Technique Notes

Fruit and vegetable food media suitable for maintaining *D. melanogaster* flies.

Kekic, Vladimir, and Sofija Pavkovic Lucic. Institute of Zoology, Faculty of Biology, Studentski trg 16, 11000 Belgrade, Serbia and Montenegro.

Drosophila melanogaster is a fruit specialist (Basden, 1954), although it may use decaying plants like tomato and onion (Atkinson and Shorrocks, 1977). Herewith we recommend four recipes for preparing media suitable for maintaining *D. melanogaster*, which represent a standard laboratory food (cornmeal medium) modified by adding fruits (banana, apple) or vegetables (tomato, carrot), without sugar and yeast. These substrates may be convenient for flies usually cultured under laboratory conditions and useful for different experimental purposes, *e.g*. when testing food preferences or life-history traits.

Table 1. Ingredients necessary for preparing food substrates convenient for maintaining *Drosophila melanogaster*. Abbreviations: N - Nipagin, E - ethanol.

Ingredients	Tomato medium	Carrot medium	Banana medium	Apple medium
distilled water	50 ml	170 ml	170 ml	170 ml
quantity of fruits/vegetables	225 ml	150 g	150 g	150 g
cornmeal	15 g	5 g	5 g	5 g
agar	3 g	2.5 g	2.5 g	2.5 g
fungicide	0.5g N/5ml E	0.5g N/5ml E	0.5g N/5ml E	0.5g N/5ml E

Before cooking mash, fruit or vegetable must be well-washed, peeled, and mixed in a blender with recommended quantity of distilled water. During winter months, when fresh tomatoes may not always be available (or is too expensive), previously mixed and frozen tomato (kept in plastic bottles in a deep-freeze) may be used.

The development of *D. melanogaster* flies kept on these substrates takes almost equal time as on the standard cornmeal-agar-yeast medium at 25 ºC (10 -12 days), except on the apple medium, where it lasts from several days up to 10 days longer. It seems to be dependent on the quantity of the sugar available in the fruit. Namely, almost the same developmental time on this fruit medium as on other mentioned substrates may be achieved if sweet sorts of apple were used or if some sugar was added. The quantity of ingredients necessary for making substrates shown in Table 1 is enough for preparing 5 culture bottles (250 ml).

In our laboratory, we successfully maintained *D. melanogaster* lines over sixty generations on the above mentioned media. We believe that these substrates may also be used in culturing some other *Drosophila* species that have similar nutritional requirements like *D. melanogaster*.

References: Atkinson, W., and B. Shorrocks 1977, Oecologia (Berl.) 29: 223-232; Basden, E.B., 1954, Trans. Roy. Soc. Edinb. 62: 602-654.

FORTRAN Program Generates Effective Artificial Courtship Song[1].

Talyn, Becky,[*] and Harold Dowse[+]. [*]Department of Biology, Denison University, Granville, OH; Current address Department of Biology, California State University, San Bernardino and University of California, Riverside; [+]Department of Biology, University of Maine, Orono.

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Examinations of animal communication functions often involve playing sounds and observing responses. Studies may use three designs: 1) one recording used repeatedly or modified to make treatments, 2) multiple exemplars per treatment using naturally-occurring differences among treatments, or 3) artificial signals that mimic natural sounds. Option three provides a unique opportunity to control each sound parameter independently, without risking pseudoreplication by exploiting one exemplar (Kroodsma, 1989). We used a FORTRAN computer program to create *Drosophila* songs with identical parameters, or differing in a single parameter. These resembled recorded songs; males and females responded to them as biologically meaningful.

GENERATED SONGS. Our program creates songs employing 22 user-set parameters (Talyn and Dowse, in press). Stochasticity is introduced, making songs more natural and rendering songs with otherwise identical parameters different in temporal structure. For example, each song begins with silence of duration 0.5 * (*inter-train interval* * (1 - *interval variation*) + 2 * *inter-train interval* * *random number range 0-1* * *inter-train interval*). Therefore the average initial silence is half the duration of the average inter-train interval, but differs depending on *random number*. This approach is used throughout the program to add variability to most parameters.

