the basis of morphological differences (Table 1). t-test values indicate that there were significant morphometrical differences between the two parent species. Trait values in hybrid individuals were intermediate of their parents. Table 2 shows average hybrid index and percentage of pure species from the six altitudinal collection sites. Hybrids were found in climatic zones where temperature fluctuations are high. Analysis of thermal range clarifies that

hybridization increases growth range of hybrid individuals (Figure 1). Increase in thermal range of hybrid will help in adaptation to wider range of growth temperatures than their parental species.

Table 1. Mean (± S.D.) values for various morphometrical traits and thermal range in *D. punjabeinsis*, *D. jambulina* and their hybrids. For significance level between hybridizing parent species, student's t- test values were used.

Trait	Parental species			Hybrid
	D. punjabeinsis	*D. jambulina*	t-test	
Wing length (mm)	2.38	2.05	**	2.20
Thorax length (mm)	1.05	1.00	*	1.01
Wing width (mm)	0.98	0.77	**	0.85
Body weight (mg/fly)	0.000815	0.001053	***	0.000872
Eye spot	0	3	***	1
Sex comb teeth (no.)	24,19	24,15	ns*	24,17
Thermal range (°C)	15-28	17-31	**	15-29

Table 2. A comparison of phenotypic plasticity of thermotolerance traits in two *Drosophila* species and their hybrids. Plasticity was quantified as coefficient of variation (CV) across complete thermal range; and on the basis of t- test acclimation capacity was compared. ** = $p < 0.01$, *** = $p < 0.001$; ns = nonsignificant.

	Heat Resistance		Cold Resistance	
Species	CV	t-test	CV	t-test
D. punjabeinsis	23.51	9.18 **	20.32	11.15 **
Hybrids	39.14	23.08 ***	43.57	46.15 ***
D. jambulina	1.80	0.71 ns	4.21	1.13 ns

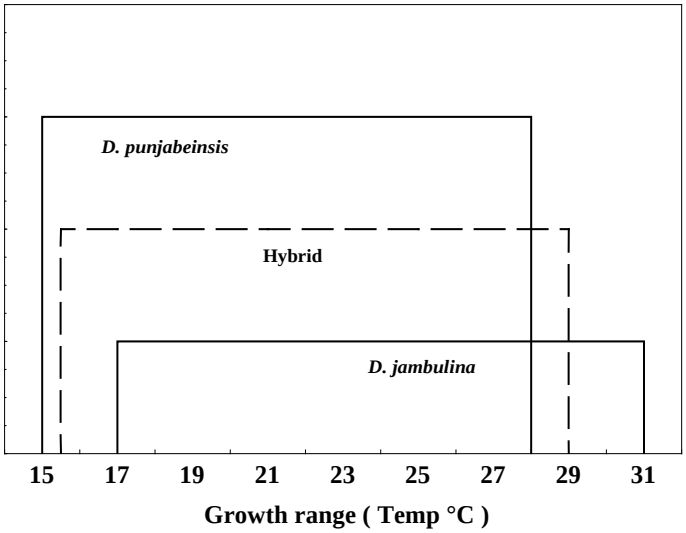

Figure 1. Developmental growth range of *D. punjabeinsis, D. jambulina*, and their hybrids.

Thermotolerance in hybrids and their parents

We assessed heat and cold tolerance in *D. punjabeinsis*, *D. jambulina* and their hybrids. There are significant differences between the two parental species based on t-test ($p = 0.001$), whereas the hybrids have intermediate values. Also, *D. punjabeinsis* shows significant geographical differences in heat and cold tolerance. We assessed plastic effects due to developmental temperatures (17 to 28°C) on thermotolerance traits. Hybrids show increased variation in thermotolerance trait than their parental species as indicated by the results of ANOVA (Table 3). Figure 2 illustrate contrasting differences in the coefficient of variation (CV) as a measure of plasticity of thermoresistance traits of *Drosophila* species and their hybrids. Plasticity is moderate in *D. punjabeinsis*, but *D. jambulina* lacks plasticity for thermal variables while hybrids have shown a high level of plasticity. These varying plasticity levels correspond with species survival in different climatic (temperate *vs*. tropical) zones.

Table 3. Effect of thermal acclimation on heat and cold resistance in D. punjabeinsis, D. jambulina and their hybrids. Significance level between control and acclimated groups are tested using student's t-test. F- statistics values are given from analysis of variance results

Species	Heat resistance			Chill coma recovery		
	Non-acclimated	Acclimated	F- value	Non-acclimated	Acclimated	F- value
D. punjabeinsis	8.21 ± 1.02	10.23 ± 0.87	4.63*	15.01± 2.00	12.68 ± 1.02	5.18*
D. jambulina	19.25 ± 0.98	20.32 ± 0.58	1.28 ns	27.02 ± 1.85	26.59 ± 0.98	2.14 ns
Hybrids	15.21 ± 2.12	21.02 ± 3.56	47.89***	20.25 ± 3.25	12.43 ± 4.11	56.87***

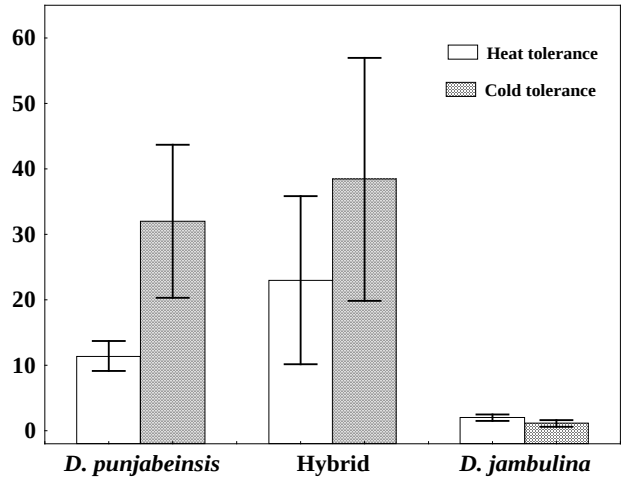

Figure 2. Bars showing coefficient of variation (CV) for thermoresistance traits due to changes in growth temperatures. SD lines demonstrate acclimatory capability of parents and their hybrids grown at 21°C and acclimated to upper and lower thermal environments.

Acclimation to thermal environments

Heat and cold tolerance were significantly affected by acclimation in hybrid individuals followed by *D. punjabeinsis*, whereas *D. jambulina* lacks acclimation effects (Figure 3). Acclimation to lower temperature significantly increases cold tolerance and decreases heat resistance in hybrids. Comparison of t-test values for control (non- acclimated) and acclimated flies shows that hybrids have high potential to acclimate or adapt to new thermal environment. *D. jambulina* lacks acclamatory capacity (t-test value = 0.71). Analysis of variance (ANOVA) indicates significant F- statistics differences on thermoresistance due to acclimation in hybrids (Table 4). Acclimation, therefore, increases resistance towards thermal stresses.

Parents vs. *hybrids fitness*

Hybrids are generally considered as less fit as compared to their parents. Study of survivorship after exposure to heat and cold was conducted for analyzing fitness. Hybrids show significantly higher overall fitness than their parental species in variable environments. Table 4 shows highest cumulative fitness for

hybrid individuals under a series of selective events. These results provide evidence for increased hybrid fitness.

Table 4. Hybrid advantage calculated as survival under different conditions during two selective environments.

Species	W_1 (Cold)	W_2 (Hot)	$W_{cumulative}$	$W_{relative}$
D. punjabeinsis	1	0.23	0.23	0.51
Hybrids	0.63	0.71	0.45	1.0
D. jambulina	0.29	1	0.29	0.64

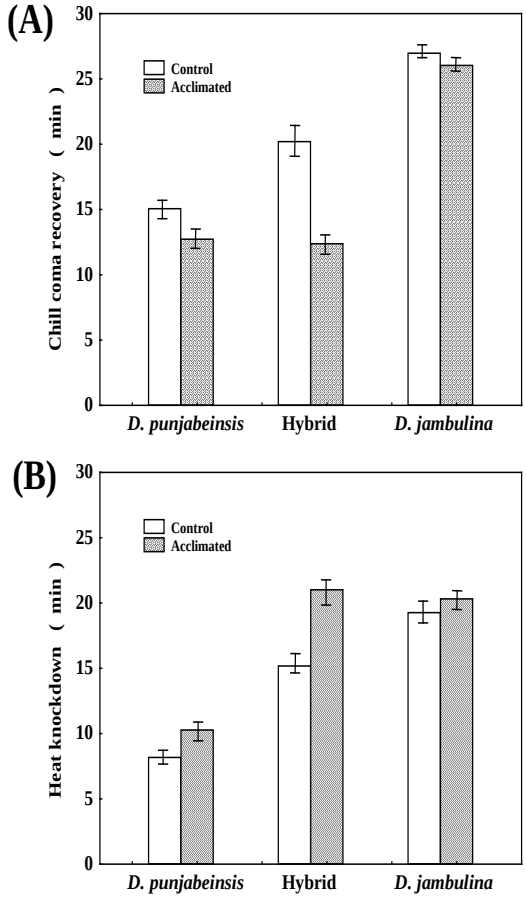

Figure 3. Relative changes in chill coma recovery (A) and heat knockdown (B) as a result of acclimation to different thermal environments in *D. punjabeinsis*, *D. jambulina*, and their hybrids.

Discussion

D. punjabeinsis and *D. jambulina* have different ecological requirements and hence are adapted to different habitats. Temperature is the main limiting factor; the former can cope with lower temperature and thus dominates in montane localities. In habitats with intermediate temperatures, both of these coexist as sympatric populations. Habitats with fluctuating temperatures are the natural laboratories for hybridization. Due to high morphological similarities between these two species, separations between parental species and their hybrids were based on differentiating characteristics based on results of crosses from laboratory hybrids. Hybridization between sibling species has been recognized to increase genetic diversity, often resulting in trait values of hybrid individuals exceeding their parents (Anderson, 1949; Ellstrand and Schierenbeck 2000; Bruke and Arnold 2001). For species having low genetic variation, hybridization can provide sufficient variation for adaptive evolution (Ellstrand and Schierenbeck 2000; O'Hanlon *et al.*, 1999). Consistent with previous studies, we found an increase in thermal tolerance variability in hybrids of *D. punjabeinsis* and *D. jambulina*.

Insects can cope with seasonal environments through phenotypic plasticity, which allows a single genotype to produce different phenotypes (Holloway *et al.*, 1997; DeWitt and Scheiner, 2004). Adaptive plastic responses for thermotolerance traits have been analyzed on the basis of short term exposures to sub-lethal conditions (hardening) and long-term exposure to conditions in the viable thermal range (acclimation) in different *Drosophila* species (Hoffmann, Sørensen and Loeschcke, 2003; Chown *et al.*, 2009). Plasticity can facilitate tolerance to a broad range of ecological conditions and spread of species to new environments. Hybrids of *D. jambulina* have high levels of thermal plasticity (measured as CV) and performed consistently better than their parents.

The degree to which an organism can acclimate is dictated by their phenotypic plasticity or the ability of an organism to change or adapt to a certain environment. Out of the two studied *Drosophila* species, *D.*

jambulina lacks acclimation capacity and thermal plasticity. Acclimation capacity of *D. punjabeinsis* represents an adaptation to a wide range of ecological conditions. Acclimation capacity of an individual might provide an understanding of how an organism can respond to an increase or decrease of habitat temperature. Studies related to hybridization effect on acclimation capacity and its association with habitat expansion have not been considered yet. Physiologists have assumed acclimation as a form of phenotypic plasticity that enhances performance of a species or individual in a particular environment (Hochachka and Somero, 2002; Prosser, 1986). Studies on hybrids of *D. serrata* and *D. birchii* provides no evidence that hybridization increases evolutionary rates and adaptation (Hercus and Hoffmann, 1999). In contrast, the results of the present study indicate that hybrids have increased variation and acclamatory capacity that leads to an increase in adaptability of a species to new ecological conditions. Acclimation is a source of phenotypic adaptation resulting in widening of regulatory range of a species (Horowitz, 2001). In the present study, results of acclimation experiments were in favor of previous reports and indicate adaptive significance of hybridization.

Significant differences were obtained in fitness of hybrid and parental species. Thermal conditions change over geographical regions. Fitness of a species is influenced by both genetic and environmental conditions; hence, fluctuation in environmental conditions can act as selective regimes, which can produce cumulative hybrid advantage. Under one or the other single selective events, parental species are more successful as they are genetically better adapted to that environment. Fitness estimation of few hybrid genotypes is equal to or greater than those of their parents suggesting a significant role of natural hybridization in adaptive evolution (Arnold and Hodges, 1995). The present study favors that hybridization cause an addition to genetic variability that can facilitate range and/ or habitat expansion of sibling species.

Hybridization in these two *Drosophila* species is, therefore, helpful for *D. jambulina* (a warm specialist species) to extend its adaptive potential, acclimation capacity, and plasticity. This expansion involved physiological adaptation and tolerance to extreme thermal conditions. The genetic variation that is necessary to increase ecological tolerance of *D. jambulina* has come from *D. punjabeinsis*. We predict that species with low potential towards adaptation to fluctuating environments can enhance their tolerance and adaptive capability via hybridization. Environmental conditions fluctuate in time and change over geographical regions. Both the genetic and environmental factors influence the fitness and adaptive potential of an organism. Thermal tolerance traits have been demonstrated as reliable tools for demonstrating climatic adaptations in populations and species of *Drosophila* (David *et al.*, 2003; Gibert *et al.*, 2001). In the present study, we used plastic responses, acclimation capacity and fitness under variable environments to assess the effect of hybridization in adaptation and improvement of survival as a measure of sibling rescue under drastic environmental conditions. Hybrid individuals are more plastic than their parental species and have increased developmental range at both extremes. Analyses of acclimation responses to temperature are more and occur quickly in hybrids. Results showed that under fluctuating environmental conditions, hybrids are more successful than their parental species as the cumulative fitness is higher for hybrids. Our results lead to conclude that hybridization in sibling species is adaptive and can lead to evolution. Therefore, hybridization between two species can be a potential stimulus for adaptation and rapidly create evolutionary novelties.

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Polyphenols as treatment at the intersection of environmental and genetic causes of Parkinson's disease in a *LRRK2* model.

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Abstract

Parkinson's disease (PD) is a chronic, neurodegenerative disorder that affects between 4 and 5 million people worldwide. The etiology of PD is both environmental and genetic. Thus far, research has shown that the reduction of environmentally triggered reactive oxygen species (ROS) levels can reduce parkinsonian symptoms in several animal models (Bonilla-Ramirez *et al.*, 2011). Furthermore, several genetic mutations including *LRRK2* (Leucine-rich repeat kinase 2) that lead to impaired mitochondrial function have been shown to impact Parkinson disease onset (Guo, 2012). We have used a fly *LRRK2* knockout model to study the role of environmental factors in PD development. *LRRK2* knockout flies were fed an antioxidant solution of polyphenols (propyl gallate, epicatechin, gallic acid, and epigallocatechin gallate) every 5 days. Polyphenol-fed flies and controls were examined using several measures of parkinsonian symptoms. Survival numbers, climbing ability, and dopamine immunohistochemistry were performed on flies at several times post-eclosion. *LRRK2* knockout flies fed with polyphenols did not show an altered lifespan, but showed a decrease in motor impairments. Treatment with polyphenols also decreased dopaminergic neuron degeneration as compared to control. It may be that polyphenols can effectively combat increased ROS due to impaired mitochondrial function in *LRRK2* mutants.

Introduction

Parkinson's disease (PD) is a chronic, neurodegenerative disorder that affects motor movement due to death of dopaminergic neurons in the substantia nigra pars compacta. Approximately 50,000 people in the U.S. are diagnosed with PD annually, and between 4 and 5 million people suffer from Parkinson's disease worldwide (Michael J. Fox Foundation, 2012). The major symptoms of PD include tremors, trembling, rigidity of limbs, slowed movement, poor posture, and issues with balance. There is no known diagnostic test or a cure for PD.

PD has both environmental and genetic origins and has been associated with defects in mitochondrial function. Defective mitochondria produce more reactive oxygen species (ROS), which are associated with aging, age-related disease, and neurodegeneration. Certain industrial environmental agents and agricultural chemicals, such as MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) or rotenone, produce PD-like symptoms by damaging mitochondria, which then produce increased levels of ROS (Jimenez-Del-Rio *et al.*, 2010). Heavy metals and paraquat (PQ) generate ROS and, in turn, parkinsonian symptoms (Jimenez-Del-Rio

et al., 2010). A study by Bonilla-Ramirez *et al.* (2011) showed that flies exposed to iron (Fe), manganese (Mn), and Copper (Cu) had reduced lifespan and locomotor activity, as well as degeneration in DA clusters due to oxidative damage. This study also showed that antioxidants (polyphenols: propyl gallate, epicatechin, gallic acid, and epigallocatechin gallate) can reduce parkinsonian symptoms caused by these ROS generators. Polyphenols exhibit neuroprotective effects based on their ability to lessen oxidative damage or by activating hormetic pathways that protect against cell toxicity (Vanzour, 2012). Therefore, ROS levels correlate with parkinsonian symptoms, and high ROS levels can be reduced using environmental antioxidants.

There is only one known gene that causes PD. Mutations in the *α-synuclein* (SNCA) gene cause an early-onset form of PD. Late-onset, or sporadic Parkinson's disease, which makes up about 90-95% of cases, is linked to the genes *Parkin*, *DJ-1* (*PARK7*), *PINK1*, and *LRRK* (*PARK8* or *dardarin*), all of which have been shown to cause to mitochondrial dysfunction (Guo, 2012). *LRRK2* is the most commonly associated gene for late-onset PD. *LRRK2* is a nuclear gene that encodes a cytoplasmic protein necessary for mitochondrial function (Smith *et al.,* 2005). The specific function of *LRRK2* is unknown, although the protein has both GTPase and kinase domains (Yue and Lachenmayer, 2011). Mutations of *LRRK2* primarily affect PD onset and can cause both familial and sporadic PD. However, the interaction is complex and the exact role of *LRRK2* in generating the disease is still unknown (Liu *et al.*, 2011).

One study by Ng *et al.* (2012) showed that the *Drosophila LRRK2* G2019S mutant (a gain of function mutation) exhibits swollen mitochondria in flight muscles as well as degeneration of dopaminergic neurons in the brain. Interestingly, the study also shows that EGCG protects against DA degeneration. Given that mitochondrial dysfunction and increased ROS production is central to the development of PD, these experiments support the notion that environmental antioxidants can be used to manipulate ROS levels in a *LRRK2* model.

The present study examined the effects of antioxidants on lifespan, motor ability, and DA degeneration in *LRRK2* knockout flies rather than the gain of function mutant used in Ng *et al.* (2012). We added a combination of polyphenols to the diet of *LRRK2* knockout flies, including propyl gallate (PG), epicatechin (EC), gallic acid (GA), and epigallocatechin gallate (EGCG). *LRRK2* knockout flies were generated using an antisense construct expressed in dopaminergic neurons in the brain. Our expectation is that the antioxidants would increase lifespan, decrease motor impairments, and decrease dopaminergic neuron degeneration, as compared to flies not fed with the polyphenol treatment. The antioxidants had a clear effect on dopaminergic degeneration and motor impairment, and no effect on lifespan.

Experimental Procedure

Fly Stock

Stocks were obtained from the Bloomington stock center. All flies raised for these experiments were fed on the standard cornmeal, molasses food and raised at 25^{o}C. We crossed *Ddc*-GAL4 flies with UAS-anti-*LRRK2* flies to generate a tissue specific LRRK knockout model. In our PD model, the F1 generation has the *LRRK2* knockout in only dopaminergic neurons (Guo, 2012).

Antioxidant Feeding

Two-three day old *Ddc*-Gal4/UAS-anti-*LRRK2* flies were administered 0.1 mM polyphenol solution in yeast paste spread on the surface of standard food in a fly bottle. The polyphenol solution consisted of a 0.1 mM solution of propyl gallate (PG), epicatechin (EC), gallic acid (GA), and epigallocatechin gallate (EGCG) in distilled water (Ortega-Arellano *et al.,* 2011). In all experiments, flies fed with the polyphenol treatment were compared to control flies that were administered yeast paste without polyphenols.

Lifespan

Eighty flies were placed into bottles spread with yeast paste with or without the polyphenol solution. Three replicates of each condition were performed. The number of live male and female flies in the vials were counted every 5 days and transferred into fresh bottles with yeast paste until no live flies remained (Bonilla-Ramirez *et al.,* 2013). The proportion of live flies was compared between flies fed with polyphenols and controls.

Climbing Assay

Two replicates of polyphenol fed and control fly bottles containing 50 flies each were used for climbing assays, which tested for locomotor impairments. Flies were placed into bottles with yeast paste and transferred every five days into fresh bottles until they were the appropriate age for testing. To perform the climbing assay, ten flies were placed in five separate, empty, clear vials with a line drawn at five centimeters. After a ten-minute rest period, the flies were gently tapped to the bottom of the vial. The number of flies that were able to climb above the five cm line in six seconds was recorded. The tests were performed three times with 1-minute intervals in between trials (Jimenez-Del-Rio *et al.*, 2010). Climbing assays were performed on flies at ages 5, 15, 20, 30, and 40 days.

Immunohistochemistry and TUNEL labeling

Dopaminergic degeneration was measured using immunohistochemistry staining on fly brains at ages 5, 15, 30, and 50 days. Flies were etherized and the heads and proboscises removed surgically. The heads were placed into 4% paraformaldehyde (PFA) to fix and then the brains were dissected under a dissecting microscope. The brains were fixed in PFA for 15 minutes and were then washed with 1× PBS. The PBS was then removed, and the brains underwent an ethanol dehydration series. The brains were then stored in 70% ethanol in the freezer. Ten replicate brains were dissected and stained for each condition.

Once removed from the freezer, the brain were rehydrated and incubated for 10 minutes at room temperature in 0.2 N HCl, then rinsed with 1× PBS. The brains were then blocked with a solution of PBS and 3% goat serum for 1 hour at room temperature. The primary antibody, 1000 fold dilution of rabbit tyrosine hydroxylase in PBGS (0.2% goat serum solution, Sigma-Aldrich, St. Louis Missouri) was added to the brains and incubated overnight. The brains were then washed extensively with PBS with 0.03% Triton X (PBTx). The secondary antibody, 1000 fold dilution anti-rabbit IgG conjugated with fluorescein in PBGS (Sigma-Aldrich, St. Louis Missouri), was added and the brains were incubated for 1 hour. The brains were then washed with PBTx. The TUNEL reaction mixture was then added and incubated at 37°C for 1 hour. The brains were then rinsed extensively in 1× PBS, mounted on a clean slide in Vectastain solution, and viewed under confocal microscopy (Feany and Bender, 2000). The number of DA clusters existing were counted and compared between polyphenol treated flies and control flies (Yang *et al.*, 2012).

Statistical Analyses

Analysis of variance (ANOVA) was used to compare groups of flies fed with polyphenols to control groups over time for lifespan, climbing assays, and dopaminergic degeneration. T-tests were preformed to compare dopaminergic death overall and degradation within specific clusters.

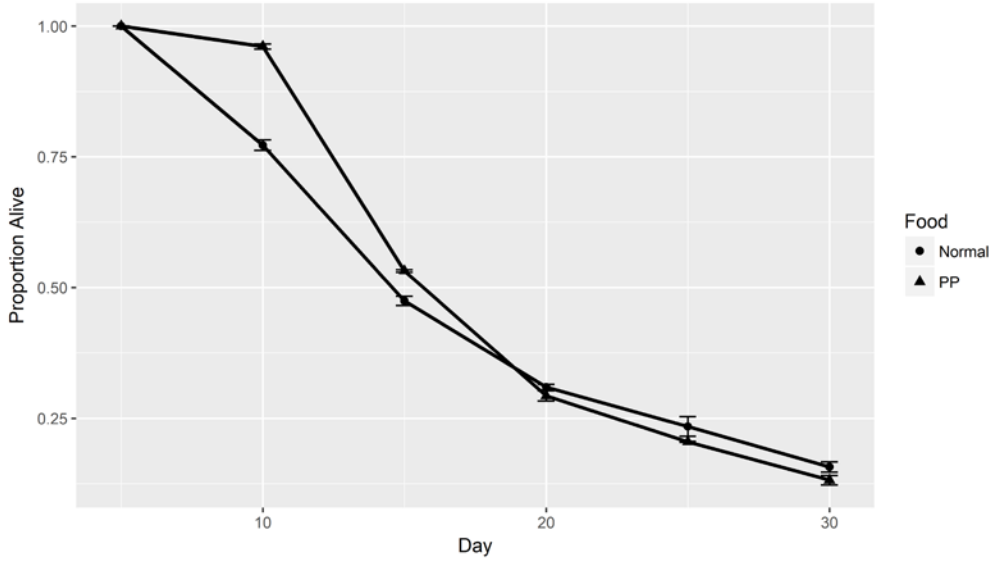

Figure 1. Polyphenol feeding did not have an impact on lifespan of *LRRK2* flies. Three replicate populations were assessed for treatment and control groups starting with 80 flies in each population and error bars are standard error of the mean.

Results

Polyphenols do not significantly increase lifespan

LRRK2 flies have a substantially reduced lifespan from those observed with wildtype flies (data not shown). Feeding a polyphenol solution to *LRKK2* knockout flies did not have an impact on lifespan of the flies (Figure 1). An analysis of the survival did not show a significant interaction between treatment and age on survival (F value = 1.66, P value = 0.21). As expected age had significant effect on lifespan ($P < 0.0001$), but treatment had no effect ($P = 0.27$).

Polyphenols significantly improve motor behavior

Polyphenols improve the climbing ability of *LRRK2* knockout flies (Figure 2). While there was no significant interaction between treatment and age and climbing (F value F = 0.089, P = 0.76), there was a significant effect of treatment on climbing ($P = 0.0005$) and age on climbing ($P < 0.0001$). Therefore, polyphenols appear to mitigate the negative impact of mitochondrial function in the LRKK2 knockout mutant.

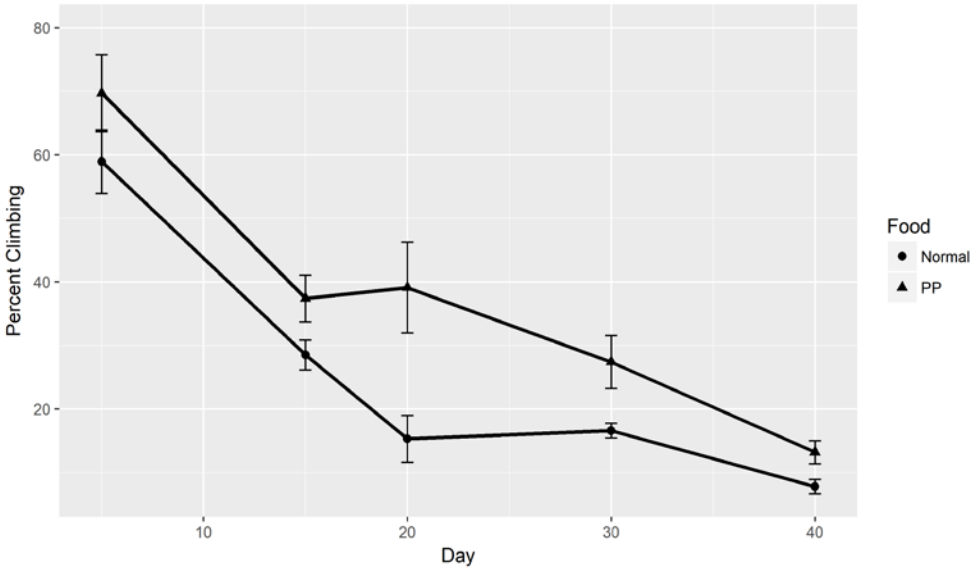

Figure 2. Polyphenols significantly improve climbing in *LRRK2* mutants. The tests were performed three times with 1 minute intervals in between trials and the average proportion of flies that climbed above the 5 cm line in 6 seconds at each age group was recorded for control and polyphenol fed flies. Data were compiled from 50 replicate flies and error bars are standard error of the mean.

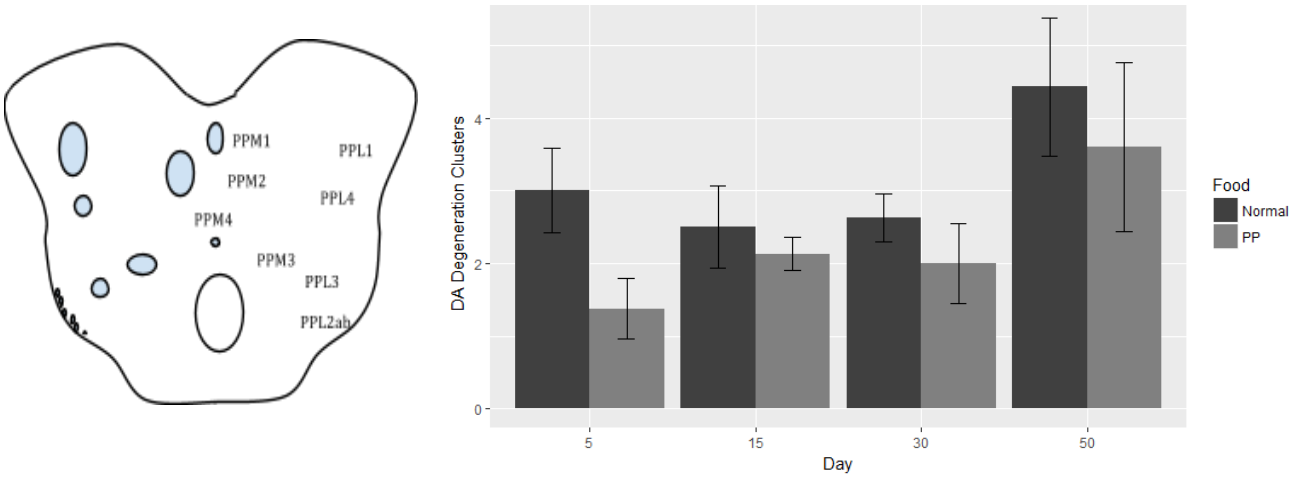

Figure 3. Impact of polyphenol treatment on *LRRK2* knockout flies over lifespan. The presence of DA and apoptotic cells was recorded, according to a map of 8 major DA clusters in *Drosophila* (3a). There was not a significant effect of polyphenols on DA degeneration over time (3b). Error bars are standard error of the mean.

Polyphenol treatment shows a small impact on dopaminergic degeneration

Brains from both treatment and control *LRRK2* knockout groups were observed for tyrosine hydroxylase staining (a dopamine marker) and for apoptotic degeneration using TUNEL staining. The expected pattern of dopaminergic neurons in the fly brain is shown in Figure 3a. Dopaminergic neurons undergoing degeneration will be obvious since they will appear yellow as compared to green dopaminergic neurons and red degenerating neurons. Both degenerating and nondegenerating dopaminergic neurons were observed; however, almost no neurons were degenerating that were not dopaminergic.

We found these data challenging to interpret. At any given time point (Figure 3b), there were no significant interactions between treatment and age on dopaminergic degeneration ($F = 0.0069$, $P = 0.934$), and there was no significant effect of the treatment on DA degeneration ($P = 0.127$), although as expected there was a significant effect of age ($P = 0.0008$). However, the average number of neurons per brain is significantly different (3.5 control and 2 for treatment, $P = 0.015$), and total overall number of degenerating neurons for an equivalent number of brains (80 *vs*. 50 averaged over three replicates, $P = 0.017$) is significantly different between control and treatment groups.

We also saw differential impacts on different clusters, although the small number of neurons in any given cluster evaluated here prevents statistical comparisons. There were many fewer degenerating neurons in cluster PPL1, PPL3, and PPL4 in the treatment groups. This differential effect on clusters has been reported before (Ng *et al.,* 2012). The time frame analysis is also problematic, since a neuron in a given brain may have degenerated before the time where the observation is done, and so it is not counted, although we did see a light increase in neurodegeneration with age. Therefore, we feel it is likely that the treatment had an impact on dopaminergic degeneration as suggested by our data and likely based on the impact of treatment we observed on climbing behavior.

Discussion

The purpose of this study was to determine if polyphenols could protect against parkinsonian phenotypes in a *LRRK2* knockout *Drosophila* model of Parkinson's disease. We examined the effect of treatment with polyphenols using a *Ddc*-Gal4/UAS-anti-*LRRK2* knockout. We hypothesized that flies fed with a solution containing multiple polyphenols (EC, PG, EGCG, GA) would have increased lifespan, decreased motor impairments, and decreased dopaminergic degeneration, as compared to flies fed without the polyphenol treatment. Polyphenol treatment did not significantly protect the lifespan of *LRRK2* flies although previous studies such as Yang *et al.* (2012) show an increase in lifespan with curcumin. The discrepancy may be a result of using the *LRRK2* knockout model versus a *LRRK2* mutation. Both gain and loss of function show Parkinson-like phenotypes. The knockout of *LRRK2* may be too debilitating beyond midlife for antioxidants to protect flies from death any longer. We did see a significant improvement in climbing assays in polyphenol fed flies versus control flies. These data is consistent with certain aspects of previous studies. Bonilla-Ramirez *et al.* (2013) showed that propyl gallate (PG) alone did not improve locomotor activity in knock-down *parkin* flies although EGCG was found to improve locomotion in both parkin and LRRK dominant mutant flies in Ng *et al.* (2012). Finally, although statistically inconclusive, we did find evidence that treatment was neuroprotective consistent with previous reports.

Even though polyphenols have exhibited neuroprotective effects, the underlying mechanisms remain unclear. Polyphenols are known to donate electrons or hydrogens to neutralize free radicals. These compounds may also induce other antioxidant enzymes such as glutathione peroxidase, catalase, and superoxide dismutase. Some literature highlights the potential role of protein and lipid kinase signaling in polyphenol function as opposed to the traditional antioxidant role as electron donors (Tsao, 2010). The lack of clarity behind polyphenols' mechanisms suggests that other factors, such as the induction of mild cell stress (hormesis), may supplement or even be necessary for their benefits. Implications of the current study and future related studies include the possible use of polyphenols as a treatment to target the intersection of genetic and environmental causes of PD. PD has multiple causes, yet no existing cure. Clarification of the mechanisms of the disease and the relationships between both causes will help develop potential dietary and pharmacological applications for polyphenols.

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RNAi effects on the alpha glycerophosphate dehydrogenase, the alpha glycerophosphate oxidase and the arginine kinase paralogs of *Drosophila melanogaster.*

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Introduction

Alpha glycerophosphate dehydrogenase (GPDH) and alpha glycerophosphate oxidase (GPO) cooperate in the adult thoracic flight muscles to drive the alpha glycerophosphate cycle producing the ATP necessary for continual flight (Sacktor, 1965; and see Figure 1 in Davis and MacIntyre, 1988). Null mutants in either enzyme, as homozygotes or hemizygotes, cannot fly. The structural genes for the flight muscle variants for the two enzymes were mapped in the 1970's and 1980's (Grell, 1967; O'Brien and MacIntyre, 1972; O'Brien and Gethman, 1973; Davis and MacIntyre, 1988). When the genome of *Drosophila melanogaster* was sequenced, two additional paralogs were discovered. Carmon and MacIntyre (2010) compared the sequences and the exon/intron structures of the three forms of GPDH (GPDH-1, 2, and 3) and GPO (GPO-1, 2, and 3). GPDH-1 and GPO-1 encode the flight muscle specific forms mentioned above, whereas GPDH-2 and 3 and GPO-2 and 3 are expressed only in the testis.

Arginine kinase (AK), like GPDH and GPO, is particularly abundant in indirect flight muscle (Lang *et al*., 1980), although low levels are also present in other tissues (James and Collier, 1988). The structural gene for arginine kinase is located 66F (Fu and Collier, 1983; Munneke and Collier, 1988) and is responsible for four alternative protein products, all of which share a common catalytic domain. The single EMS-induced null is due to an amino acid substitution (L182Q) in the common domain. It is an embryonic lethal which precludes assessing its role in flight muscle energetics. There are also two additional paralogs of AK that are only expressed in the testis.

The relevant information on each of the nine paralogs is shown in Table 1. All forms of each enzyme are evolving under purifying selection indicating they are functionally important in the fly.

To further assess the functional roles of the paralogs for GPDH, GPO, and AK, we have inactivated them with RNAi's from the Vienna collection. These RNA lines are also listed in Table 1. To drive the expression of the RNAi's, we have used two Gal4 constructs, one with a tubulin promoter (tub-gal4) and one with a promoter from the *bag of marbles* gene (bam-gal4) (M^{c}Kearin and Spradling, 1990). The former should drive the RNAi's in most if not all cells, whereas the latter should do so in the male germ line. We have assessed three different phenotypes reflecting the actual and possible roles of GPDH and GPO during

development. They are: viability, flight ability, and male fertility and examined in the progeny of Gal4 and RNAi lines.

Table 1. Information on the paralogs of GPDH, GPO and AK.

Paralog	Genome Number	Map Location	RNAi*
GPDH-1	CG 9042	26A	KK105359
GPDH-2	CG 3215	59C	GD4707
GPDH-3	CG 31169	94A	KK101299
GPO-1	CG 8256	52C	KK110608
GPO-2	CG 2137	43D	GD4813
GPO-3	CG 7311	34D	GD41235
AK-1	CG 32031	66F3-4	GD34036
AK-2	CG 5144	66F3	GD 6047
AK-3	CG 4546	89A5	GD 11344

*VDRC stock number

Materials and Methods

Viability is scored as "+" in the tables below if the expected numbers of Gal4;RNAi progeny survive to adulthood. If they do not survive to the adult stage, they are scored as "lethal" in the viability column. To assess flight ability 10-20 adults are placed in a vial which is stored at room temperature overnight. The next day the vial is rotated on a platform shaker for 5 minutes (the "exercise period") and the flies are dumped onto a sheet of white paper. The number of flies that have flown away in a 20 second interval is then recorded over the number of flies originally in the vial. To determine male fertility, five males with the Gal4-RNAi genotype are placed in a vial with virgin females from a dpy^{ov1} stock. Three to five replicates are set up and scored for the eventual emergence of *dpy* plus progeny after 14 days. The number of vials producing progeny is then recorded.

Table 2. RNAi inactivation of single paralogs.

RNAi-Gal4	Viability	Flight Ability	Male Fertility
GPDH-1-tub	lethal	n.d.*	n.d.*
GPDH-1-bam	+	10/10	3/3
GPDH-2-tub	+	10/10	0/3
GPDH-2-bam	+	10/10	0/3
GPDH-3-tub	+	9/10	3/3
GPDH-3-bam	+	10/10	3/3
GPO-1-tub	+	10/10	3/3
GPO-1-bam	+	10/10	3/3
GPO-2-tub	+	10/10	3/3
GPO-2-bam	+	10/10	3/3
GPO-3-tub	+	10/10	3/3
GPO-3-bam	+	9/10	3/3
AK-1-tub	lethal	n.d.*	n.d.*
AK-1-bam	+	10/10	3/3
AK-2-tub	+	10/10	3/3
AK-2-ban	+	10/10	3/3
AK-3-tub	+	10/10	0/3
AK-3-bam	+	10/10	0/3

*Can't be done

Results and Discussion

I: RNAi inactivation of single paralogs

Each of the three forms of the three enzymes was subjected to its RNAi driven by either the tubulin- Gal4 or the bag of marbles-Gal4. The results are shown in Table 2.

Inactivating GPDH-1 with its RNAi driven by the ubiquitous tubulin promoter results in pre-adult lethality in keeping with its pivotal role in intermediary metabolism. Similarly, inactivating AK-1 with its RNAi driven by the tubulin promotor results in embryonic lethality. The only other specific effect is male sterility when either GPDH-2 or AK-3 are inactivated by their respective RNAi driven by either tubulin-Gal4 or by Gal4 driven by the bag of marbles promoter active in the testis. No effect on flight ability was detected when a GPDH, GPO, or AK paralog was inactivated.

II: RNAi inactivation of pairs of paralogs for each enzyme

In order to determine if paralogs of the same enzyme will compensate in any of the three phenotypes, we drove the RNAi's for pairs of the paralogs with either tubulin-Gal4 or the bag of

marbles-Gal4. Double mutant stocks of RNAi; tub-Gal4/ In(3LR)TM3,Sb and RNAi; bam-Gal4 were constructed when the RNAi and tub-Gal4 combinations are viable and fertile. Flies from these stocks were then crossed to flies from the second RNAi stock and the appropriate F1's were scored for the three phenotypes. The results are presented in Table 3.

The expectation here is if the paralogs can compensate for one another, flies carrying both RNAi's activated by Gal4 should show a more extreme phenotype than flies with either RNAi alone. This does not seem to be the case when the double RNAi combinations are viable and fertile. Hence it is clear that the paralogs are affecting distinct and separable functions from one another.

Table 3. RNAi inactivation of paralog pairs-same enzyme.

RNAi's-Gal4	Viability	Flight Ability	Male Fertility
GPDH-1&2-tub	lethal	n.d.*	n.d.*
GPDH-1&2-bam	+	9/10	0/3
GPDH-1&3-tub	lethal	n.d.*	n.d.*
GPDH-1&3-bam	+	10/10	3/3
GPDH-2&3-tub	+	10/10	0/3
GPDH-2&3-bam	+	9/10	0/3
GPO-1&2-tub	+	20/20	3/3
GPO-1&2-bam	+	10/10	3/3
GPO-1&3-tub	+	8/10	3/3
GPO-1&3-bam	+	10/10	3/3
GPO-2&3-tub	+	10/10	3/3
GPO-2&3-bam	+	10/10	3/3

*Can't be done

III: RNAi inactivations of paralogs for different enzymes

Davis and MacIntyre (1988) reported that a combination of a particular GPDH-1 and a GPO-1 null mutant resulted in synthetic lethal phenotype, emphasizing the importance of the alpha glycerophosphate cycle, not only in the operation of the flight muscles but also during pre-adult development. Proceeding from that observation, we wondered if any combination of RNAi's against paralogs of GPDH and GPO would produce synthetic lethals or more extreme phenotypes should they be participating in the alpha glycerophosphate cycle during development. Thus, we produced several such combinations driving their expression once again with tubulin-Gal4 or bag of marbles-Gal4. The data we obtained are presented in Table 4.

Double RNAi's where one is GPDH-1 driven by tubulin-Gal4 are invariably lethal and thus are not shown in Table 4. Also, the male sterility effected by the RNAi against GPDH-2 when driven by either tubulin-Gal4 or bag of marbles-Gal4 was observed and apparently not affected by inactivation of any of the GPO paralogs. In summary we observed no instance of either suppression or enhancement of viability, flight ability, or male fertility in any other combination of paralogs of GPDH or GPO inactivated by their corresponding RNAi's. This again indicates the paralogs of the two enzymes are acting independently of one another during development.

IV: Effect of RNAi inactivation of AK3 on sperm development

When either tubGAL4 or bamGAL4 drivers are crossed to an AK3-RNAi stock, male offspring with both GAL4 and RNAi elements are completely sterile. RT-PCR of RNA extracted from testes of these males using primers specific to the AK3 transcript reveal an absence AK3 transcripts in both qualitative (Figure 1a) and quantitative (Figure 1b) assays. The fold change for AK3 transcripts from testes of normal (Sb/AK3 RNAi) versus knockdown (bamGAL4/AK3 RNAi) siblings shows that the transcript is reduced to extremely low levels in the knockdown. The specificity is also shown by the fact that transcript levels of AK2 are essentially the same between the two genotypes. Further, phase contrast images of testes from these males reveal early stages of spermatogenesis are normal, although no normal mature sperm accumulate in the seminal vesicle (Figure 1d). Closer examination reveals that the process of individualization of the sperm is never completed (Figure 1c). There appears to be a failure of the individualization complex (Fabrizio *et al*., 1973) to complete the process of converting the sixty four spermatocytes contained within the cyst to sixty four individual spermatids. In this case, the testis-specific paralog AK3 does play a critical and specific role in sperm development. Furthermore, the effectiveness of the bamGAL4 driver suggests that transcription

occurred pre-meiotically, while the effects upon sperm function occurred post-meiotically. This is a situation similar to that seen for the testis-specific paralog of another gene, Gld2 (Sartain *et al*., 2011).