Pulses are sine waves of set frequency, with a parabolic amplitude envelope of set duration and frequency. Pulse trains are organized with set inter-pulse interval and pulses per train, and inter-pulse interval cycles (cycles reviewed in Alt, *et al.* 1998 and others). Sine trains are sine waves of set amplitude, frequency, and duration. Parameter values used were published or measured averages for *D. melanogaster* (Talyn and Dowse, in press). Average inter-pulse interval was set at 44 ms instead of 28 ms.

PLAYBACK EXPERIMENTS. We verified the effectiveness of artificial vs. recorded songs by playing each song to two groups of ten pairs simultaneously (40 pairs/song; 5 artificial and 15 recorded songs). We measured courtship effort (CE), the average proportion of unmated males courting, and mating amount, the cumulative number of pairs.

To verify that flies interpret artificial songs as courtship, we used the observation that males increase movement and court nearby males during playbacks (Crossley *et al.,* 1995). We observed one winged and one wingless male under a microscope (12×), and counted wing flicks using event recorder software written in Turbo Basic® by H.D. Playback sets consisted of one-minute each of three artificial sounds – pulse, sine and white noise – presented to different males in all 6 orders.

RESULTS. Females responded to generated song as a biologically meaningful signal. Mating amount was consistent between recorded and artificially generated treatments, both higher than silence (Figure 1a). Wingless males courted more when played recorded song than during no-song controls (Tukey post-hoc test, df = 104, p = 0.039). However, CE did not differ during generated vs. recorded songs (p = 0.143) or any other combination.

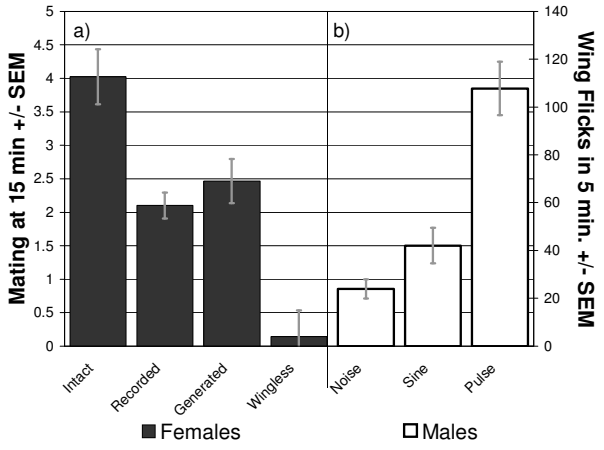

Figure 1. a) Females respond to recorded and artificial courtship song. Both elicit significantly more mating with wingless males than in the absence of playback. b) Males respond to pulse and sine song as biologically relevant signals.

Males responded to artificial song by wing flicking at a cohabitating male. More flicking occurred during pulse or sine than during white noise (Figure 1b; Bonferoni adjusted paired t-test), indicating that both sounds stimulated the auditory system.

SUMMARY. This program generated songs that males and females recognized as meaningful signals. Females mated during artificial and recorded songs. Males responded to pulse and sine components. Importantly, the program could generate many animal sounds, particularly those with amplitude, rather than frequency, modulated structures. With some simple modifications, it could generate more complex sounds, allowing researchers to avoid problems encountered when using recorded song playbacks.

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Drosophila melanogaster as an *in vivo* model for somatic cell genotoxicity of chemicals by Comet assay.

Siddique, H.R., A. Dhawan[1], D.K. Saxena, and D. Kar Chowdhuri[2]. Embryotoxicology Division, [1]Developmental Toxicology Division, Industrial Toxicology Research Centre, P.O. Box 80, M.G. Marg, Lucknow – 226 001, U.P., India. [2]Corresponding Author: Embryotoxicology Division; Industrial Toxicology Research Centre; P.O. Box No. 80; M.G. Marg; Lucknow 226 001; Uttar Pradesh; India; Tel: +91-522-2227586; Fax: +91-522-2228227.

Issues of animal use and care in toxicology research and testing have become one of the fundamental concerns for both science and ethics. Emphasis has been given for the use of alternatives to mammals in testing, research, and education.

For several decades *Drosophila* has been in use widely as an insect model, because of its well-elucidated genetics and developmental biology. Moreover, its use has been recommended by

the European Centre for the Validation of Alternative Methods (ECVAM), whose goal is to promote the scientific and regulatory acceptance of alternative methods, which reduce, refine, or replace the use of laboratory animals (Festing *et al.,* 1998). In the recent years, *Drosophila* has evolved as one of the model organisms in toxicological studies (Kar Chowdhuri *et al*., 1999, 2001; Mukhopadhyay *et al.,* 2002).

Table 1. Comet parameters to show DNA damaging potential of EMS, MMS, CPM and ENU in *Drosophila* gut cells

Groups	Tail DNA (%)	OTM (arbitrary unit)	Tail Length (□m)
Control	8.67 ± 0.47	0.70 ± 0.04	5.23 ± 0.31
EMS: 0.05mM	8.28 ± 0.46^{ns}	0.68 ± 0.02^{ns}	5.46 ± 0.36^{ns}
EMS: 0.50mM	14.72 ± 0.50**	1.31 ± 0.03**	17.90 ± 0.33**
EMS: 5.00mM	17.01 ± 0.95**$^{\$}$	2.97 ± 0.11**$^{\$}$	31.06 ± 0.65**$^{\$}$
MMS: 0.05mM	8.61 ± 0.69^{ns}	0.69 ± 0.04^{ns}	4.99 ± 0.31^{ns}
MMS: 0.50mM	12.38 ± 0.60**	1.15 ± 0.04**	14.88 ± 0.47**
MMS: 5.00mM	14.95 ± 0.65**$^{\#}$	1.69 ± 0.07**$^{\$}$	24.33 ± 0.38**$^{\$}$
ENU: 0.05mM	8.06 ± 0.70^{ns}	0.64 ± 0.42^{ns}	5.54 ± 0.31^{ns}
ENU: 0.50mM	7.06 ± 0.66*	0.60 ± 0.05 ^{ns}	5.07 ± 0.50^{ns}
ENU: 5.00mM	12.85 ± 0.53**	1.37 ± 0.05**	17.65 ± 0.34**
CPM: 0.05mM	8.06 ± 0.70^{ns}	0.64 ± 0.043^{ns}	5.92 ± 0.56^{ns}
CPM: 0.50mM	12.80 ± 0.62**	1.57 ± 0.6**	22.56 ± 0.67**
CPM: 5.00mM	14.89 ± 0.65**$^{\#}$	1.94 ± 0.08**$^{\$}$	26.85 ± 0.53**$^{\$}$

Values are mean ± S.E. of 100 counts. Ns = non-significant vs control ($p > 0.05$); *= vs. control $p < 0.05$; ** = vs. control $p < 0.001$; $^{\#}$ = vs. ENU $p < 0.02$; $^{\$}$ = vs. ENU $p < 0.001$. Ethyl methanesulphonate (EMS), Methyl methanesulphonate (MMS), Cyclophosphamide (CPM) and N-Ethyl-N-nitrosourea (ENU).

The single cell gel electrophoresis (SCGE) assay, also known as the Comet assay, is one of the most promising genotoxicity tests developed in the recent years, for assessment of DNA damage in single cells. Its simplicity and the ability to be performed in cells of any tissue have made it an acceptable tool for biomonitoring and genetic toxicology.

Our earlier studies have shown the usefulness of *Drosophila melanogaster,* as an *in vivo* model for assessment of genotoxicity of a synthetic pyrethroid insecticide – cypermethrin using the alkaline Comet assay (Mukhopadhyay *et al*., 2003).