Table 4. RNAi inactivation of pairs of paralogs-different enzymes.

RNAi's-Gal4	Viability	Flight Ability	Male Fertility
GPDH-1&GPO-1-bam	+	20/20	3/3
GPDH-1&GPO-2-bam	+	9/10	3/3
GPDH-1&GPO-3-bam	+	20/20	3/3
GPDH-2&GPO-1-tub	+	20/20	0/3
GPDH-2&GPO-1-bam	+	10/10	0/3
GPDH-2&GPO-2-tub	+	18/20	0/3
GPDH-2&GPO-2-bam	+	18/20	0/3
GPDH-2&GPO-3-tub	+	20/20	0/3
GPDH-2&GPO-3-bam	+	17/20	0/3
GPDH-3&GPO-1-tub	+	10/10	3/3
GPDH-3&GPO-1-bam	+	20/20	5/6
GPDH-3&GPO-2-tub	+	20/20	3/3
GPDH-3&GPO-2-bam	+	19/20	3/3
GPDH-3&GPO-3-tub	+	10/10	3/3
GPDH-3&GPO-3-bam	+	10/10	3/3

There is, however, the issue of the RNAi's against GPDH-3, all three GPO paralogs, and AK-2. Since we did not observe any phenotype associated with them when Gal4 is driven by the tubulin and bag of marbles promoters, we cannot be sure they are inactivating their respective messenger RNA's. We have no reason, *a priori*, to suspect them, especially given the dramatic mutant phenotypes associated with the GPDH-1, GPDH-2, AK-1, and AK-3 RNAi's. Also, when Gal4 is driven by the muscle specific promoter actin-88F, all of the GPDH RNAi's, including GPDH-3 and all three GPO RNAi's, produce offspring that are unable to fly. Similarly, the GPO-1 RNAi and AK-1 RNAi, when Gal4 is driven by another muscle specific promoter, mef-2, induced flight-lessness in the Gal4;RNAi containing off-spring. However, in the cases of GPDH-3 and the GPO RNAi's, independent assessment of their knockdown effects on their mRNA's, as was done with AK-3, should be made.

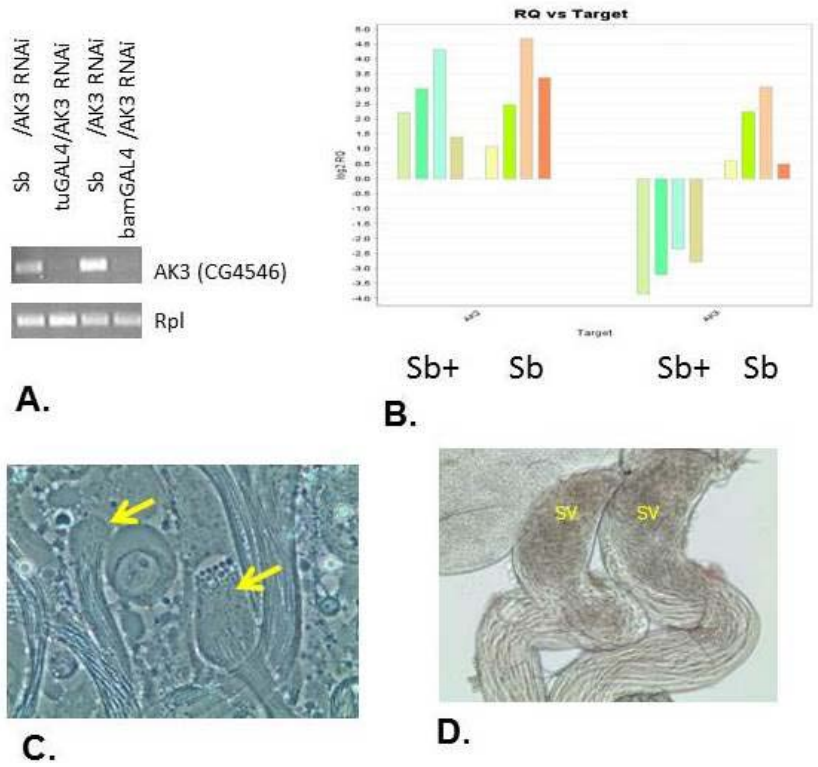

Figure 1. RNAi knockdown of AK3. **A,** Qualitative RT-PCR of AK3 transcripts. RNA from testes of Sb/AK3 RNAi, tuGAL4/AK3 RNAi siblings and Sb/bamGAL4, bamGAL4/AK3 RNAi siblings was used to generate cDNA, which was amplified with primers specific to AK3 (upper panel) or primers specific to Rpl 32.(lower panel). **B,** Quantitative RT-PCR of AK3 transcripts. RNA from testes from four individual Sb/bamGAL4 and four individual bamGAL4/AK3 RNAi siblings was used for RT-PCR using primers specific to AK2, AK3, and RpL32. Reactions were performed on an ABI StepOne Plus platform and comparative CT analysis done with the StepOne software. **C,** Phase contrast images of testis from bamGAL4/AK3 RNAi males. Arrows indicate individualization complexes on cysts of 64 spermatids. **D,** Seminal vesicles of bamGAL4/AK3 RNAi males. No mature sperm are present.

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Effect of temperature on the development time and pupation height of *Drosophila*.

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Temperature is important for the development of insects and other animals. Since insects are cold-blooded organisms, temperature is the most important environmental factor that can influence insect behavior, distribution, development, survival, aging, and reproduction (Mikasa and Narise, 1980; Gillooly *et al.*, 2002; Régnière *et al.*, 2012; Kelly *et al.*, 2013; Danjuma *et al.*, 2014). I chose *Drosophila* (fruit flies) to study the effect of temperature on development time, because they are easy to handle, well understood, possess a short life cycle of just two weeks, and are easy to keep in large numbers. They show complete metamorphosis. There are ~2000 species of *Drosophila* in the world. The average lifespan of *Drosophila* is about 50 days. A female can lay hundreds of fertilized eggs during her brief lifespan. *Drosophila* is also an excellent model organism to study human diseases.

Since temperature affects the development and survival of *Drosophila*, I chose a few laboratory populations to test the developmental time, pupation height, and number of flies emerged.

To perform the experiment, I used the following materials and equipment: 10-15 flies of Oregon-R and Canton-S (3-5 day old individually laboratory-mated female *Drosophila* for each temperature condition); fly food (containing yeast, sugar, agar, cornmeal, water, methylparaben, and ethanol); incubators to keep the flies for testing the development time [18°C, 25°C, and 29°C]; CO_2 chamber used when sorting; and microscope to examine flies.

I used 10-15 flies (males and females) from the main stock and individually transferred them into a fresh vial, while they were sleeping. I prepared three sets (replicates) of vials for each temperature setting (9 total vials with 10-15 flies each). I put these vials into incubators with temperatures set at 18°C, 25°C, and 29°C for 2 days. After 2 days, the flies were taken out from the vials and were observed throughout the developmental stages from egg/embryo until adult. I also observed the pupation height, which was based on the number of pupae resting on the side of the vials. In addition, I also counted the number of flies emerged. Data was analyzed and a graph was made on Microsoft Excel.

I tested if different temperature regimes affect the development time of *Drosophila* and found that at 18°C, flies grow much slower (development time ~20 days) as compared to 25°C (development time ~9 days) and 29°C (~7 days) (Figure 1). I performed these experiments in triplicate and found the same results. I also observed that at lower temperatures the size of the flies is much larger compared to higher temperatures. In addition, I also observed the lowest pupation height at lowest temperature (18°C; Figure 2). Furthermore, I noticed that at lower temperatures flies show less lethality compared to higher temperature.

The above-mentioned results suggest that at lower temperatures *Drosophila* develop slowly. These results also show that at a favorable temperature insects will grow much faster. It is possible that lower temperatures may decrease the rates of depletion of energy substrates (Koštál *et al*., 2016). More lethality observed at high temperatures in our experiments could be due to the accumulation of metabolic waste products or a higher rate of water loss, which is usually greater at later stages of development (Davidson,

1944). Based on my observations and results, I conclude that temperature plays a crucial role in the development of *Drosophila* and may affect the development of other insects, in general. Future experiments using natural and laboratory populations will be employed to test the adaptability and genetic factors on development time and aging.

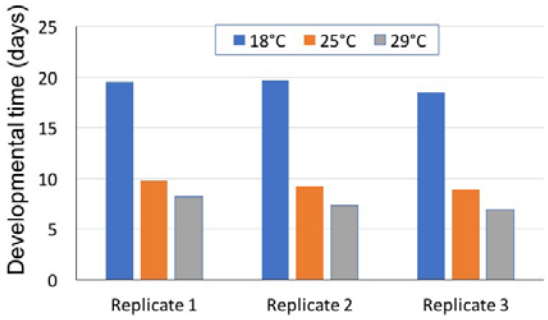

Figure 1.

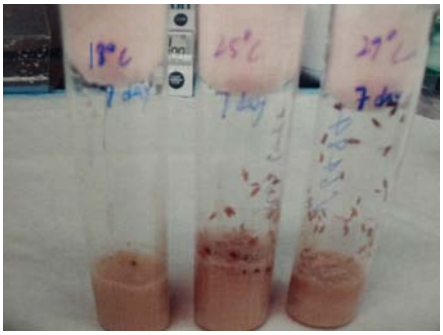

Figure 2.

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Inferring the evolutionary significance of chromosomal inversion polymorphism: insight from *Drosophila* model.

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Abstract

Inversions play a major role in shaping evolutionary processes like speciation and adaptation, by suppressing recombination between advantageous genes spanning inversion breakpoints. Chromosome inversion polymorphism is mostly found to be associated with disease pathogenesis in human beings; however, due to various study limitations, the molecular mechanism behind the appearance of this mutational change is poorly understood. A wealth of information was generated especially on frequency and distribution of chromosomal inversions in species populations using various *in vivo* systems. In addition, their evolutionary significance has been established by gaining knowledge from various cytogenetics and behavioral studies conducted using these model systems. To this respect, *Drosophila* is considered to be one of the popular model organisms as it carries polytene chromosomes in one of its larval stages. Also it shares 75% of genome homology with humans and the basic cellular and biological processes are found to be conserved amongst both of them, thus clearly showing the significance of the study outcome of this model in

understanding human disease biology and evolution. In the present article, we have reviewed and compiled the research outcome of chromosomal inversion polymorphism studies carried out in different *Drosophila* species to determine the significant role played by inversions at organismal level. This article aims to provide an insight in understanding the genotype and phenotype architectural changes occurring through inversion polymorphism and their evolutionary consequences in humans as well as other organisms. Keywords: Mutation**,** Chromosome inversion polymorphism, *Drosophila*, Life history traits, Adaptation, Speciation

Introduction

The genome of an organism is susceptible to changes that may occur due to different biological, physical, or biochemical processes. Mutation can be defined as any random change in the gene sequence of an organism that may or may not affect the normal functioning of the body (Ohta, 1992; Kimura *et al*., 1994; Alberts *et al*., 2000). Mutations can occur due to exposure to some environmental mutagens, which may alter the functioning of any gene, leading to a diseased condition. Inversion is a kind of mutation in which two breaks occur in one chromosome and the region between the breaks rotates 180 degrees before rejoining with the two end fragments. Inversions fall into two classes: pericentric inversions include a centromere, while paracentric inversions do not include a centromere. Pericentric inversions may reduce fertility by producing unbalanced gametes that carry insertions, deletions, and chromosomes with either zero or two centromeres (Kirkpatrick, 2010).

According to the demographic theory proposed by Mettler *et al*. (1977), inversions are categorised into four types: i) common cosmopolitans are those that occur in many populations at a frequency greater than 5%; ii) rare cosmopolitans, too, are those that are present in many populations, but at frequency usually less than 5%; iii) recurrent endemics are those that occur in only a few individuals in the same or adjacent populations; iv) unique endemics are those that are recorded only once. Inversions can either occur as homozygous wherein the sequence is inverted on both chromosomes (Inv/Inv) or there is standard sequence on both chromosomes (ST/ST), or they can be heterozygous (ST/Inv) wherein there is inverted sequence in one chromosome only (Singh and Mohanty, 1990). Heterozygous inversions are easily identifiable as they lead to formation of an inversion loop while pairing, and the same has been thoroughly studied in certain Dipterans using their polytene chromosomes. The recombination in case of heterozygous inversions may result in the formation of non-viable gametes and as a consequence, the probability of recombination decreases. In this situation, the genes located within the inversion loop undergo no or fewer changes, thus escaping them from the effect of environmental change. It has been proved experimentally in *Drosophila* (Singh and Mohanty, 1990) that inversion heterozygosity present in one chromosome enhances the crossing-over rate in other chromosomes, whereas decreases crossing-over not only within the inverted loop, but also in its adjacent region. Moreover, a correlation has been established between the crossing-over frequency and the DNA fragments involved within the inversion breakpoints (Singh and Mohanty, 1991). Unlike deletions and duplications, inversions do not change the overall amount of the genetic material, thus organisms carrying inversions are found to be viable without showing any particular abnormalities at the phenotypic level (Griffiths *et al*., 2000). In some cases, when one of the chromosomes breaks within a functional gene then that inversion breakpoint is considered to be a lethal gene mutation.

Inversions occur as a normal phenomenon in natural populations of different species, and inversion polymorphisms are known to have important phenotypic and evolutionary consequences in humans (Caceres *et al*., 2015). Frequency of inversion polymorphism has been studied in species populations of different geographical origins and is found to be species–specific. In an attempt to study the evolutionary forces leading to primate evolution, Feus *et al*. (2005) compared the inversions present in the genome of chimpanzees and humans and could trace the role of inversion in the process. Nine pericentric inversions are identified to be the potential candidates in the speciation events of hominids and chimpanzees and occurred approximately five-six million years ago (Kehrer *et al*., 2005). Breakpoint analysis of those inversions strongly evidenced that specific high copy repetitive elements play a major role in bringing changes in the genome architecture during hominoid evolution (Kehrer *et al*., 2005; Guillen and Ruiz, 2012).

Several inversions in the human population have been identified playing a role in disease etiology, for instance, a 48 kb inversion was found to be linked to the Emery-Dreifuss muscular dystrophy (Broman *et al*.,

2003), 17q21 to neurodegenerative disorder (Skipper *et al*., 2004; Stefansson *et al*., 2005; Webb *et al*., 2008), and 16p11 to asthma and obesity (Gonzalez *et al*., 2014). Another common paracentric inversion polymorphism 8p was found to have substantial clinical impact (Giglio *et al*., 2001; Broman *et al*., 2003). Equally important, an inversion on chromosome 17 is thought to affect fertility and is noted to be positively selected in European populations (Stefansson *et al*., 2005). Inversions on chromosome four and eight are found to be involved in translocations, and such rearrangements in the chromosome drastically affect the expression of some important genes spanning the inversion breakpoints (Giglio *et al*., 2001; Broman *et al*., 2003). On chromosome 19, a 415 kb human polymorphic inversion (HsInv0379) was studied by Puig *et al*. (2015), which disrupts and inactivates a transcription factor gene. As an outcome, a new transcript of that gene is created and the manifestation of this change has been observed at the phenotypic level. However, the molecular mechanism behind the occurrence of these inversions and their role in disease pathogenesis is poorly understood. Therefore, research efforts are being continuously made on various model and non-model organisms to explore more in this field.

The genus *Drosophila* has been extensively researched in various fields including genetics, evolution, and medical biotechnology, which makes it an ideal model system. It is one of those model organisms whose genome has been completely sequenced in twelve species. The species of this genus, like other Dipterans, harbours polytene chromosomes in their salivary gland of third instar larvae, gut cells, ovary nurse cells, follicle cells surrounding ooctyes, and fat body cells (Zhimulev and Koryakov, 2009). However, most of the cytogenetic studies have been carried out using the salivary gland cells.

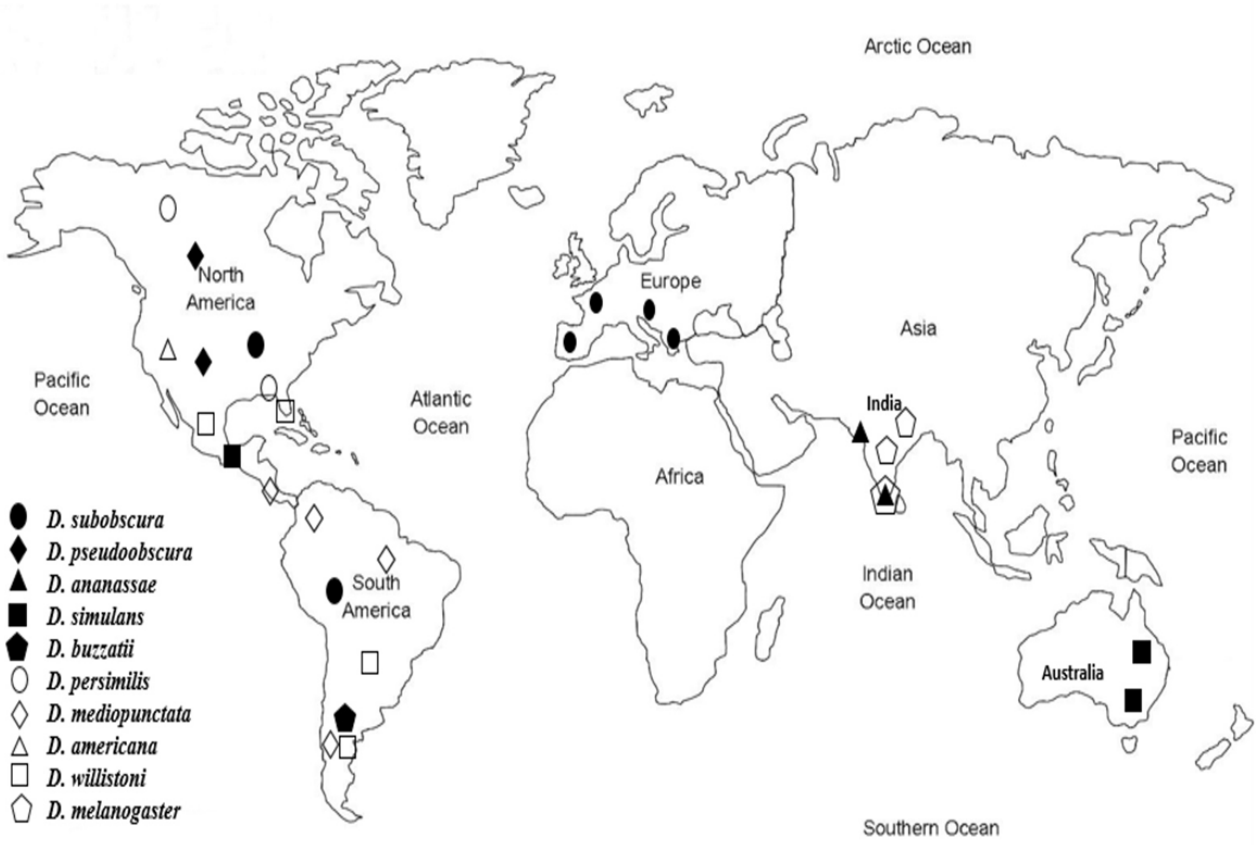

Figure 1. Shows the *Drosophila* species used in inversion polymorphism studies worldwide.

Small number of chromosomes and presence of polytene chromosomes in its salivary gland cells make this model instrumental in cytogenetic studies. A number of experiments involving inversion frequencies, their association and co-existence have been carried out in various *Drosophila* species worldwide such as *D.*

ananassae, D. willistoni, D. robusta, D. pseudoobscura, etc. (Figure 1) in order to understand their consequences and role in different evolutionary processes like adaptation and speciation (Dobzhansky, 1970; Rieseberg *et al*., 1999; Noor *et al*., 2001; Kirkpatrick and Barton, 2005). Chromosomal inversion polymorphism has been extensively studied in one of the cosmopolitan species, *D. ananassae*, and a total number of 70 paracentric inversions were reported. This species shows unusual mutational properties due to presence of pericentric inversions and translocations and carry three of the most common cosmopolitan inversions (subterminal or alpha in 2L, terminal or delta in 3L, basal or eta in 3R) in its worldwide populations (Kaufman, 1936; Singh, 1970). Singh (1989) studied inversion polymorphism in 12 Indian natural populations of *D. ananassae* and concluded that chromosomal polymorphism is adaptively important and populations undergo genetic divergence as a consequence of their adaptation to varying environments. The populations of this species are known to show high population sub structure across the whole distribution range. Inversion frequency is known to vary along with geographical, latitudinal distribution of different population of *Drosophila* species as found by Dobzhansky and co- workers in a species known as *D. willistoni* (Dobzhansky, 1947). Their findings suggest that chromosomal polymorphism is more prevalent amongst populations at the centre of geographical distribution rather than in marginal populations (Hoffmann and Rieseberg, 2012). The same pattern of adaptation of polymorphism was also reported by Carson (1949) in *D. robusta*. They hypothesised the prevalence of homoselection owing to low levels of inversion polymorphism and thus higher adaptability amongst the marginal population, whereas in the central population the level of inversion polymorphism is high and heteroselection is favored resulting in higher adaptation (Singh, 2001). Similar kinds of studies performed in *D. pseudoobscura* infer that inversion polymorphism is adaptive and balanced due to higher Darwin fitness of inversion heterozygotes. Studies were also done to find the association of inversion polymorphism heterozygosity with the genes located within and also in the vicinity of the inversion. A linkage study was done in Japanese populations of *D. melanogaster* between the *Adh* locus and 2L inversion and both of them were found to be positively correlated (Watanabe and Watanabe, 1977). The sequence specificity at the inversion breakpoints was also studied by some workers after advanced molecular biology techniques had been introduced (Kehrer *et al*., 2005).

The overall studies on occurrence and maintenance of inversion polymorphism in natural populations of *Drosophila* are found to be associated with an organism's fitness and adaptation to different ecological niches. The present review is a compilation of advantageous role played by different inversion polymorphisms in affecting both phenotype and genotype of an organism.

Effect of Inversion on Life History Traits

It is evidenced that inversion polymorphism affects various life history traits in different organisms including *Drosophila, Anopheles,* and *Neurospora*. The traits affected by inversion polymorphism have been enlisted in Table 1. Inversion polymorphisms in *Drosophila* have been found to be associated with several life-history traits, *e.g*., body size, shape, pigmentation, and bristle number. (Bertran *et al*., 1998; Rodriguez *et al*., 1999; Dahlgaard *et al*., 2001). Body size and bristle number again are found to be linked with several factors like reproductive success and longevity of an organism (Robertson, 1957; Santos *et al*., 1988; Das *et al*., 1994; Norry and Loeschcke, 2002; Iriarte *et al*., 2003). The correlation between body pigmentation and chromosomal inversion was studied by Hatadani *et al*. (2004) in *D. mediopunctata*. This species possesses three dark spots in its abdomen and this color pattern is genetically determined mainly by the second chromosome, which is highly polymorphic for inversions. Body color was also studied in *D. americana* and similar results were observed suggesting that body color is an adaptive trait (Wittkopp *et al*., 2011). Experiments carried out in *D. mediopunctata* and *D. buzzati* populations suggest that inversion polymorphism affects the wing size and body shape (Iriarte *et al*., 2003; Hatadani *et al*., 2004). There are research reports showing inversion polymorphism affecting different developmental traits such as larva and pupa developmental time period (Norry *et al*., 1995; Iriarte and Hasson, 2000; Singh, 2008). A link between the frequency of inversion polymorphism (2L: alpha inversion) and presence of extra scutellar bristles has also been found by Das *et al*. (1994) among five Indian natural population of *D. ananassae* belong to different eco-geographic regions.

Table 1. Literature showing examples of chromosomal inversion polymorphism affecting different traits of *Drosophila* species.

Species	Inversion Reported	Chromosome Arm	Role of inversion	Reference
D. ananassae (India)	4 Cosmopolitan Paracentric inversions	2L, 2R; 3L; 3R	Population genetics; Positive correlation between inversion and extra bristles	Das *et al.* 1994;Singh 1998; Singh & Singh 2008
D. mediopunctata	2 Paracentric inversions	2nd	Role in color polymorphism, Wing size and shape	Hatadani *et al*. 2004
D. melanogaster (India)	23 Paracentric inversion	2L; 2R; 3L; 3R	Inversion frequency varies with latitudes; Epistatic interaction between unlinked inversions	Das & Singh 1991a; Singh & Das 1991b
D. melanogaster (Europe)	Five Cosmopolitan Paracentric inversion	2L; 2R; 3L; 3R; 3R	Change in inversion frequency with respect to seasonal fluctuations suggesting a role in adaptation	Gonzalez & Mensua 1987 ;Refusta & Rubio1990
D. melanogaster (Japan)	27 Paracentric One pericentric	2L; 2R; 3L; 3R; X, 3L	Comparison of changes and similarities between the inversion frequencies: Studying the evolutionary forces	Inoue & Watanabe 1979
D. pallidosa	One Paracentric inversion	2R	Species divergence from *D. ananassae*	Singh *et al.* 2012
D. persimilis	Three inversions	XL; XR; 2nd	Species divergence from *D pseudoobscura*.	McGaugh & Noor 2012
D. subobscura (Europe)	Eight inversions	2 in autosomes, 1 in sex chromosome	Genetic uniformity maintained across wide latitudinal gradient	Simo *et al*. 2012
Drosophila (Other *Drosophila* species)	Both paracentric and pericentric	Both on autosomes and X-chromosome	Body size, Wing shape, Resistance to heat and cold stress, Development time, Reproductive success	Hoffmann & Rieseberg 2008

Role of Inversion Polymorphism In Adaptation

Inversion polymorphism is known to play an important role in thermal adaptation. Several studies on *Drosophila* show a correlation among changes in inversion frequencies to the recent climate changes. Those studies infer that widespread species may adapt in response to the 0.2ºC increase in temperature per decade in the past 30 years (IPCC, 2007). There is also literature evidence showing a strong correlation between change in temperature and *Drosophila* species distribution. A clinal pattern exhibited by the *In (3R)P* inversion has been detected in *D. melanogaster* along the eastern coast of Australia over more than 20 years (Anderson *et al*., 2005; Umina *et al*., 2005). This inversion increases sharply from a low frequency in the temperate south of Australia to be close to fixation in tropical populations. Inversions are also known to harbor many heat shock response genes of Hsp family (Molto *et al*., 1992; Rego *et al*., 2010). Similar studies have been conducted in *D. subobscura,* which harbors a large number of inversion polymorphisms in its five large acrocentric chromosomes (Rezende, 2010). In *D. subobscura*, the chromosome O, which is homologous to the third and second chromosome of *D. melanogaster* and *D. pseudoobscura*, respectively, is the longest and most polymorphic chromosome, carrying about 40 natural chromosomal arrangements and maximum number of overlapping and non-overlapping inversions (Krimbas and Loukas, 1980; Krimbas, 1993). In a study done on

South and North American population of *D. subobscura*, it was observed that behavioral thermoregulation and heat tolerance are "coadapted" (Dolgova *et al*., 2010). The different arrangements on chromosome O determine thermal preference of adults as observed in a laboratory temperature gradient study. The cold climate *Drosophila* flies with standard arrangement on 'O' chromosome (O_{st}) with respect to inversion polymorphism prefer lower temperature than their warm-climate counterparts carrying different inversion polymorphisms (O_{3+4} and O_{3+4+8}) (Dolgova *et al*., 2010). Thus, it is concluded that in *D. subobscura* thermal preference/ heat tolerance and chromosomal inversion polymorphism maintain parallel latitudinal clines throughout worldwide populations.

The reason behind this latitudinal gradient may be due to correlated selection rather than genetic correlation (Dolgova *et al*., 2010; Calabria *et al*., 2012). It has been observed that flies carrying the warm-climate chromosome arrangement (O_{3+4}) have higher basal protein levels of Hsp70 than their cold-climate O_{st} counterparts and also possess more thermal tolerance, but a link between thermal tolerance and levels of Hsp70 could not be established (Molto *et al*., 1992; Calabria *et al*., 2012). Research was also being carried out to estimate the effect of April 2011 heat wave amongst the European population of *D. subobscura* (Rodrıguez *et al*., 2013). The result revealed that thermal stress affects the genetic makeup of the organism as a larger number of inversions were observed on the chromosome O of this species in warm climate in comparison colder climate. It is evidenced from these studies that evolution of chromosome inversion polymorphisms in *D. subobscura* has been found to be fast and reversible, which may be due to eco-climatic variations. In a similar study to explore more about genetic divergence at chromosome O, six candidate genes for thermal adaptation were selected and analysed for their chromosomal arrangements with respect to O_{3+4} chromosomal polymorphism in two populations of *D. subobscura* (Pegueroles *et al*.,2013). The results showed extensive gene flux outside the inverted region which supports the local adaptation hypothesis, while significant genetic differentiation was found within the inverted region. However, high levels of gene flow were detected for all six genes when comparing the same arrangement among populations, and the maintenance of these inversions in the local populations favors the '*local adaptation'* hypothesis over the '*coadapted genome'* hypothesis.

Impact of climatic changes on the genetic composition was also evidenced in a study conducted in *D. pseudoobscura* by Dobzhansky and later on in various other species, *e.g*., *D. persimilis*, *D. funebris*, *D. flavopilosa*, *D. robusta*, *D. melanica*, *D.melanogaster*, and *D. mediopunctata* (Rezende *et al*., 2010). The outcomes of all these studies show the adapting nature of chromosomal inversion polymorphism across species with respect to their geographical distribution (Rezende *et al*., 2010). Balanya *et al*. (2003) provided unambiguous evidence that selection on the chromosomal inversion polymorphism of *D. subobscura* must be strong and in that selection procedure, environmental factors associated with latitude probably play an important role. Warm-climate chromosome rearrangements are increasing in frequency in European populations of *D. subobscura* (Orengo and Prevosti, 1996; Rodriguez *et al*., 1996). The same trend has been reported in populations of South and North America, indicating warm-climate inversions are increasing in frequency at higher latitudes in all continents (Balanya *et al*., 2006). The above results suggest that variations in genetic markers including chromosomal polymorphism can be helpful to determine the impact of climatic change on the genetic makeup of populations at different locations (Rezende *et al*., 2010). The genetic uniformity of chromosomal inversions was studied by Pedro *et al*. (2012) in European population of *D. subobscura* to see the effect of latitudinal gradient on the genetic content of the inversion polymorphism of the species. Their findings were in accordance with the *local adaptation hypothesis* of Dobzhansky as very low genetic differentiation was observed amongst the flies taken from a wide geographical latitude difference suggesting that the population maintained its genetic uniformity across the wide latitude.

With increasing industrialisation the level of pollution is getting increased day by day in nature. Heavy metal pollutants affect natural population of organisms in many ways. They may cause selection pressure which may lead to genetic adaptation (Reznick and Ghalambor, 2001; Loxdale, 2010). Studies regarding the deteriorating effect of pollutants on the natural population of insects go back to the times of Dobzhansky (1971) who studied the effect of DDT a well-known pesticide, on the third chromosome of *D. persimilis* and *D. pseudoobscura.* In *D. melanogaster* of Katasunuma, Japan, stark changes in the frequency of inversions on 2nd chromosome were observed along with an increase in the frequency of lethal second chromosome. Watanabe *et al.* (1975) studied these changes and tried to establish its relation with the increased use of pesticides and the changing environmental conditions. However, any conclusive link between

these proposed reasons could be established. In a study done on *D. subobscura* the link between stress and role of inversion in adapting to the stress condition was established (Kenig *et al*., 2015). In a laboratory maintained population of *D. subobscura*, it was shown that resistance to the harmful effect of lead contamination was higher in populations originating from a more polluted locality. Also amongst the urban population the frequency of inversion polymorphism was higher due to presence of large ecological niche in such environment (Singh, 2008; Kenig *et al*., 2010). The results showed that initial difference in inversion polymorphism between populations remained in laboratory throughout experiment and in case of lead contamination has been increased significantly (Kenig *et al.,* 2015). Populations originating from a polluted environment had a higher level of inversion polymorphism and better ability to adapt and evolve. So both historical events as well as selection procedures are important for bringing evolution as both of them take part in adaptation.

Role of Inversion in Speciation

Speciation can be defined as gradual changes in the genome of an organism over a long period of time that leads to significant changes causing species divergence. Inversions are known to play a major role in speciation. The possible mechanism of inversion in speciation was explained by proposing two models (White, 1978; King, 1993). Firstly, the traditional under-dominance model which says that recombination between rearranged chromosomes generates gametes carrying chromosomal duplications or deficiencies. The gametes produced as a result of such process are in viable and cause sterility in the individual, thus creating a reproductive barrier between populations or species that differ for this rearrangement. However, this model cannot be accepted owing to the fact that heterozygotes are more fit and a rearrangement that causes a large reduction in the fitness of heterozygotes is unlikely to become established in the first place. Now the question arises that despite gene flow species do persist. So to answer the reason behind the existence of species, a second model was proposed which suggests that there is reduced rate of recombination between the inverted and non-inverted region of the chromosome by which the genes present in the inverted region remain conserved (White, 1978; King, 1993, Noor *et al*., 2001; Rieseberg, 2001). Literature proposes that chromosomal inversions partition the genome into regions protected from gene flow by reducing recombination over long stretches, which may lead to speciation (Noor *et al*., 2001; Barrientos *et al*., 2002; Navarro and Barton, 2003; Kirkpatrick and Barton, 2006).

Another mechanism by which inversions may facilitate speciation is the accumulation of alleles that contribute to reproductive isolation between populations connected by gene flow (Hoffmann *et al*., 2008). A comparative cytogenetic study using polytene chromosomes was done by Singh *et al*. (2012) in two sibling species *D. ananassae* and *D. pallidosa*. Both the species though share the same ecological niche but are reproductively isolated due to behavioral isolation. The experiment revealed the presence of inversion loops in hybrid progeny, thus suggesting a change in the order of gene arrangement which might be leading to species differentiation (Singh *et al*., 2012). Similar study was carried out on the sister species *D. pseudoobscura* and *D. persimilis*, which harbor different chromosomal arrangements with respect to inversions present on XL, XR, and 2nd chromosome, and the genes present within the inverted regions may be responsible for reproductive isolation amongst these two species (McGaugh, 2012).

Though there is absence of gene flow, the subdivided population acquires genetic variations by exposing themselves to different geographical regions, which may lead to population differentiation and species divergence. The evolutionary forces leading to speciation have also been studied by several scientists. The outcome of these studies reveal that mutations in the form of chromosomal inversions are found to be the cause of genomic divergence (Rieseberg, 2001; Faria and Navarro, 2010; Gompert *et al*., 2012; Guerrero *et al*., 2012; Feder *et al*., 2014). Similarly, rapid chromosomal evolution with respect to inversion polymorphism has been reported in *D. mojavensis*, where natural selection is found to play a major role in increasing the number of fixed inversions in a new environment (Gullean and Alfredo, 2012). However, it has been observed that favorable genes within the inversions are more favorable together in their natal habitat than they would be present individually (Nosil and Feder, 2011). The divergence pattern and the recombination rate have been studied in a pair of parapatric species *D. pseudoobscura* and *D. persimilis* and their sympatric outgroup species *D. miranda*. The results of this study revealed that within the XL and chromosome two inversions, *D.*

persimilis and *D. pseudoobscura* share a deeper ancestor than they do with *D. miranda* (Stevison, 2011; McGaugh, 2012). Though they have been diverged approximately 0.5–0.85 Ma (Aquadro *et al.*, 1991; Hey and Nielsen, 2004; Stevison, 2011), part of their genome carry a signature of more recent hybridization (Machado *et al*., 2002; Hey and Nielsen, 2004; Machado *et al*., 2007; Kulathinal *et al*., 2009; Stevison, 2011).

Literature also suggests a role of inversion at the beginning of sex chromosome evolution (Ming and Moore, 2007). This has been clearly evidenced by studying genetic variability that during the early evolution of mammalian Y a series of overlapping inversions progressively extended the size of non-recombining portions of Y (Lahn and Page, 1999). The same has been explained under sex-antagonist selection model (Kirkpatrick, 2010). Therefore, inversion helps to isolate the Y chromosome from X chromosome genetically and make Y chromosome as an asexual genetic unit. As a consequence of which Y evolves as a functional male determining chromosome in mammals and flies. Charlesworth *et al*. (2005) also suggested that chromosomal rearrangements such as inversion increase recombination suppression in sex chromosomes thus playing a role in maintaining differentiation amongst sex determining genes located on X and Y chromosomes.

Analysis of inversion breakpoints may provide an insight into the occurrence of the inversion polymorphism. Different chromosomal rearrangements including transposable elements (TE), segmental duplications(SDs), short sequence repeats, or chromosomal breakages involving single and double staggered breaks at the breakpoint region are known to play a significant role in generation of inversions (Guillen and Ruiz, 2012). Several attempts have already been made by scientists to understand this random or non-random association of specific sequences present at the breakpoint region of fixed inversions between different species. For instance, the chromosome two of *D. mojavensis* has been well studied for the breakpoint analysis of seven fixed inversions in this chromosome (Guillen and Ruiz, 2012). Similar study has also been done in *D. buzzati* and the probable reason for occurrence of these inversions has been highlighted in both the studies (Calvete *et al.*, 2012). However, the results of these studies do not provide a concurrent proof of the link between the existence of molecular markers (TE, SDs, *etc*.) at the breakpoint region and the corresponding inversion. The question as to whether the presence of these specific repetitive sequences at the inversion breakpoint has been exploited by the organisms to adapt to the changing environment by creating inversions remains unanswered and requires deeper research.

Conclusion

The overall study outcome as carried out in different parts of the world, suggest a major role of inversion polymorphism in life history trait variations, adaptation, and speciation. The species-specific nature of inversions makes them instrumental also in species identification. Although a lot of research on inversion polymorphism has been performed in world-wide populations of *Drosophila* and other arthropods, further exploration is needed to understand the basis of their occurrence, selective existence, and their role in bringing changes in gene-genome organisation of different eco-geographic species populations. With the technological advancement, genetic mapping has become less time consuming, inaccurate and provides us detailed information about mutation or changes leading to disruption or inactivation of specific genes towards a better understanding of directional selection, selective gene adaptation during speciation. The examination of inversion breakpoints has unfolded a higher level of understanding so as to gain in-depth knowledge for the reason for occurrence of repetitive sequences at the region flanking the inversions. Therefore, the cytological and the molecular studies of chromosomal mutations including inversions may altogether help in bridging the gaps between phenotype effects and genotype changes that occur in the organisms.

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Checklist of Drosophilid species so far described and recorded from the Darjeeling hill areas, West Bengal, India.

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Systematic position:

PHYLUM: ARTHROPODA
CLASS: INSECTA
SUBCLASS: PTERYGOTA
DIVISION: ENDOPTERYGOTA
ORDER: DIPTERA
SUBORDER: BRACHYCERA
SUPER FAMILY: EPHYDROIDEA
FAMILY: DROSOPHILIDAE

Subfamily Steganinae

I . Genus *Leucophenga* Mik

1. *Leucophenga rimbikiana* Singh & Gupta, 1981

II. Genus *Stegana* Meigen

2. *Stegana shirozui* Okada, 1971

Subfamily Drosophilinae

III. Genus *Dettopsomyia* Lamb

3. *Dettopsomyia argentifrons* Okada, 1956
4. *Dettopsomyia nigrovittata* Malloch, 1924

IV. Genus *Drosophila*

i. Subgenus *Dorsilopha* Sturtevant

5. *Drosophila busckii* Coquillett, 1901

ii. Subgenus Sophophora

6. *Drosophila melanogaster* Meigen, 1830
7. *Drosophila neoelegans* Gupta & Singh, 1977
8. *Drosophila ananassae* Doleschall, 1858
9. *Drosophila bipectinata* Duda, 1923
10. *Drosophila malerkotliana* Parshad & Paika, 1964
11. *Drosophila parabipectinata* Bock, 1971
12. *Drosophila lucipennis* Lin, 1972
13. *Drosophila tristipennis* Duda, 1924
14. *Drosophila unipectinata* Duda, 1924
15. *Drosophila immacularis* Okada, 1966
16. *Drosophila pulcherella* Tan, Hsu & Sheng, 1949
17. *Drosophila suzuki indicus* Parshad & Paika, 1964
18. *Drosophila takahashii* Sturtevant, 1927
19. *Drosophila trilutea* Bock & Wheeler, 1972
20. *Drosophila kurseongensis* Gupta & Singh, 1977
21. *Drosophila nepalensis* Okada, 1955
22. *Drosophila gundensis* Prakash & Reddy, 1977
23. *Drosophila kikkawai* Burla, 1954
24. *Drosophila punjabiensis* Parshad & Paika, 1964
25. *Drosophila trapezifrons* Okada, 1966
26. *Drosophila suborosa* Kumar & Gupta, 1992

iii. Subgenus *Drosophila* Fallén

27. *Drosophila neomakinoi* Gupta & Singh, 1981
28. *Drosophila novazonata* Gupta & Dwivedi, 1980
29. *Drosophila parazonata* Gupta & Dwivedi, 1980
30. *Drosophila mediobandes* Dwivedi & Gupta, 1980
31. *Drosophila trisetosa* Okada, 1966
32. *Drosophila ramamensis* Dwivedi, 1979
33. *Drosophila bimorpha* Singh & Gupta,1980
34. *Drosophila guptai* Dwivedi, 1979
35. *Drosophila tetradentata* Singh & Gupta, 1980
36. *Drosophila peniclubata* Singh & Gupta, 1980
37. *Drosophila dominici* Dwivedi, 1981
38. *Drosophila immigrans* Strutevant, 1921
39. *Drosophila annulipes* Duda, 1924
40. *Drosophila fuscicostata* Okada, 1966
41. *Drosophila maryensis* Gupta & Dwivedi, 1980

42. *Drosophila setitarsa* Gupta & Dwivedi, 1980
43. *Drosophila pentastriata* Okada, 1966
44. *Drosophila paralongifera* Gupta & Singh, 1981

iv. Subgenus *Siphlodora* Patterson and Mainland

45. *Drosophila* (*Siphlodora*) *lacertosa* Okada, 1956
46. *Drosophila* (*Siphlodora*) *repleta* Wollaston, 1858

v. *Incertae sedis*

47. *Drosophila mukteshwarensis* Joshi, Fartyal and Singh, 2005
48. *Drosophila teresae* Pradhan, 2015

V. Genus *Hypselothyrea* de Meijere

49. *Hypselothyrea guttata* Duda, 1926

VI. Genus *Hirtodrosophila* Duda

50. *Hirtodrosophila fascipennis* Okada, 1967

VII. Genus *Liodrosophila* Duda

51. *Liodrosophila okadai* Dwivedi & Gupta, 1979

VIII. Genus *Lordiphosa* Basden

52. *Lordiphosa acutissima* Okada, 1956
53. *Lordiphosa aurantifrons* Okada, 1984
54. *Lordiphosa neokurokawai* Singh & Gupta, 1981
55. *Lordiphosa parantillaria* Kumar & Gupta, 1990
56. *Lordiphosa coei* Okada, 1966

IX. Genus *Nesiodrosophila* Wheeler & Takada

57. *Nesiodrosophila lindae* Wheeler & Takada, 1964

X. Genus *Scaptodrosophila* Duda

58. *Scaptodrosophila minima* Okada, 1966

XI. Genus *Scaptomyza* Hardy

59. *Scaptomyza tistai* Kumar & Gupta, 1992
60. *Scaptomyza clavata* Okada, 1973
61. *Scaptomyza parasplendens* Okada, 1966

62. *Scaptomyza graminum* Fallén, 1823

63. *Scaptomyza pallida* Zetterstedt, 1847

64. *Scaptomyza elmoi* Takada, 1970

65. *Scaptomyza himalayana* Takada, 1970

XI. Genus *Zaprionus* Coquillett

Subgenus *Zaprionus*

66. *Zaprionus indianus* Gupta, 1970

Chromosomal inversion polymorphism analysis of a population of *Drosophila polymorpha* of the Serra do Tabuleiro State Park, Brazil.

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The Atlantic Rainforest comprises a vegetal formation that covers a great territory of Brazil and also parts of Paraguay and Argentina (Ishiy *et al*., 2009). Due to its coastal location, this is one of the ecosystems that presents a higher risk of extinction. However, this Biome consists on one of the most important reservoirs of biodiversity on the planet. Because of this, the genetic analysis of insect populations as bioindicators in the remaining areas of Atlantic Forest in Santa Catarina State (Brazil) is very timely and is of the utmost importance for the understanding of the Biodiversity of this ecosystem, providing critical information for establishing conservation policies.

One way for assessing genetic diversity is through the analysis and characterization of chromosomal inversion polymorphisms in natural populations. In natural populations of the genus *Drosophila*, chromosomal inversion polymorphisms are frequently observed forming inversion loops, and in high frequency. The most probable explanation of this phenomenon is that while the recombination index is reduced between the alleles contained inside an inversion, the alleles contained inside it are maintained as a block that pass through generations as a unit. This block may be under natural selection and co-adapted to a particular climatic/geographic condition and, thus, they may provide reproductive advantage to his carrier (Dobzhansky, 1970; Anderson *et al*., 2005; Kirkpatrick and Barton, 2006). In addition, in *Drosophila*, the presence of chromosomal inversions provoke no reduction of fertility (Krimbas and Powell, 1992).

Heterozygous chromosomal inversions are easily observable in polytene chromosomes, present in the salivary glands of the larvae of flies. The polytene chromosomes present a series of bands of heterochromatin and puffs that are species specific, so the characterization and localization of the inversion loops are possible. *Drosophila polymorpha*, the species studied in this work, have 18 paracentric inversions described so far (Rohde and Valente, 1996; Cordeiro *et al*., 2014).

The objective of this study was to analyze the polytene chromosome polymorphisms of a *Drosophila polymorpha* population of a primary formation of the Serra do Tabuleiro State Park (27°44'48"S; 48°48'43"W), Brazil (Figure 1). This park is the biggest conservation area of the Santa Catarina State, comprising almost 1% of his territory. The flies were captured in the field using traps designed by Roque *et al*. (2011) and cultured in the laboratory. Isolines from single females of *D. polymorpha* were founded, using the medium described by Bizzo *et al*. (2012). Species were determined and confirmed from the analysis of the F1 male genitalia.

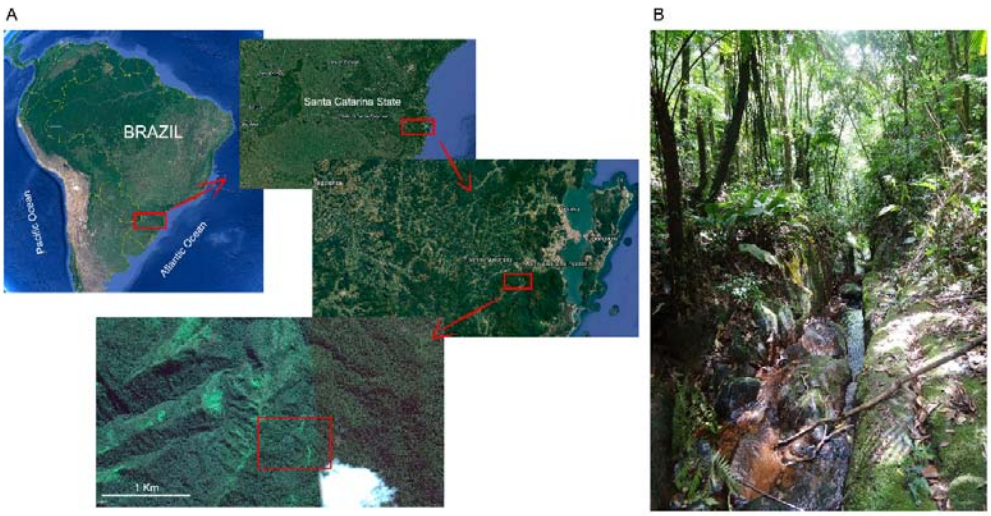

Figure 1. Characterization of the study site. A) Map of the study site in Brazil, showing Santa Catarina State. Map of Santa Catarina State, highlighting the collection area at Santo Amaro da Imperatriz, with satellite images of the region limited by the red rectangles (Reference: http://maps.google). B) Typical collecting site.

Next, polytene chromosome preparations were obtained from third instar larvae and photographed for analysis of heterozygous chromosomal inversions. Three chromosomal inversion polymorphisms were detected, all located on the right arm of chromosome 2.

These results reveal that there is a well-established inversion chromosomal polymorphism, which allows characterizing these continental populations and contrasting them with other conservation units, both continental and insular. This indicates the need for stabilizing effective conservation policies in this park.

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A geometric analysis of the macronutrient needs of *Drosophila suzukii* larvae.

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Abstract

The nutritional needs of animals largely depend on their ecology and habitat. Phenotypes and general performance often depend on the synergistic influence of multiple nutrients. These effects are currently studied within the geometric framework of nutrition. Contrary to its close relative *Drosophila melanogaster*, the invasive Spotted-Wing *Drosophila*, *Drosophila suzukii*, attacks fresh, undamaged fruit devoid of microbial growth. Different oviposition habits suggest different nutritional needs by the two species. We investigated the combined influence of carbohydrate and protein concentrations on the larval performance of a *D. suzukii* population. Proportions of individuals that survived until the adult stage were maximal at intermediate protein and low sugar concentrations. Larval development was shortest under high protein diets. Observations on this population are congruent with what is known of *D. suzukii* larval ecology, as ripening, undamaged fruit is generally poor in sugars and proteins in comparison to ripe, yeast-colonized fruit. We discuss the limitations

of fly nutrition experiments based on laboratory food, such as ours, where the natural dynamics of microbial growth is neglected.

Introduction

Animals need to acquire a variety of resources to develop and reproduce. The relative proportions of nutrients, as well as their absolute amounts, influence phenotype and ultimately fitness (Raubenheimer *et al.*, 2009). Importantly, numerous nutrients have synergistic effects: the effect of given resource on animal phenotype has different effect when another resource is abundant or scarce (Jacobs *et al.*, 2009). This observation has led to the formulation of the geometric framework of nutrition (*i.e.*, nutritional geometrics), whereby several types of nutrients are jointly studied in a multi-dimensional space (Raubenheimer *et al.*, 2009). This framework has, for example, allowed to disentangle the effect of caloric restriction and protein intake on longevity in *Drosophila melanogaster*, the ubiquitous fly encountered in kitchens and laboratories world-wide (Lee *et al.*, 2008).

The spotted-wing *Drosophila*, *Drosophila suzukii*, our focal organism, is a particular species among Drosophilid flies as it is able to oviposit in undamaged fruit whose skin it pierces with the aid of a large, serrated ovipositor (Atallah *et al.*, 2014). Current understanding of *Drosophila suzukii* ecology indicates larvae develop in conditions that differ from those of species such as *D. melanogaster*. Larvae of *D. suzukii* and *D. melanogaster* would hence have distinct but partly overlapping nutritional niches. First, both species can be found in ripe fruit, if the skin barrier has been broken, but *D. suzukii* also oviposits in unripe fruit (*e.g.*, Swoboda-Bhattarai and Burrack, 2015) that do not contain high concentrations of sugars yet (Prasanna *et al.*, 2007) and are free from microbial growth. Second, unlike *D. suzukii*, *D. melanogaster* often oviposits on decaying fruit that can be very ripe (*i.e.*, with high sugars levels) and, most importantly, largely colonized by bacteria and yeast (Becher *et al.*, 2012; Rombaut *et al.*, 2017). Accordingly, the behaviors of female flies that seek oviposition sites are different in the two species. *D. melanogaster* responds to yeast volatiles (Oakeshott *et al.*, 1989; Becher *et al.*, 2012), whereas *D. suzukii* responds to fruit volatiles (Karageorgi *et al.*, 2017; Swoboda-Bhattarai *et al.*, 2017). Female attraction to yeast relates to the beneficial effects it has on larval nutrition: in several *Drosophila* species yeast is the main source of proteins of larvae (Starmer and Fogleman, 1986; Anagnostou *et al.*, 2010; Becher *et al.*, 2012). In *D. suzukii*, yeasts do associate with adults (Hamby *et al.*, 2012), but their role in larval ecology is poorly known. It is nonetheless reasonable to assume *D. suzukii* mothers inoculate larval medium by depositing yeast cells during oviposition, a form of pseudo-vertical transmission. Based on female behavior, we hypothesized that *D. suzukii* larvae would have access to lower yeast concentrations than those of *D. melanogaster*, because *D. melanogaster* eggs are deposited in fruit where yeast has already had time to develop and reach high densities, while yeast concentration would be lagging behind when *D. suzukii* eggs hatch. This hypothesis translates into the prediction that the performance of *Drosophila suzukii* larvae would be best in nutritive media with low or intermediate protein concentrations, not benefiting from high protein concentrations. Similarly, as ripening fruit is usually poorer in sugars than ripe fruit, we expected *D. suzukii* larvae to perform poorly on - or at least not benefit from - high sugar concentrations.

To investigate the nutritional needs of *Drosophila suzukii* larvae, we used the nutritional geometric framework (Raubenheimer *et al.*, 2009). As for most other *Drosophila* studies within this framework, we chose to vary carbohydrate and protein availability. Proteins availability was manipulated by varying the proportion of dead yeast in the medium recipe. Carbohydrates were manipulated by varying the amount of saccharose (*i.e.*, short-chained carbohydrates, sugar) in medium recipe. Yeast cells also contain long-chained carbohydrates; therefore, yeast amounts also affected carbohydrate concentrations, which was taken into account in medium composition calculations. Larval performance was assessed by recording the proportion of larvae that reached the adult stage (*i.e.*, survival to emergence) and time to adult emergence (*i.e.*, developmental rate).

Material and Methods

Biological material

Drosophila suzukii is native from Asia and has invaded Europe, North and South America in the last 10 years (Adrion *et al.,* 2014, Fraimout *et al.,* 2017). Females are known to oviposit on fresh, undamaged fruits (Lee *et al.,* 2015). Eggs hatch within 24 h; time until adult emergence ranges from 10 days to 4 weeks, depending on conditions.

We used a *D. suzukii* population founded with *ca*. 30 females captured in Southern France, near Montpellier, a year earlier (*ca*. 20 generations). As population size was kept small - on average less than 30 reproducing females - we expect drift to have occurred and some genetic diversity to have been lost. Besides, microsatellite analyses have revealed low genetic diversity in natural populations around Montpellier (Fraimout *et al.,* 2017). Despite a lack of genetic variation, it is possible the population had adapted (in evolutionary terms) to laboratory conditions, an unfortunate caveat that our study shares with the other studies on *Drosophila* nutritional ecology we compare our results to (Rodrigues *et al.,* 2015).

Table 1. Recipe and macronutrient composition of the 25 nutritional media used in the experiment. In addition to water, yeast and sugar (*i.e.*, saccharose), recipe contained 3 g of Agar and 1.5 g of Nipagin (diluted in alcohol that evaporates during medium preparation). Note the diet used to maintain our *D. suzukii* population was close to treatment number 9.

Treatment number	Yeast mass (in g)	Sugar mass (in g)	Water in mL	Concentration in proteins	Concentration in carbohydrates	Proportion of carbohydrates brought by yeast input
1	37	0	300	0.053	0.015	1.000
2	37	5.2	300	0.052	0.030	0.499
3	37	10.4	300	0.052	0.044	0.332
4	37	20.7	300	0.050	0.071	0.200
5	37	41.4	300	0.047	0.122	0.111
6	18.5	2.6	300	0.028	0.016	0.499
7	18.5	7.8	300	0.027	0.031	0.249
8	18.5	18.1	300	0.027	0.061	0.125
9	18.5	38.9	300	0.025	0.115	0.062
10	18.5	80.3	300	0.022	0.206	0.031
11	9.3	3.9	300	0.014	0.016	0.250
12	9.3	9.1	300	0.014	0.032	0.125
13	9.3	19.4	300	0.014	0.062	0.063
14	9.3	40.1	300	0.013	0.117	0.031
15	9.3	81.6	300	0.012	0.210	0.016
16	4.6	4.5	300	0.007	0.016	0.125
17	4.6	9.7	300	0.007	0.032	0.062
18	4.6	20.1	300	0.007	0.063	0.031
19	4.6	40.8	300	0.006	0.118	0.016
20	4.6	82.2	300	0.006	0.212	0.008
21	2.3	4.9	300	0.004	0.017	0.062
22	2.3	10	300	0.004	0.033	0.031
23	2.3	20.4	300	0.003	0.063	0.016
24	2.3	41.1	300	0.003	0.119	0.008
25	2.3	82.6	300	0.003	0.213	0.004

Experimental methods

Larvae were reared in vials containing one of 25 different nutritional treatments (Table 1). We varied the concentration in proteins and carbohydrates by manipulating the amount of sugar (*i.e.*, saccharose) and dead bakers' yeast, *Saccharomyces cerevisiae*, in the medium recipe. This recipe was based on standard fly food initially designed for *Drosophila melanogaster*. Our choice of treatments was guided by the idea that laboratory foods are richer in nutrients than those encountered by flies in the wild. By consequent, we mostly explored concentrations of nutrients lower than that of standard medium. The macronutrient composition of the medium on which our *D. suzukii* population was maintained before the experiment was similar to treatment number 9 (Table 1), but also contained fresh banana. Banana was excluded from experiment's recipes in order to allow comparison of our results with those from other studies.

We used known composition in proteins and carbohydrates in yeast provided by our supplier to convert yeast quantities into protein and carbohydrates concentrations (1 g yeast contained 0.49 g of proteins and 0.14 g of digestible, long-chained carbohydrates). Note that we further distinguish total carbohydrates from the fraction of carbohydrates provided by yeast input (see statistical methods and results). Media also contained agar for consistency and nipagin to prevent the development of mold (Table 1). It should be noted that with this type of dietary manipulation, water content in the medium decreases as macronutrient concentration increases. The effect of water availability is, therefore, confounded with that of high macronutrient availability.

An experimental unit consisted of a 39 mm diameter drosophila vials with 15 mL of medium. Each received 40 *D. suzukii* eggs that we manually transferred from oviposition plates (*i.e.*, petri dishes with a medium made of grape juice and agar, exposed to females for *ca.*12 h). Note that manipulating eggs, rather than allowing females to naturally oviposit on experimental media, was mandatory in order to control for larval density and ensure all eggs came from females exposed to similar conditions. Unfortunately numerous eggs were killed in the process as *D. suzukii* eggs are notoriously more fragile than those of *D. melanogaster*, explaining the moderate rates of survival to adulthood reported below. Each treatment consisted of 3 replicates spread in 3 blocks each initiated 24 h apart. To assess fly development, experimental units were checked daily for newly emerged flies, which were removed from the vials and sexed. The adults collected and frozen during this experiment were unfortunately lost, preventing their phenotyping. The experimental room was maintained at 23°C under a 14 L : 10 D photoregime.

Statistical methods

We used standard linear models to analyse the response of survival and age at adult emergence to macronutrients concentrations. Protein and carbohydrate concentrations were square-root transformed, the proportion of carbohydrates contributed by yeast was log transformed. Initial models contained the term describing the proportion of carbohydrates from yeast, the terms describing nutrient contents and their squared value (*i.e.*, factor^2) so as to permit non-linear relationships between factors and responses, as well as 2nd order interactions between nutrient composition terms. Basic model hence had the form:

$$\begin{aligned}\text{Trait} = {} & \sqrt{\text{Proportion.proteins}} + (\sqrt{\text{Proportion.proteins}})^2 + \sqrt{\text{Proportion.carbohydrates}} + (\sqrt{\text{Proportion.carbohydrates}})^2 \\ & + \sqrt{\text{Proportion.carbohydrates}} * \sqrt{\text{Proportion.proteins}} * + (\sqrt{\text{Proportion.carbohydrates}})^2 * (\sqrt{\text{Proportion.proteins}})^2 \\ & + \sqrt{\text{Proportion.carbohydrates}} * (\sqrt{\text{Proportion.proteins}})^2 + \sqrt{\text{Proportion.proteins}} * (\sqrt{\text{Proportion.carbohydrates}})^2 \\ & + \text{Log}(\text{proprotion.carbohydrates.from.yeast}) + \varepsilon.\end{aligned}$$

For the analysis of development time we also included a factor describing sex-ratio, the proportion of females among the adults that emerged. Using a backward selection procedure we removed non-significant terms starting from highest order interactions. Data distribution complied with the main assumptions of the linear model, namely independence, homoscedasticity and normality of the residuals. All analyses were carried out with the statistical software JMP 12.1.

Data presented in Figures 1 and 2 are heat-maps fitted over values predicted by the final models for each of the 25 nutritional treatments. Predicted values are presented, rather than raw data, so as to facilitate comparison with other nutritional geometric studies in *Drosophila* that used the same method.

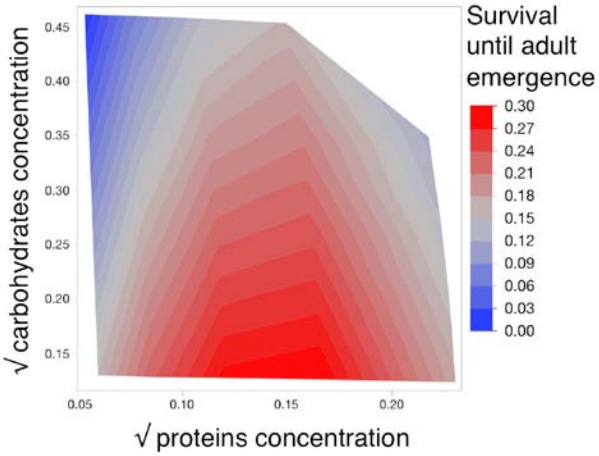

Figure 1. Combined effects of the concentrations of proteins and carbohydrates on the proportion of eggs that developed until the adult stage. Protein and carbohydrate concentrations were square-root-transformed. There were 25 treatments, each replicated 3 times.

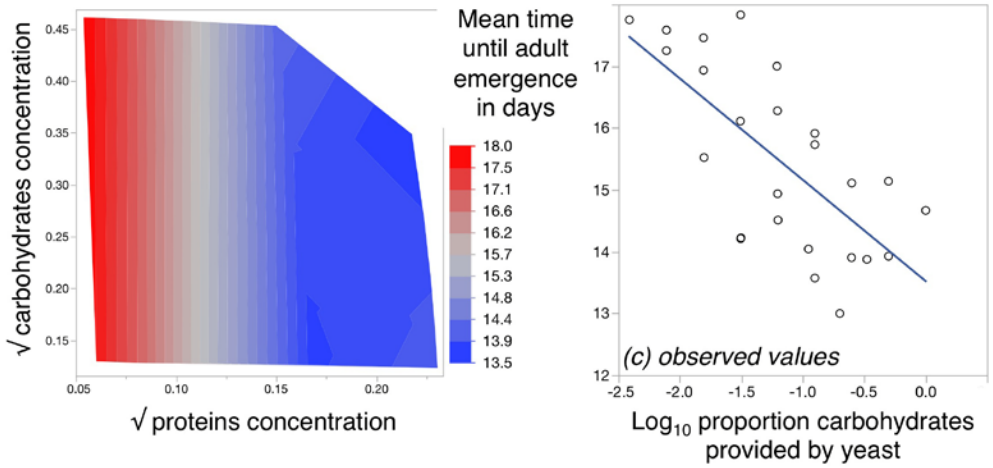

Figure 2. Combined effects of the concentrations of proteins and carbohydrates on the time until adult emergence. Protein and carbohydrate concentrations were square-root-transformed; proportion of carbohydrates from yeast was log-transformed. There were 25 treatments, each replicated 3 times.

Table 2. Statistical analyses of (a) the proportion of eggs that developed into adults, (b) the average time between oviposition and adult emergence. Proteins and carbohydrates concentrations were square-root-transformed; proportion of carbohydrates from yeast was log-transformed. We used standard linear models and only present final models, without non-significant terms.

Trait	Factors	D.F.	F	*p* value
(a) proportion of eggs that developed into adults	Carbohydrates concentration	1	17.7	< 0.0001
	Proteins concentration	1	10.7	0.002
	Proteins concentration ^{2}	1	23.4	< 0.0001
	Error	71		
(b) Time until adult emergence	Proteins concentration	1	132	< 0.0001
	Proteins concentration ^{2}	1	17.9	< 0.0001
	Proportion of carbohydrates from yeast	1	5.62	0.021
	Carbohydrates concentration	1	1.33	0.25
	Error	64		

Results

Survival to adulthood

The proportion of eggs that developed until adulthood decreased as total carbohydrate concentration increased (Figure 1, Table 2a). Survival peaked at intermediate protein concentrations showing this macronutrient has a non-monotonous effect on this trait. There was no significant effect of the proportion of carbohydrates from yeast on survival. Overall, the proportion of eggs that produced adults never exceeded 31%, which is partly due to egg mortality during their manual transfer to nutritive media.

Time until adult emergence

The average time until adult emergence decreased when the concentration of proteins in the diet increased (Figure 2a, Table 2b). In contrast, neither the concentration of carbohydrates, nor sex-ratio had a significant effect time until adult emergence. As the proportion of carbohydrates brought by yeast increased, there was a marginally significant acceleration of development (Table 2b, Figure 2b).

Relationship between survival and development speed

Treatments that produced many survivors also tended to favor fast development (correlation r = -0.37, P = 0.066) (Figure 3 dashed line). It appeared that treatments with the greatest protein concentrations had opposite effects on survival and developmental rate. Excluding from the analysis the 3 diets with more than 10% proteins and 10% carbohydrates (squares in Figure 3) greatly improved the correlation between the two traits (correlation r = -0.67, P = 0.001) (Figure 3 solid line). Comparison of Figures 1 and 2 indeed show they mostly differ in the top right part of the panels (*i.e.*, high proteins and carbohydrate concentrations), where survival is poor but development fast.

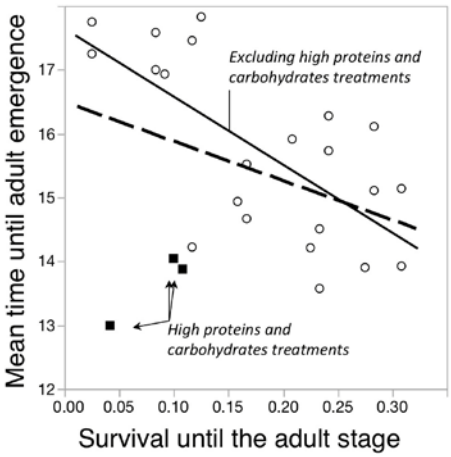

Figure 3. Relationship between survival until the adult stage and time until adult emergence. Each point (circle and square) corresponds to a diet treatment. Squares indicate treatments with protein and carbohydrate concentrations above 10%. Full line indicates the relationship between the two traits including all treatments; dashed lines indicate relationship excluding the 3 treatments with protein and carbohydrate concentrations above 10%. Correlations are there to highlight how treatments with high protein and carbohydrate concentrations depart from the general inverse relationship between survival and developmental rate.

Discussion

The study of larval response to variation in carbohydrate and protein concentrations in the nutritive medium revealed different effects on survival until the adult stage and time until emergence. Survival was maximized with an intermediate protein concentration and decreased as carbohydrates increased in concentration (Figure 1). However, there was no visible effect of carbohydrates on time until adult emergence, whereas greater protein concentrations led to faster development (Figure 2).

Congruence between field ecology and laboratory reaction norms

Drosophila suzukii is famous for having a different ecology from that of most Drosophilid species, and, in particular, from *Drosophila melanogaster* (Cini *et al.*, 2014). A notable feature is its ability to lay eggs in healthy, unripe fruit (Atallah *et al.*, 2014) and, therefore, exploit resources not yet colonized by bacteria and yeast, at least before egg deposition. As yeasts are the main source of dietary proteins of *Drosophila* larvae,

we expected *D. suzukii* to maintain good performance even in media with low proteins (i.e., yeast) concentrations (see detailed reasoning in the introduction). This prediction is met by our results on survival, which is maximized at intermediate protein concentration (Figure 1). Another prediction was that *D. suzukii* larvae would not perform well in high sugar conditions as ripening fruit, where *D. suzukii* females would lay eggs, contain less sugar than ripe fruit. Again, this prediction was matched by our survival data: high sugar content had a detrimental effect on the proportion of eggs that developed as adults. These results may be compared to those of a recent study on *D. suzukii* nutrition (Silva-Soares *et al.,* 2017) that also used the geometric framework and similar macronutrients concentrations in larval diets. In both experiments, larval survival was optimal at intermediate protein concentration. However, in the study by Silva-Soares *et al.* (2017) carbohydrate concentration had no significant effect on survival, while higher concentrations were detrimental in our study (Figure 1). Speed of development responded similarly proteins concentration in the two studies; even though there was a marginal difference when concentrations were maximal as reduced performance is only noticeable in the previous study (note quadratic terms are significant in both experiments). Comparison with similar experiments on *D. melanogaster* may reveal whether the patterns we and Silva-Soares *et al.* (2017) observed are specific to *D. suzukii*. It appears that both survival and development speed in the two species respond differently to protein and carbohydrate availability. In a recent study that used methods similar to ours (Rodrigues *et al.,* 2015), survival of *D. melanogaster* larvae until the adult stage improved with protein concentration and showed only limited decrease at high protein concentration (see Figure 1A in Rodrigues *et al.,* 2015). Effects on developmental rate were also contrasted between our observations and the *D. melanogaster* study. We observed a monotonic acceleration of development with increasing protein concentrations, whereas in *D. melanogaster* this trait was optimal at intermediate protein concentrations (see Figure 1B in Rodrigues *et al.,* 2015). It, therefore, appears that the congruence between our results and the available knowledge on *Drosophila* ecology is best for the rate of survival: for this trait *D. suzukii* performed best in media poorer in proteins than did *D. melanogaster*. The congruence between predictions and observations is less for speed of development, which is not as important to fitness than survival and is, therefore, under weaker selective pressure. It is important to note that the study by Rodrigues *et al.* (2015) contained treatments with greater concentrations of proteins than ours. Their range of conditions should, therefore, have permitted the detection of reduced larval performance at high protein concentrations. We can, therefore, conclude that, at least for the studied populations, nutritional landscapes of *D. suzukii* and *D. melanogaster* are likely different. *D. suzukii* and that survival reaction norms match what is known of the oviposition habits of gravid females.

Treatments that combined high protein and high carbohydrate availability were detrimental for survival but not for developmental speed (Figure 3). In all other treatments, greater survival associated with faster development. The surprising pattern at high protein concentration could be interpreted as an alternative developmental strategy triggered by diet composition (Pigliucci, 2005). It is frequent to observe effects of the proportion of yeast in the diet of *Drosophila* larvae on their phenotype (Anagnostou *et al.,* 2010), or on that of the adults they produce (Fellous and Lazzaro 2010). The adaptive value of this phenotypic plasticity may even be discussed (Gould and Lewontin, 1979). Sometimes, different developmental trajectories produce different combinations of traits that nonetheless have similar fitness (Schmidt *et al.,* 2012). But in our case, it is unlikely that faster development, at the cost of greater mortality (black squares in Figure 3), reflects adaptive plasticity. A slightly faster development (here 1-2 days) probably cannot offset the cost of reduced survival (here ± 50%). However, our results suggest that larval food composition may affect important parameters of fly demography. It is thus possible that population structure (*i.e.*, the relative proportion of individuals from different age classes) may be affected by nutritional composition of the available resources (de Roos and Persson, 2013). The growth of populations feeding and developing on fruits with distinct macronutrient composition may then be limited by constrains exerted at different stages of the life-cycle (Nicholson, 1957), with consequences at the community level (Miller and Rudolf, 2011).

Challenges with laboratory studies of insect nutrition

Our approach has a caveat that is common to most studies of nutrition in *Drosophila* flies; namely, we did not re-create the microbial environment in which flies normally develop. We did manipulate dead yeast concentration, but in nature yeasts are alive and their concentration responds to larval feeding (Stamps *et al.,*

2012). It is clear that adults *D. suzukii* and *D. melanogaster* have different relationships with fruits and microbes: if both species can be attracted to yeast volatiles at least for adult feeding (Becher *et al.*, 2012) only *D. suzukii* responds to fruit volatiles (Keesey *et al.*, 2015, Swoboda-Bhattarai *et al.*, 2017). However, our understanding of fly-microbe relationships in the natural environment is for the moment shallow. Some studies on adult feeding ecology have shed light on the trade-off between feeding and oviposition (Lihoreau *et al.*, 2016; Plantamp *et al.*, 2017), or the reliance of adults on yeast for nutrient acquisition (Becher *et al.*, 2012; Yamada *et al.*, 2015). However, larval ecology remains poorly known. Other unidentified environmental factors - beyond macronutrient concentration - probably influence the development of *Drosophila* larvae. For instance, it is unknown whether the feeding apparatus of *D. suzukii* is different from that of other *Drosophila* species; one could indeed hypothesize their greater ability to chew through firm, unripe fruit flesh. The difference between the nutritional environments used in lab assays and that encountered by flies in nature may explain some hard-to-interpret results: for example, why do *D. melanogaster* females sometimes prefer to lay their eggs in environments that do not seem optimal for larval development (Rodrigues *et al.*, 2015). In addition to effects of yeast symbionts, bacterial symbionts involved in processing and assimilating nutrients greatly differ between lab and wild conditions (Chandler *et al.*, 2011; Chandler *et al.*, 2014), increasing the mismatch between lab conditions and the environment to which flies are adapted. In the case of *D. suzukii*, which oviposits on ripening fruit still attached to the host plant, ripeness, and, therefore, sugar concentrations may be dynamic and vary during larval life. Along these lines, we believe yeast growth during fruit infestation (Stamps *et al.*, 2012), and the subsequent variations of proteins concentrations, is one of the most important phenomena that is not taken into account in the geometric framework of nutrition.

Relationships between laboratory and field phenomena are further blurred by the genetic adaptation that occurs during domestication (Hoffmann *et al.*, 2001; Fragata *et al.*, 2014), as may have been the case in our study. This is, however, a problem met by other *Drosophila* studies to which we compare our results. Similarly, most studies only test a single population or strain per species, even though intra-specific genetic variation is very common for plant-exploitation traits in herbivores (*e.g.*, Jaenike, 1985; Fellous *et al.*, 2014) and interactions with symbiotic microbes *(e.g.*, Fellous *et al.*, 2012).

An unexpected result was the response of developmental rate to the proportion of carbohydrates that came from yeast input (Figure 2b). Carbohydrates are generally separated into short- and long-chained carbohydrates that correspond to readily accessible sugars and storage molecules such as starch, respectively. In our experiment, yeast provided long-chained carbohydrates in complement to the saccharose (a type of sugar) of our medium recipe. We cannot explain the effect of the proportion of carbohydrates provided by yeast, but relate it to the stoichiometry of nutrients involved in physiological processes (Jacobs *et al.*, 2009). As such, it is in favor of the geometric framework of nutrition (Raubenheimer *et al.*, 2009). Along these lines, variations of unidentified nutrients - and the discrepancy between lab environments and natural microbial communities - may explain why in another recent study *D. suzukii* larvae developed more slowly in yeast-rich medium than on real fruits (Jaramillo *et al.*, 2014), while in our experiment yeast enrichment accelerated development.

The nutritional framework of nutrition is a powerful tool to study insect physiology. However, the caveats discussed above - such as the dynamic nature of the microbial symbionts that provide nutrients and the existence of unidentified environmental factors - show artificial diets may not be adequate to understand the ecology of *Drosophila* flies. We believe this objective will be better met with alternative methods using real fruit and naturally occurring communities of microbes. In the wild, *D. suzukii* is frequently found associated with the yeast *Hanseniaspora uvarum* (Hamby *et al.*, 2012), which prompts for an in-depth study of the nature of this symbiosis.

Conclusions

We studied the performance of *D. suzukii* larvae across a range of nutritional conditions following the geometric framework of nutrition (Raubenheimer *et al.*, 2009). Our main results - that survival peaked at intermediate protein concentration in the larval diet and that carbohydrates are detrimental - are congruent with the behavior of *D. suzukii* females that are known to oviposit in sugar-poor fruit, not yet colonized by yeast. However, in retrospect, we identified a number of factors that may limit the significance of this type of

experiment for *in-natura* processes. Overcoming these challenges will be necessary to better understand the nutritional ecology of *Drosophila* flies.

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Developmental homeostasis reflected in symmetry of cell death in the *Bar* eye of *Drosophila*.

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One of our recent interests is the identification of genetic modifiers of cell death in compound eye facets using the sequenced strains of *Drosophila* developed by Mackay and her colleagues (Mackay *et al*., 2012; DGRP strains available from the Bloomington *Drosophila* Stock Center; for earlier pilot data see Thompson *et al*., 2015). But these experiments also provide an opportunity to explore a somewhat unrelated phenomenon: the degree of symmetry in the extent of developmentally-patterned cell death. Using *Drosophila* eyes carrying the mutation *Bar*, deviations from symmetry are a potential measure of developmental homeostasis – the compensations required to generate a symmetrically bilateral body plan. Cell death in *Bar* eyes is clearly variable, with phenotypes in our experiments ranging from less than 50 to over 300 facets per eye. Our hypothesis is that, in spite of this tendency to vary, there is developmental coordination within an individual that tends toward symmetrical expression in facet number.

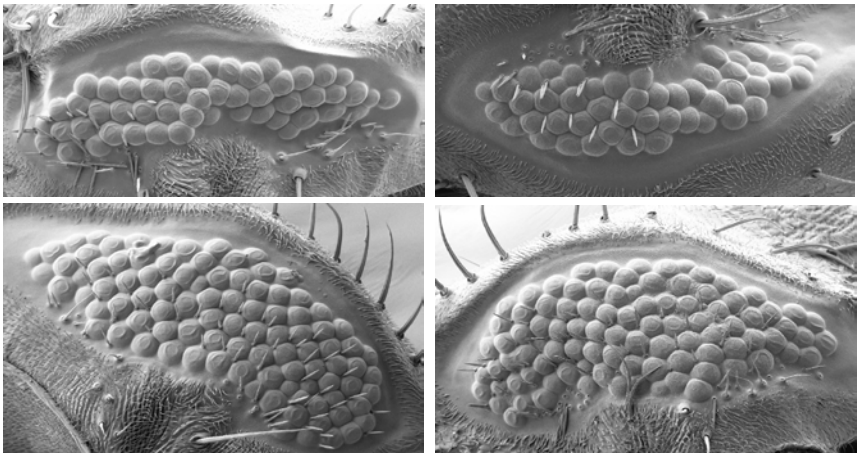

Figure 1. Representative pairs of *Bar* eyes from an F_1 male from #25745 (top row) and a male from # 25185 (lower row).

We have chosen two DGRP strains that yield quite different numbers of facets when heterozygous in males carrying the sex-linked mutation *Bar*. The average facet number from strain #25745 is 71 ± 13 (n = 80), while for #25185 it is 169 ± 44 (n = 62). We are able to get such precise facet numbers by using the Zeiss NEON 40EsB scanning electron microscope to image individual eyes (Figure 1). Inbred females carrying the balancer *Basc* with the dominant sex-linked *Bar* eye mutation were mated to males from each of two of the DGRP lines that had shown quite different F_1 facet numbers in earlier crosses. F_1 males carry the *Bar* mutation and are heterozygous for eye facet number modifiers from a sequenced DGRP line. Individual heads were removed with a razor blade and bisected between the eyes. Pairs of eyes were mounted on SEM plugs, air dried for several days, and then coated with gold-palladium in a Hummer 6 sputter coater. High resolution images were taken of each eye at an average magnification of 350×. Facet numbers were then counted in duplicate by several participants. This is clearly excessive replication, but, for this step in our project, it allowed each participant to become directly involved

in measurements and have their results compared to those of others. Variation associated with individual counters was not significant, and the analyses presented here were done on the average of each eye's counts.

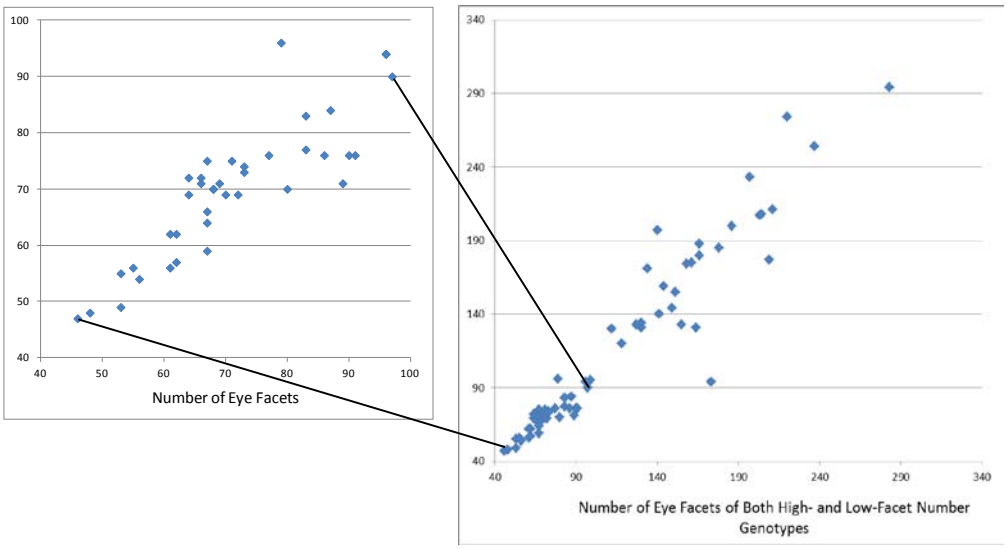

Figure 2. Facet numbers from matings with strain #25745 (left graph), showing how symmetrical the expression is, even over a range of phenotypes in which facet number approximately doubles. The graph at the right shows data from both genetic backgrounds, with the lines indicating where the first set of data are located on the second graph.

Even though there was the expected difference in the degree of cell death expressed by the two heterozygous sequenced genotypes, the symmetry of expression was remarkable. The pairs of eyes in Figure 1 are representative. To quantify this more clearly, Figure 2 plots the two eyes (randomly as "left" and "right"). The tightness of fit to a slope of 1.0 (actually, 1.06 in these data) for this wide range of facet numbers is due to close symmetry of cell death levels in individual pairs of eyes. Within-fly developmental homeostasis for cell death occurs between sides of the fly. This is perhaps especially surprising, since many key aspects of Dipteran development show cell autonomy, as seen for example in the distinct sexual dimorphism in male-female mosaics, *i.e.*, gynandromorphs.

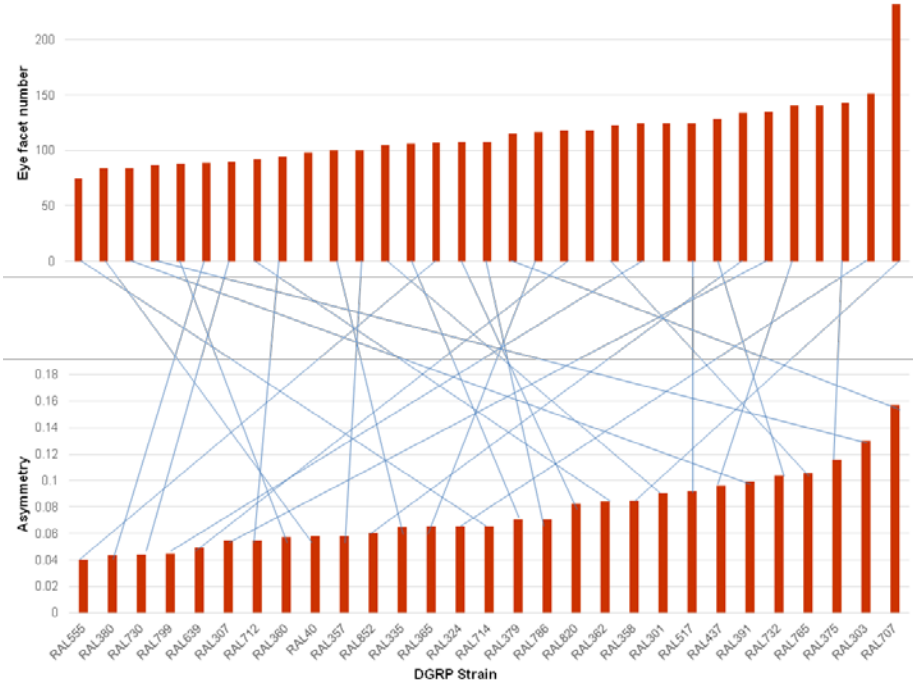

Figure 3. Lack of correlation between DGRP backgrounds affecting *Bar* eye facet number (top) and fluctuating asymmetry (FA) below.

One early prediction was that the degree of symmetry would be inversely correlated with the number of facets. There would be more randomness in eye facet counts as the number of facets increased. Symmetry might happen by chance if the number of facets is low, but symmetry would decline if facet number was higher. This is a reasonable prediction given the fact that, even within the same genotype, there is fly-to-fly variation in *Bar* gene expression. Indeed, there is some hint of that in these data, with more spread as facet number increases (Figure 3). But, overall, these data show that symmetry is not directly correlated with facet number when fluctuating asymmetry (FA) is compared to average facet number for a large series of DGRP F_1 data sets. To quantify deviations from symmetry within each strain background, we calculated fluctuating asymmetry (FA) = |one side – the other side|/(the sum of the two sides * 0.5). Developmental homeostasis, reflected in a high level of cell death bilateral symmetry, must be a process that over-rides the cell autonomous expression seen in other aspects of *Drosophila* development.

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Guide to Authors

Drosophila Information Service prints short research, technique, and teaching articles, descriptions of new mutations, and other material of general interest to *Drosophila* researchers. The current publication schedule for regular issues is annually, with the official publication date being 31 December of the year of the issue. The annual issue will, therefore, include material submitted during that calendar year. To help us meet this target date, we request that submissions be sent by 15 December if possible, but articles are accepted at any time. Receipt by 31 December is a firm deadline, due to printer submission schedules.

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Green, R.L., 1998, Heredity 121: 430-442.

Waters, R.L., J.T. Smith, and R.R. Brown 1990, J. Genet. 47: 123-134.

Note the initials are before each name except for the senior author.

Mutation Notes

The *Drosophila melanogaster straw* locus is allelic to *laccase2*.

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In the course of our studies on the *apterous* locus (Gohl *et al*., 2008; Gohl and Müller, unpublished), we have isolated a PiggyBac insertion in the nearby *laccase2* gene (CG42345). The new insert *PBac{WH}laccase2*$^{5151\text{-}37A}$ was obtained by mobilization of *PBac{WH}ap*^{f00451} and it was molecularly mapped to near the 3' end of the *laccase2* gene (see Figure 1). The enzyme Laccase2 is part of the catecholamine pathway leading to pigmentation and sclerotization of the adult fly cuticle (Riedel *et al*., 2011). It oxidizes dopamine to dopamine quinone, which, in the presence of the Yellow protein, polymerizes to form black melanin. Laccase2 enzyme also oxidizes N-β-alanyldopamine to a quinone, which mediates cuticle protein cross-linking (sclerotization) (Riedel *et al*., 2011).

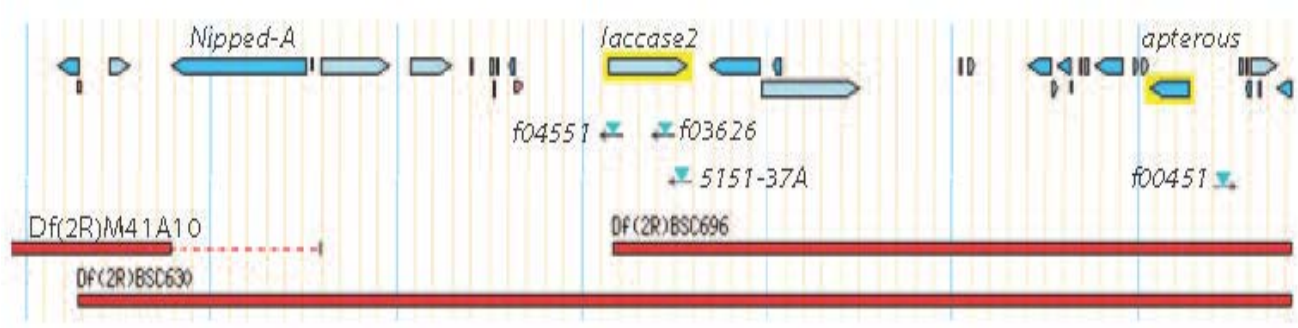

Figure 1. 700-kb genomic interval surrounding the *laccase2* locus at the base of chromosome arm 2R (adapted from FlyBase). In the upper part of the panel, gene spans of all loci in the region are shown. Genes *Nipped-A*, *laccase2*, and *apterous* are highlighted. Below the gene spans, all PiggyBac insertion sites mentioned in the text are indicated. At the bottom of the panel, the complete length of deficiencies *Df(2R)BSC630* and *Df(2R)BSC696* are depicted by a red bar. For the much larger deletion *Df(2R) M41A10,* only the approximate position of its distal break point is shown.

In order to genetically characterize the new allele, complementation crosses among stocks *PBac{WH}laccase2*$^{5151\text{-}37A}$*/SM6, PBac{WH}laccase2*^{f04551}*/CyO, PBac{WH}laccase2*^{f03626}*/CyO, Df(2R)M41A10/ CyO, Df(2R)BSC630/CyO,* and *Df(2R)BSC696/CyO* were set up (insertion sites and deficiency breaks are depicted in Figure 1). The results indicated that *laccase2* alleles *PBac{WH}laccase2*^{f04551}, and *PBac{WH}laccase2*^{f03626} are lethal. The new allele *PBac{WH}laccase2*$^{5151\text{-}37A}$ is viable over deficiency as well as over both of the two lethal *PBac{WH}* alleles (see Table 1). Such trans-heterozygous flies show a consistent pigmentation defect reminiscent of *yellow* null alleles: bristles and wings lose their characteristic dark color. However, and in contrast to *yellow*, pigmentation on abdominal tergites remains largely unchanged (data not shown). Similar phenotypes were previously obtained by RNAi-mediated knockdown of the *laccase2* gene (Riedel *et al*., 2011).

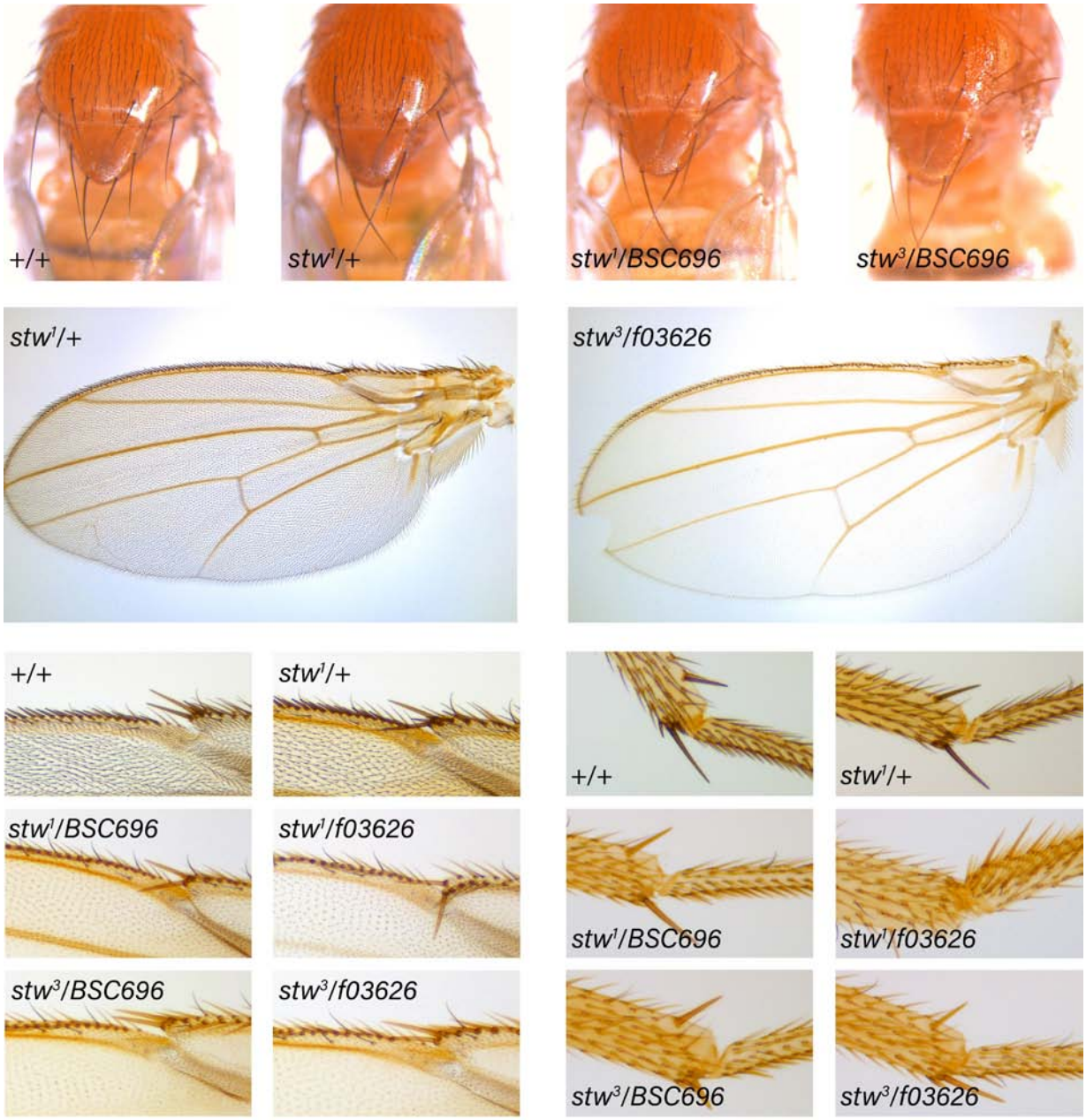

Figure 2. Adult phenotypes of *stw*^{1} and *stw*^{3}. In the top 4 pictures, the change in thoracic bristle pigmentation is shown. Bristle color in OregonR (+/+) and heterozygous *stw*^{1} (*stw*^{1}/+) flies is clearly darker than in *stw*^{1} (*stw*^{1}*/BSC696*) and *stw*^{3} (*stw*^{3}*/BSC696*) hemizygotes. In the middle of the panel, the difference in appearance of the wing blade between heterozygous *stw*^{1}/+ and *stw*^{3}*/f03626* flies is documented. Note the yellowish appearance of the blade in *stw*^{3}*/f03626* as compared to *stw*^{1}/+. Wing blade *stw*^{3}*/f03626* is slightly damaged at the tip. Wings were dissected and embedded in Hoyer's before images were taken. At the bottom left, blow-ups of proximal-anterior wing margins are shown. Note the loss of dark pigment in hemizygous (*stw*^{1}*/BSC696*, *stw*^{3}*/BSC696*) or trans-heterozygous (*stw*^{1}*/f03626*, *stw*^{3}*/f03626*) *stw*^{1} and *stw*^{3} conditions as compared to OregonR (+/+) or *stw*^{1}/+. At the bottom right, blow-ups of adult legs are depicted. Bristles acquire a distinct yellowish color in hemizygous (*stw*^{1}*/BSC696*, *stw*^{3}*/BSC696*) or trans-heterozygous (*stw*^{1}*/f03626*, *stw*^{3}*/f03626*) *stw*^{1} and *stw*^{3} conditions as compared to OregonR (+/+) or *stw*^{1}/+. Pictures of the same morphological structures were taken under identical illumination conditions.

Table 1. Results of complementation crosses.

Genotype	Viability	straw phenotype
Df(2R)M41A10 / Df(2R)BSC630	lethal	
Df(2R)M41A10 / Df(2R)BSC696	viable	no
Df(2R)M41A10 / PBac{WH}laccase2^{f04551}	viable	no
Df(2R)M41A10 / PBac{WH}laccase2^{f03626}	viable	no
Df(2R)BSC630 / Df(2R)BSC696	lethal	
Df(2R)BSC630 / PBac{WH}laccase2^{f04551}	lethal	
Df(2R)BSC630 / PBac{WH}laccase2^{f03626}	lethal	
Df(2R)BSC696 / PBac{WH}laccase2^{f04551}	lethal	
Df(2R)BSC696 / PBac{WH}laccase2^{f03626}	lethal	
PBac{WH}laccase2^{f03626} / PBac{WH}laccase2^{f04551}	lethal	
Df(2R)M41A10 / PBac{WH}laccase2$^{5151\text{-}37A}$	viable	no
Df(2R)BSC630 / PBac{WH}laccase2$^{5151\text{-}37A}$	viable	yes*
Df(2R)BSC696 / PBac{WH}laccase2$^{5151\text{-}37A}$	viable	yes*
PBac{WH}laccase2^{f04551} / PBac{WH}laccase2$^{5151\text{-}37A}$	viable	yes
PBac{WH}laccase2^{f03626} / PBac{WH}laccase2$^{5151\text{-}37A}$	viable	yes
Df(2R)M41A10 / stw^{1}	viable	no
Df(2R)BSC630 / stw^{1}	viable	yes
Df(2R)BSC696 / stw^{1}	viable	yes
PBac{WH}laccase2^{f04551} / stw^{1}	viable	yes
PBac{WH}laccase2^{f03626} / stw^{1}	viable	yes
Df(2R)M41A10 / stw^{3}	viable	no
Df(2R)BSC630 / stw^{3}	viable	yes
Df(2R)BSC696 / stw^{3}	viable	yes
PBac{WH}laccase2^{f04551} / stw^{3}	viable	yes
PBac{WH}laccase2^{f03626} / stw^{3}	viable	yes

* These flies also show an *apterous* wing phenotype because the *PBac{WH}laccase2$^{5151\text{-}37A}$* chromosome has retained insert *PBac{WH}ap^{f00451}* and *Df(2R)BSC630* and *Df(2R)BSC696* also take out *apterous* (see Figure 1).

PBac{WH}laccase2^{f04551} / CyO (B#18785), Df(2R)M41A10/SM1 (B#*741), Df(2r)BSC630 / CyO (B#25705), Df(2R)BSC696 / CYO* (B#*26548), stw^{1}*(B#*412)* and *lt^{1} rl^{1} stw^{3}* (B#1056 were obtained from the Bloomington stock center. *PBac{WH}ap^{f00451} and PBac{WH}laccase1^{f03626} were* purchased from the Exelixis stock collection at Harvard Medical School.

The first *straw* allele (*stw^{1}*) was discovered by Calvin Bridges exactly 100 years ago in 1917 (Morgan *et al*., 1925). According to FlyBase, the *straw* locus has not been annotated yet. But information available on FlyBase suggests that *straw* could be allelic to *laccase2*: (1) *straw* has been mapped to the base of 2R but distal to *Df(2R)M41A10*; (2) the described *straw* phenotype is very similar to what we have observed for our new allele *PBac{WH}laccase2$^{5151\text{-}37A}$*. This assumption was borne out by our observations obtained by complementation crosses between *straw* alleles *stw^{1}* and *stw^{3}* and deficiencies and *laccase2* alleles shown in Figure 1 (see Table 1):

– *Df(2R)M41A10* complements both *straw* alleles but neither *Df(2R)BSC630* nor *Df(2R)BSC696* do. Hemizygous *stw^{1} and stw^{3}* flies are well viable and show the typical straw phenotype (see Figure 2).

– Importantly, the *straw* alleles are also not complemented by *PBac{WH}laccase2^{f04551}* and *PBac{WH}laccase2^{f03626}* (see Figure 2). In trans-heterozygous flies, pigmentation is lost in wings and bristles.

In conclusion, these complementation tests demonstrate that *straw* is allelic to *laccase2*. Therefore, we propose that according to established *Drosophila* nomenclature practices, the gene name of CG42345 should be changed to *straw*, as it was first called in the Morgan lab in 1917. Our observation "Bridges" the historical gap in understanding the molecular nature of the *straw* mutants discovered 100 years ago.

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History of the FM7 balancer chromosome.

Merriam, John[1]. Revised by Scott Hawley and Danny Miller, 1-22-2014. Figure courtesy of Angie Miller. [1]Molecular Cell and Developmental Biology, University of California, Los Angeles 90095.

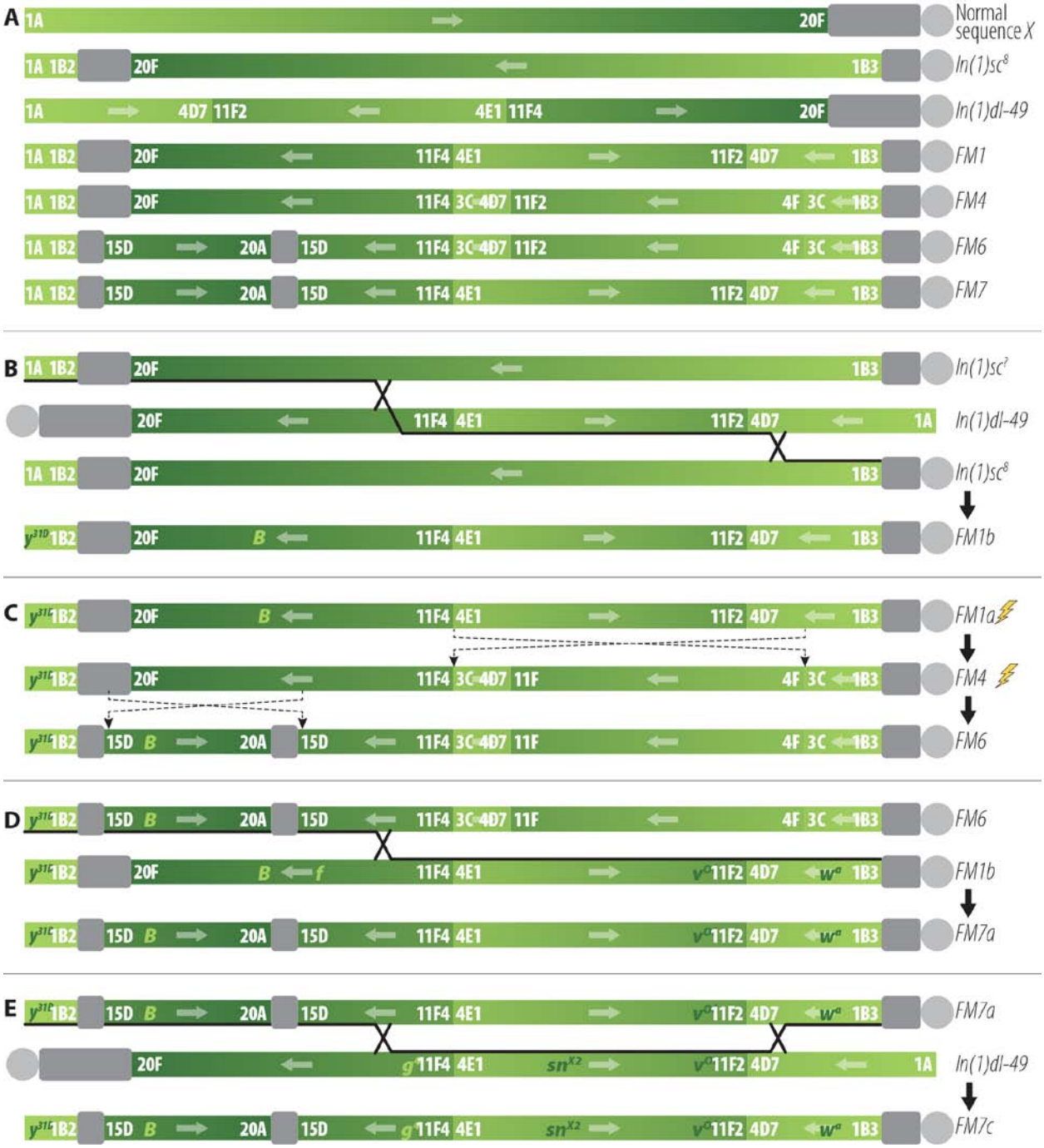
Like all modern balancer chromosomes in *D. melanogaster*, *FM7* was constructed from a series of progenitor balancer chromosomes. Inversion heterozygotes, but not homozygotes, suppress crossing over at the inverted portion of the chromosome. Sturtevant (1913) discovered the first example of an inversion, which he named *In(3R)C*, for crossover suppressor in the right arm of chromosome 3. This inversion reverses much of the distal third of the chromosome (from section 92D1 to 100F2) so that the chromosome sections in the right arm distal to the break at 92D1 are in the order centromere–100F2–92D1–telomere, and it suppresses crossing over for this region (from the marker *Dl* (*Delta*) to the telomere). Muller (1918) used *In(3R)C* to make the first permanent heterozygous stock, or "balanced stock", with the marker *Bd*, an allele of the *Serrate* (*Ser*) gene, that has both a dominant wing notching and recessive homozygous lethality. In the language of its time, this stock was "pure breeding"—all progeny had the same phenotype and genotype of their parents—because *Bd/Bd* and *In(3R)C/In(3R)C* homozygotes did not survive, leaving only *Bd/In(3R)C* heterozygotes each generation. In order for this stock to remain heterozygous each generation there must be suppression of crossing over, as it keeps the wild-type Bd^+ allele from *In(3R)C* from being placed onto the *Bd* chromosome, which would allow for recovery of *Bd+/Bd* non-*In(3R)C* progeny.

The importance of this example was instantly recognized, leading to the identification of dominant crossover suppressor lines for all the linkage groups, and it was applied to maintaining mutant alleles with poor viability and/or multiply-marked chromosomes. Because *Drosophila* stocks cannot be maintained through frozen lines, essentially all of the thousands of mutant alleles in different genes now available must be maintained in balanced stocks without selection. The balancer chromosomes responsible have improved to contain multiple inversions for more complete crossover suppression, as well as a dominant marker for identification and recessive lethal or sterile mutants to prevent the stock from becoming homozygous for its balancer and losing the mutant allele.

Along with balancing mutant alleles, the inverted chromosomes also became essential in screens for new mutants. Muller (1928) recovered a balancer on the *X* (or 1^{st}) chromosome, *In(1)Cl*, also carrying the visible markers *sc v f* (all recessive) and *B* (dominant), as well as a lethal allele in an unknown gene. The middle two-thirds of this chromosome was inverted from 4A5 to 17A6. Maintained as the "ClB" stock, it formed the basis for Muller's assay to determine the fraction of sperm that carried a new *X*-linked lethal mutation after exposure to X-rays, work for which he received the Nobel Prize in 1946. Balancers were also used to identify lines with segregating recessive lethal mutations following mutagenesis, which could be identified as stocks which only gave heterozygous mutagenized-chromosome/balancer progeny. One highlight example is a 1980 paper by Nusslein-Volhard and Wieschaus describing their identification of the embryonic patterning genes through lethal alleles, for which they received the Nobel Prize in 1992.

Both the stock-maintenance and selective-screening uses of balancers depend on their effectiveness in suppressing crossing over. The goal of balancer construction has, therefore, been to add multiple inversions in order to cover as much of the chromosome as possible. *In(1)Cl* suppressed crossing over for most *X* regions except most proximally, but was less useful because of its own recessive lethality. *In(1)dl-49*, the second *X*

inversion discovered, is homozygous and hemizygous viable and fertile, although it inverts only the middle third of the *X* so it acts in a polar fashion, suppressing crossing over in the middle and distal thirds but allowing crossing over proximally. Muller led an extensive effort to generate X-ray induced variants for altered phenotypes of genes and for altered chromosome rearrangements. Often the examples were both—the frequent *scute* (*sc*) mutations turned out to be inversion or translocation rearrangements through breakpoints in the centric heterochromatin that repaired next to the distally located *sc* gene, inactivating the *sc* gene through position effect. One example is *In(1)sc*^{8}, an entire-arm inversion that eliminated all single crossovers but allowed the less frequent double crossovers to survive, which facilitated the movement of markers such as w^{a} and *B* into and out of *In(1)sc*^{8}. This, of course, meant it was not a very good balancer by itself.



Although some multiply-inverted combinations were induced by irradiation, *X* chromosome balancer design and construction took a major step forward through the use of crossing over to place the centrally located *In(1)dl-49* inside of *In(1)sc*^{8}. This was not easy and somewhat paradoxical, because *In(1)dl-49* does not allow double crossovers to occur on the X, yet it takes a double crossover, one on each side of *In(1)dl-49*, to get it into the *In(1)sc*^{8} chromosome. Muller found a way, however, possibly by using a triploid line carrying a metacentric attached *X* chromosome isogenic for *In(1)dl-49* with *In(1)sc*^{8} as the free *X* chromosome (Muller, 1934). Alternatively, triploid females are known to have elevated rates of crossing over, particularly at the terminal and centric ends of chromosome arms, so it is not unlikely that the necessary double crossover occurred in triploids (Dan Lindsley, personal communication); this latter alternative is shown in Figure 1B.

By 1953, the doubly inverted chromosome *In(1)sc*^{8}*+In(1)dl-49* marked with y^{31d} sc^{8} w^{a} lz^{s} and *B* existed; its synthesis was credited to Schultz and Curry by Lindsley and Grell (1967) and it was named *First Multiple 1* (*FM1*) by Lewis and Mislove (1953). *FM1* functions well as a balancer except in the presence of additional chromosome rearrangements, such as heterozygous autosomal inversions, which will boost crossing over elsewhere [the "interchromosomal effect" (Joyce and McKim, 2011)], that increased the frequency of double crossovers proximal to the *dl-49* interval inside *sc*^{8}. Attempts to improve its crossover suppression by X-irradiation succeeded only in forming a partial reinversion of the *dl-49* interval, the *FM4* variant (Figure 1C), which looks complex in cytology but does not suppress double crossovers for most of the arm inside the *sc*^{8} inversion. Further irradiation of *FM4* produced a better version, the *FM6* variant, which contained a new inversion from 15E to 20A that suppressed double crossovers proximally but still allowed them distally (Figure 1C).

To expedite the production of an effective balancer chromosome, *FM1* was crossed to *FM6* and a recombinant was chosen that merged the best features of both (Figure 1D). The process was not difficult, because the *FM1/FM6* genotype is homozygous for *In(1)sc*^{8} and has normal levels of crossing over for most of the *X* (Sturtevant and Beadle, 1936). From *FM1,y* w^{a} v^{Of} *f B/FM6,*y^{31d} *dm B* females, only a single crossover (distal to the *dl-49* interval and recognized by the differential markers w^{a}, v^{Of} and dm^{+} from *FM1* and f^{+} from *FM6*) was required to obtain the stock referred to as *FM7a* [*i.e.*, *In(1)sc*^{8}*+In(1)dl-49+In(1)15E-20A,*y^{31d} w^{a} v^{Of} *B*] (Merriam, 1969). In the presence of autosomal rearrangements, *FM7a* effectively suppresses crossing over along the entire *X*, but because both *FM7a* males and homozygous females are viable and fertile, a sterile balancer (*i.e.*, without fertile homozygotes) was needed. To that end, *FM7a* was further converted by recombination with *In(1)dl-49* lz^{s} to produce *FM7b* [*In(1)sc*^{8}*+In(1)dl-49+In(1)15E-20A,* y^{31d} w^{a} lz^{s} v^{Of} *B*] and with *In(1)dl-49,* sn^{X2} v^{Of} g^{4} *f* to produce *FM7c* [*In(1)sc*^{8}*+In(1)dl-49+In(1)15E-20A,*y^{31d} w^{a} sn^{X2} v^{Of} g^{4} *B*] (Figure 1E) (Merriam and Duffy, 1972). The lz^{s} and sn^{X2} female sterile mutants were induced on *In(1)dl-49*-bearing chromosomes, so their entry into *FM7* was possible by a double crossover between homozygous *In(1)dl-49* sequences. The *FM7a* and *FM7c* balancers (plus customized reporter additions) are the two most widespread *X* chromosome balancers in use today.

References: Use the references section in FlyBase to identify the cited literature.

Technique Notes

Creation of artificial berries as a tool to study reproduction in *Drosophila suzukii.*

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Abstract

Drosophila suzukii is an invasive fruit fly species that has spread from Southeast Asia to North America and Europe. The introduction of the species has resulted in massive crop loss among soft skinned fruits due to the species' rare ability, among *Drosophila*, to lay eggs directly into ripe fruit. Chemical and biological control methods have not shown clear indications of successfully suppressing population growth. In an attempt to better understand what properties make cultivars of fruits either more or less susceptible to parasitism by *D. suzukii*, artificial berries were created as a means to provide uniform berries in which a single chemical or morphological feature could be manipulated. By controlling the medium and incubation periods, chemical composition and berry firmness, respectively, were successfully varied. Artificial berries showed attractiveness and reproductive viability similar to those of real blueberries. Further, artificial berries in which sugar content was manipulated indicated that sugar content is important for berry selection; however, sugar is not the only factor that effects selection. Key Words: *Drosophila suzukii*; Oviposit; Fecundity; Artificial Berry; Crop Protection

Introduction

The spotted wing fruit fly, *Drosophila suzukii*, is an invasive pest that targets thin-skinned fruits such as blueberries, raspberries, cherries, and grapes (Lee *et al.*, 2011; Walsh *et al.*, 2011; Asplen *et al.*, 2015). While most other drosophilids lay their eggs in rotting fruit, the ability of *D. suzukii* to use ripe fruit, via a specialized serrated ovipositor, makes them a serious problem for market growers (Bellamy, Sisterson, and Walse, 2013; Kinjo *et al.*, 2013; Steffan *et al.*, 2013). The commercial impact of *D. suzukii* has been considerable, with estimated crop losses of 20-50%, and the potential for upwards of $500 million in lost revenue annually (Bolda, Goodhue, and Zalom, 2010; Walsh, *et al.*, 2011; Cini, Ioriatti, and Anfora, 2012). Efforts to develop control methods have included baits (Cha *et al.*, 2012; Hamby *et al.*, 2013; Cha, Landolt, and Adams, 2017), insect predators (Mazzetto, 2016, Woltz *et al.*, 2014), and pesticides. However, it is unclear if baits can depress populations sufficiently to protect crops, biological controls have not been proven effective, and the pest's use of ripe fruit makes pesticide application problematic (Bruck *et al.*, 2011).

Determining which factors play significant roles in oviposition choice and reproductive success may aid in applying current, and developing new, detection and management tools. Head to head tests have indicated that fruits such as raspberries are more attractive as oviposition sites than other potential targets (Bellamy *et al.*, 2013). Further, characteristics such as firmness, sugar content, size, and pH have been tested to determine if certain varietals or ontogenic stages of the fruit may be more susceptible than others (Gong *et al.*, 21016, Lee *et al.*, 2015, Kinjo, 2013, Lee *et al.*, 2011). While these studies have provided critical information that growers may be able to use in developing or switching to more resistant crops, it remains difficult to isolate individual characteristics while controlling for others. Using real berries, for instance, makes it possible to compare cultivars for the effect of firmness on oviposition (Kinjo, 2013, Lee *et al.*, 2015), but there are many other differences between varietal treatments that may be affecting these results. Similarly, as fruit ripens, firmness, size, color, and sugar content can all change independently of each other making it difficult to assign significance to a specific factor. The use of artificial substrates whose contents can be controlled and manipulated factors has to date relied on simple sheets that may have their own limitations. *D.*

suzukii are known to use visual cues such as color and size in their oviposition choices, both of which are difficult to reproduce using filled petri dishes and other two-dimensional target choices (Kirkpatrick *et al.*, 2016; Takahara and Takahashi, 2017). Finding a way to make an artificial fruit that allows both the physical components (*e.g.*, size, shape, and firmness) and chemical components (*e.g.*, sugar content, acidity) to be simultaneously and independently manipulated may help us get a better grasp of the relative importance of these factors.

This paper provides a description of a technique, borrowed from molecular gastronomy, to create artificial fruit (in this case blueberries) with defined and controllable physical and chemical characteristics. The effectiveness of these artificial berries was then tested to investigate their attractiveness and viability as oviposition sites.

Materials and Methods

Fly cultures

We used a laboratory cultured population of *D. suzukii* that was originally started from wild-caught flies on the Saint Joseph's University campus (Philadelphia, PA) in 2014. Stocks were kept in 15 mL vials containing fly food (Formula 4-24 Instant Drosophila Media), yeast, several blueberries, and an aqueous 0.5 % propionic acid solution to hydrate the fly food. Fly stocks were kept at 21^{o}C under 16:8 L:D conditions with stocks transferred to fresh food vials weekly.

Artificial berry production

Artificial berries can be created through the addition of sodium alginate to a homogenous liquid medium at a ratio of 1 gram of sodium alginate for every 100 mL of medium. Adding the sodium alginate to the medium while it is being mixed (in this case during the blending of the real blueberries) ensures consistent and thorough mixing throughout. The solution is then added, drop-wise with a wide mouthed syringe (the size and amount dispensed determined the size of the resulting berries), into a 1% calcium chloride water bath. The resulting spheres are then left in the bath for 30 minutes, removed, and washed with cold water to remove excess salt from the surface. Spheres may be kept refrigerated and used within two days. Three different berries were produced: pure blended blueberries, blueberries plus 10% additional sugar, and sugar water at equal concentration to the real berries as determined via the USDA's database for food nutrients (Agricultural Research Service 2016). To test our ability to modify the berries firmness, soak times were varied in a separate set of production trials to investigate its effect on resulting berry firmness. Firmness of the berries (both real and artificial) was determined by measuring the maximum force needed to pierce the berry with a thin screw. Force was determined using a Vernier pressure sensor (Vernier Software and technology, Beaverton OR), which was driven into the berries using a Velmex BiSlide (Velmex, Inc., Bloomfield, NY) positioner. Maximum force was determined using LoggerPro (Vernier software and Technology, Beaverton OR).

Single female assays

Virgin, two-day-old male and female pairs were placed into vials and observed until they copulated. Directly following the completion of copulation, the females were placed, individually, into a vial containing three treatment berries. Five days later the females were removed from their vials and total cumulative counts of adult offspring were made between day 14 and 21, post-copulation. Differences between treatments were determined *via* a one-way ANOVA with LSD *post-hoc* analysis.

Population box assays

To assess both oviposition choice and treatment effect on reproductive success, groups of mixed sex flies were placed in population boxes and presented with a choice of different berry types. Each clear acrylic population box was 46 cm × 21 cm in size, with mesh ports at each end to allow airflow through the box. Each box also contained ten threaded openings in the floor into which 100 mL glass jars could be screwed at an even spacing in a 2 × 5 pattern. To keep the box humid and prevent the berries from drying out, two 500 mL beakers, and the two center jars, were filled with water. The remaining eight jars contained six each of

either real blueberries, artificial blueberries, artificial blueberries with an additional 10% cane sugar, or spheres made of a medium of 10% cane sugar solution. Each box contained two jars of each the above treatments. The location of each jar was semi-randomized, with no two jars of the same treatment placed next to each other.

Fifty adult flies were counted under CO_2 anesthesia, then spread out evenly throughout the box. The number of flies in each jar was then recorded once/day over five days (and subsequently averaged), after which the jars were removed from the box and the contents (minus flies) were transferred to individual vials. Total cumulative counts of emerging adult offspring were recorded for a week-long period starting 14 days after the flies were initially introduced to the boxes. The percentage of the total population found on each berry type was averaged between replicate jars within each box, as were the offspring counts, producing one data point per box for each treatment. The box trials were replicated eight times. Differences between treatments were determined via a one-way ANOVA with an LSD *post-hoc* test. Percentages of parent flies on each berry type were arcsin transformed before statistical testing to meet the requirements of ANOVA.

Results

Changes in soak time in the calcium chloride bath were found to modify the firmness of the berries with increased soaking leading to increased firmness. Soak time and firmness followed a very linear relationship (linear regression, $r^2 = 0.69$, $F = 86.97$, $p < 0.001$) with a 4× increase in soak time leading to an ~4× increase in firmness. However, our firmest artificial berries were 24% less firm than real berries.

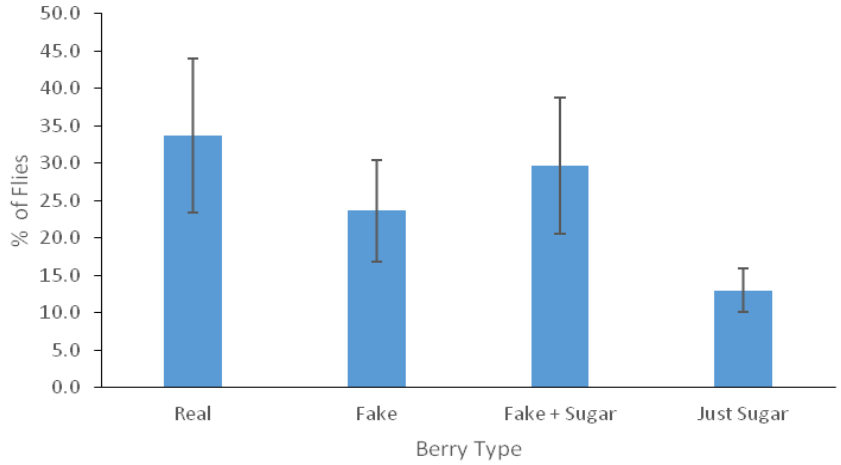

Figure 1. Average (± standard deviation) percentage of fly population found on each type of berry (average of two sub-replicate jars, averaged over five daily observations).

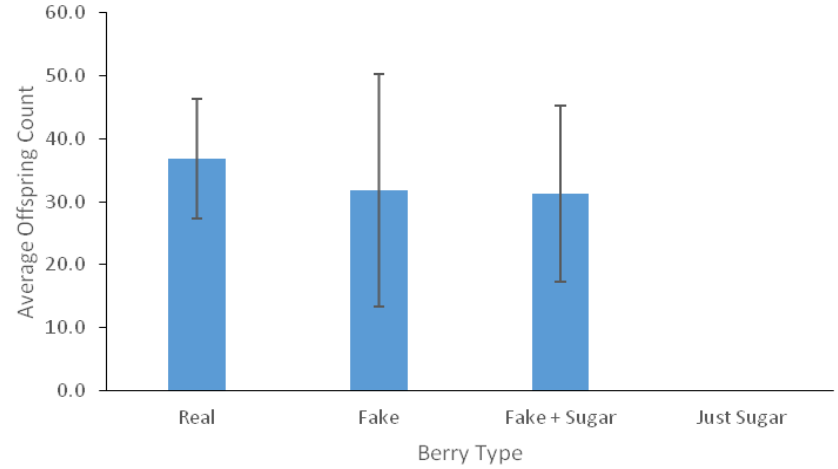

Figure 2. Average (± standard deviation) offspring counts (average of two sub-replicates per box) for each population box berry treatment.

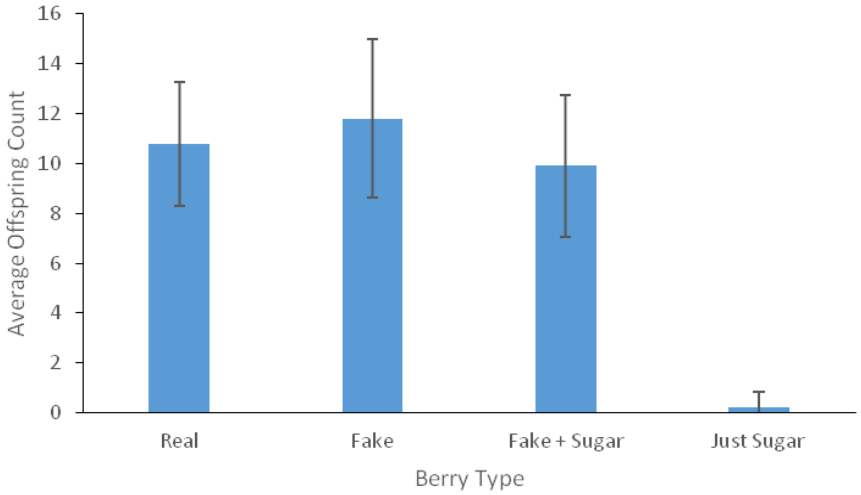

Figure 3. Average (± standard deviation) offspring counts (average of two sub-replicates per box) for individual female berry treatment.

Population box assays showed significant differences among treatments for number of adult flies on each type of berries (one-way ANOVA df = 3, F = 10.7, $p < 0.001$), with significant differences between the real berries and both the fake and sugar water treatments (Figure 1). Offspring counts similarly showed significant differences between treatments (one-way ANOVA df = 3, F = 14.5, $p < 0.001$), but this was driven almost solely by the lack of offspring in the sugar water treatment (Figure 2).

Offspring production from individual females with access to different berry types showed significant differences (one-way ANOVA df = 3, F = 47.9, $p < 0.001$) with the same pattern of production seen in the box assays: no difference in production between treatments except the sugar water berries (Figure 3).

Discussion

This paper illustrates a new way to produce artificial berries for studies on the reproductive biology of *D. suzukii*, a species of drosophila that relies heavily on ripe fruit for oviposition. Both the chemical and physical characteristics of these berries were manipulated to isolate specific aspects of oviposition choice and larval developmental success. We have shown that the attractiveness and ability to support eggs and larvae of *D. suzukii* were the same between real and artificial berries.

Comparisons of real, artificial, and sugar water berries indicated that adult flies were using more than sugar content to determine oviposition suitability. While similar numbers of adult flies were found on real and artificial (made with pure blueberry juice) berries, significantly fewer flies were found on the berries made with pure sugar water with a brix level equal to the real berries. Interestingly, while flies preferred artificial berries made with juice over sugar water, the flies preferred artificial berries with added sugar. Therefore, it appears that though flies were influenced by components other than sugar, sugar levels did play a role in their choice. The need for additional (+10%) sugar in the artificial berries to match the attractiveness of real berries may indicate that there is a factor not perfectly recreated in the artificial berries that the sugar-spike helps to overcome. That said, the levels of attractiveness were very similar.

Once oviposition occurred there were no differences among berry types, aside from the sugar water treatment, in terms of offspring production. This indicates that the conditions necessary for egg viability and larval development were successfully recreated in our artificial berries. Further, the same pattern was seen in both the population boxes and the individual female assays.

The attractiveness and reproductive viability of these artificial berries, along with the ability to manipulate many of their characteristics, makes them a useful tool in determining fruit susceptibility and control methodologies for ripe-fruit seeking pests. While this study only investigated the effects of sugar and juice content, it is certainly possible to isolate and manipulate a wide range of other chemical characteristics that may vary among varietals. As the technique described here can be used for any liquid it would be possible to add, subtract, or modify real juice or artificial mixtures. It may be possible, therefore, to determine the exact chemical cues adults use to choose oviposition sites. Further, the same approach can provide

information on the environment necessary for egg and larval development, which may in turn provide additional control options.

While such chemical manipulations are possible using 2D petri-dish sheets of juice infused agar or other materials (*e.g*., Takahara and Takahashi, 2017), our berries look very much like real berries which may provide some advantages. Admittedly, more work remains to increase the firmness of the artificial berries to match that of their real counterparts. We are, however, able to manipulate berry size along with their firmness, providing more realism and the ability to independently vary physical parameters separate from chemical characteristics. This capability may be of particular importance when studying the effect of fruit ontogeny where different aspects may vary at different time points. Overall, we have found that this method of producing artificial berries provides an easy and versatile approach to studying the reproductive and nutritional needs of an important agricultural pest species.

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Trap model for capturing *Drosophila suzukii* (Matsmura, 1931).

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Abstract

The monitoring of the Spotted Wing Drosophila relies on a variety of correlating factors, necessary for an effective detection of population density of a given place. The type of trap is one of said factors, and

several prototypes have been developed in countries that have had their fruit crops harshly damaged by this pest. However, although there are countless models made from different materials and arrangements, there is still no one standard model to be followed. Based on the studies that have displayed the most effectiveness in capturing *Drosophila suzukii*, a trap model targeting an effective monitoring was developed, made from durable and easily accessible materials, at a low cost for producers.

Introduction

The *D. suzukii* (Matsmura, 1931) (Diptera: Drosophilidae) species, known as the Spotted Wing Drosophila (SWD), is an Asian native species that has been spreading rapidly throughout North America and Europe since 2008 (Hauser, 2011; Cini *et al*., 2012;), causing major economic losses, especially to blueberry, blackberry, cherry, raspberry, and strawberry crops (Bolda *et al*., 2010; Burrack *et al*., 2013). The first record in South America dates back to 2013, in Brazil (Deprá *et al*., 2014; Schlesener *et al*., 2014; Geisler *et al*., 2015), and the fly has recently been detected in Uruguay (González *et al*., 2015).

Drosophila suzukii has a high reproductive potential and it can rapidly disperse to new areas (Cini *et al*., 2012). A recent study indicated a high environmental adequacy, particularly in temperate and subtropical regions, such as found in the Asian, European, North and South American continents, all of which already have records for the species, and potential occurrences in Oceania and Africa, places that have not yet recorded occurrences, but that do have propitious environmental conditions for the fly's establishment (Dos Santos *et al*., 2017).

Spotted Wing Drosophila females present an ovipositor with two rows of serrated teeth, enabling the laying of eggs inside ripe or ripening fruits (Lee *et al*., 2011). Once the egg is deposited, the eclosion of larvae occurs and they begin feeding upon the endocarp, as a secondary damage may be caused by the pathogens infestation, accelerating fruit decomposition and possibly compromising the entire crop (Walsh *et al*., 2011; Anfora *et al*., 2012).

Detecting the pest early and accompanying the evolution of its populations is fundamentally important when it comes to crop management and making decisions regarding the necessity of control. Regular monitoring is the first step for a successful integrated pest management (IPM) program (Gallo *et al*., 2002), and it is particularly important for *D. suzukii*, since the pest has been recently introduced and its expansion and distribution are irregular (Basoalto *et al*., 2013). The monitoring of SWD includes a trap-and-lure system, which uses visual and olfactory stimuli for capture (Iglesias *et al*., 2014). Therefore, the proper design for a species ought to consider the fly's color, shape, and structure (Cini *et al*., 2012), as well as density and trap positioning, around and within the crop area (Basoalto *et al*., 2013). A trap for the SWD must be effective, lasting, and have a low acquisition cost (Lee *et al*., 2012) so that, when combined with an attractant solution, it will lead to effective monitoring.

The development of different types of traps for capturing the SWD has been targeted by many researchers in the last few years. Traps using PET (Polyethylene terephthalate) bottles were the first ones used for prototypes, but today there are other versions, amongst commercial models and/or the ones developed by researchers that seek not only to be efficient for *D. suzukii*, but also to be selective to non-target organisms.

Some studies suggest that red and black traps can be more attractive to the SWD (Basoalto *et al*., 2013; Renkema *et al*., 2014) in orchards that have mature and similarly colored fruits (Renkema *et al*., 2014). Lee *et al*. (2012) reason that traps that share the same color as the host fruits may be in disadvantage if visual concurrence occurs, but this may also be an advantage, if the flies are more sensitive to the color of said fruit. The attractiveness of colors might be affected by the combination of volatile cues, as well as physical contrast with the environment (Lee *et al*. 2013). The authors tested different trap colors and verified that the yellow trap caught more flies than black, clear, and white traps, and the red trap caught more flies than the clear trap. Although such factor is not yet quite established, the red color has been widely used.

Several design models already tested presented an ample variation regarding *D. suzukii* capture. As for the area of the orifice through which flies enter, results demonstrate that traps that have a larger entry area are more effective than the ones with a smaller area (Landolt *et al*., 2011; Lee *et al*., 2012). In a following study, an increase of the entry area captured more individuals and it was more selective for certain types of

trap when population density was low, but the progressive entry area enlargement had diminishing returns, particularly for commercial traps (Renkema *et al*., 2014).

As for the position of the entry orifices, traps that had side holes captured more flies than the traps with a top entry (both with and without tents for shading and protection against rain) (Lee *et al*., 2013). For orifice size there are protocols that, generally, suggest an entry size between 1/8 and 3/16 of an inch on the side of the collecting flask, one inch apart from one another, and a three inch portion ought to be kept with no holes, in order to pour in the attractant when trap maintenance is due (OSU, 2011). Such dimensions mostly avoid the entering of non-target organisms that are larger than SWD, although they are not selective to insects as big or smaller. Traps with a larger surface area for exposing the attractant also seem to form an important element, since 90 cm² surface traps captured 12% more *D. suzukii* compared to 40 cm² surface traps (Lee *et al*., 2013).

Besides considering physical aspects, the ideal trap must be economically viable, easy to use, and durable (Renkema *et al*., 2014), since it is not just a monitoring tool for an integrated management program, it can also be used as a control method, providing mass capture and resulting in the suppression of a local population by a large number of traps.

Notwithstanding a series of factors necessary for attracting *D. suzukii* that are common knowledge amongst researchers, there is still no consensus about a standard trap model to be used. Therefore, aiming to register the seasonal activity of the species in Brazil, and based on the studies already performed by other researchers, a trap model for capturing *D. suzukii* was developed at the Insect Biology Laboratory from the Eliseu Maciel School of Agronomy, Universidade Federal de Pelotas, RS, Brazil. In addition to a prototype that sought to unite the characteristics that were most effective regarding capturing this drosophilid, the model also meant to better the cost-benefit relationship, so it could be used by producers and researchers alike (Figure 1). The trap was named SWDTRAP.

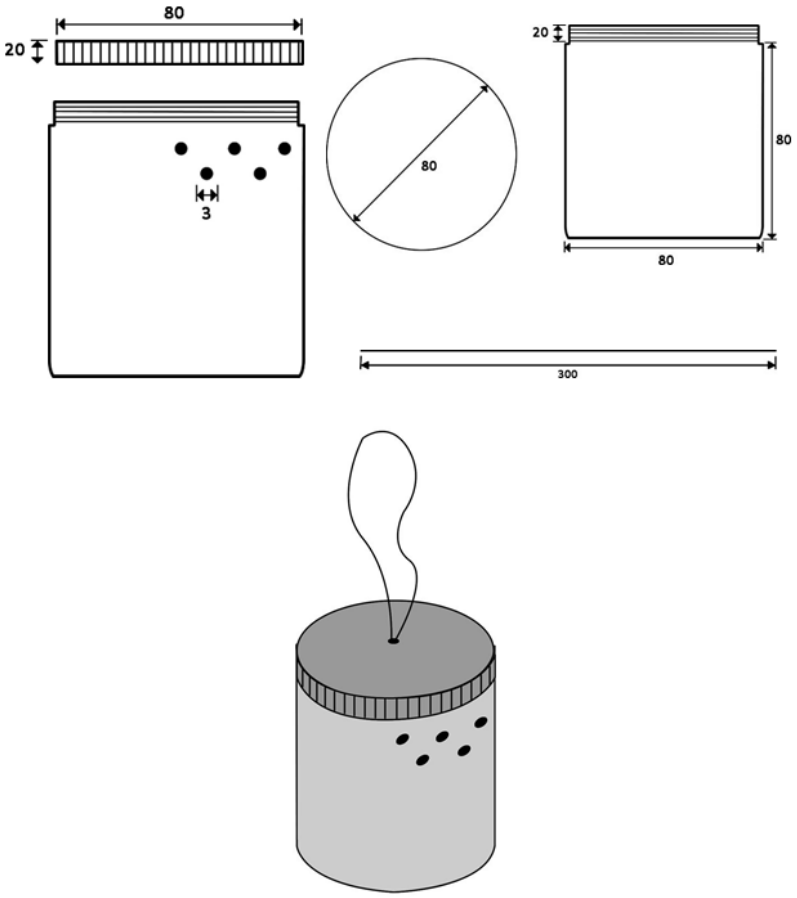

Figure 1. Illustrative drawing of the SWDTRAP (Scale: millimeter).

SWDTRAP design

Traps were made of virgin polypropylene plastic 500 mL vessels (Cloroplast®) closed with a screw cap (Figure 1). Polypropylene is a white, opaque, thermoplastic polymer, characteristically a material that has low cost, it is versatile, and particularly chemically resistant to solvents (Bonelli *et al*., 2001). This resistance to chemical abrasion is a relevant characteristic considered when developing a prototype, because the type of attractant substance used as bait may cause desiccation, followed by vessel breakage. For instance, baits

that use vinegar as well as wine are basically composed of acetic acid and ethanol, respectively. Also, traps are exposed to climate variations, which also reduce its lifespan.

Figure 2. New SWDTRAP trap for capturing *Drosophila suzukii* in guava (A), blackberry (B), and blueberry (C) crops.

On the side of the vessel, 16 three millimeter orifices were cut, and approximately three inches were left with no holes, so attractant liquid could be poured inside the vessel. Traps were painted red (Suvinil® multi use spray, 54631753 color), and before painting the vessels were sanded with a fine grain sandpaper, as to better allow the paint to adhere. At the center of the cap an orifice was cut and one millimeter diameter nylon thread was introduced (D Tools®), in order to suspend the trap at the orchard. In order to stop rainwater from falling inside the trap through the gap between the cap's orifice and the nylon thread, a few drops of instant glue were placed (Super Bonder Gel Loctite®) as a sealing agent.

The proposition of this new trap to be used in monitoring *D. suzukii* constitutes an alternative to other models that have already been developed, with the objective of producing a prototype that could be easily managed, made from readily found materials, low cost, resistant to field conditions, with the option for storage so it could be reused later. This model is already being used for monitoring SWD in upcountry Pelotas, Rio Grande do Sul, Brazil, for commercial blackberry (*Rubus* sp), blueberry (*Vaccinium myrtillus*), strawberry

(*Fragaria* x *ananassa*), surinam cherry (*Eugenia uniflora*), strawberry guava (*Psidium cattleianum*), and guava (*Psidium guajava*) crops (Figure 2).

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Rearing method for *Drosophila suzukii* and *Zaprionus indianus* (Diptera: Drosophilidae) on artificial culture media.

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Introduction

The Spotted Wing Drosophila (SWD), *Drosophila suzukii* (Matsumura, 1931) (Diptera: Drosophilidae), is an alien species whose main characteristic is an ability to infest intact fruit, perforating it in order to lay eggs and allowing the possibility of larval development.

Since *D. suzukii* was identified in a Californian raspberry field in 2008 (Hauser, 2009), it has spread rapidly throughout North America (Walsh *et al*., 2011; Hauser, 2011), Europe (Cini *et al*., 2012), and South America (Deprá *et al*., 2014), causing damage to a series of commercial fruit crops, wild fruits, and ornamental plants (Lee *et al*., 2015; Schlesener *et al*., 2015; Arnó *et al*., 2016). A recent study indicates environmentally adequate areas in Oceania and Africa where a particular predisposition to the occurrence of *D. suzukii* is found, although there has been no record of the species occurring in those continents. Models indicate that -

due to the environmental conditions - those areas are prone to the establishment of this species in case of future invasion (Dos Santos *et al.*, 2017).

The SWD displays a preference for soft, fragile tegument fruits such as blackberries (*Rubus* sp.), cattley guava (*Psidium cattleianum* Sabine), cherries (*Prunus* sp.), raspberries (*Rubus idaeus* L.), strawberries (*Fragaria x ananassa* Duch.), surinam cherries (*Eugenia uniflora* L.), grapes (*Vitis* spp.), amongst others (Bolda *et al*., 2010; Burrack *et al*., 2013).

Drosophila suzukii presents some morphological characteristics that allow it to be easily identified from other *Drosophila* species of the *melanogaster* species group. Males display a dark spot along the front edge of each wing and two rows of combs on the first and second tarsal segments of the first pair of legs, whilst females have a doubly serrated and narrow ovipositor, lined with robust sclerotized teeth (Kansawa, 1939; Walsh *et al.*, 2011; Vilela and Mori, 2014). The species has a short biological cycle, with high biotic potential and overlapping generations (Emiljanowicz *et al.*, 2014) and a preference for mild weather (Mitsui *et al.*, 2010).

The African fig fly, *Zaprionus indianus* Gupta, 1970 (Diptera: Drosophilidae), is also an invasive species with a high colonization potential, spreading rapidly throughout tropical regions, probably due to the intensification of world fruit trade (Stein *et al.*, 2003). This species presents a significant capacity for survival in anthropized settings, with a high biotic potential, and it is considered to be one of the most abundant species amongst drosophilid community members in Brazil (Silva *et al.*, 2005). The African fig fly has a yellow/brownish color, and it can be identified by the presence of longitudinal white stripes with narrow black borders over the head and thorax (Vilela, 1999).

Zaprionus indianus presents a generalist feeding behavior, where most of its nutrition comes from bacteria and yeast found in damaged and/or decomposing fruit, particularly the *Candida tropicalis* (Commar *et al.*, 2012) yeast. This substrate is used in feeding for adults, oviposition, and larval development (Vilela *et al.*, 2000), thus considered a secondary pest. Nevertheless, this species is the main pest to fig (*Ficus carica* L., var. Roxo de Valinhos) crops, since it lays eggs through ostiole of the fruit, while it has just started ripening. Hence the name 'African fig fly' (Vilela *et al.*, 2000).

The aforementioned species are of great importance in fruit production, since both cause damage to economically significant crops. In order to perform studies in the species mentioned before, it is essential to use a defined, low cost, fly culture medium to keep these species under laboratory conditions, and obtain a large number of flies all year around. In this context, we present a recipe of food media and raising protocol to breed *D. suzukii* and *Z. indianus* in the laboratory.

Materials and Methods

Drosophila suzukii and *Z. indianus* stocks were established in the Insect Ecology Lab, in the Ecology, Zoology, and Genetics Department from the Biology Institute in the Federal University of Pelotas (UFPel). Fly cultures were conditioned in B.O.D. (Biochemical Oxygen Demand) climate chambers at 23 $\pm$ 1ºC for *D. suzukii* and 25 $\pm$ 1ºC for *Z. indianus*, 70 $\pm$ 10% relative humidity (RH), and 12:12h photoperiod.

Collecting flies

Adults of *D. suzukii* were obtained from infested fruits. Blackberry fruits (*Rubus* spp.) were collected in a rural property located in Rincão da Caneleira, Pelotas, Rio Grande do Sul, Brazil (31°38'20"S and 52°30'43"W). After collection, fruits were placed in a thermal box and sent to the lab, where they were weighed and individualized in plastic containers (150 mL) with a screened lid and a fine layer of vermiculite (1 cm). Fruits were kept in a climate-controlled room at 23 $\pm$ 2°C temperature, 70 $\pm$ 10% RH, and a 12:12 h photoperiod, until adult flies emerged. After emerging, assumed *D. suzukii* couples were kept in glass vials (8.5 × 2.5 cm) containing the food media (see below) and covered by hydrophilic cotton plugs (Figure 1 A). Couples were kept for a 10 day period, so they could copulate and oviposit, followed by specific confirmation of specimens by diagnosed taxonomic characteristics (Vlach, 2013), aided by an optical stereomicroscope.

The flies that originated from the *Z. indianus* stock came from infested strawberry fruits collected in Pelotas, Rio Grande do Sul state, Brazil (31°40'49"S and 52°26'14"W).

Table 1. Fly food ingredients to culture *D. suzukii* and *Z. indianus*

Ingredients	Quantity
Distilled Water	1000 mL
Bacteriological Agar	8 g
Brewer's Yeast	40 g
Cornmeal (medium sized grains)	80 g
Fine sugar	100 g
Propionic acid	3.0 mL
Methylparaben (10%)*	8 mL

*Methylparaben: 0.8 g dissolved in 8 mL of ethanol 99,9%

Fly medium recipe and medium preparation

The fly medium recipe used to culture *D. suzukii* and *Z. indianus* described here (Table 1) is a modified fly medium referred to as standard cornmeal-yeast-glucose-agar medium by Matsubayashi *et al.* (1992), traditionally used to culture *Drosophila* species at several *Drosophila* labs in Japan.

The preparation of the fly medium has the following steps: 1) add 1/3 of the distilled water (330 mL) to the cornmeal and leave it to rest; 2) mix the brewer´s yeast, agar, and the remaining water (2/3 of water), and cook at high heat, constantly stirring, until boiling; 3) add the hydrated cornmeal and keep stirring until boiling; 4) finally, adding the fine sugar and keep stirring over mild heat until it boils once more; 5) remove the pot from heat and immediately add the propionic acid and methylparaben solution. Allow the solution to cool slightly; 6) dispense the media into the glass vials (8 mL medium per vial); 7) wait for cooling and vapor release of excess humidity of the vials under room temperature. Cover the vials with a fine cotton cloth, to avoid contamination by other drosophilids. 8) Afterwards, plug the vials with hydrophilic cotton. If necessary, the fly media can be stored in hermetically sealed plastic bags in refrigerator at 5 $\pm$ 2°C temperatures for up to 30 days.

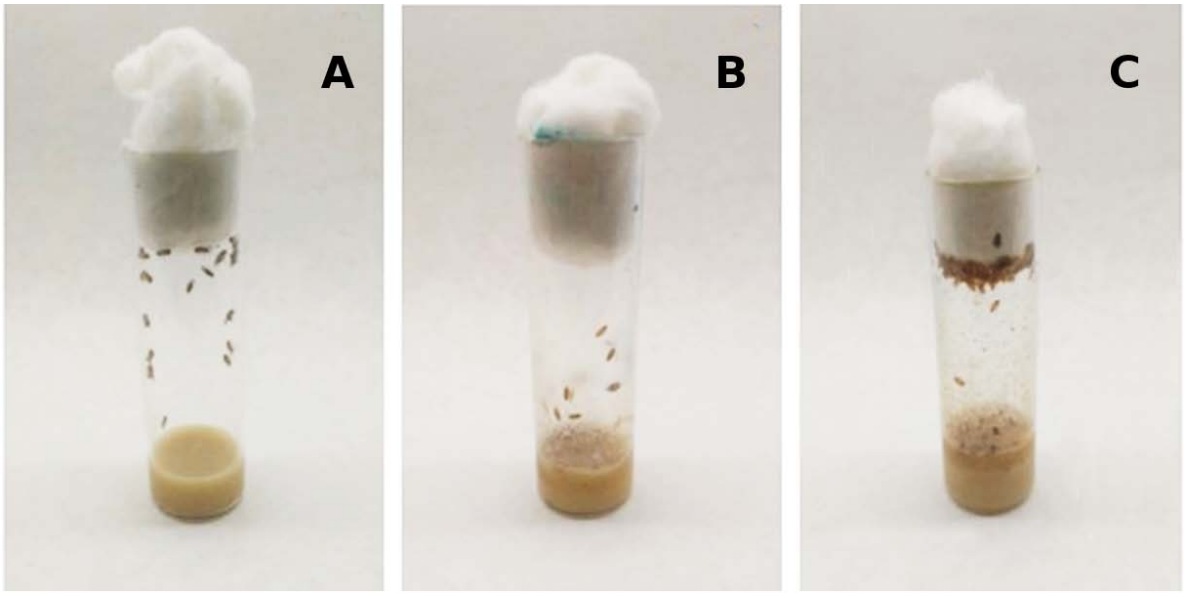

Figure 1. Rearing *D. suzukii* at the laboratory. A) Adults in oviposition stage; B) fly culture media with early stages of development, larvae, pre-pupae and pupae; and C) fly culture showing empty pupae.

Culturing fly

To keep *D. suzukii* and *Z. indianus* cultures in good health, maintenance ought to be done twice a week, consisting in the transference of recently emerged flies, those still on a pre-oviposition period or on early stages of laying eggs, into new vials (Figure 1A), and eliminating senile subjects and tubes where the

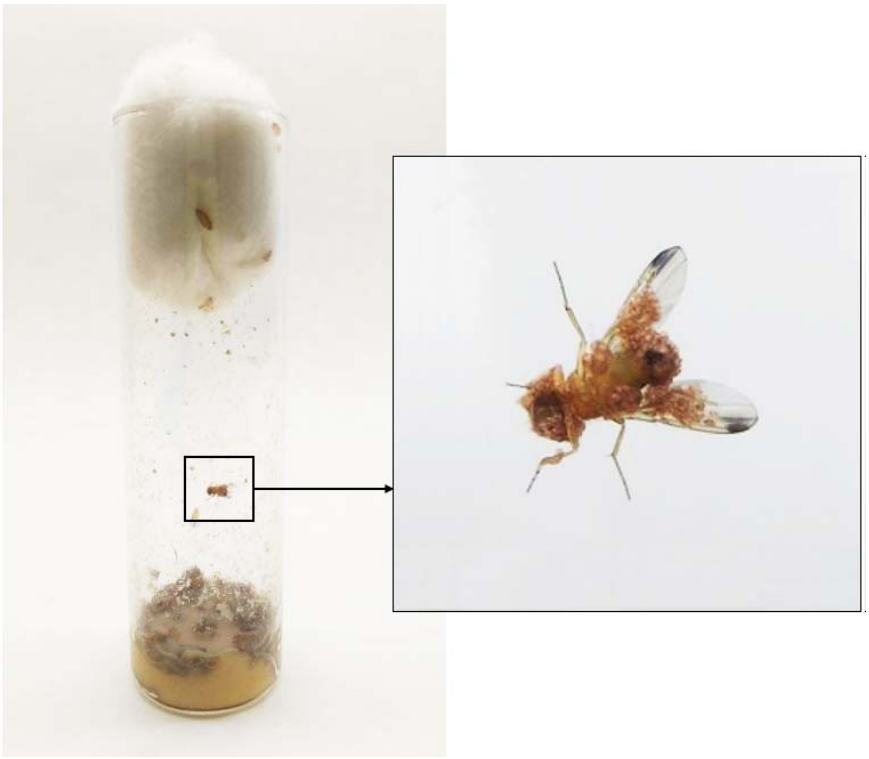

Figure 2. Rearing *D. suzukii* at the laboratory. Fly culture infected with mites (left). In detail, a male infected with mites.

majority of flies has already emerged. At each of the removal of flies it is important to properly mark the tubes, and the recipients of new subjects dated accordingly, as to maintain an organized culture.

Flies as old as three days are placed into new food vials (Figure 1A), which will be a source of humidity, substrate for egg-laying, and food for larvae and adults. Flies will remain in this environment for up to seven days, after which they can be transferred or discarded (Figure 1B). Keeping flies inside the vials for a longer period is not recommended, because it will be overcrowded, with consequent competition for food and loss of insect quality. The optimum density of adults per vial will be that in which the number of flies may occupy, at most, half the surface of the medium. If the aim is to get a lot of progeny or to obtain vials showing the developmental life cycle, make a new replica every 3-5 days.

Cultures that do not present further adult emergence must be discarded (Figure 1C). The discarding must also be done when the presence of mites adhered to the flies body is identified (Figure 2). The occurrence of mites is ordinary in colonies, but that may lead subjects to be not as fit, because of the stress induced by the mites. The presence of mites can be minimized by eliminating contaminated culture vials and cleaning the benches and instruments with alcohol 70%. The use of cotton plugs will minimize the dissemination of mites to the laboratory; make sure that the plugs fit tight to the culture vial.

Cleaning the glass vials must be performed on a weekly basis. The remainder of the fly media is removed with tweezers and spatula, and the vials are soaked into a hot sodium hypochlorite (3%) solution for up to 24 h. Afterwards, the vials are rinsed with boiling water and placed on test tube racks to drain, and later dried in lab stoves (at 35°C), where they remain until they are once again used.

Results and Discussion

Drosophila suzukii average development time (egg to adult) was 11 days at 23°C. Emiljanowicz *et al.* (2014) have observed a longer period (12.8 days), most likely due to the lower temperature (22°C) and medium composition. Andreazza *et al.* (2016) used a modified medium from Emiljanowicz *et al.* (2014) and obtained adults in 11 days at 25ºC temperature. In a study that sought to compare natural (blueberry) and artificial yeast based diet, it was observed that the development time was shorter when flies were fed a natural diet, and the average development periods were 10.6 and 11.7 days, respectively (Jaramillo *et al.*, 2015).

The *Z. indianus* species took 14 days to fully develop (egg to adult) at 25°C. The development time in artificial diets based on banana and yeast varied between 12.7 and 28.8 days at 28°C and 18°C, respectively (Nava *et al.*, 2007). With a natural diet (fig), the African fig fly develops in an average of 17 days (Pasini and Lúcio, 2014).

The fly food and rearing method here described is suitable for the rearing of both species, since it provides adequate conditions for biological development, furthermore presenting unlikelihood of contamination by other drosophilids and opportunistic microorganisms (mites, fungi, and bacteria). In the event of contamination, the problem is easily dealt with, by eliminating the contaminated cultures, with no consequence to the remainder culture. Rearing methodology using plastic boxes as cages tends to be more prone to be contaminated by other drosophilids, since there is a larger surface in contact with the external environment. Also, detection and elimination of the contamination demands disposing of many more subjects, since the entire cage ought to be eliminated (Andreazza *et al.*, 2016). Furthermore, compartmentalization of fly cultures in several vials facilitates the removal of different biological stages of the fly for lab bioassays.

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A new climbing assay protocol for *Drosophila melanogaster.*

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Introduction

Rapid iterative negative geotaxis (RING) in *Drosophila melanogaster* is a frequently employed behavioral assay for addressing questions on motility in relation to ageing, nutrition, and in use of the fly as a disease model. The protocol employs tapping the flies down in a vial and observing the number of flies that climb up to certain level to arrive at a climbing index (Todd *et al.*, 2004; Gargona *et al.*, 2005; Nicole *et al.*, 2009; Charles *et al.*, 2012; Rakshith *et al.*, 2013). The modified RING assay design described in the present study is a variant, wherein a 25 cm tube containing flies is rotated by a half-turn using a motor at fixed programmable intervals to elicit negative geotaxis. This rotation repeatedly places the flies at the bottom of the tube and elicits climbing. Similar studies have been carried out by Sean *et al.*, 2016 to measure genetic variation in response to gently induced exercise.

Keywords: *Fly*, climbing index, negative geotaxis.

A. B.

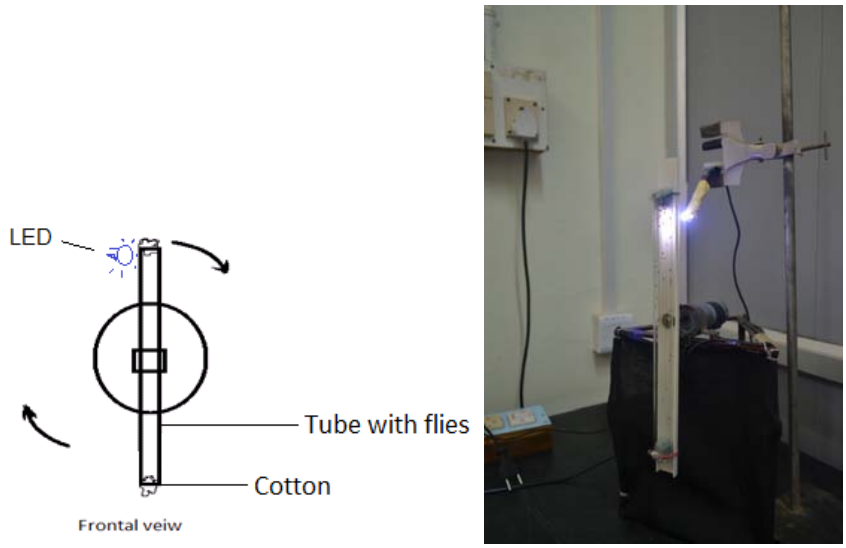

Figure 1. (A) Rotor-RING assay. A schematic representation of the apparatus is provided in A. The rotor rotates clockwise at intervals of 2 minutes. (B) Photograph of the apparatus.

Material and Methods

Fly stocks (Canton-S) obtained from *Drosophila* stock centre, University of Mysore, was maintained in the laboratory as an inbred stock. The stock was maintained at 22°C. Two-day-old flies were used for studies. A motor-operated apparatus with a 25 cm tall tube, fixed in the middle, was made to rotate a half turn of 180^{o} (Figure 1). Two-day-old *Drosophila melanogaster* (Canton-S) flies were used. The interval between successive rotations is programmable in the apparatus. The flies which are at the top of the tube will be at the bottom when the tube makes a half rotation. This induces their innate instinct of negative geotaxis and the flies start climbing up. The tube is designed for the flies to climb a distance of 25 cm. The tube was divided into 4 equal parts using a marker. Time taken for a half rotation is 2 sec. The imparted centrifugal force is negligible and the time between two successive turns was 2 minutes. Climbing index was calculated by

counting the flies which were present in the upper most quadrant. The experiment was conducted for 30 min which thus completes 15 cycles. For each experiment 50 flies were taken. The experiments were carried out before noon. All the experiments were carried out in three sets. Males and females were considered separately for the experiment.

Table 1. Showing number of flies in different divisions of the tube.

Number of rotations	Division 4	Division 3	Division 2	Division 1 Uppermost
1		1	2	47
2				50
3	4		2	44
4	3		2	45
5			1	49
6	2		2	46
7		1		49
8	1		1	48
9		1		49
10	1		1	48
11		1		49
12	1	1	2	46
13	3		2	45
14	2	2		46
15	2	1		47

Observations

Flies that are placed at the bottom of the tube tend to move up due to negative geotaxis. Repetitive stimulation of 15 cycles placed the flies in the bottom of the tube and evoked the flies to climb repeatedly. The time taken for one session of training was 30 minutes following which the flies rested. Climbing index was calculated by counting the number of flies manually (Table 1).

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Antioxidant properties of fruit/vegetable substrates suitable for culturing *Drosophila melanogaster*.

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In year 2000, we established four *Drosophila melanogaster* strains reared on substrates cooked with fruits and vegetables (banana - B strain; apple - A strain; T - tomato strain; and C- carrot strain), which represent resources used for feeding in natural conditions (Shorrocks, 1972). After culturing flies for more than 60 generations,we have published those recipes as suitable for maintaining *D. melanogaster,* as well as species with similar nutritional requirements (Kekić and Pavković-Lučić, 2003). Later, we have used those strains in experiments devoted to the influence of nutrition on morphological characteristics, cuticular

chemistry, life-history, and behavioral traits (Pavković-Lučić and Kekić, 2010; Trajković *et al*., 2013; Pavković-Lučić *et al*., 2016; Trajković *et al*., 2017a, b).

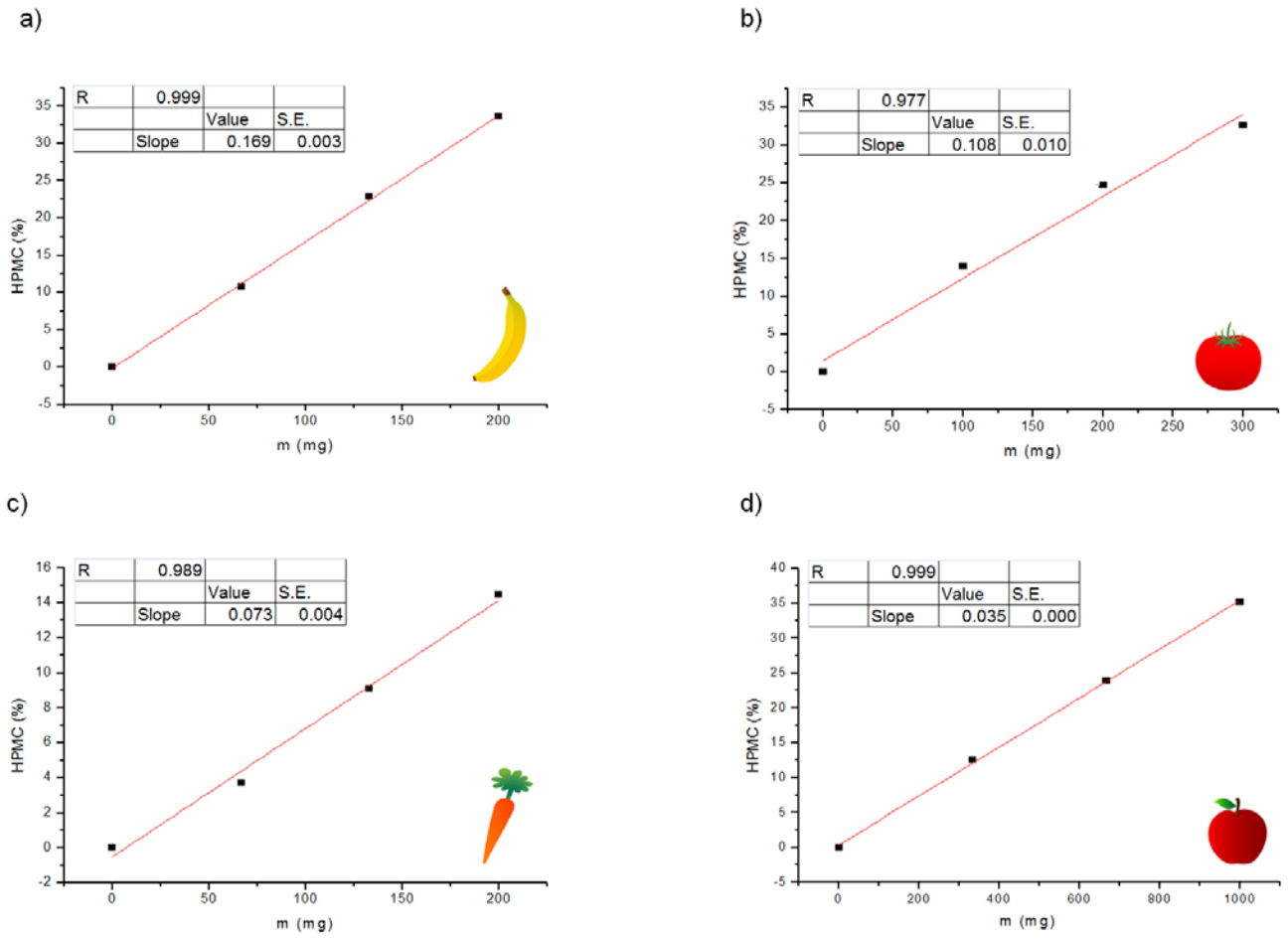

Figure 1 (a-d). Antioxidant properties of four substrates used for *D. melanogaster* culturing: banana substrate (a), tomato substrate (b), carrot substrate (c), and apple substrate (d), measured by HPMC assay.

Chemical analysis of the aforementioned food revealed differences in their protein content and C/N ratio (Trajković *et al*., 2017a). Since fruits and vegetables are sources of phytochemicals which act as antioxidants (Slavin and Lloyd, 2012), we have recently tested antioxidant properties of the diets using an electrochemical assay based on direct current polarography. The polarographic assay applied was selected as fast, reliable, and low cost alternative to spectrophotometric antioxidant assays commonly applied in analysis of food and biological samples. Moreover, polarographic monitoring of HPMC current decrease allowed colored and turbid samples, usually unacceptable in spectrophotometric assays. Polarographic i-E curves were obtained using the Princeton Applied Research 174 Polarographic analyzer and recorded on Houston Instrument Omnigraphic 2000 X-Y recorder. The dropping time of working dropping mercury electrode (DME) (with capillary characteristics of m = 2.5 mgs^{-1} at mercury reservoir height of 75 cm) was programmed on time 1 s, while current oscillations were damped with low pass filter of instrument set at 3 s. Saturated calomel electrode (SCE) was used as reference and platinum foil as auxiliary electrode. The initial potentials were 0.10 V *vs* SCE. Potential scan rate was 10 mV/s. Clark & Lubs (CL) buffer of pH 9.8, used as supporting electrolyte, and initial solution for HPMC assay were obtained as described previously by Gorjanović *et al*. (2013a). Before each i-E curve recording, initial solution as well as solution after addition of aliquots of each analyzed samples was deaerated using pure gaseous nitrogen. Inert atmosphere was kept in

the cell during each i-E curve recording. Decrease of anodic limiting current originating from HydroxoPerhydroxoMercury(II) complex [Hg(O2H)(OH)] formation in alkaline solutions of hydrogen peroxide at potential of mercury dissolution upon gradual addition of antioxidants was observed. The assay based on that decrease was optimized (Sužnjević *et al*., 2011) and applied on wide variety of food and biological samples (Gorjanović *et al*., 2013b). Dependence of decrease of HPMC anodic limiting current on volume or mass of gradually added complex samples has been followed and plotted. The slope of the starting linear part of that plot was considered as a measure of antioxidant (AO) activity. The activity was expressed as percentage of anodic limiting current decrease per volume or mass of complex samples added (%/mL or %/mg).

Based on the results, fruit/vegetable substrates could be arranged according to their antioxidant properties, from the highest to the lowest, in the following way: banana (0.169 ± 0.003%/mg) > tomato (0.108 ± 0.001%/mg) > carrot (0.073 ± 0.004%/mg) > apple (0.035 ± 0.001%/mg). We must emphasize that we always peel an apple before cooking the substrate. This could be an explanation for its low antioxidant activity, since the majority of compounds with high antioxidant potentials are found in the apple skin (Wolfe *et al*., 2003). Further, cooking destroys part of the AOs, so our results can not be compared to those obtained for fresh fruit. Nevertheless, information considering differences in antioxidant properties of aforementioned diets could be useful for further experimental purposes.

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Creation and standardization of methods for ethological analysis of *Drosophila melanogaster*: preference test and immobilization stress.

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Introduction

The many fundamental biological mechanisms conserved between *Drosophila* and *Homo sapiens* justify the study of *Drosophila* in many biological fields, including neurobiology of learning, memory, and stress (Jennings, 2011). As observed in different vertebrates, like *Rattus norvegicus*, *Mus musculus*, or *Danio*

rerio, *Drosophila* react to uncontrollable and inescapable stress exposure with decreased escape or avoidance named as "learned helplessness" (Vollmayr and Gass, 2013). In this regard, a study showed that individuals of *Drosophila melanogaster* expressed "learned helplessness" when exposed to thermal shocks as "punishment" for stopping their locomotion for one second (Yang *et al*., 2013). The presence of "learned helplessness" in *Drosophila* indicates the existence of complex mechanisms in these organisms allowing the study of the neurobiology of stress (Vollmayr and Gass, 2013).

Additionally, chronic or acute stress is one of the environmental factors involved in the development of psychiatric disorders (Joca *et al.*, 2003). Preclinical studies aiming to characterize treatments for psychiatric disorders usually test the anti-stress properties of a substance in laboratory animals submitted to inescapable stress such as immobilization or forced swimming (Lino-de-Oliveira *et al*., 2001; Lino-de-Oliveira *et al*., 2005). Indeed, it was already observed that "learned helplessness" in *D. melanogaster* is reduced by previous treatment with lithium (Ries *et al*., 2017) indicating that, as in vertebrates, the response of flies to stress is ameliorated with the treatment with antidepressants. Together, present evidence indicates that *D. melanogaster* may be useful for the discovery of new psychotropic medications.

Studies on behavior and drug testing in vertebrate animals have been criticized in the field of ethics and animal welfare (Huet and Haan, 2014). Currently, there are initiatives aiming to decrease the quantity or replace the vertebrates, by invertebrates, for example, in biomedical studies. These initiatives are known as 3Rs: reduction, refinement, and replacement (Huet and Haan, 2014). Therefore, the present study is aimed to describe the behavior of *D. melanogaster* against different stressful stimuli targeting the standardization of its use in neurobiology of stress and antidepressants. For that, adult flies of *D. melanogaster* were bred in four palatable media and the behaviors of these animals were analyzed in two different situations: a test of preference for palatable food and an immobilization stress. Immobilization stress was used as inescapable stress in studies using rats (Ueyama *et al*., 1997; Lino-de-Oliveira *et al*., 2001). The hypothesis is that the flies will prefer the food where they were bred and, after the stress exposure, they will reduce their preference or the search for food.

Material and Methods

Behavioral experiments were performed using wild male and female *D. melanogaster*. The stocks were obtained from Stock Center Tucson (Arizona, U.S.A.), and they were maintained in the Drosophilids laboratory at the Federal University of Santa Catarina. The animals were maintained under natural temperature and lighting. The adults were removed from the stock of the laboratory and were bred in a medium made of corn. Initially, each glass contained five males and five females, and they were maintained in different media. These studies comprised three different pilots. In all of them, flies were submitted to tests of preference for palatable foods and immobilization stress.

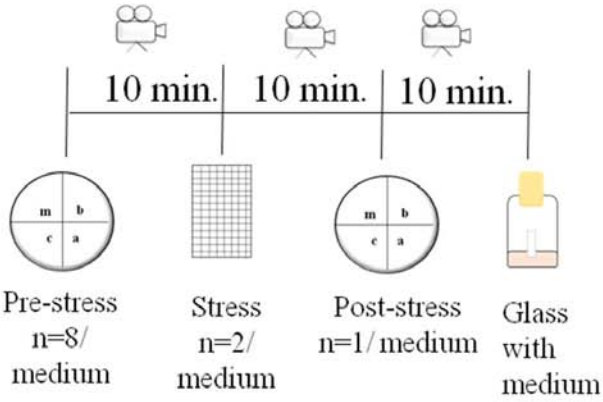
In the first pilot, flies were bred in four different types of media: alcohol, banana, molasses, and complete media around seven days before the tests (Figure 1a). 24 hours before the tests, flies were put in a glass containing only agar medium (1%) for the food privation. The tests were realized in three consecutive steps (pre-stress, stress, and post-stress) to flies of each medium (Figure 1b). A Petri dish divided in four quadrants was used to test the preference of substrate. Three quadrants were filled with agar and one quadrant with palatable medium (Figure 1c). In the pre-stress step, after 24 hours of food privation, eight flies of each medium were placed in the Petri dish (n = 32). To put the flies in the Petri dish they were anesthetized with CO_2 and handled with tweezers. Before the next step, flies were anesthetized again. Only a male and a female of each medium were used in the stress and post-stress steps (n = 8). Other flies used in the pre-stress went back to the glass with medium. Immobilization stress was induced putting the flies into a hole made in agar in a 96-well plate for PCR (Figure 1b). The hole was made with a tube of 2 millimeters of diameter. The post-stress was done as the same way of pre-stress. All steps were filmed for 10 minutes.

Alcohol was selected as one of the bred medium, because it is known that drosophilids have adaptations to live in environments containing this substance. However, we observed that the concentration of alcohol used here (14%) was not adequate to create the animals because many flies died in this medium. Despite that they can survive in places containing ethanol, in natural environment flies can avoid places with concentration higher than 5% (Devineni and Heberlein, 2013). Therefore, this exposure can be intermittent in

a

Medium	Ingredients	Proportions
Complete medium	Water	79 mL
	Molasses	7 mL
	smashed banana- "caturra" type	6 g
	Alcohol	14 mL
	Agar	1 g
Banana	Water	75 mL
	smashed banana- "caturra" type	25g
	Agar	1 g
Molasses	Water	92.5 mL
	Molasses	7.5 mL
	Agar	1 g
Alcohol	Water	86 mL
	Alcohol	14 mL
	Agar	1 g

b



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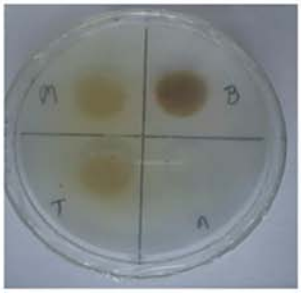


Figure 1. a. Ingredient proportions used in each medium. b. Timeline of the test. c. Petri dish used for the preference test.

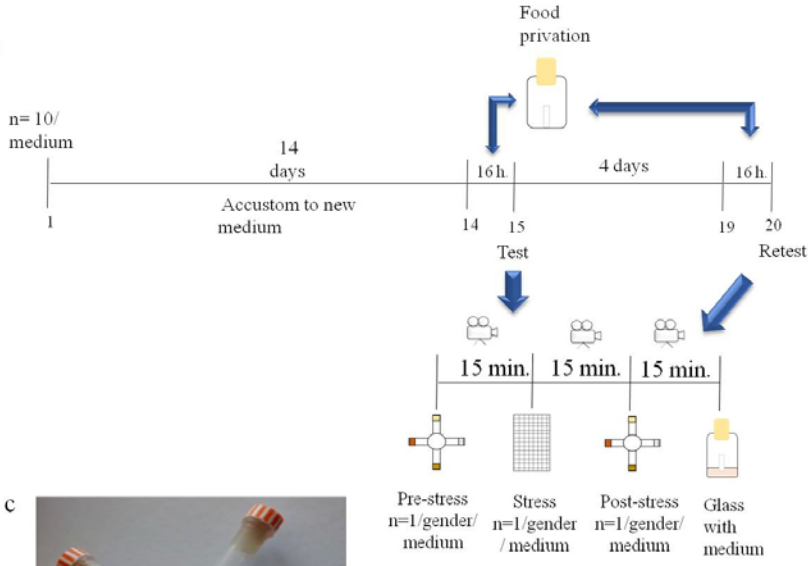
contrast to laboratory conditions. The time and the method for food privation were not adequate, because many flies died in this period. Krashes and Waddell (2008) maintained flies without food for a period of 16 to 20 hours in a glass contained only a filter paper soaked in water. In the next pilots this methodology was used. The use of CO_2 and the handling of the flies with tweezers were also difficulties to maintain all the animals alive during the tests. Apparatus and methods used to observe the animal's behavior were a problem. Flies and medium in the Petri dish showed overlapping of colors and it did not allow a good visualization of the behaviors in the videos. The apparatus used in immobilization stress was not good, because the hole made in the agar was small in comparison to size of the flies. This hole had 0.2 cm of diameter and adults of *D. melanogaster* has 0.1 cm in length (Pitnick and García-Gonzalez, 2002). The locomotion of the flies and the withdrawal of the apparatus using tweezers became difficult. Besides this, flies attached on the agar, because did not have a protection "between" the agar and the animals.

In the second pilot, the flies were created in three types of media: banana, molasses, and complete medium, and they were maintained on this medium for two weeks before the tests (n = 10/medium) (Figure 2a). An apparatus made by students of the laboratory was used for the preference test. This apparatus was named "plus maze for preference test" and each tube of these maze contained 1.5 mL of the media: banana, molasses, complete medium, and agar (Figure 2c). The tests were also realized in three consecutive steps (pre-stress, stress, and post-stress). A female and male of each medium was used in the tests (n = 6). Before this, flies were deprived of food for 16 hours (Krashes and Waddell, 2008). They were placed into a glass containing a filter paper soaked in water (Figure 2b). For the preference tests (pre and post-stress), flies, individually, were placed into the plus maze and it was covered with a cover slip (Figure 2b). Immobilization stress was induced by putting the flies into a hole made in agar in a 96-well plate for PCR and this was made with a tube of 3 millimeters of diameter. All steps were filmed for 15 minutes (Figure 2b). To transfer the flies between different places, they were anesthetized with CO_2 and handled with tweezers. After five days, a retest was made using the same flies and following the same protocol. Behavioral catalogs were developed to the preference and to immobilization tests and after analysis of the films we observed that the size of the plus

a

Medium	Ingredients	Proportions
Complete medium	Water	79.5 mL
	Molasses	14.5 mL
	Smashed banana- "caturra" type	6 g
	Agar	1 g
Banana	Water	75 mL
	Smashed banana- "caturra" type	25 g
	Agar	1 g
Molasses	Water	92.5 mL
	Molasses	7.5 mL
	Agar	1 g

b



c

Figure 2. a. Ingredient proportions used in each medium. b. Timeline of the test and retest. c. Plus maze used for the preference test.

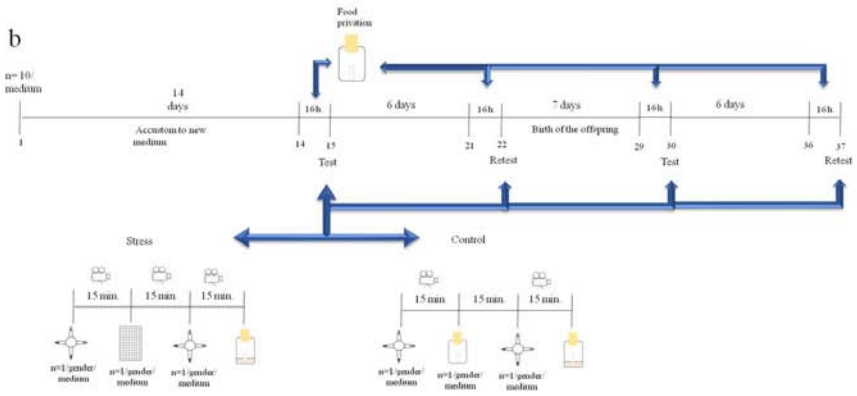
maze was not satisfactory, because the animals spent more time in the center of the apparatus and did not explore the arms with food. In behavioral studies with rats, for example, it is common to use a plus elevated maze. The size of these apparatuses agrees with our hypothesis. Pellow *et al*. (1985) used a plus elevated maze with arms of 50 cm in length for adult rats with 20 cm in length. The plus maze used in our study had arms with 4.5 in length. With the proportions used for rats, the flies used in this work would be 1.8 cm in length, however, adults of *D. melanogaster* are 0.1 cm in length (Pitnick and García-González, 2002). Das *et al*. (2016) said that starvation can contribute to the expression of memories associated to sugar. Among all the six animals tested on this pilot, immobility on the center of the plus maze was the behavior observed with more frequency in the videos. Three animals explored the arms of the plus maze, but this exploration did not occur in the pre- and post-stress steps; therefore, the arm exploration can be random. Using CO_2 to anesthetize the animals was another factor that would increase the time of immobility and decrease the plus maze exploration. This method to anesthetize is used in some studies with drosophilids (Lefranc and Bundgaard, 2000; Zimmerman *et al*., 2008), but the repeated exposure to CO_2 can influence the behavior of these animals. Immobilization stress can be another factor that decreased the locomotion of the flies, mainly during the post-stress steps. It is known that learned helplessness exists in *D. melanogaster* (Yang *et al*., 2013) and it can collaborate to reduce the locomotion and the looking for palatable food in the plus maze.

In the third pilot, flies were bred in four media: alcohol, banana, molasses, and complete medium (Figure 3a). They were maintained on these media for two weeks until start of the tests (n = 10/medium). Before the tests these flies were deprived of food for 16 hours, as in the second pilot (Figure 3b). To the preference test a small plus maze was created (Figure 3c). Flies were divided into two groups: control and stress. Two flies (male and female) of each medium and each group were tested (n = 16). For the stress group, the tests were performed in three consecutive steps: pre-stress, stress, and post-stress steps. Flies of the control group, on the second step, were placed into the glass for food privation (Figure 3b). On the pre- and

a

Medium	Ingredients	Proportions
Complete medium	Water	70 mL
	Molasses	9 mL
	Smashed banana- "caturra" type	6 g
	Alcohol	15 mL
	Agar	1 g
Banana	Water	70 mL
	Smashed banana- "caturra" type	30 g
	Agar	1 g
Molasses	Water	91 mL
	Molasses	9 mL
	Agar	1 g
Alcohol	Water	94 mL
	Alcohol	6 mL
	Agar	1 g

b



c

Figure 3. a. Ingredient proportions used in each medium. b. Timeline of the test and retest to stress and control groups. c. Plus maze used for the preference test.

post-stress steps, flies, individually, were placed into the plus maze and it was covered with a cover slip. The immobilization stress was made in the same way as the second pilot. After each couple generated offspring, a male and female of each medium/group were also tested (n = 16) following the same protocols used for their parental. All steps were filmed for 15 minutes (Figure 3b). To transfer the flies between the "house glasses" to the "privation food glass" they were anesthetized with CO_2 and handled with tweezers. To transfer them between the steps of the tests, an entomological aspirator was used. A polystyrene box was used to do the films with less light interference on the animal behaviors. After seven days, retest was made using the same flies and following the same protocol (Figure 3b). The new plus maze, besides not having the ideal size yet, was better than that used on the second pilot, because it was smaller than the first. Although animals still carry out behaviors of immobility, the exploitation of the arms of the plus maze was more expressive than that observed in the second pilot. Knowing that *D. melanogaster*, as other insects, had positive phototropism (Gao *et al*., 2008) we did the videos inside a polystyrene box. It was an improvement in relation to the other pilots, because the light interference on the behaviors of the flies was lower than in the first and second pilots. The use of the aspirator had pros, because the flies were not exposed many times to CO_2, and cons, because using the entomological aspirator was more difficult to maintain the animal in the center of the plus maze to begin the test. After analyzing the videos, it could be observed that the time spent in locomotion on the plus maze increased and less animals died during the tests, compared to other pilots. Barron (1999) suggested that behavior studies avoid CO_2 to anaesthetize the animals, because CO_2 can change the behavior of them. However, among the eight parental flies that were exposed to immobilization stress, six increased the time of immobility. It was observed that the arms of the plus maze were explored with more frequency than in the second pilot. The same animals reduced their arm exploration after stress steps. The environment that insect larvae and adults live in can influence behaviors, such as food preference, of these animals (Abed-Vieillard and Cortot, 2016). But, only 7 of 32 animals used in tests explored more often the arm containing the same food of their "house glass". *Drosophila* has a preference to concentrations up to 5% of ethanol, looking for places with odor or food containing this substance (Kaun *et al*., 2011). But, during the preference test, the arm containing alcohol medium did the flies explore the less. The other arms, containing media with sugar, were more explored. *D. melanogaster* could be good species to study anhedonia, but to confirm this hypothesis a

larger sample number must be used in the behaviors tests. Beside this, a second method to stress flies could be efficient to obtain a better result, too.

The results of the three pilots of this study showed that *D. melanogaster* is sensitive to alcohol and avoids this substance or dies when in concentrations higher than 5%. Besides this, when in starvation, flies of this species shows preference for substrates that contain palatable food, such as sugar. Results also indicate that *D. melanogaster* is sensitive to stressful situations, like related to immobilization and CO_2 anesthesia, and these situations could modify their behaviors to looking for food. This study shows a preliminary analysis of behavior of *D. melanogaster*. It is important to establishment of models for replacement of vertebrates in behavioral studies. However, to confirm the hypothesis it is necessary for a greater behavior analysis, with higher sample number.

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Mapping *Drosophila* phototaxis behavior mutants; Possibly extend method to genetic diseases in human families.

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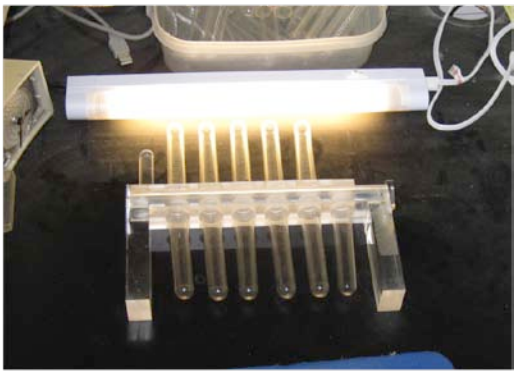
Recombination based mapping traditionally measures distances between traits or markers by recombination fractions in order to map them. An alternative recombination based approach, based on localizing crossovers in intervals defined by markers having known locations, efficiently positions traits, including those previously difficult to map, to a chromosome section between such markers. This approach makes use of the fact that many gametes will carry a chromosome with either no crossover or only a single crossover. The class of non-crossover chromosomes can be used to map a trait to a specific chromosome ("not independent assortment" of the trait with reciprocal parental gametes). The class of gametes with a single crossover, identified by a change in phase at only a single interval between known markers, divides the chromosome into left and right portions; the sum of such divisions, over several progeny with single crossovers, can be used to localize the trait to a specific interval. For discrete dichotomous traits, such an approach is significant with relatively few progeny. For quantitative, or polychotomous, or weakly penetrant traits, the reciprocal single crossover-gametes for an interval can be typed for systematic differences in

phenotype, leading to left-right assignments of the locus relative to crossovers. Here I show *Drosophila* non-phototactic behavior mutants can be successfully mapped using this approach.

Phototaxis counter current apparatus,designed by Seymour Benzer



- Flies normally run towards light when agitated; flies that move to the top are shifted to the next tube, reach last tube on right after 5 moves to the light in 5 trials.

Figure 1.

y cv v f car parents
+ + + + +
(lower X chromosome has non-photactic mutant)

- Sort progeny on counter-current apparatus for photoaxis behavior (progeny sort into tubes 0-5)
- Score numbers of each crossover type in each tube
- Graph results: compare photaxis behaviors by their graph patterns. Map which interval changes pattern between black and white
- The non-crossover graphs show the different parental phototaxis behaviors
- The reciprocal single crossover progeny in each of the 4 intervals will show a parental behavior; the crossover in that interval will show the mutant to be either left or right of the crossover
- The results in all 4 intervals agree to the location of the mutant

Figure 2.

Males segregating the *norpA* mutation

00000 (white) and 11111 (black) are reciprocal non-crossover types (=y cv v f car and + + + + +, respectively)

01111 (white) and 10000 (black) are reciprocal single crossover types in the y-cv interval. Mutant maps there (crossover in interval can be to either side of mutant, therefore no difference in white and black graphs).

Crossovers in all 3 other intervals point to mutant at their left, confirming location.

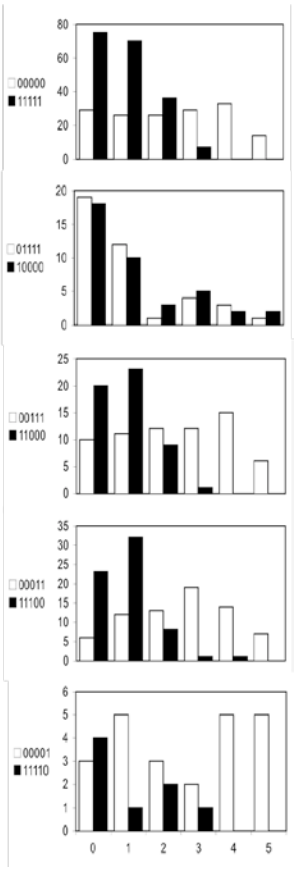


Figure 3.

Males segregating the *tan* mutation

00000 (white) and 11111 (black) are reciprocal non-crossover types (=y cv v f car and + + + + +, respectively)

01111 (white) and 10000 (black) are reciprocal single crossover types in the y-cv interval. Mutant maps to the right of that crossover location

00111 and 11000 are crossovers in the cv-v interval. Mutant maps there.

Crossovers in other 2 intervals point to mutant at their left, confirming location.

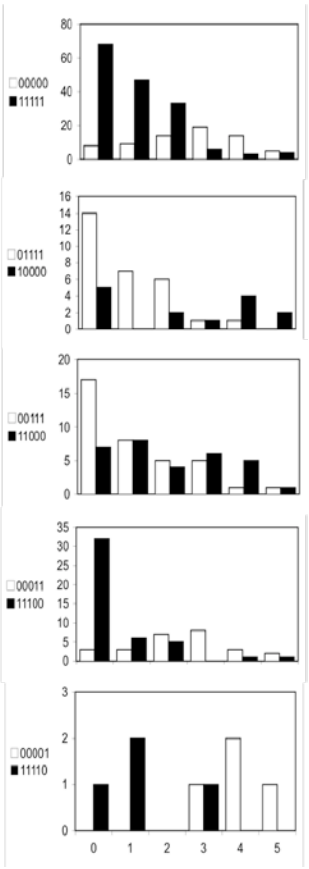


Figure 4.

The paper of Benzer (1967) is considered as the origin of neurogenetics. He identified four recessive X-linked mutants on the basis of abnormal phototactic behavior of failing to run towards light when agitated (Figures 1 to 4).. Two of these mutants, *tan* and *lozenge*, had additional phenotypic changes as well. In order to identify the genes involved and/or to confirm the importance of the *tan* and *lozenge* loci to phototaxis, the causative mutants were mapped using recombination with a well-marked X chromosome (*y cv v f car*) from a normal phototaxis background (Merriam and Benzer, 1969). Because the mapping data for the original four mutants are not available, the crosses were redone here for the *tan* and *norpA* mutants with stocks obtained from Bloomington. The *norpA* mutant was recovered in a follow up screen for non-phototaxis mutants using an apparatus shown below (Merriam unpublished, cited in Hotta and Benzer, 1970; and Benzer, 1973). The *norpA* gene is located at 1-7 between *y* and *cv*; the *tan* gene is located at 1-27 between *cv* and *v*. These two cases provide sufficient examples for the method of using recombination to identify the locations of risk alleles for behavioral, quantitative, or otherwise individually non-obvious phenotypes. Data are available on request.

Table 1. The predicted frequency of sperm carrying 0, 1, 2, 3, 4 or 5 crossovers per autosome, obtained from applying the calculations of Figure 3 to the observed exchange frequencies in Figure 2. The % do not always sum to 100 as explained in Figure 2. *means some possible but less than 1%.

Chromo-some	% gametes with each number crossovers					
	0	1	2	3	4	5
1	7	26	35	22	6	*
2	10	32	36	18	4	*
3	12	35	36	15	2	
4	18	43	31	7	*	
5	16	41	33	8	*	
6	17	41	32	6	*	
7	17	41	32	6	*	
8	23	48	27	3		
9	20	44	30	6		
10	22	46	28	4		
11	22	47	28	4		
12	18	27	33	25		
13	26	49	24	1		
14	49	50	1			
15	25	48	23	1		
16	26	48	24	2	*	
17	25	48	25	2		
18	30	50	20	*		
19	25	50	25	*		
20	30	50	20	*		
21	50	50				
22	47	48	2			

How many crossovers are observed/expected in human chromosomes?

Because of the abundance of DNA markers every human family has the potential to be fully informative for every chromosome, *i.e*., every chromosome can be well-marked for heterozygosity with sufficient intervals analyzed to recognize every crossover. To determine whether such an approach would be feasible for mapping human traits, I estimated the frequency of non-crossover, single crossover, and multiple crossover bearing gametes for human chromosomes from the literature on the observed distribution of chiasmata in sperm (Table 1).

Those recombination levels are in the range to make this a workable approach for human family studies with three generations or more. Their application to studies on common disorders may be helpful in finding high risk low frequency causative alleles or in assessing the relative importance of chromosome sites that are associated with risk alleles.

Literature cited: Benzer, S., 1967, Proc. Natl. Acad. Sci., USA 58: 1112; Benzer, S., 1973, Scientific American 229: 24; Hotta, Y., and S. Benzer 1970, Proc. Natl. Acad. Sci., USA 67: 1156; Merriam, J., and S. Benzer 1969, Genetics 61: s40.

The use of 3D printing to facilitate *Drosophila* behavior research.

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Studying and quantifying *Drosophila* behavior is critical for understanding their role in ecologically important interactions. For example, knowing the food choice behavior of the agricultural pest *D. suzukii* can provide valuable information regarding their management and control (Lee *et al.*, 2011a), while other *Drosophila* species may act as vectors for the transport of animals, such as nematodes, which can themselves have profound impacts on ecosystems (Kiontke and Fitch, 2013). To study these and other behaviors in a controlled laboratory setting requires a number of different and specific experimental apparatuses, whose creation can be an exercise in compromise. Off-the shelf arenas (petri dishes, vials, *etc*.) are rarely the optimal size or shape and are often difficult to modify (*e.g.*, the addition of entrances, dividers, or gates) due to their materials (*i.e.*, glass or brittle plastic). Adding to that the need to control opacity, color, optical qualities, or texture leaves the experimenter with the choice of making do with sub-optimal equipment or spending considerable resources constructing a custom fabricated item. With the availability and affordability and ease of desktop 3D printing equipment, however, this is no longer the case.

Consumer-level 3D printers and design software are revolutionizing many areas of scientific investigation (Chen, 2012, Lipson and Kurman, 2013, Chia and Wu, 2015). While previously only available in well-funded engineering labs, the recent availability of affordable units that can print in multiple types of plastic, and at sub-millimeter resolutions, means that researchers can quickly and easily design and print anything from replacement parts for larger devices to the purpose-built chambers needed for biological research, such as behavioral studies with *Drosophila*.

In this note we demonstrate the functionality of this on-demand, rapid prototyping and production system to support research into: 1) *D. suzukii*'s feeding preferences; and 2) *D. suzukii*'s ability to act as vectors for nematode dispersal. Each device could easily be adapted for use with other insect species.

Case study 1. *Feeding choice by* D. suzukii

Determining the characteristics that attract *D. suzukii*, a significant agricultural pest, to particular food sources may provide useful information for their control (Lee *et al.*, 2011a). The chambers designed and built for this experiment provide advantages over the classic population box (Dobzhansky, 1947; Lewontin, 1965; Caravaca and, Lei 2016). First, they allow for increased replication due to their size and affordability. Second, they provide for easier and more effective collection of flies that have chosen a treatment.

The chambers provide a simple two-choice environment (though they could easily be modified to provide more choices) that is small enough to allow full exploration by the flies, but still large enough that their choices are clearly evident. Further, flies can be effectively isolated with their choice without fear of losing specimens while capping jars or chasing them with a suck-tube.

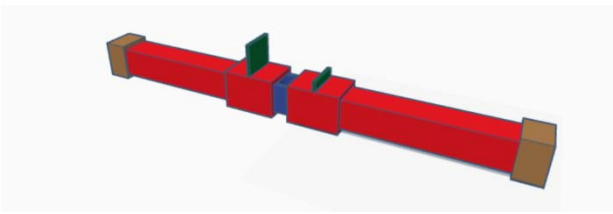

Figure 1. Rendering of the choice chamber. Gates (green) are shown in both the open and closed positions. The central addition port (where flies are added at the start of a trial) is shown here (in blue) before the gates would be joined.

The chamber (Figure 1), printed in transparent polylactic acid (PLA) plastic, consists of two tubes (25 mm × 25 mm × 140 mm) each with a removable cap at one end and a gate at the other. Food choices are placed in each of the capped ends and the tubes are joined by a connector with a port on top allowing for the addition of flies equidistant from both choices. The gates are then pushed together to close the port and seal the two tubes together. After the trial is finished the gates are dropped, sealing any flies into the tube with their choice.

Tests on this design were run using twenty flies per replicate (n = 20), with three different blueberry (BB) type comparisons (Table 1) alternating the sides on which each choice was located. Fake blueberries were made with pure pureed blueberries and fashioned into blueberry mimics. This technique, described elsewhere in this volume (McDevitt *et al*., 2018), allows for future studies of specific chemical and physical characteristics of berry food or oviposition choices. After 24 hours, the gates were dropped and the flies in each chamber counted.

Table 1. Treatment choices, average % (+/- SD) of flies in each treatment's side.

Choice 1	% near choice 1	Choice 2	% near choice 2
Thawed BB	87 (10)	Nothing	13 (10)
Fake BB	74 (14)	Nothing	26 (14)
Thawed BB	53 (16)	Fake	47 (16)

For these tests, individual chi-square analyses were run for each replicate with an assumption of no preference for either side. Thawed BB treatments were significantly preferred ($p < 0.05$) 18/20 times, while the fake blueberries were significantly preferred 13/20 times, and the fake *vs*. real trials found no significant difference between choices in any of the replicates.

These tests demonstrated to us that the chamber design was small enough for flies to find the food choices, that there were no inherent preferential regions leading to artefactual distributions (no side was preferred), and that flies could be successfully introduced into and isolated from the chambers where their food choices reside. While our PLA versions did not allow for visualization of flies during the trial, it is possible to have these printed in clear acrylic, which would permit real-time visual monitoring and recording of fly movement during the trials if appropriate.

Case study 2. Drosophila *as a vector for the external transport of nematodes*

Nematodes, such as the model organism *Caenorhabditis elegans*, have been well-studied in the lab but surprisingly little is known about their ecology (Kiontke and Sudhouse, 2006). Wild nematode species are often found associated with other animals, including insects such as *Drosophila,* where their attachment is thought to be a primary means for their dispersal to new ecological niches (Lee *et al.,* 2011b). When food is scarce and conditions unfavorable, *C. elegans* will enter into a developmental life stage called the dauer stage (Hu, 2007). Dauer larvae carry out an interesting dauer-specific behavior called nictation that involves the attachment of the worm to a substrate, such as fungi (Lee *et al.,* 2011b) and the waving of their heads. It has been shown that nictation leads to a higher incidence of the nematode attaching to an insect vector (Lee *et al.,* 2011b). However, much is not known about these interactions, specifically the frequency of attachment, rate of transfer, and any species-specific effects on that rate from either worms or their insect vectors. Such relationships may provide important information for biocontrol of *Drosophila*, nematodes, and other pests (Lee *et al.,* 2011a).

To determine the rate at which an individual fly would pick up, move, and drop off a nematode at a new location, we designed and printed small chambers that housed a single fly (*D. suzukii*) along with source and sink environments for the worms (*C. elegans*). The design provides clear viewing of the fly and the worms from above the chamber, while allowing illumination from the bottom, using a previously described imaging platform (Churgin *et al.,* 2017). This was done by using the parts of the petri dish on which the worms are grown, or carried to, as both the base (dish) and top (lid) of the chambers (Figure 2). The design and construction of the chamber provides for a new, clean viewing port for each replicate while providing fly-tight seals at both the top and bottom. A gate allows the fly's movement to be limited to the source side for a controlled amount of time before exposing them to the sink.

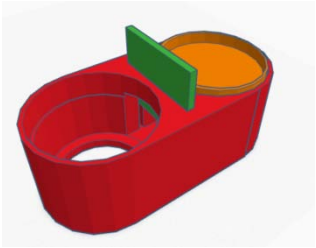

Figure 2. Rendering of the nematode vector chamber. Gate (green) is shown in the open position allowing the fly to move from the source to the sink side. The petri lid (orange) is shown on one side as it would sit, inverted, becoming the top window. The other side shows how the floor is open allowing light to pass up through the floor to backlight the worms for visualization. The actual chamber was printed in opaque black PLA.

Initial trials were conducted where ~7500 *C. elegans* dauer larvae were placed into the source side, which contained a 40 mm petri dish with nematode growth medium covered in medical gauze, a nictating substrate that was previously described (Lee *et al.*, 2011). A single *D. suzukii* fly was added to the source side for 1 hour, the gate was opened for ~10 minutes allowing for the fly to travel to the sink side of the device (which contained a fresh 40 mm dish of medium with no worms). After another hour, the number of worms on the sink side of the device was easily quantified as any worms present on the sink plate had to have been carried there by the fly. This was repeated for 7 trials. In only 1 of the 7 trials were worms transferred (two worms), suggesting that transfer is very infrequent, which is supported by another study (Lee *et al.*, 2011).

These initial experiments, using the custom chambers, illustrate that worms are carried by flies, and that the worms can be easily visualized on both the source and sink plates. Further, we found that worm dispersal via fly likely occurs in the range of single digits per fly per hour, at most. This low rate took place even in a chamber which should exacerbate the chances of this happening due to its small volume increasing the potential for contact between flies, worms, and substrates. This study is, however, in its infancy and has only looked at *D. suzukii*. Future experiments will alter the time the flies are exposed to the source, allowing for a temporal analysis of the frequency of transfer, and will compare different fly species to determine if these can affect the rate of transfer. Moreover, these experiments could be expanded to genetic studies using different fly species or *C. elegans* strains.

Discussion

Using easy to design software and a desktop 3D printer, we were able to custom-build effective research equipment that would otherwise have been impossible or too costly to produce. Using our Makerbot2 printer and free online design software (tinkercad.com), we were able to test many designs, often taking less than an hour from idea to production. Changes in gate configuration, sizes, shapes, opacity, color, and other parameters were tested, modified, and ultimately replicated for pennies a prototype (a $40 spool of PLA was more than enough to fuel the entire prototyping and production run of 20 choice chambers). We know of no other way that equipment such as this could have been developed and built as economically and quickly without this new technology. While our printer, a 5-year-old Makerbot2, is a high-end prosumer model (the latest version retails for $2,499), there are many less expensive models from a range of different companies (Makezine) that should be able to produce similar products, and even a model such as ours can pay for itself quite quickly.

Being able to set the dimensions of the equipment based on what was best for the experiment and not what supplies were available allowed us much more freedom, and likely resulted in better experimental results. While we continue to improve our designs, the current versions have been made available to use, modify, and print from the website thingiverse.com (items 2735670 and 2735695), or if researchers do not have access to a printer, the current designs can be produced by a third-party 3D production house such as shapeways.com (https://www.shapeways.com/shops/inator-devices), which will produce uploaded designs in a range of materials ranging from metals to various forms of plastic. We hope that the continued development of easy to use and affordable 3D printing will help drosophilists continue to learn more about these important, but often difficult to study organisms.

References: Caravaca, J.M., and E.P. Lei 2016, JoVE 109; Chen, A., 2012, Chronicle of Higher Education (www.chronicle.com/article/3-D-Printers-Arent-Just-for/134440); Chia, H.N., and B.M. Wu 2015,

J. Biol. Eng. 9: 4; Churgin, M.A, S.K. Jung, C.C. Yu, X. Chen, D.M. Raizen, Fang-Yen C. Elife 2017, May 31: 6; Cini, A., C. Ioriatti, and G. Anfora 2012, Bull. Insectology 65(1): 149-160; Dobzhansky, Th., 1947, Evolution 1: 1-16; Hu, P.J., 2007, Wormbook Aug 8: 1-19; Kiontke, K., and D.H.A. Fitch 2013, Current Biology 23: R862-864; Kiontke, K., and Sudhouse 2006, Wormbook Jan 9: 1-14; Lipson, H.M., and D. Kurman 2013, *Fabricated: The New World of 3D Printing*. Indianapolis, IN: Wiley; Lee, J.C., D.J. Bruck, A.J. Dreves, C. Loriatti, H. Vogt, and P. Baufeld 2011, Pest Manag. Sci. 67: 1349-51; Lee, H., M.K. Choice, D. Lee, H.S. Kim, H. Hwang, H. Kim, S. Park, Y.K. Paik, and J. Lee 2011, Nat. Neurosci. Nov 13: 107-12; makezine.com/comparison/3dprinters/; McDevitt, D., S. McRobert, and J. Fingerut 2017, Dros. Inf. Serv. 100: this issue.

Culture medium for flower-breeding drosophilids.

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Despite more than 6,800 species of drosophilids are known (Bächli, 2017), very few species are easily maintained in the laboratory and could be used as research models. Among the reasons, it is relatively difficult to keep some lineages, particularly when they have specific developmental requirements. Flower-breeding drosophilids are one of these species that could not be maintained in the laboratory and extremely depends on living flowers. In this context, the recipe presented below was developed to rear flower-breeding drosophilids that do not grow in already described medium, such as the ones from Bizzo *et al.* (2012), Markow and O'Grady (2006), Marques *et al.* (1966), Schmitz (2016), and Vaz *et al.* (2014).

This recipe was developed and has been used in the *Laboratory of Genetic Diversity and Evolution* of *Universidade Federal de Pelotas* (Rio Grande do Sul, Brazil), prepared with flower extracts from *Brugmansia suaveolens* (Solanaceae) and *Ipomoea alba* (Convolvulaceae). However, it can be performed using extracts of other flower species, or even from mushrooms. It proved to be successful for raising *D. bromelioides*-like species (new species not yet properly described, named '*tipo III*' by Schmitz (2010) belonging to the *Drosophila bromeliae* group, from *Drosophila* genus) and *D. denieri* (belonging to the *Phloridosa* subgenus from *Drosophila* genus). Both species depend on flowers to develop their life cycle (Brncic, 1983; Schmitz, 2010). In our tests the lineages were maintained until F4 generation, in good performance.

The preparation of the medium uses common ingredients and the flowers can be kept in the freezer until their use, overcoming the natural periodicity of the blooms. Also, the equipment used is accessible making the routine preparation of this medium very cheap and easy.

Medium ingredients

0.22 g of agar
0.45 g of sugar
0.02 g of dry yeast biological
0.01 g of methilparaben
10ml of distilled water
1 macerated flower

Procedure

- Mix agar and 5 ml of distilled water and wait 10 min
- Add the remaining distilled water, the sugar, and the dry yeast
- Mix everything and boil on the microwave three times for 1 min

- Add the methylparaben and mix again
- Add the macerated flower and mix
- Transfer the medium to clean vials and let the medium cool protected from contaminants
- The use of a folded tissue in the medium is recommended

Comments

The flowers were collected and frozen at -10ºC, or lower, in identified bags with flower species and collection date. The adults of P generation were collected with entomological aspirator in the mature flowers of *B. suaveolens* (used for *B. suaveolens* medium) and in *I. alba* (used for *I. alba* medium). The adults of P generation were placed in small vials containing the culture medium. These groups of flies are difficult to identify only using stereomicroscope. So, yellow adults (*Drosophila bromeliae* species group) were separated from black adults (*Phloridosa* subgenus species group). Adult females were placed separated in the medium for *B. suaveolens* and for *I. alba* and let oviposit for 5 days. After that, the P generation females were stored in 90% ethanol. Adult males, from all generations, were identified through the analysis of the terminalia using the technique from Wheeler and Kambysellis (1996), modified by Bächli *et al.* (2004). After the first day of transference of the adults to a new medium, it was placed two drops of the flower extract prepared with 5 ml of distilled water, to feed the larvae and adults. All media were kept in a temperature and humidity controlled chamber (±25°C, 60% r.h.) reaching to the F4 generation in both kinds of media (*B. suaveolens* and *I. alba*) in good performance.

Acknowledgments: The authors thank to João Henrique Figueredo Oliveira for stimulus to develop this medium and to CNPq for fellowship and research grants.

References: Bächli, G., 2017, TaxoDros: The database on taxonomy of drosophilidae. Available at: http://www.taxodros.uzh.ch/. Accessed in: Dec 30th 2017; Bächli, G., C.R. Vilela, S.A. Escher, and A. Saura 2004, Fauna Entomologica Scandinavica; Brncic, D., 1983, In: *Genetics and Biology of* Drosophila vol. 3d. (Ashburner, M., H.L. Carson, and J.N. Thompson, jr., eds.). Academic Press; Markow, T.A., and P.M. O'Grady 2006, Drosophila: *A Guide to Species Identification and Use*. Elsevier; Marques, E.K., M. Napp, H. Winge, and A.B. Cordeiro 1966, Dros. Inf. Serv. 41: 187; Schmitz, H.J., 2010, Genética, Ecologia e Evolução de drosofilídeos (Insecta, Diptera) associados a flores. PhD thesis in Genetics and Molecular Biology, Universidade Federal do Rio Grande do Sul, Brazil.

Guide to Authors

Drosophila Information Service prints short research, technique, and teaching articles, descriptions of new mutations, and other material of general interest to *Drosophila* researchers. The current publication schedule for regular issues is annually, with the official publication date being 31 December of the year of the issue. The annual issue will, therefore, include material submitted during that calendar year. To help us meet this target date, we request that submissions be sent by 15 December if possible, but articles are accepted at any time. Receipt by 31 December is a firm deadline, due to printer submission schedules.

Manuscripts, orders, and inquiries concerning the regular annual DIS issue should be sent to James Thompson, Department of Biology, University of Oklahoma, Norman, OK 73019. Telephone (405)-325-2001; email jthompson@ou.edu; FAX (405)-325-7560.

Submission: Manuscripts should be submitted in Word, with pictures preferably in *jpg. To help minimize editorial costs, proofs will not be sent to authors unless there is some question that needs to be clarified or they are specifically requested by the authors at the time of submission. The editor reserves the right to make minor grammatical, spelling, and stylistic changes if necessary to conform to DIS format and good English usage. Color illustrations will appear black and white in the printed version but will be in color in the electronically-accessible version on our web site (www.ou.edu/journals/dis).

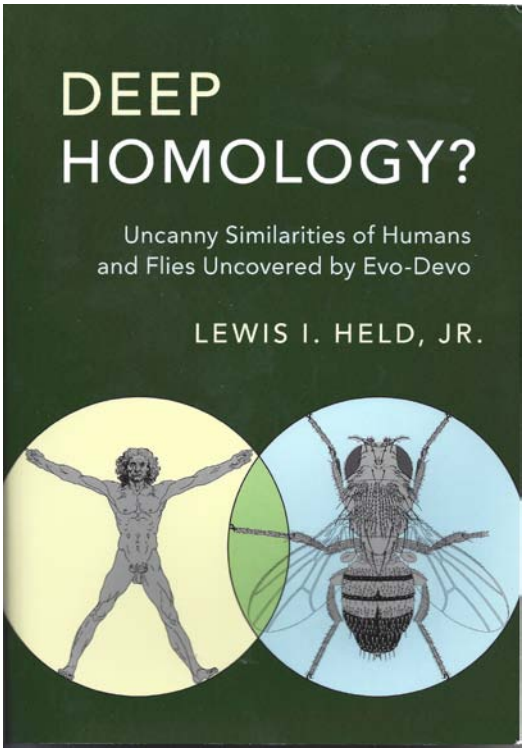
Citation of References: Citation should be by name and date in the text of an article (Smith, 1989; Jin and Brown, 1990; Waters *et al*., 1990). At the end of the article, references should be listed alphabetically by senior author, listing all authors with initials, date, journal, volume and page numbers. Titles will not be included except for books, unpublished theses, and articles in press. An example format is:

Green, R.L., 1998, Heredity 121: 430-442.

Waters, R.L., J.T. Smith, and R.R. Brown 1990, J. Genet. 47: 123-134.

Note the initials are before each name except for the senior author.

New Book Announcement



Deep Homology? Uncanny Similarities of Humans and Flies Uncovered by Evo-Devo.

Held, Lewis I., Jr. 2017, 272 pp. Cambridge University Press, ISBN-978-1-107-14718-8 (hardback) and 978-1-316-60121-1 (paperback). $39.99.

Discoveries about the extent of genetic homologies between humans and *Drosophila* are revolutionizing our understanding of human development and disease. That is a theme of this wonderful new book. *Deep Homology?* is information-dense, yet still enjoyably readable. It is almost like a scientific detective story, in which large amounts of data build a solid and important picture. Detail and readability make a challenging combination for a writer to master, but Lewis Held does it very well. I must admit, however, that I was not surprised. I know many have enjoyed and learned from his earlier books, *Models for Embryonic Periodicity*, *Imaginal Discs*, *Quirks of Human Anatomy*, and *How the Snake Lost Its Legs*. In addition to being accessible to both undergraduates and professional biologists, Lewis's work is always supported by extensive detail. Indeed, over 2500 sources in the reference section give a convenient introduction to important literature. There are also extensive illustrations that, like those in his earlier books, make complex ideas clearer. By drawing together strands from a diverse published literature, *Deep Homology?* brings to light mechanisms and relationships that deepen our understanding of the shared genetic history of species. As we learn more about the biological elements that humans and *Drosophila* share, we come to appreciate more deeply our fundamental connectedness.

James Thompson, DIS editor.

Teaching Notes

Effect of maternal age on recombination rate in *Drosophila melanogaster.*

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Abstract

Fitness is defined as the reproductive success of an organism in producing offspring and passing their genes to the next generation (Hedrick, 2011). Many genetic factors can affect fitness, including selection, mutation, and migration, but the most ubiquitous is recombination, which is caused by the exchange of genetic material between chromosomes during meiosis. Numerous studies have examined the role of recombination in evolution, including conflicting reports on the interaction of age and recombination rates (see discussion in Hudson and Kaplan, 1985; Brooks, 1988; Otto and Michalakis, 1998; Hunter *et al*. 2016). Here, we examine how maternal age affects the rate of X-chromosome recombination in *Drosophila melanogaster*. Our hypothesis is that recombination rates are positively correlated with maternal age. If this is true we would expect to see an increase in the rate of recombination as maternal age increases. Such a correlation was observed in this study.

Introduction

Recombination, which involves exchanges of genetic material between homologous chromosomes during meiosis, is a significant source of genetic variation that can influence fitness (Roeder, 1997; McDonald, Rice and Desai, 2016). Changes in positions of gene alleles on a chromosome caused by recombination could affect gene expression and lead to increases in fitness, which can assist organisms in adapting faster to changing environments (Badyaev, 2005; Presgraves *et al*., 2005). Negative effects can also arise, however, such as increases in the rate of chromosomal rearrangements if recombination occurs by unequal crossing over (Lupski, 1998). In addition, it is sometimes reported that recombination is not always influenced by environmental or biological factors (Otto and Michalakis, 1998). On the contrary, many organisms, including yeast, nematodes, and fruit flies, experience alterations in recombination rates due to environmental and biological factors, including temperature, nutrition, and age (Plough, 1917, 1921; Neel, 1941; Brooks and Marks, 1986; Parsons, 1988; Barnes *et al*., 1995; Mancera *et al*., 2008; Rodgers-Melnick, *et al.* 2014; and references in Dollard *et al*., 2016).

Fitness is usually assumed to decrease with age, leading to a reduction in progeny numbers over time (Stearns, 1992; Partridge and Barton, 1993). As age increases, therefore, the likelihood for genes to be passed to new generations decreases. Many studies using *D. melanogaster*, mice, hamsters, and humans also support the hypothesis that recombination rates can be affected by maternal age (Plough, 1917, 1921; Redfield, 1966; Kong, *et al.* 2004; Bleazard *et al*., 2013; Hunter and Singh, 2014; Campbell, *et al*., 2015; Martin *et al*., 2015; Hunter *et al.*, 2016). Despite extensive research on this topic, however, disagreements still exist as to whether recombination rates increase, decrease, or do not change with increased maternal age (see a discussion of this topic and references in Hunter *et al*., 2016).

Four issues among multiple studies have led to little consensus as to how recombination rates change with increasing maternal age in *D. melanogaster*. First, different strains were used in these studies, making it difficult to determine if the effect of maternal age on recombination is correlated with age or genetic background. Second, some studies allowed repeated matings, which could result in an increase in the rate of recombination unrelated to age (Priest *et al*., 2007; Hunter *et al*., 2016). Third, many studies focused on progeny from single females, while others counted progeny from groups of females. Finally, the influence of

maternal age on recombination rates is not uniform across the entire genome; certain regions of the genome or chromosomes have higher frequencies of recombination compared to other regions (Lercher and Hurst, 2002; Fiston-Lavier *et al.*, 2010).

In this study, we investigated the hypothesis that X-chromosome recombination rates in *D. melanogaster* are positively correlated with maternal age, with older females having higher recombination rates compared to younger flies.

Materials and Methods

A fly stock possessing two X-linked genetic markers, [white eyes (*w*) and singed small bristles (sn^3)], was used in this study, plus the Canton-S (CS) wild type stock, which has red eyes and straight bristles. As shown in Figure 1, parental virgin females from the *w* sn^3 stock were crossed with males from the CS stock, and single F1 *w* sn^3/+ + virgin females were crossed with two *w* sn^3/Y sibling males per vial. The F2 progeny were then scored for recombination. Female F2 recombinants would be *w* +/*w* sn^3 (white eyes with straight bristles) or + sn^3/*w* sn^3 (red eyes with singed bristles) genotypes. Recombinant F2 males would have a genotype of *w* +/Y (white eyes with straight bristles) or + sn^3/Y (red eyes with singed bristles). In addition non-recombinant females are *w* sn^3/*w* sn^3 (white eyes with singed bristles) or + +/ *w* sn^3, (red eyes with straight bristles), while non-recombinant males are *w* sn^3/Y (white eyes and singed bristles) or + +/Y (red eyes and straight bristles). These crosses are detailed in Figure 1.

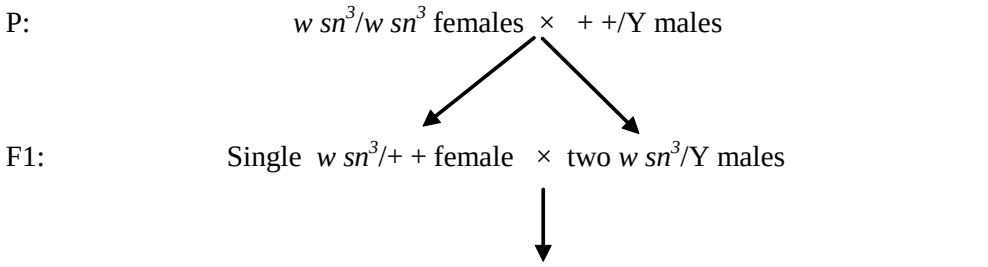

Figure 1. Crossing scheme for this experiment.

In this study, 31 total runs of flies were carried through the crossing scheme of Figure 1, with each run consisting of 10 aging vials each established two days apart. For the first aging vial in each run, one virgin F1 female was mated to two F1 males. After two days females were transferred to new vials and males were discarded. Hence, the F1 females were given two days to lay eggs and were then transferred to a fresh vial. This procedure continued for twenty days (10 vials for each run) for all 31 runs, with no additional matings beyond the first two days. Regression analysis was conducted using the PRISM statistical program to determine if recombination rate does significantly increase with maternal age.

Results

A total of 5239 progeny were scored for recombination (4289 non-recombinants and 950 recombinants). Table 1 details the number of progeny (non-recombinants and recombinants) for the twenty days of the 31 runs. Table 2 shows the percent recombination for each two-day period.

As seen in Figure 2, the slope of the best-fit line is significantly different from a slope of zero ($p = 0.001$). The average recombination rate increased from 15.54 percent in days 1-2 to 21.59 percent in days 19-20, resulting in an overall significant increase in recombination rate of approximately six percent. These results support our hypothesis that recombination rates increase with maternal age.

Table 1. The number of recombinant and non-recombinant progeny obtained from 31 lines.

Days	Total Progeny	Non-Recombinants	Recombinants
2	817	690	127
4	717	625	92
6	463	379	84
8	386	320	66
10	519	429	90
12	379	301	78
14	650	509	141
16	620	465	125
18	424	334	90
20	264	207	57

Discussion

The objective of this study was to determine if maternal age affects the rate of X-chromosome recombination. Although it is well known that as maternal age increases nondisjunction events increase (Hunt and Hassold, 2001), it is less clear how maternal age affects recombination. As previously stated, the results of studies relating to maternal age and recombination rates have been inconsistent, with reports of decreasing, increasing, or non-changes in rates with increased age. The results of this study clearly support the hypothesis that as maternal age increases, recombination rate also increases in *D. melanogaster*. Similar results were reported by Bridges (1915), Priest *et al*. (2007), Hunter and Singh (2014), and Hunter *et al.* (2016) for *Drosophila* and by Bleazard *et al*. (2013), Campbell *et al*. (2015), and Martin *et al*. (2015) for humans.

Table 2. The recombination rate for each two day period of the 31 runs. Recombination rate was determined by dividing the number of recombinant flies from a two day span by the total number of flies from the same two day span. For example, there was a total of 817 flies from days 1-2 and 127 recombinants (see Table 1), resulting in 127/817 = 15.54 percent recombination.

Day	Recombination Rate (%)
2	15.54
4	12.83
6	18.14
8	17.10
10	17.34
12	20.58
14	21.69
16	21.16
18	21.23
20	21.59

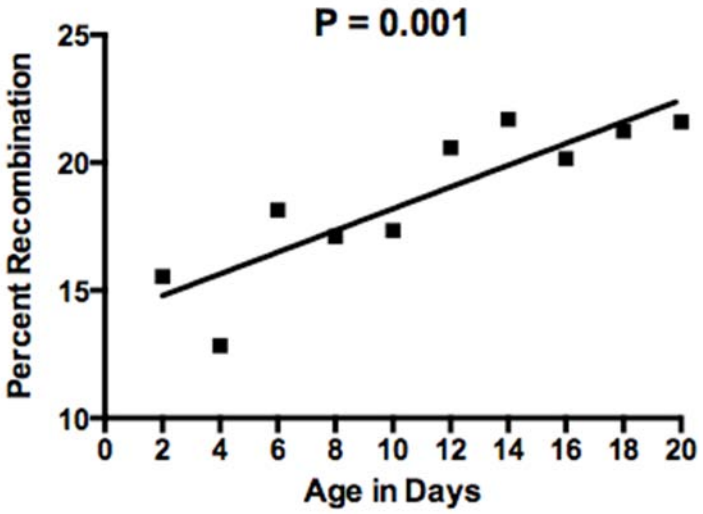

Figure 2. Recombination rates graphed with a line of best fit.

One possible consequence of increased recombination rates with age is an increase in positive selection for advantageous combinations of gene alleles on a chromosome with age. Positive selection could then lead to advantageous gene interactions in future generations. Therefore, if recombination rates increase with age, positive selection could also increase, allowing organisms to adapt more quickly to changing environments. Yet, recombination is also known to be mutagenic (Lercher and Hurst, 2002). For example, unequal recombination can lead to extra or missing base pairs that cause genetic disorders in humans (Nakamoto *et al*., 2002). Recombination can, therefore, be beneficial or detrimental to fitness.

Another factor that might affect recombination rates is the genetic background. Genetic backgrounds have been shown to affect recombination, by decreasing or increasing recombination rate by up to two-fold (Brooks and Marks, 1986; Stevison, 2011; Hunter *et al*., 2016). Hence, it would be important to test additional genetic backgrounds, other than the CS and *w sn*^{3} stocks that were used in this study, on the effect of aging on

recombination rates. Such experiments could assist future studies of how recombination rates and aging are influenced by genetic background.

One other interesting trend was observed in this study. In almost every run females refrained from laying eggs for two to four days, before resuming oviposition. One explanation for this observation may be that the females needed additional nutrients for the metabolically expensive process of oviposition (Chapman and Partridge, 1996). This gap in oviposition may give females time to build up the necessary nutrients to resume oviposition. This interesting phenomenon should be studied in more depth.

A class discussion of the results of this study might include: 1) Why was recombination and aging only tested in females in this study? There is no recombination in male *D. melanogaster* (Morgan, 1914). 2) Are there genes that are known to directly affect rates of recombination? Yes, including RAD51 in yeast, mice, *Drosophila*, and humans (for a discussion of this topic, see Baker and Hall, 1976; Shinohara *et al*., 1993; Staeva-Vieira *et al.*, 2003).

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Reversion of the *Bar* (*B*) mutation in the Basc X chromosome of *Drosophila melanogaster* by unequal crossing over.

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The dominant, X-linked, *Bar* (*B*) mutation was isolated in 1914 by Sabra Colby Tice as a change in the structure of the eye of *D. melanogaster* from round (wild type) to a narrow bar of eye tissue in homozygous females and hemizygous males, and as less extreme *Bar*-eyes in heterozygous, B/B^{+}, females

(Tice, 1914; for a more detailed description of the *B* mutation, see Lindsley and Zimm, 1992; Wolfner and Miller, 2016).

May (1917) observed that the *B* mutation could revert to wild type (*Bar* eyes to round eyes) and proposed that the reversion mechanism was either "…a reversible chemical reaction between two compounds one of which is more stable than the other." Or "…partial non-disjunction." (May, 1917). Based in part on observations by Zeleny (1919, 1921, 1922) that the *B* to B^+ reversions only occurred in females, Sturtevant (1925) proposed that these reversions were due to unequal crossing-over events that occur in about 1 in 1,000 to 2,000 flies (also see Sturtevant and Morgan, 1923). Bridges (1936) confirmed cytologically that the *B* mutation was associated with a tandem duplication, which we now know arises by crossing over between two *B104* transposable DNA elements (Tsubota *et al*., 1989).

Unequal crossing-over events are important in *Drosophila* and other higher organisms, including humans. For example, red-green color blindness in humans can be caused by an unequal crossing-over event (Nathans *et al*., 1986), and humans with triplications of a segment of chromosome 17 have a more extreme form of Charcot-Marie Tooth disease than those with duplications (Liu *et al*., 2014). Unequal crossing-over events have also given rise to extra copies of genes that can evolve into new functions, including globins (Shen *et al*., 1981), as well as extra and missing DNA base pairs, which can lead to human disorders (Nakamoto *et al*., 2002)

Based on the above reports, we tested the hypothesis that reversions of *B* to B^+ in Basc X chromosomes are caused by a loss of one duplication at the *B* locus due to unequal crossing-over events in homozygous (Basc/Basc) diplo-X females, but not in heterozygous (Basc/+) females or hemizygous males that have a single X chromosome (Basc/Y). To test this hypothesis, we performed the following three sets of crosses.

In these crosses, parental Basc/Basc females and Basc/Y males have *Bar* eyes that are also white-apricot in color (the *a* in Basc stands for the w^a mutation), Basc/+ parental females have a reduced *Bar*-eyed phenotype, and C(1)DX, *y f* parental females are marked with the *y* (*yellow* body color) and *f* (*forked*, short bristles) mutations and contain two X chromosomes attached to a single centromere. In Cross 1, the F1 progeny are Basc / Y patroclinous males (either *B*/Y or B^+/Y) that receive their X chromosome from their fathers and their Y chromosome from their mothers, and C(1)DX, *y f* matroclinous females, that get their attached-X chromosome from their mothers and their Y from their fathers. See Lindsley and Zimm (1992) for a more detailed description of the mutations (*y* and *f*), compound-X chromosome, and Basc balancer chromosome used in this study.

Cross 1: C(1)DX, *y f* / Y females × Basc / Y males

The F1 progeny were scored for *B*/Y *Bar*-eyed males and B^+/Y round-eyed, white-apricot (revertant) males. Each B^+ revertant male, if isolated, would be mated to virgin C(1)DX, *y f* / Y females to make sure the round phenotype bred true into males in the F2 generation. We predict, however, that no B^+ revertant males will be recovered from this cross.

Cross 2: Basc / Basc females × Basc / Y males

In cross 2, since *B* has been reported not to revert in males (Zeleny 1919, 1921, 1922; Sturtevant 1925), any F1 B^+ / Y males should arise by unequal crossing over in parental females. Each of the presumptive B^+ / Y males were mated to C(1)DX, *y f* females to make sure the round phenotype bred true into males in the F2 generation. We predict that B^+ revertant males derived from unequal crossing over in Basc/Basc parental females will be recovered in this cross at a frequency of about 1 in 1,500 F1 males

(Sturtevant 1925). We should also recover some *B/B*$^+$ F1 females that have less extreme *Bar*-eyed phenotypes.

Cross 3: Basc / *B* females × Basc / Y males

In cross 3 the revertant F1 progeny from parental females were scored as *B*$^+$ / Y, red-eyed, males (from the *B* containing X chromosome in parental females), or as *B*$^+$ / Y, white-apricot eyed, males from the Basc chromosome in parental females. The non-revertant *B* males were Basc / Y (bar and white-apricot eyes) and *B* / Y (bar and red eyes). We expected no *B*$^+$ / Y, white-apricot, revertants from cross 3, since crossing-over events do not occur in males, and the Basc X chromosome in the parental heterozygous females (Basc / +) contains multiple inversions that cover the entire chromosome. Recombination events in inversion heterozygotes are not recovered in progeny because they contain extra or missing segments of the X chromosome and/or extra or missing centromeres (see references in Woodruff *et al*., 2013).

The results from Crosses 1, 2 and 3 are shown in Table 1. As predicted, *B*$^+$ revertants were only recovered in Cross 2, and the results of Cross 1 and Cross 3 were significantly different from Cross 2. These results support the hypothesis that *B* revertants are caused by loss of a segment of the X chromosome due to unequal crossing-over events.

Table 1. Observed results for the recovery of *B* non-revertants and *B*$^+$ revertants.

Crosses	Number of *B*$^+$ Revertants	Number of *B* Non-Revertants
Cross 1: C(1)DX, *y f* / Y females × Basc / Y males	0	4,976[a,b]
Cross 2: Basc / Basc females × Basc / Y males	6	4,755[a,c]
Cross 3: Basc / *B* females × Basc / Y males	0	5,250[b,c]

[a] Fisher exact test P, one tail = 0.01; [b] Fisher exact test P, two tail = 1.00; [c] Fisher exact test P, one tail = 0.01.

Class discussions of the results of this study might include: 1) *Drosophila* geneticists might look at the results of this study and say of course Morgan, Sturtevant, and Bridges, the founders of *Drosophila* genetics, were right about the mechanism of reversion of the *B* mutation. But they were not always right. For example, Morgan (1912, 1914) reported that *D. melanogaster* males do not undergo recombination. Yet, Bridges and Morgan (1919) recovered male recombination events in a 1912 experiment (see discussion in Woodruff and Thompson, 1977). Maybe these recombination events were associated with transposable DNA elements, such as P elements that are known to cause breakage events that can lead to recombination of markers in *D. melanogaster* males (Henderson, Woodruff and Thompson, 1978). 2) Are there other examples in the literature of unequal recombination events leading to genetic disorders in humans? Yes: see examples in Lupski (1998), including the blood disorder β-thalassemia.

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Inf. Serv. 96: 241-245; Zeleny, C., 1919, J. Gen. Physio. 2: 69-71; Zeleny, C., 1921, J. Exper. Zool. 34: 203-233; Zeleny, C., 1922, Genetics 7: 1-115.

Measuring narrow-sense heritability in *Drosophila melanogaster* using inbred strains.

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For a trait to evolve by either natural or human selection, the phenotypic variation of the trait must be inherited, *i.e.* be due to genetic variation. The fraction of total variation in a trait due to genetic variation is called the heritability of the trait. In addition, the best measure of whether a trait will evolve or respond to selection is narrow sense heritability (h^2), the fraction of the total variation due to the additive effects of genes. Dominance and gene × environmental interactions also affect quantitative traits and heritability values (for discussions of heritability, see Falconer and Mackay, 1996; Roff 1997; Allendorf and Luikart 2007; Hedrick, 2011).

Three possible ways to estimate the h^2 of a quantitative trait are: 1) trait correlations between parents and their offspring, where h^2 is equal to the regression slope of mid-parent values to offspring values; 2) comparing concordance of traits in monozygotic *versus* dizygotic twins, where h^2 is equal to two times the monozygotic concordance minus dizygotic concordance; 3) and using the results of selection experiments, where h^2 is equal to the response of selection divided by the selection differential (see Falconer and Mackay, 1996).

Everett *et al.* (2016) estimated h^2 for bristle number in *Drosophila melanogaster* by comparing midparent numbers to offspring numbers and observed a h^2 of 0.05 for females and 0.04 for males. In addition, Woodruff and Thompson (2005) estimated h^2 of sternopleural bristle number by selecting for increased bristle numbers over eight generations and observed h^2 values of 0.11 for females and 0.15 for males in non-inbred lines.

In this study, we estimated h^2 for sternopleural bristle numbers using three highly inbred lines of *D. melanogaster* (see sternopleural bristles in Chyb and Gompel, 2013, and in Figure 2 of Everett *et al*., 2016). We used a modified version of the methods of Possidente and McQuade (2015), who estimated h^2 for body size using inbred lines of *D. melanogaster*. The advantage of using such highly inbred, homozygous, lines to measure h^2 is that variation among individuals within the same line is due entirely to non-genetic effects, while dominance effects are eliminated (see discussions of this topic in Falconer and Mackay, 1996; Possidente and McQuade, 2015). With inbred lines, h^2 is equal to the genetic variance (V_G) divided by the sum of genetic variance and environmental variance (V_E) (Possidente and McQuade, 2015), *i.e*.,

$$h^2 = V_G/(V_G + V_E),$$

where V_G can be calculated using half the difference in means squared of the inbred lines examined, divided by 2 ($V_G = 0.5(((Mean^1 – Mean^2)/2)^2)$), and V_E for a given inbred line can be calculated using the standard deviation squared of that line ($V_{E1} = SD_1^2$) (Possidente and McQuade, 2015). To properly estimate h^2 you need to use the pooled estimate of V_E by calculating the average V_E for two populations of the same sample size ($V_E = (V_{E1} + V_{E2}) * 0.5$). To detail this process, we will walk through the calculation of h^2 for sternopleural bristle number using two theoretical inbred lines of *D. melanogaster*, IB_1 and IB_2.

IB_1 males had a mean sternopleural bristle number of 16.00 bristles, with a standard deviation (SD) of ± 2.58, while IB_2 males had a mean of 25.31 bristles with a SD of ± 3.25. Hence,

$IB_1\ V_E = SD^2 = 2.58^2 = 6.66$ $IB_2\ V_E = SD^2 = 3.25^2 = 10.56$
Total $V_E = (V_{E1} + V_{E2}) * 0.5 = (6.66 + 10.56) * 0.5 = 8.61$
$V_G = 0.5 * [((Mean_1 – Mean_2)/2)^2] = 0.5 * [((16 – 25.31)/2)^2] = 10.84$
$h^2 = V_G /(V_G + V_E) = 10.84/(10.84 + 8.61) = 0.56$

We used these formulas to calculate narrow-sense heritability in the following three inbred lines of *D. melanogaster*:

1) yIB females and males (marked with the yellow-body, sex-linked, *y* mutant and inbred by brother sister matings for 336 generations).
2) C(1)DX, *y f* females (marked with *y* = yellow bodies and *f* = forked bristle mutants) and w^{1118} (white eyed mutant) males (inbred by brother sister matings for 136 generations).
3) and C(1)DX, *y w f* females (marked with *y* = yellow bodies, *w* = white eyes and *f* = forked bristle mutants) and Binscy males (*B* = narrow eyes mutant and inbred by brother sister matings for 12 generations).

For detailed discussions of the mutant genes and chromosome rearrangements used in this study see Lindsley and Zimm (1992). A total of 38 flies were scored for bristle numbers from each side of males and females for each inbred line.

The mean (± SD) for sternopleural bristle numbers in males and in females of each inbred line, and the h^2 values for each inbred line comparison, are given in Table 1. What is clear from Table 1 is that estimations of h^2 using comparisons of different inbred stocks is not constant (varying from 0.004 to 0.640), suggesting that heritability is influenced by differences in genetic variation present in differrent stocks, strains and populations. Yet, Falconer and Mackay (1996) state that estimates of heritability tend to be similar in different populations.

Table 1. Means (± SD) and narrow sense heritability (h^2) values for sternopleural bristle numbers in comparisons of values in males and in females of three inbred lines of *D. melanogaster*.

	Mean (± SD) of Bristles	P	h^2
Male Comparisons			
yIB vs w^{1118}	17.18 (± 2.38); 16.69 (± 1.66)	0.41	0.004
yIB vs Binscy	17.18 (± 2.38); 27.24 (± 3.55)	<0.0001	0.581
w^{1118} vs Binscy	16.69 (± 1.66); 27.24 (± 3.55)	<0.0001	0.640
Female Comparisons:			
yIB vs C(1)DX, y f	17.65 (± 1.88); 21.05 (± 2.90)	<0.0001	0.195
yIB vs C(1)DX, y w f	17.65 (± 1.88); 20.61 (± 2.41)	<0.0001	0.204
C(1)DX, y f vs C(1)DX, y w f	21.05 (± 2.90); 20.61 (± 2.41)	<0.0001	0.298

Thirty-eight flies were scored for steronopleural bristle numbers for each line. P values are from t-tests using the Prism program.

In many cases in Table 1 the lowest estimations of h^2 are for inbred lines with similar mean bristle numbers (for example in yIB *vs* w^{1118} males, where h^2 was 0.004) and the highest estimations of h^2 are observed in lines with different mean bristle numbers (for example in w^{1118} *vs* Binscy males, where h^2 was 0.640). Also notice that the highest estimations of h^2 were in comparisons of Binscy males with other males (h^2 = 0.581 and 0.640) and C(1)DX, *y w f* females compared to C(1)DX, *y f* females and yIB females (h^2 = 0.298 and 0.204). Finally, the estimations of h^2 in this study, except for yIB *vs* w^{1118} (h^2 = 0.004), were higher than those estimated by comparing midparent to offspring (h^2 = 0.05) and by selection responses (h^2 = 0.11 and 0.15) (Woodruff and Thompson, 2005; Everett *et al*., 2016). Other reported estimations of h^2 for abdominal bristle numbers from parent-offspring regressions are about 0.51 (Falconer and Mackay, 1996).

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Lack of chromosome breakage and altered sex ratios by copper sulfate in *Drosophila melanogaster.*

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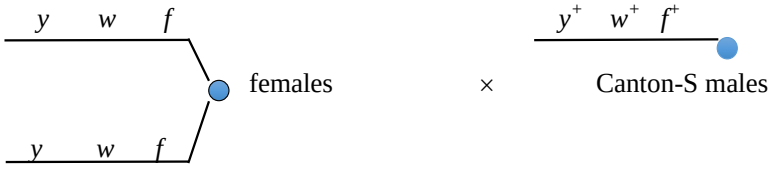
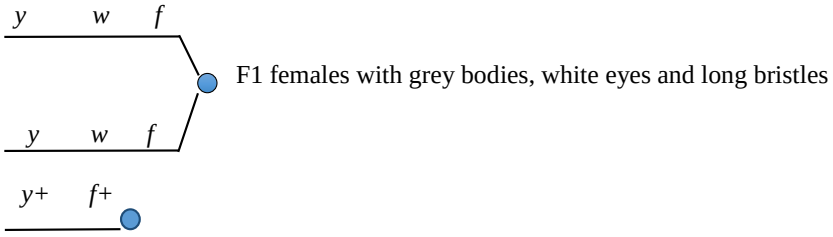
Although copper plays an important metabolic role in all organisms, high concentrations can have toxic and mutagenic effects (Pra *et al.*, 2008; Balinski and Woodruff, 2017, and references therein). One common source of excess copper concentrations in the environment is copper sulfate, which is a fungicide used to kill bacteria, fungi, and snails and is a potential producer of genetic damage in exposed humans (National Pesticide Information Center: http://npic.orst.edu/factsheets/cuso4gen.html). Copper sulfate induces chromosome breakage events in mice and increases the rate of recessive sex-linked lethal mutations in *Drosophila melanogaster* (Law, 1938; Agarwal *et al*., 1990). It is the objective of this study, therefore, to determine if copper sulfate induces chromosome breakage events in the model system *D. melanogaster*. It is our hypothesis that this chemical will significantly increase X-chromosome breakage events. Since copper can alter sex ratios (Niklasson *et al*., 2000), we also investigated the ability of copper sulfate to alter sex ratios and the recovery rate of XXX (triplo-X) female progeny.

We screened for the ability of copper sulfate to induce chromosome breakage by treating adult wild-type (Canton-S) *D. melanogaster* males with 0.5 mM of copper sulfate mixed in *Drosophila* instant food (Wards Natural Science) and mating these males to C(1)DX, *y w f* / Y females possessing two X chromosomes attached to a single centromere and the recessive genetic markers *y* (yellow body color), *w* (white eyes), and *f* (forked bristles). We have previously observed that 0.5 mM of copper sulfate is just below the toxic level for *D. melanogaster* males (Balinski and Woodruff, 2017, and unpublished results). The attached-X chromosome and visible mutants are further discussed in Lindsley and Zimm (1992).

As shown in the mating scheme below, y^+ flies have wild-type grey body color, w^+ flies have wild-type red eyes, and f^+ flies have wild-type long bristles. The Y chromosomes in females and males will be ignored, since we did not identify Y-chromosome breakage events. This assay was previously used to identify chemical-induced and gamma-ray-induced chromosomal breakage in *D. melanogaster* males (Blount and Woodruff, 1986; Woodruff and Russell, 2011).



If no X-chromosome breakage occurs, the F1 females will have yellow body color, white eyes, and forked bristles. If, for example, a break occurs in males between y^+ and w^+ and another break between w^+ and f^+, the rejoined fragment (y^+ and f^+) will be recovered in F1 females, resulting in grey body color, white eyes, and long bristles, as shown below.



Other F1 female phenotypes can occur from different breakage events in the parental male X chromosome, including w^+ f^+ fragments, which give yellow bodies, red eyes, and long bristles, y^+ w^+ fragments that result in grey bodies, red eyes, and forked bristles, and y^+ fragments that produce grey bodies, white eyes, and forked bristles (see Figure 1 in Woodruff and Russell, 2011).

Since all of these breakage events result in females with extra X-chromosome fragments, this assay has been called the hyperploidy test (Auerbach, 1976). In addition, XXX females that are grey in body color, have red eyes, and long bristles were observed in this study, independently of chromosome breakage events.

We hypothesized that 0.5 mM of copper sulfate would significantly increase our historical laboratory frequency of spontaneous chromosome breakage events (2/97,895 = 0.002%) (Woodruff and Russell, 2011), suggesting that exposure to copper sulfate in humans may cause genetic changes. As a positive control, we previously observed that 2,010 rads of gamma rays induced two breakage events out of 1,521 flies using this hyperploidy assay (2/1,521 *vs* the spontaneous frequency of 2/97,895; P = 0.001) (Woodruff and Russell, 2011). We also predicted that copper sulfate will alter sex ratios by reducing the number of hemizygous F1 males that inherit treated X chromosomes. In addition, we determined if copper sulfate treatment alters the recovery of XXX F1females as compared to the untreated controls; some XXX F1 flies may be due to recovered breakage events that do not include the y^+, w^+ or f^+ markers.

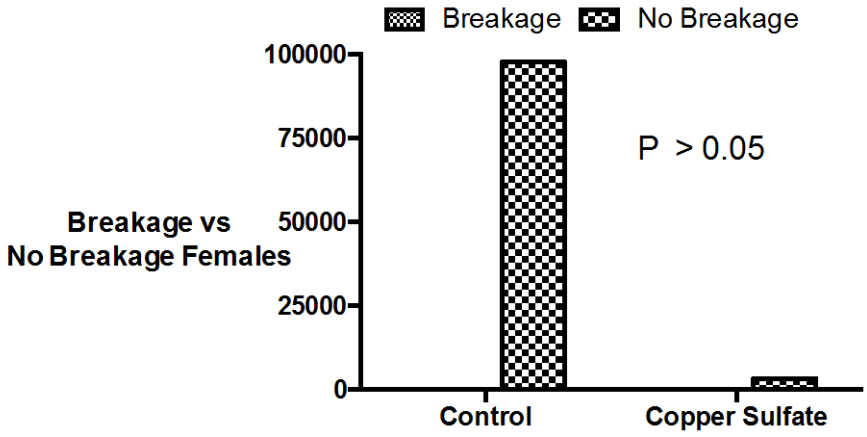


Figure 1. Chromosome breakage events in the presence and absence of copper sulfate.

Copper sulfate did not induce chromosome breakage events in this study (two breakages in 97,895 flies in our historical laboratory control and no breakage events in 3,335 flies in this study; P > 0.05; see Figure 1). In addition, sex ratios were not altered by copper sulfate (treatment: 2,784 males and 2,119 females recovered, 0.57 males to total progeny; control: 4,438 males and 3,335 females recovered, 0.57 males to total progeny; P = 0.75; see Figure 2). Finally, we observed 16 F1 XXX females out of 2,312 total females in the

control crosses, whereas 40 XXX females were recovered out of 3,375 total females in the treated flies (P = 0.09). Hence, copper sulfate did not significantly change the recovery of XXX females (see Figure 3).

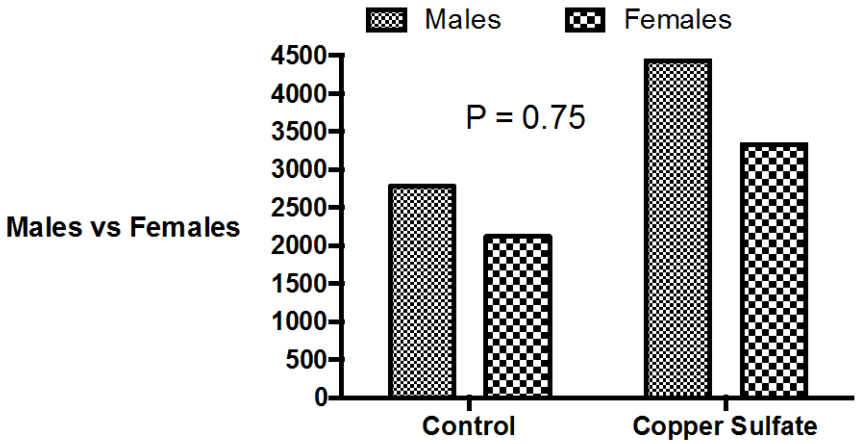


Figure 2. Sex ratios in the presence and absence of copper sulfate.

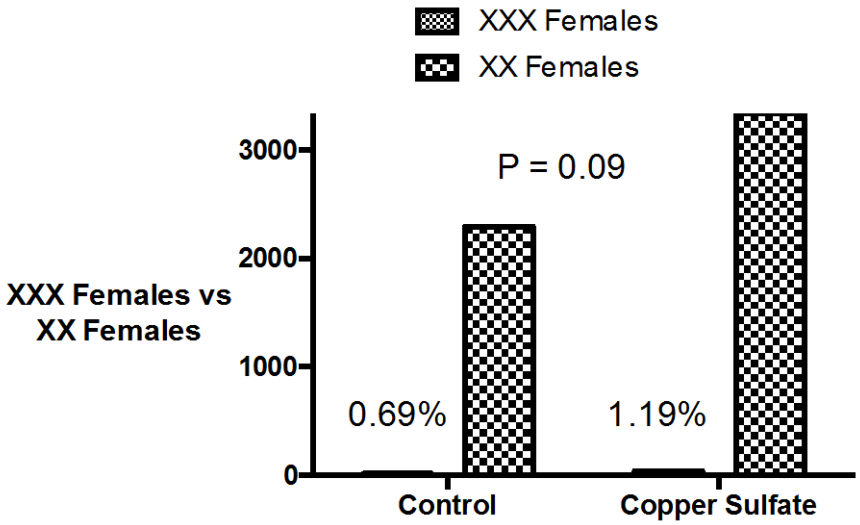


Figure 3. The recovery of XXX females in the presence and absence of copper sulfate.

In summary, copper sulfate did not cause chromosome breakage, did not alter the sex ratio, and did not alter the recovery of XXX females. It would be of interest to increase the number of copper sulfate treated flies in a follow up experiment to increase the recovery of low frequency breakage events. It would also be of interest to follow the procedure of larval injections and immersion of eggs with copper sulfate, which was observed to increase the frequency of recessive sex-linked lethal mutations in *D. melanogaster* (Law, 1938), to see if these procedures induce chromosome breakage events.

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An undergraduate cell biology lab: Western Blotting to detect proteins from *Drosophila* eye.

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Abstract

We have developed an undergraduate laboratory to allow detection and localization of proteins in the compound eye of *Drosophila melanogaster*, a.k.a fruit fly. This lab was a part of the undergraduate curriculum of the cell biology laboratory course aimed to demonstrate the use of Western Blotting technique to study protein localization in the adult eye of *Drosophila*. Western blotting, a two-day laboratory exercise, can be used to detect the presence of proteins of interests from total protein isolated from a tissue. The first day involves isolation of proteins from the tissue and SDS-PAGE (sodium dodecyl sulfate-polyacrylamide) gel electrophoresis to separate the denatured proteins in accordance to their molecular weight/s. The separated proteins are then transferred to the Nitrocellulose or Polyvinylidene difluoride (PVDF) membrane in an overnight transfer. The second day lab involves detection of proteins (transferred to the membrane) using Ponceau-S stain, followed by immunochemistry to detect the protein of interest along the total protein transferred to the membrane. The presence of our protein of interest is carried out by using a primary antibody against the protein, followed by binding of secondary antibody which is tagged to an enzyme. The protein band can be detected by using the kit, which provides substrate to the enzyme. The protein levels can be quantified, compared, and analyzed by calculating the respective band intensities. Here, we have used fly eyes to detect the difference in level of expression of Tubulin (Tub) and Wingless (Wg) proteins in the adult eye of *Drosophila* in our class. The idea of this laboratory exercise is to: (a) familiarize students with the underlying principles of protein chemistry and its application to diverse areas of research, (b) to enable students to get a hands-on-experience of this biochemical technique. Keywords: *Drosophila melanogaster*, eye, Western Blot, protein estimation. localization of proteins, SDS-PAGE gel electrophoresis.

Introduction

Recent educational research on teaching biology to undergraduates has raised concerns about how traditional approaches in large classes fail to reach many students and thereby emphasized on the need for more hand-on experiential learning instructions (Puli and Singh, 2011; Tare *et al.*, 2009; Tare and Singh, 2008; Uman and Singh, 2011; Wood, 2009; Woodin *et al.*, 2009). One of the hallmarks of the modern day science education is experiential learning, which allows students to get a hands-on-experience to understand latest scientific research and concepts. In modern day undergraduate curriculum, research is an important component of habits of inquiry and learning (Puli and Singh, 2011; Tare *et al.*, 2009; Tare and Singh, 2008; Uman and Singh, 2011). Efforts have been channeled to develop a repertoire of laboratory courses to expose undergraduates to modern day biology concepts and techniques used in biomedical research. The new text books provide exhaustive and detailed information through movies and illustrations on how proteins play a role in a biological function and what approaches can be used to determine their localization as well as

quantitate them using Western Blot approach. Despite the utility of animations and videos the best way of learning is through hands-on experiential learning (Puli and Singh, 2011; Tare *et al.*, 2009; Tare and Singh, 2008; Wood, 2009). However, it comes with a cost of time and resources. We devised a laboratory to introduce students to the Western Blot technique, its principle and applications, which will allow students to determine presence or absence of a protein in a particular tissue and how to semi-quantitatively estimate a protein in a sample. Furthermore, this exercise can be finished in two laboratory sessions with some preparation done prior to the demonstration to the students (Figure 1).

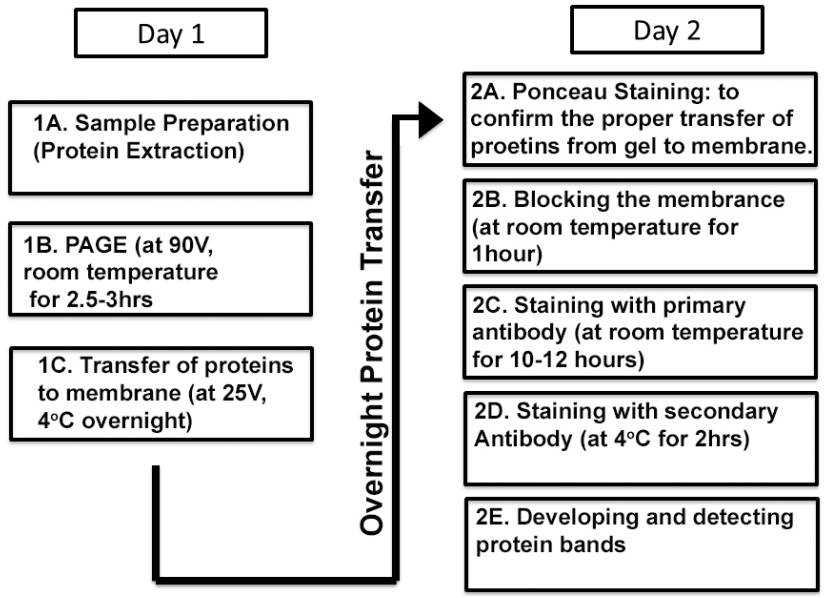

Figure 1. Schematic presentation of time line for Western Blot analysis. We have developed a two-day western blot protocol for undergraduate laboratory course. This strategy will allow demonstration of this modern day technique to undergraduate students.

We have developed this laboratory exercise to study the ubiquitously expressed Tubulin (Tub) and Wingless (Wg) protein in the adult eye of *Drosophila*. The *Drosophila* model is highly versatile as it is easy to rear flies in masses in a short period of time (Puli and Singh, 2011; Singh *et al.*, 2012; Tare *et al.*, 2009; Tare and Singh, 2008). Furthermore, there are several eye specific mutants available in flies if you want to show comparison of gene expression among various genetic backgrounds. *Drosophila* model can be easily used to demonstrate protein isolation, detection and quantitation.

Western Blotting technique (or immunoblotting) was first described by Towbin *et al.* (Towbin *et al.*, 1979). Since then, this technique has become one of the widely used techniques in the field of basic sciences. Western blot is a highly sensitive biochemical technique, which uses the property of monoclonal/polyclonal antibodies (highly specific in nature) to bind to their respective antigens. It is mainly used for the detection, presence/absence, and finding differences in the expression level of a particular protein, or in characterization of proteins (Kim, 2017). Western blot involves isolation/identification of specific proteins of interest from tissue samples, or mixture of proteins extracted from cells, which are later quantified, normalized, denatured (in order to convert their complex structure of protein into its simpler forms). The denatured polypeptides in the protein sample are then separated on the gel based on their size, molecular weight (Kilo-Daltons (kDa), using SDS-PAGE (denatured) gel electrophoresis (Kim, 2017; Mahmood and Yang, 2012). The gel containing the separated protein bands is then placed onto the nitrocellulose or PVDF membrane, and the protein bands are then electrophoretically transferred from the gel on to the membrane. These membranes are then subjected first to the blocking step (5% Bovine Serum Albumin, BSA, in order to prevent non-specific binding of antibodies onto the membrane). After the blocking step, the membranes are treated and incubated with both primary (specific to the target protein), and secondary antibodies (specific to the primary antibody, covalently bound/labeled with enzymes). The enzyme becomes active upon availability of its chromogenic substrate and causes a color reaction. The development of a colored product (using these set of specific enzyme and substrate reactions) is detected and analyzed using gel documentation (BioSpectrum 500) system.

Table 1. List of reagents and solutions used in the Western Blotting lab.

Solutions	Volume	Composition	Preparation/Catalog No.
4X Separation Buffer	500ml	Tris Base – 90g Sodium dodecyl sulfate (SDS) – 2g	Adjust the pH to 8.8 with HCl and make up the volume to 500ml with autoclaved water. Store at room temperature.
4X Stacking buffer	500ml	Tris Base- 30.25g Sodium dodecyl sulfate (SDS) - 2g	Adjust the pH to 6.8 with HCl and make up the volume to 500ml with autoclaved water. Store at room temperature.
1X Tris / Glycine / SDS buffer (Running Buffer)	1Liter	10X Tris/Glycine/SDS buffer - 100ml Autoclave Water - 900ml	100ml of 10X Tris/Glycine/SDS buffer is dissolved in 900 ml autoclaved water. Store at room temperature.
1X Tris / Glycine buffer (Transfer buffer)	1Liter	10X Tris/Glycine buffer - 100ml Methanol - 200ml Autoclaved water - 700ml	100ml of 10X Tris/Glycine buffer and 200ml of methanol is dissolved in 700ml of autoclaved water. Store at 4°C.
1X TBST (TBS Tween-20 buffer)	1Liter	20X TBS Tween-20 buffer (readymade) - 50ml Autoclave Water – 950ml	50ml of 20X TBS Tween-20 buffer is dissolved in (Make up the volume with) 950ml autoclaved water. Store at room temperature.
70% Ethanol	100ml	Reagent Alcohol - 70ml Autoclave Water - 30ml	70ml Reagent Alcohol is dissolved in 30ml of Autoclave Water and stored at room temperature.
5% BSA (Bovine Serum Albumin)	10ml	BSA – 0.5g 1X TBS Tween-20 buffer (TBST) – 10ml	0.5g BSA is dissolved in 10ml of 1XTBST and stored at 4°C.
10% APS (Ammonium persulfate) (freshly prepared)	1ml	APS – 0.1g Autoclave water – 1ml	0.1g APS dissolved in 1 ml of autoclave water in a sterile tube.
TEMED (Tetramethylethyl enediamine)	20gm (26ml)	Ready to use	Fischer Scientific, Cat. #BP150-20
2X Sample Buffer (Laemmli buffer) Concentrate	1 vial	Ready to use	SIGMA, Cat. #S3401
Phenylmethanesulfonyl fluoride (PMSF)	1ml	PMSF – 0.035g Isopropanol – 1ml	0.035g PMSF is dissolved 1 ml isopropanol in a sterile tube and stored at room temperature.
1% Glacial Acetic Acid	100ml	Glacial Acetic Acid – 1ml Autoclave Water – 99ml	1ml Glacial Acetic Acid is dissolved in 99 ml autoclaved water and stored at room temperature.
40%Acrylamide / bisacrylamide (29:1)	1Liter	Ready to use	Fischer Scientific, Cat. #BP1406-1

Protocol

The entire methodology of Western Blotting can be divided into four major steps: (1) Sample preparation, (2) SDS-PAGE gel electrophoresis, (3) Transfer of proteins to the membrane, and (4) Identification of a protein from a total protein sample using immunochemistry.

DAY I

1. Sample Preparation

We have used *Drosophila* adult eyes as the tissue source for the total protein isolation. We used fruit flies as they are easy to rear and large number of flies can be generated in a small time window as life cycle if just 12 days long at room temperature (Singh *et al.*, 2012; Tare *et al.*, 2013). The biological samples, ~25 adult fly heads are first separated from their respective adult fly bodies using sterilized tweezers and are collected in labelled tubes that are kept on ice. To each tube, 50 µl of Laemmli 2× Concentrate Sample Buffer, (SIGMA, Cat.# S3401-1VL) and 3 µl of Phenyl methane sulfonyl fluoride (PMSF) (SIGMA, Cat. #P7626-5G), a protease inhibitor, is added. The tubes are labelled and the samples are macerated thoroughly with a sterilized pestle. They are then boiled at 100°C for 10-15 minutes and are immediately kept on ice for 10 minutes. The samples are then subjected to centrifugation for 10 minutes at 10,000 rpm and then snap chilled on ice again for 10 minutes. The supernatant is transferred into a labelled fresh tube, which is later stored at a low temperature of -20°C. The total protein concentration in a sample is determined by calculating absorbance at 280 nm wavelength using spectrophotometer (Nanodrop) along with the control (2× sample buffer can be used as control. The samples are then normalized by calculating the amount of protein required for a total concentration of 30 or 40 µg/ml per well and diluting it by adding 2× sample buffer (loading a total volume of 10 µl per well).

Table 2. Recipe for preparation of SDS-PAGE gel. The reagents required for preparation of a 10% gel are mentioned below.

4X Separating Gel	Volume req. for preparing 1 gel	Volume req. for preparing 2 gels
40% Acryalmide/bisacrylamide (29:1)	1.25 ml	2.5 ml
4X separation buffer	1.25 ml	2.5 ml
Autoclaved water	2.5 ml	5 ml
10%APS (freshly prepared, stored at 4°C)	50 µl	100 µl
TEMED (stored at 4°C)	5 µl	10 µl
4X Stacking Gel	**Volume req. for preparing 1 gel**	**Volume req. for preparing 2 gels**
40% Acryalmide/bisacrylamide (29:1)	0.25 ml	0.5 ml
4X stacking buffer	0.625 ml	1.25 ml
Autoclaved water	1.625 ml	3.25 ml
10%APS (freshly prepared, stored at 4°C)	25 µl	50 µl
TEMED (stored at 4°C)	2.5 µl	5 µl

2. SDS-PAGE Gel electrophoresis

In order to save time, we sometimes use precast gels (Mini Protean stain free precast gels from Bio-Rad). For casting gels, the two glass plates are washed first with autoclaved water and cleaned with 70%

ethanol (Reagent Alcohol, Fisher Scientific, Cat. #A962-4) for setting them up in the gel apparatus. The polyacrylamide gels are formed by polymerization of acrylamide and bis-acrylamide (bis, N,N'-methylene-bis-acrylamide, Fischer Scientific, Cat. #BP1406-1). Polymerization is initiated by Ammonium Persulfate (APS) (Fisher Scientific, Cat. #BP179-100) and TEMED (Tetramethylethylenediamine) (Fisher Scientific, Cat. #BP150-20). TEMED accelerates the rate of formation of free radicals from persulfate and these in turn catalyze polymerization. Therefore, the 4× separating gel mixture is prepared first and mixed well before addition of both APS and TEMED. APS is added first followed by addition of TEMED (Table 2). The gel components are mixed thoroughly to ensure homogenous solution. The gel mixture (without any further delay) is poured inside the space (up to 70% of total size of glass plate) present between the two glass plates. Around 350 µl of 70% ethanol (or just needed enough to cover the surface) is poured on top of the polymerizing gel to prevent the gel from coming in contact with air, which may trigger rapid polymerization of only the upper part of the gel. After the gel has polymerized (after ~ 35 minutes), the 70% ethanol is poured out, and the top of the gel is washed thoroughly with autoclaved water.

The 4× stacking gel mixture is prepared in a similar fashion as 4× separating gel mixture, and the volume and concentration of chemicals required to prepare a 4× stacking gel is mentioned in Table 2. Once prepared, 4× stacking gel mixture is poured on top of the polymerized 4× separating gel using a micropipette. The combs are inserted slowly just to make sure no bubbles are trapped inside and the gel is left undisturbed to complete the polymerization process. Once the gel has completely polymerized, the gel plates are fitted inside (lower glass plate facing inside) the gel cassette (containing red-positive and black-negative electrodes). The gel cassette is then lodged inside the electrophoresis unit that contains 1× Tris/Glycine/SDS (1× TG-SDS) (10× Tris/Glycine/SDS Buffer, BIO-RAD, Cat. #161-0732) buffer (Table 1). The top of the gel cassette unit is also filled with the 1× TG-SDS buffer, which is required to complete the circuit. The combs are then taken out and wells are washed nicely with 1× TG-SDS buffer (to remove any loose pieces of acrylamide, which if left untreated, can block the wells during the gel run). The normalized protein samples are mixed with 2× Laemmli Concentrate Sample Buffer to make up the total volume to 10 µl, which is then loaded into the respective wells of the gel. A molecular weight marker (Precision Plus Protein Standards Kaleidoscope (BIO-RAD, Cat. #161-0375) is loaded (~4.5 µl) adjacent to the experimental samples in order to get an idea about the size or molecular weight of the protein of interest (Kim, 2017; Mahmood and Yang, 2012; Weber and Osborn, 1969), which is measured in Kilo-Daltons (kDa). The gel is then subjected to electrophoresis using power supply unit at 90V for 2.5-3 hours.

3. Transfer of proteins to the membrane

The 1× Tris Glycine buffer (or TG, transfer buffer) (10× Tris/Glycine Buffer, BIO-RAD, Cat. #161-0734) is prepared according to Table 1 and is kept at 4°C for pre-cooling. Both Nitrocellulose or PVDF membranes (Immun-Blot PVDF: BIO-RAD, Cat. #162-0177) can be used during the transfer process, but PVDF membranes are more durable, hydrophobic, chemically more inert (as compared to nitrocellulose membranes), which increases their potential to bind more to protein (Bass *et al.*, 2017). The PVDF membrane is cut to the size of the gel and is soaked in methanol for 5-10 mins. The membrane, 2 filter papers, 2 sponges are then transferred into 1× Tris/Glycine buffer (Table 1) in order to equilibrate them before the transfer process. The glass plates (containing polymerized gel) are taken out from the gel cassette. The upper plate is removed slowly followed by removal of stacking gel gently from the rest of the gel and the gel is poured with 1× Tris/Glycine buffer to equilibrate.

Preparation of transfer sandwich (to be carried out overnight): It is performed in a tray containing 1× Tris/Glycine (transfer buffer, Table 1). The sequence for sandwich formation is as follows - The black side of the sandwich apparatus is placed down in the tray (containing 1× transfer buffer), followed by a sponge (wetted in 1× transfer buffer) and a rectangular piece of white filter paper (wetted in 1× transfer buffer). The gel is placed and is covered by placing the nitrocellulose or PVDF membrane onto the gel (make sure no bubbles are trapped inside). The bubbles are removed by rolling a glass rod on the gel and membrane. Onto the gel and the membrane, another piece of white filter paper (wetted in 1× transfer buffer) is placed, followed

by another sponge (wetted in 1× transfer buffer). The sandwich is locked afterwards. The sandwich is then placed hinge down, with its black side towards the black side (cathode-negative) of the transfer apparatus. The transfer apparatus is filled with a small ice pack and pre-cooled 1× transfer buffer filled up to the brim. The transfer process is performed in a cold room at 25 Volts (V), at 4°C for overnight or at 60V, 4°C for 2 hours.

DAY II

After the transfer process is done, the membrane is carefully taken out from the transfer electrophoresis unit and washed three times with autoclaved water (2 minutes each). The membrane is then treated two times with 1% Glacial acetic acid solution (ARISTAR, Cat# BDH3094-2.5LG) for 5 minutes each. The membrane is then stained with Ponceu-S staining solution (SIGMA, CAT. #P7170-1L), while shaking for 5-10 minutes, and is further de-stained with 1% Glacial acetic acid (protein bands are clearly visible at this stage). Ponceau-S stain marks the protein bands. However, if the bands are not clearly visible, it doesn't always mean that it won't show any signal during the developing process, because West Dura developing kit is a lot more sensitive than Ponceau-S stain. It can detect approximately 100 ng of protein per band (Ness *et al.*, 2015). The membrane is then washed three times with autoclaved water (10 minutes each) and is then equilibrated with 1× TBST (20× TBS Tween-20 Buffer, Thermo Scientific, Cat. #28360) solution three times (10 minutes each).

4. Identification of a protein from a total protein sample using immunochemistry

Primary and Secondary Antibodies: The membrane is first blocked with 5% Bovine Serum Albumin (BSA) (Fisher Scientific, Cat. # BP1600-100) prepared in 1× TBST for 1 hour at room temperature and is then incubated with primary antibody-Monoclonal Anti-α-Tubulin antibody (1:12000) produced in mouse (SIGMA, Cat. # T5168), Monoclonal Anti-Wingless (1:500) produced in mouse (DSHB, 4D4) prepared in 5% BSA, 1× TBST, overnight at 4°C or 3-4 hours at room temperature (depending on the time available to instructor). The membrane is then washed three times with 1× TBST (10 minutes each, more or less number of washes depends on the antibody used) and is further treated with secondary antibody (Goat anti-Mouse IgG-HRP 1:5000) (Santa Cruz Biotechnology, Cat. Sc-2005) prepared in 5% BSA, 1× TBST for 30 minutes at room temperature or for 2 hours at 4°C. After secondary antibody treatment, the membrane is washed three times with 1× TBST, 10 minutes each to remove any extra unbound antibody left on the membrane to avoid nonspecific signal.

Developing protein bands and detection: The Super Signal West Dura Extended Duration Substrate Kit (Thermo Scientific, Cat. #34076) (highly sensitive in nature) is used for developing the protein bands. The kit allows detection of even mid-femto gram of antigen by oxidizing luminol based chemiluminescent substrate for Horseradish peroxidase (HRP) detection. Equal volumes of SuperSignal West Dura Stable Peroxide Buffer (Prod. # 1859025) and SuperSignal West Dura Luminol/Enhancer Solution (Prod. #1859024) (~1 ml) are mixed together in a tube to form the developing solution and is applied on to the membrane. The membrane is then shaken manually, just to make sure the developing solution covers the entire surface of the membrane. The membrane is incubated with developing solution for 5 minutes at room temperature and the solution is drained afterwards. The membrane is then analyzed and imaged in a Gel Documentation System (UVP BioSpectrum 500 Imaging System with LM-26 and BioChemi 500 Camera f/1.2, S/N021110-001) with exposure time (limit range from 5 sec to 1 minute, and longer if necessary).

Advantages of using Western blotting technique

1. One of the challenges of teaching a laboratory course is the willingness of the institution to invest in setting up the lab. Therefore, the use of cost- and time-effective exercises can facilitate easy implementation of these laboratory programs. The solutions used for the Western Blot analysis are commercially available and are inexpensive.

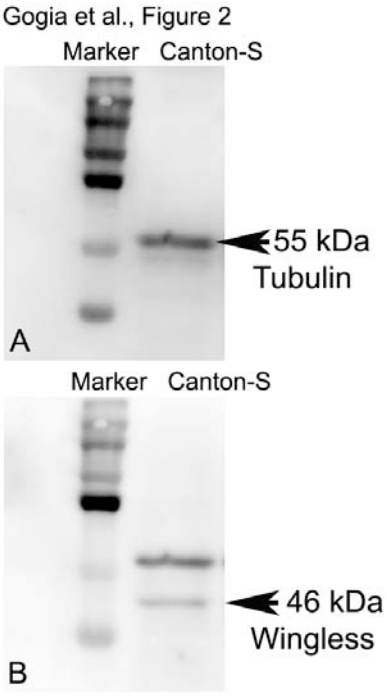

Figure 2. Western Blot Analysis to detect proteins. Total protein sample isolated from wild-type adult eye of *Drosophila* were separated by SDS-PAGE electrophoresis. The (A) Tubulin and (B) Wingless protein were detected using the Tubulin and Wingless antibody. (A) A band corresponding to 55 kDa molecular weight, was detected which corresponds to Tubulin. (B) A band corresponding to 46 kDa molecular weight, corresponding to Wingless (Wg) was detected. Images were captured using the BioSpectrum® 500 Imaging System. The same blot was first used to detect Tubulin. It was then stripped and used for detecting Wingless protein.

2. It is challenging for undergraduate students to learn these technique from books, animations, or tutorials. This teaching note will help develop experiential learning opportunities for students and learn this technique in an easy and effective manner.

3. The first step of Western blot involves separation of total proteins using SDS page electrophoresis. Therefore, this lab can be coupled with the SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) laboratory. Thus, in two days you can demonstrate two techniques as it just adds one more lab for identification of a protein using immunohistochemistry (as transfer of proteins from gel to membrane can be done overnight). Moreover, the procedure has incubation steps. The time between the incubations can be utilized for interaction with lab instructor, clarifying the concepts and class discussions.

4. The students get general overview of Western Blotting technique, which is highly sensitive and can detect as little as 0.1ng of protein. This exercise provides hands-on experience of this technique starting from sample preparation to visualizing the proteins onto the membrane.

5. The technique employs use of antigen-antibody reactions (highly specific in nature) and thus has the capability of detecting the protein of interest even from a mixture or a solution containing 300,000 diverse range of proteins. It can also help detect the immunogenic responses (caused by bacteria or viruses), or can be used to study regulation of genes known to cause asthma, allergy (García-Sánchez and Marqués-García, 2016), or for detection and diagnosis of deadly diseases like human immunodeficiency virus (HIV) (Feng *et al.*, 2017).

6. This Western blot analysis utilizes standard protein chemistry and is easy to demonstrate in a undergraduate laboratory setup as it does not need educational demonstration kits that minimize the exposure of students to details. It will add to the skill set of students and will help develop a core of trained individuals suitable for academics or industrial settings.

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Real time quantitative PCR to demonstrate gene expression in an undergraduate lab.

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Abstract

The objective of this teaching note is to develop a laboratory exercise, which allows students to get a hands-on experience of a molecular biology technique to analyze gene expression. The short duration of the biology laboratory for an undergraduate curriculum is the biggest challenge with the development of new labs. An important part of cell biology or molecular biology undergraduate curriculum is to study gene expression. There are many labs to study gene expression in qualitative manner. The commonly used reporter gene expression studies are primarily qualitative. However, there is no hands-on experience exercise to quantitatively determine gene expression. Therefore, it is necessary to design a laboratory exercise that enables the students to carry out cell or molecular biological assays in the desired time. Here we report a laboratory where we can introduce students to gene expression using the real time Quantitative Polymerase Chain Reaction (RT-qPCR) by comparative C_T method to analyze expression of genes in *Drosophila* tissues. Keywords: *Drosophila melanogaster*, eye, real time quantitative PCR, gene expression.

Introduction

A challenging situation emerging with fast paced growth on the research front in various disciplines of Biology is to introduce emerging new concepts into the undergraduate curriculum too (Puli and Singh, 2011; Tare *et al*., 2009; Tare and Singh, 2008; Usman and Singh, 2011; Wood, 2009). Interestingly, central dogma of molecular biology is an age old and time-tested concept that has been delivered in the undergraduate classroom. Even though the basic concept about central dogma is that genetic information of an organism or a cell is stored in nucleic acid DNA, which is then transcribed into single stranded RNA, and finally translated to protein but the strategies to study gene expression (qualitatively and quantitatively) have been evolving to date. The conventionally used approaches to deliver this curriculum in laboratory class are to use reporter gene expression, immunohistochemistry, or using protein trap lines. However, the majority of these techniques are qualitative, or to some extent semi-quantitative, in nature. Therefore, there are not many quantitative approaches to determine or compare levels of gene expression among different tissues that can be used for classroom demonstration.

Drosophila melanogaster has not only served as a workhorse for research but has also been exploited to develop undergraduate laboratory classes. The short life cycle of 12 days, high reproductive ability of fly, and a long repertoire of genetic tools have made this a very useful model for undergraduate classroom (Puli and Singh, 2011; Singh *et al*., 2012; Tare *et al*., 2009; Tare and Singh, 2008). *Drosophila* can be used to visualize gene expression by employing techniques like enhancer trapping, epitope tagging, antibody staining, or gene trapping. All these methods are qualitative in nature and are laborious and time consuming (Puli and Singh, 2011; Tare *et al*., 2009; Tare and Singh, 2008). In multicellular organisms, including flies, differential gene(s) expression along spatial levels of gene(s) expression in different cells/ tissues and temporal axis generates diversity in cell types and patterning. Furthermore, the gene expression also varies under different experimental conditions. Therefore, quantification of gene expression has been a crucial aspect of modern day biological research. There are different ways to quantify gene expression: as a validation of protein levels (Kim *et al*., 2008), as a validation of the extent of transcription of a gene (Pal *et al*., 2007), to study differences in gene expression between the diseased and the normal state (Ren *et al*., 2007), change in expression of cells exposed to chemical substances (Woods *et al*., 2008), quantification of non-coding RNA gene expression (Calin *et al*., 2007), and as a diagnostic tool (Paik *et al*., 2004). Real time quantitative PCR (RT-qPCR) has served as one of the modern day workhorses to perform such quantitative analysis. Hence, we are introducing here the use of Real time quantitative Polymerase Chain Reaction (RT-qPCR) technique to help students to investigate gene expression quantitatively along the temporal as well as spatial axes.

Protocol

This RT-qPCR laboratory designed for undergraduate curriculum has been divided into two labs/classes. The first day, students extract total RNA from the tissue, run RNA on a gel, and determine quality and concentration of RNA. They also prepare cDNA from mRNA in the first lab. On the second day, they run qPCR and analyze data (Figure 1). Thus, we have divided this qPCR into four steps: (1) Sample preparation and RNA isolation, (2) RNA to cDNA conversion, (3) qPCR, and (4) Analysis.

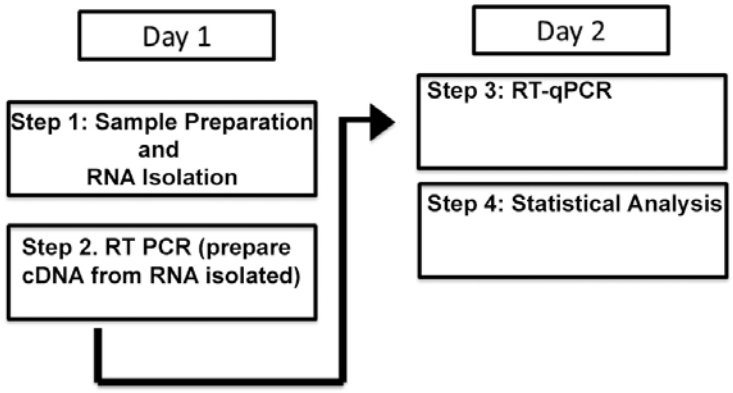

Figure 1. Schematic presentation of time line for Real Time Quantitative Polymerase Chain Reaction (RT-qPCR). We have developed a two-day RT- qPCR protocol for undergraduate laboratory course. This strategy will allow demonstration of this modern day technique to undergraduate students.

1. Sample Preparation and RNA Isolation

Drosophila melanogaster, flies, were obtained from the Bloomington Stock Center, Indiana; http://flystocks.bio.indiana.edu. The third instar larvae were selected and dissected in Phosphate Buffered Saline (PBS, containing 137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, pH 7.4; Dulbecco and Vogt, 1954) using sharp forceps (Electron Microscopy Sciences Cat. No. # 72707-01). We isolated the eye-antennal imaginal discs to study the expression level of desired genes. Twenty pairs of third instar eye-antennal imaginal disc (n = 40) were collected in microcentrifuge tubes upon dissection. The imaginal discs were stored in RNAlater (Thermo Fisher, Cat. No. # AM7024) solution.

The tubes were briefly centrifuged and RNAlater solution was completely removed. We added 500 µl of TRIzol Reagent (Thermo Fisher, Cat. No. # 15596926), which is used to isolate good quality RNA from tissue samples. TRIzol is a monophasic solution, primarily consisting of phenol and guanidine isothiocyanate along with other proprietary components, which was used for homogenization of tissue. Chloroform was

added and homogenate was allowed to separate into a clear upper aqueous layer. 200 µl of the aqueous phase was transferred to RNA clean and Concentrator™ (Zymo research, Cat. No. R1080) columns, and the recommended protocol was followed, *i.e.*, solution was passed through the RNA binding buffer, which binds RNA to the desired columns. Then RNA wash buffer was added to remove all the impurities from the column. Finally RNA was eluted in 20 µl of molecular grade water (DNase/RNase free) and collected in a separate tube. The molecular grade water serves as the elution buffer. It releases the RNA from the column. Quality of RNA as well as concentration of RNA was determined by calculating absorbance at 260 nm (A260) and 280 nm (A280) wavelengths using Nanodrop 2000 spectrophotometer (Thermo Scientific). Good quality samples had A260/ A280 ratio greater than 2 and a peak at 260 nm.

2. RNA to cDNA Preparation

On Day 1 of the lab, the reverse transcription (RT) reaction is used to prepare cDNA from RNA. RNA concentration for each sample was calculated using the absorbance values obtained from Nanodrop. For RT reactions, 200 ng total RNA was used for each reaction. RNA was heated at 65°C for 10 minutes. Then, it was snap chilled on ice for 3 minutes, followed by a short spin of 10 seconds. It was tapped five times, after which again a short spin of 3 minutes was given, and RNA was ready for RT PCR reaction. We used first-strand cDNA synthesis kit (GE healthcare, Cat# 27926101) to generate cDNA from the isolated RNA. This kit has NotI-(dT)18 bi-functional primer, which can be used to selectively prime mRNA with poly(A) tail and is designed to generate full-length first strand cDNA. The kit master mix is comprised of: RNA 9 µl (for 200 ng concentration), first strand mix- 5 µl, DTT- 1 µl, oligo (dt) primer-1 µl. The mix is incubated at 37°C for 1 hour, then 135 µl molecular grade water is added and solution is incubated again for 5 min at 98°C for enzyme inactivation. Half of the volume(s) are used for negative RT reaction without using oligo(dt) primers, *i.e.*, first strand mix- 2.5 µl, DTT- 0.5 µl, molecular grade water: 68 µl. Solution was incubated for 5 min at 98°C for enzyme inactivation to which 4.5 µl of RNA was added.

Table 1. Recipe for PCR reaction.

Ingredient	Per reaction (µl)
SYBR green reagent	12.5
Forward/reverse primer mix (50 mM each)	0.625
Molecular biology grade water	9.25

3. Quantitative Real Time Polymerase Chain Reaction

On Day 2 of the lab, we performed the RT-qPCR reaction. It was performed using iQ ™ SYBR® Green Supermix (Bio-Rad) and Bio-Rad iCycler (Bio-Rad). Following master mix was prepared, accounting to one to two additional reactions per gene (Table 1). To each well of 96-well plate, 23 µl of master mix (Bio-Rad, Cat # 223-9941) was added, followed by addition of 2 µl of cDNA (generated in Step 2). Plate was sealed using sealing strip (MSBI001 Bio-Rad). A brief spin (up to 1,500 rpm) was performed on a centrifuge equipped with a 96-well plate adapter. Then PCR plate was placed in iCycler and PCR was performed as per the manufacturers' protocol. Typically, step 1: 95°C for 3 minutes (1 cycle). This step is performed for initial denaturation of the double stranded cDNA to the single strands and loosen secondary structures in single stranded DNA. The step 2 comprise of 40 cycles of 30 seconds at 95°C and 30 seconds at 60°C. This is an annealing step during which DNA amplification occurs, and as DNA keeps on amplifying so does SYBR® Green get intercalated to (binds into) the DNA double helix. This alters the structure of the dye and causes it to fluoresce more. Thus, along the course of time as the concentration of DNA increases, so does the intensity of fluorescence. An instrument that combines thermal cycling with fluorescent dye scanning capability can measure this change in the fluorescent intensity. Fluorescence is plotted against the cycle number, and finally RT-qPCR generates an amplification plot that represents the accumulation of product over the duration of the entire PCR reaction. This way real time quantification of gene product is done, and Ct values were recorded and then using pre designed template (Figure 3A) fold change was calculated.

4. Analysis

There are various methods in which RT-qPCR data can be reported including absolute or relative expression level. Absolute expression provides the exact copy number of data *via* a standard curve (Chen *et*

al., 2005). The data are presented as copy number per cell. As in relative PCR, data are presented in reference to another gene often represented as the internal control. We perform absolute quantification only when precise copy number of gene is required, for example, calculation of viral load (Niesters, 2001). The disadvantage of absolute PCR is the increased effort to generate a standard curve.

Various strategies have been developed to represent the relative gene expression level data such as efficiency correction method, sigmoid curve fitting method, and comparative C_T method (also known as the $2^{-\Delta\Delta C_T}$ method). C_T method is best among all (Livak and Schmittgen, 2001). Though despite being the best method so far, one should not forget that the comparative C_T method makes several assumptions, including that the efficiency of the PCR is close to 1 and the PCR efficiency of the target gene is similar to the internal control gene (Livak and Schmittgen, 2001).

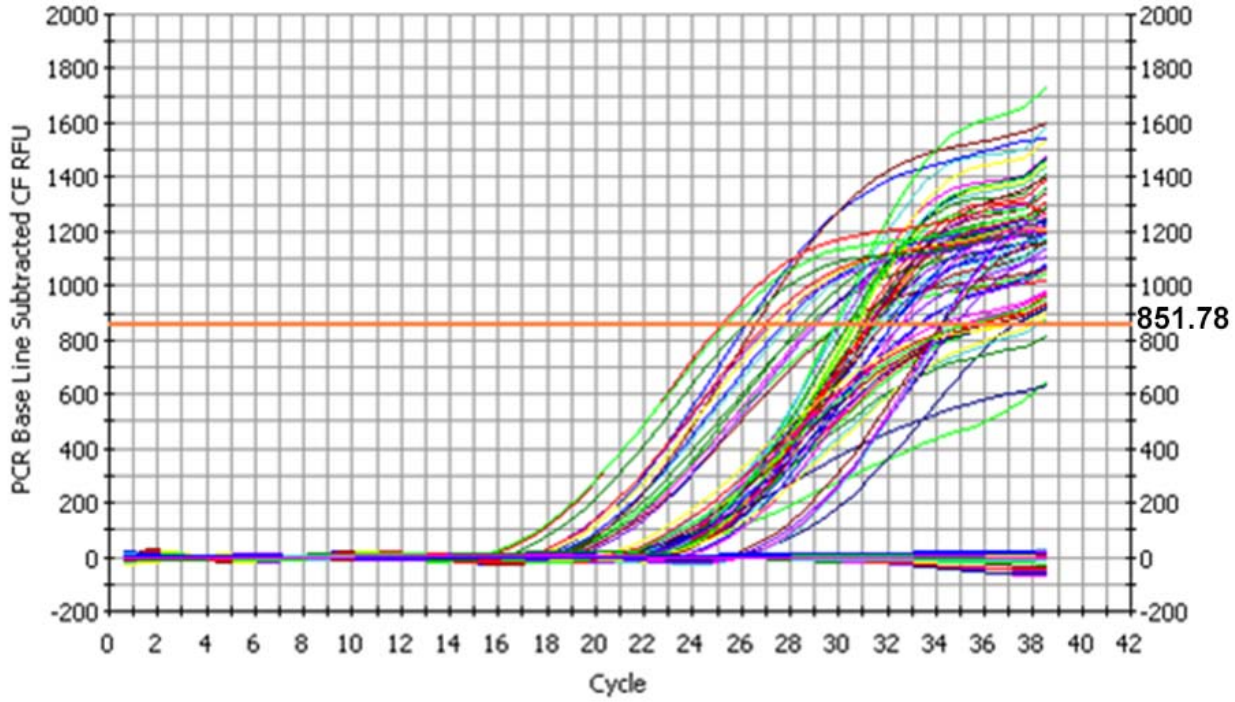

Figure 2. Demonstration of Real-time qPCR output by the calculation of C_T values. Data from a typical real-time PCR output run (40 cycles) in our. The point at which the curve intersects the threshold (horizontal orange line), which corresponds to 851.78, is the C_T value.

The quantitative endpoint for real-time quantitative PCR is the threshold cycle (C_T). The C_T is defined as the PCR cycle at which the fluorescent signal of the reporter dye crosses an arbitrarily placed threshold (Figure 2). By presenting data as the C_T, one ensures that the PCR is in the exponential phase of amplification. The numerical value of the C_T is inversely related to the amount of amplicon in the reaction (*i.e.*, the lower the C_T, the greater the amount of amplicon) (Schmittgen and Livak, 2008).

C_T method presents data as a fold change in gene expression.

Equation 1

$$\text{Fold change} = 2^{-\Delta\Delta CT}$$

This equation is used to compare gene expression in two different samples (Sample A and Sample B); each sample is related to internal control gene, which could be a house keeping gene like GAPDH. Sample A can be the treated/ experimental sample, whereas Sample B is untreated (control). Also, Sample A can be the diseased (experimental) form and Sample B then can be normal/wild-type state (control). Sample A can be affected with Virus, and Sample B is not. Expanding the equation 1 in its full form:

Equation 2

$$2^{-\Delta\Delta CT} = [(C_T \text{ gene of interest} - C_T \text{ internal control}) \text{ sample A} - (C_T \text{ gene of interest} - C_T \text{ internal control}) \text{ sample B}]$$

Statistical analysis was performed using two-way analysis of variance (ANOVA) and Student's t-test for independent samples. Samples were run in triplicates ($n = 3$). Statistical significance was determined with 95% confidence ($p < 0.05$). Calculating such values for a variety of genes could be cumbersome at the undergraduate level, so we have developed a predesigned template for Microsoft Excel that can be used for undergraduates to calculate the final value with precision. It will also help them to calculate Standard deviation and P values. The results were graphed using Microsoft Excel (Figure 3A). We compared the expression levels of a gene *suppressor of stellate like protein* (*ssl*) between control and an experiment where we targeted the expression of a regeneration cascade gene in the third instar larval eye imaginal disc. We found that *ssl* levels were downregulated ~10 folds as compared to the wild-type third instar larva (Figure 3B). These results provided a quantitative estimate to our prior results from RNA Sequencing approaches. It further demonstrates that RT-qPCR can be an excellent tool for comparing gene expression levels.

Advantages

1. RT-qPCR is less time consuming and less cumbersome.

2. RT-qPCR has the ability to monitor the progress of PCR reaction as it occurs in real time. It also has the ability to precisely measure the amount of amplicon at each cycle. This allows highly accurate quantification of the amount of starting material in samples.

3. RT-qPCR can produce quantitative data with an accurate dynamic range and does not require post-amplification manipulation (Morrison *et al*., 1998).

4. RT-qPCR assays are 10,000- to 100,000-fold more sensitive than RNase protection assays (Wang and Brown, 1999), 1000-fold more sensitive than dot blot hybridization (Malinen *et al*., 2003), 10 times more sensitive then Agarose gel electrophoresis (Fellahi *et al*., 2016), and can even detect a single copy of a specific transcript (Palmer *et al*., 2003).

5. In addition, RT-qPCR assays can reliably detect gene expression differences as small as 23% between samples (Gentle *et al*., 2001) and have lower coefficients of variation (SYBR® Green at 14.2%; TaqMan® at 24%) than end point assays such as band densitometry (44.9%) and probe hybridization (45.1%) (Schmittgen *et al*., 2000).

Conclusion

The majority of the laboratory exercises taught in the undergraduate laboratories are either from commercially developed expensive kits or other conventional experimental labs that do not emphasize communication skills through the use of graphs. Our laboratory exercises (Puli and Singh, 2011; Tare *et al*., 2009; Tare and Singh, 2008), including this one, address this problem and are designed to expose students to

basic lab skill sets (a) involving RNA extraction and cDNA preparation, (b) Preparing sample for RT-qPCR, (c) Analyzing real time RT-qPCR data.

Acknowledgment: This laboratory exercise was designed in the Department of Biology, at the University of Dayton. ASM is supported by graduate program of the University of Dayton. AS is supported by NIH1R15GM124654-01 and Stem Catalyst Grant.

A

	ssl		House keeping Gene
	control	Experiment	18.9
CT	25	27.7	19.7
	24.7	27.6	22.1
	25.2	28	19.2
HOUSE GEN	20.23333	19.83333	19.9
DIFFERENCE	-4.7666667	-7.8666667	20.4
	-4.4666667	-7.7666667	18.2
	-4.9666667	-8.1666667	19.2
2^ΔΔCt	0.03673587	0.00428447	19.8
	0.04522716	0.00459198	19
	0.03198043	0.00348007	19.5
Average	0.03798116	0.00411884	20
Ratio	0.9672131	0.11280522	17.7
	1.190779	0.12090164	17.9
	0.84200791	0.09162631	17.5
Average	1	0.10844439	16.5
St Dev	0.17668207	0.01511701	16.6
P Value		0.01236584	18.3

B

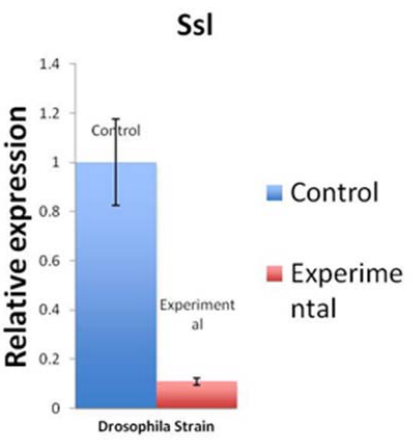

Figure 3. Graphical representation showing relative fold change in expression level of gene *suppressor of stellate like protein* (*ssl*) between control and experimental third instar larval eye imaginal disc. (A) A Microsoft Excel sheet template was designed with pre-inserted formulas to make necessary calculations, *i.e.*, fold change, Standard deviation, and P values. This will allow undergraduates to insert C_T values in the template and the rest of the calculations will be done automatically. Using this excel sheet, gene *suppressor of stellate like protein* (*ssl*) levels in *Drosophila* third instar larval eye imaginal disc (Control: wild-type, Canton S larval eye imaginal disc) were compared to that of experiment where we targeted the expression of a regeneration cascade gene in the third instar larval eye imaginal disc. (B) Note that *ssl* levels were down-regulated ~10 folds as compared to the wild-type third instar larval eye imaginal disc.

References: Calin, G.A., C.-g. Liu, M. Ferracin, T. Hyslop, R. Spizzo, C. Sevignani, M. Fabbri, A. Cimmino, E.J. Lee, S.E. Wojcik *et al.*, 2007, Cancer Cell 12: 215-229; Chen, C., D.A. Ridzon, A.J. Broomer, Z. Zhou, D.H. Lee, J.T. Nguyen, M. Barbisin, N.L. Xu, V.R. Mahuvakar, M.R. Andersen *et al.*, 2005, Nucleic Acids Research 33: e179-e179; Fellahi, S., M.E. Harrak, J.H. Kuhn, G. Sebbar, E.A. Bouaiti, K. Khataby, O.F. Fihri, M.E. Houadfi, and M.M. Ennaji 2016, BMC Research Notes 9: 231; Gentle, A., F. Anastasopoulos, and N.A. McBrien 2001, Biotechniques 31: 502, 504-506, 508; Kim, Y.W., R.J. Zhao, S.J. Park, J.R. Lee, I.J. Cho, C.H. Yang, S.G. Kim, and S.C. Kim 2008, British Journal of Pharmacology 154: 165-173; Livak, K.J., and T.D. Schmittgen 2001, Methods 25: 402-408; Malinen, E., A. Kassinen, T. Rinttila, and A. Palva 2003, Microbiology 149: 269-277; Morrison, T.B., J.J. Weis, and C.T. Wittwer 1998, Biotechniques 24: 954-958, 960, 962; Niesters, H.G.M., 2001, Methods 25: 419-429; Paik, S., S. Shak, G. Tang, C. Kim, J. Baker, M. Cronin, F.L. Baehner, M.G. Walker, D. Watson, T. Park *et al.*, 2004, New England Journal of Medicine 351: 2817-2826; Pal, S., R.A. Baiocchi, J.C. Byrd, M.R. Grever, S.T. Jacob, and S. Sif 2007, The EMBO Journal 26: 3558-3569; Palmer, S., A.P. Wiegand, F. Maldarelli, H. Bazmi, J.M. Mican, M. Polis, R.L. Dewar, A. Planta, S. Liu, J.A. Metcalf *et al.*, 2003, Journal of Clinical Microbiology 41: 4531-4536; Puli, O.R., and A. Singh 2011, Dros. Inf. Serv. 94; Ren, Z., A. Shin, Q. Cai, X.-O. Shu, Y.-T. Gao, and W. Zheng 2007, Breast Cancer Research 9: R2-R2; Schmittgen, T.D., and K.J. Livak 2008, Nature Protocols 3: 1101; Schmittgen, T.D., B.A. Zakrajsek, A.G. Mills, V. Gorn, M.J. Singer, and M.W. Reed 2000, Analytical Biochemistry 285: 194-204; Singh, A., M. Tare, O.R. Puli, and M. Kango-Singh 2012, Dev. Dyn. 241: 69-84; Tare, M., O.R. Puli, S.M. Oros, and A. Singh 2009, Dros. Inf. Serv. 92: 174-180; Tare, M., and A. Singh 2008, Dros. Inf. Serv. 91: 160-164; Usman, M., and A. Singh 2011, Journal of STEM Education: Innovations and Research 12: 58-66; Wang, T., and M.J. Brown 1999, Analytical Biochemistry 269: 198-201; Wood, W.B., 2009, Annu. Rev. Cell Dev. Biol. 25: 93-112; Woods, D.C., C. Alvarez, and A.L. Johnson 2008, Gynecologic Oncology 108: 632-640.

Available Stock List

University of Chile

Stock list of species and strains available in the laboratory of Ethology, Genetics and Evolution of Behavior, Faculty of Medicine, University of Chile, Santiago.

Del Pino, F[a]., and R. Godoy-Herrera[a]. [a]Laboratorio de Etología, Genética y Evolución de la Conducta. ICBM, Facultad de Medicina, Universidad de Chile, P.O.Box 8389100, Independencia 1027, Santiago, CHILE.

A list of *Drosophila* species and strains kept in our laboratory is provided below. The purpose is to inform Drosophilists around the world about the stocks available in our laboratory. We indicate the origin (country and region) where adults that formed the strains were collected. Secondly, we provide information on the breeding sites from which the adults that formed some of the strains emerged. The information could be useful to ecological geneticists. Some neurological mutants are also listed. Several other endemic and cosmopolitan species and strains living in Chile and other countries in South America that we have available are also listed. When appropriate, we have provided geographical coordinates where we made the collections.

Species and strains	Country of origin	Geographical coordinates
1.- *D. melanogaster*		
Wild type strains		
Oregon R-c*	USA	
Canton - S **	GERMANY	
La Florida	CHILE	33° 27′ 00″ S
Til-Til[¶]	CHILE	33° 05′ 00″ S
Til-Til[‡]	CHILE	33° 05′ 00″ S
Trana[ŧ]	CHILE	35° 52′ 00″ S
Valdivia[ß]	CHILE	39° 48′ 00″ S
Autosomic mutants		
ebony [11] (*e*[11])		
vestigial (*vg*[1])		
taxi (*tx*)		
dumpy (*dp*)		
brown (*bw*)		
Sex-linked mutants		
white (*w*[1])		
white (*w*[5])	BRASIL	
yellow (*y*[A66c])		
Neurological strains		
rutabaga (*rut*) **		
atonal (*ate*[1])		

orco**		
Syn^{97cs} **		
2.- *D. simulans*		
Wild type strains		
Til-Til[¶]	CHILE	33° 05′ 00″ S
Buchupureo[€]	CHILE	36° 08′ 00″ S
3.- *D. buzatti*		
Wild type Strains		
Providencia	CHILE	33° 27′ 00″ S
Til-Til[¶]	CHILE	33° 05′ 00″ S
4.- *D. pavani*		
Wild type strains		
Chillán	CHILE	36° 36′ 00″ S
La Florida	CHILE	33° 27′ 00″ S
Los Angeles	CHILE	37° 28′ 00″ S
Pelequén[¶]	CHILE	34° 25′ 00″ S
5.- *D. gaucha*		
Wild type strains		
Campos de Jourdan	BRASIL	22° 44′ 22″ S
Mutant strains		
yellow-white†		
6.- *D. mesophragmatica*		
Wild type strains		
Huaraz	PERÚ	9° 31′ 38″ S
7.- *D. virilis*		
Wild type strains		
Santiag	CHILE	33° 27′ 00″ S
8.- *D. funebris*		
Wild type strains		
Kentucky	USA	37° 30′ N

*Strain sent from The University of Columbia in 1952, USA

** = Strain sent by Dr. B. Gerber, Prof. Martin Heisenberg laboratory, Germany.

[¶] Til-Til = Collected from decaying fruits of *Opuntia ficus-indica*

[‡] Til-Til = Collected from decaying grapes of *Vitis vinífera* fruits (Seedless variety).

[ʂ] Trana = Collected from decaying grapes of *Vitis vinífera* (País variety).

[ß] Valdivia = Collected from decaying fruits of *Malus domestica* fruits (Golden delicious variety).

[€] Buchupureo = Collected from decaying fruits of *Prunus persica* (Beetraga variety).

[¶] Pelequén = Collected from decaying fruits of *Opuntia ficus-indica*

†Spontaneous.

58th Annual *Drosophila* Research Conference

The 58th Annual *Drosophila* Research Conference was held on 29 March – 1 April 2017 at the Town & Country Resort & Convention Center, San Diego, CA. The Conference Organizers were Leanne Jones, Chair, Doris Bachtrog, Claude Desplan, and Amy Kiger. The conference was sponsored by The *Drosophila* Board in association with the Genetics Society of America, 9650 Rockville Pike, Bethesda, MD 20814-3998.

Opening Remarks

Leanne Jones, Welcome

R. Hawley: Larry Sandler Award Winner Talk

Opening Session Talk

Sean Carroll: The making and unmaking of the animal kingdom.

Plenary Lectures (in presentation order)

Bruno Lemaitre: The *Drosophila-Spiroplasma* interaction as a model to dissect the molecular mechanisms underlying insect endosymbiosis.

Virginie Courtier-Orgogozo: The mutation behind species evolution.

Peter R Hiesinger: Simple rules in neural circuit assembly.

Irene Miguel-Aliaga: How the gut talks and listens.

Buzz Baum: Tissue refinement: a noisy path to order.

Francois Payre: Orchestrating the proliferation differentiation switch of adult intestine stem cells.

Marcos Gonzalez-Gaitan: Asymmetric signaling endosomes in asymmetric division.

Julia Zeitlinger: Why the pause? Catching RNA polymerase II *in vivo*.

Marta Zlatic: Circuits principles of memory-based behavioral choice.

Erika Bach: Stem cell homeostasis in the *Drosophila* testes.

Nitin Phadnis: The conflicts that shape genomes, cells and species.

Julius Brennecke: The piRNA pathway – a small RNA based genome defense system.

Workshops

PI Early Career Forum
Organizers: Guy Tanentzapf and Amy Bejsovec

Ecdysone Workshop
Organizers: Rebecca Spokony and Elizabeth Ables

Integrating Research and Teaching at PUIs Using *Drosophila melanogaster* as a Model Organism
Organizers: Afshan Ismat, Norma Velazquez Ulloa, and Judy Leatherman

Wound Healing and Regeneration
Organizers: Adrian Halme and Rachel Smith-Bolton

Feeding Behavior, Nutrition and Metabolism
Organizers: Tânia Reis and William W. Ja

Everything You Ever Wanted to Know About Sex
Organizers: Mark Van Doren, Michelle Arbeitman, and Artyom Kopp

Navigating the Career Decision Making Process
Organizer: Sonia Hall

Spotlight on Undergraduate Research
Organizers: Eric Stoffregen, Kimberly A. Carlson, Jennifer Jemc Mierisch, and Catherine Silver Key

Drosophila Microbiome
Organizers: Will Ludington, Brooke McCartney, and Nichole Broderick

Developmental Mechanics
Rodrigo Fernandez-Gonzalez and Guy Tanentzapf

Biogenic Amines and Behaviors
Sonall A. Deshpande, Seth Tomchik, and Kyung-An Han

The North American *Drosophila* Board

The Board's duties include: overseeing community resource centers and addressing other research and resource issues that affect the entire *Drosophila* research community. The Board also administers the finances for the annual North America *Drosophila* Research Conference and its associated awards, and it chooses the organizers and the site of the annual meeting. The Board consists of nine regional representatives and four international representatives, who serve 3-year terms. The three elected officers are President, President-Elect, and Treasurer. In addition, the Board has *ex officio* members who represent *Drosophila* community resources or centers. For more information about the Board and the summaries of the annual Board meetings, see: Fly Board under the News menu at the FlyBase web site: flybase.bio.indiana.edu.

Drosophila Board Membership 2016 - 2017

President: Laura Johnston (Columbia University, New York, NY)

President-Elect: Deborah Andrew (Johns Hopkins School of Medicine, Baltimore, MD)

Past-President: David Bilder (University of California, Berkeley)
Past-President: Ken Irvine (Waksman Institute, Rutgers University, Piscataway, NJ)
Past-President: Amy Bejsovec (Duke University, Durham, NC)

Treasurer: Deborah Andrew (Johns Hopkins School of Medicine, Baltimore, MD)

Regional Representatives:

New England: Giovanni Bosco (University of Arizona, Tucson)
Heartland: Michael Galko (University of Texas MD Anderson Cancer Center, Houston)
Midwest: Bing Zhang (University of Missouri, Columbia)
Mid-Atlantic: Chris Rushlow (New York University, NY)
Southeast: Andrea Page-McCaw (Vanderbilt University Medical Center, Nashville, TN)
California: Amy Kiger (University of California – San Diego, CA)
Great Lakes: Scott Barolo (University of Michigan, Ann Arbor)
Mountain: Sarah Certel (University of Montana, Missoula)
Canada: Esther Verheyen (Simon Fraser University, Burnaby, Canada)

International Representatives:

Asia: Li-Mei Pai (Chang Gung University, Taiwan)
Australia/Oceania: Gary Hime (University of Melbourne, Australia)
Europe: Sarah Bray (University of Cambridge, England)
Latin America: Juan Riesgo-Escovar (Mexican National Autonomous University, Mexico)

Primarily Undergraduate Institution Representative:

Alexis Nagengast (Widener University, Chester, PA)

***Ex Officio* – Representing *Drosophila* Resources:**

Norbert Perrimon (FlyBase; Harvard University)
Susan Celniker (BDGP; Lawrence Berkeley National Laboratory, Berkeley)
Thom Kaufman (Bloomington Stock Center & FlyBase; Indiana University)
Kevin Cook (Bloomington Stock Center & Nomenclature Committee; Indiana University)
Patrick O'Grady (Drosophila Species Stock Center; UC Berkeley)
Maxi Richmond (UCSD Drosophila Species Stock Center; University of California, San Diego)
Jim Thompson (Drosophila Information Service; University of Oklahoma)
Liz Perkins (Harvard TRiP; Harvard University)
Hugo Bellen (Bloomington Stock Center Advisory Committee & P Element Project; Baylor College of Medicine)
Allan Spradling (P-Element Project; HHMI/Carnegie Institute)
Stephanie Mohr (Director, DRSC/TRiP; Harvard University)

Jonathan Zirin (Asst. Director, DRSC/TRiP; Harvard University)
Scott Hawley (Nomenclature Committee; Stowers Institute for Medical Research)
Lisa Meadows (VDRC; Vienna, Austria)
Masanobu Itoh (DGRC, Kyoto; Kyoto, Japan)
Toshiyuki Takano-Shimizu (DGRC, Kyoto; Kyoto, Japan)
Chuck Langley (At-large; University of California, Davis)
Brian Oliver (FlyBase Advisory Board; NIH)

Genetics Society of America:

Lynn Cooley, GSA Board of Directors (lynn.cooley@yale.edu)
Suzy Brown, Senior Director (sbrown@genetics-gsa.org)
Tracy DePellegrin, Executive Director (tracey.depellegrin@thegsajournals.org)