Due to relatively small cell size, a number of changes were made in the general protocol of the Comet assay. Low melting point agarose was used at 1.5% (0.75% final concentration) in contrast to 1% (0.5% final concentration) generally used and recommended (Tice *et al.*, 2000). A major modification made in the present study was in the composition of lysing solution and the time for unwinding and electrophoresis as compared to that used by Bilbao *et al.* (2002). Dimethyl sulphoxide (DMSO) was removed from the lysing solution, which is conventionally added to scavenge radicals generated by the iron released from hemoglobin (Singh *et al*., 1988). No such heme groups are present in *Drosophila*. An earlier study has also shown that a dietary concentration of over 0.3% DMSO was cytotoxic to *Drosophila melanogaster* (Nazir *et al*., 2003a). Although, Bilbao *et al.* (2002) used 2-h lysis and 20-min unwinding and electrophoresis of neuroblast cells of *Drosophila,* we optimized the experimental condition and reduced the time of unwinding and electrophoresis to 10 and 15 min. respectively, resulting in improvement in the performance of the assay (Mukhopadhyay *et al*., 2003).

In the present study, we used four DNA damaging agents, ethyl methanesulphonate (EMS), methyl methanesulphonate (MMS), cyclophosphamide (CPM), and N-ethyl-N-nitrosourea (ENU) at 0.05, 0.5 and 5.0mM concentrations to evaluate the efficacy of the Comet assay in somatic cells (gut cells) of *Drosophila*. All the chemicals showed a dose dependent increase in the DNA damage as evidenced by an increase in the Comet parameters viz. Olive tail moment (arbitrary units), tail length (μm) and tail DNA (%) (Table 1). While the lowest concentration of the chemicals did not induce any significant DNA damage ($p > 0.05$) in the cells from exposed *Drosophila*, a significant induction ($p < 0.05$) in DNA damage was observed in the two higher concentrations (0.5mM and 5.0mM) of all

the chemicals except ENU (Table 1). When comparisons among these four chemicals were made in the context of their DNA damaging potential, we observed that EMS, MMS, and CPM have the higher DNA damaging potential as compared to ENU, which appears to be the least DNA damage-inducing agent.

This study indicates *Drosophila melanogaster* as a model for *in vivo* genotoxicity assessment and also shows the usefulness of the modified method of comet assay for the evaluation of *in vivo* genotoxicity in somatic cells of *Drosophila melanogaster.*

References: Bilbao, C., J.A. Ferreiro, M.A. Comendador, and L.M. Sierra 2002, Mutation Research 503: 11-19; Festing, M.F.W., V. Baumans, D.R. Combes, M. Halder, F.M. Hendriksen, B.R. Howard, D.P. Lovell, G.J. Moore, P. Overend, and M.S. Wilson 1998, Alternatives to Laboratory Animals 26: 283-301; Kar Chowdhuri, D., D.K. Saxena, and P.N. Viswanathan 1999, Pesticide Biochemistry and Physiology 63: 15-25; Kar Chowdhuri, D., A. Nazir, and D.K. Saxena 2001, Journal of Biochemistry and Molecular Toxicology 15: 173-186; Mukhopadhyay, I., A. Nazir, K. Mahmood, D.K. Saxena, M. Das, S.K. Khanna, and D. Kar Chowdhuri 2002, Cell Biology and Toxicology 18: 1-11; Mukhopadhyay, I., D. Kar Chowdhuri, M. Bajpayee, and Dhawan Alok 2003, Evaluation of *in vivo* genotoxicity of cypermethrin in *Drosophila melanogaster* using alkaline comet assay. Mutagenesis (in press); Nazir, A., I. Mukhopadhyay, D.K. Saxena, and D. Kar Chowdhuri 2003a, Toxicology Mechanisms and Methods 13: 147-152; Singh, N.P., M.T. McCoy, R.R. Tice, and E.L. Schneider 1988, Experimental Cell Research 175: 184-191; Tice, R., E. Agurell, D. Anderson, B. Burlinson, A. Hartmann, H. Kobayashi, Y. Miyamae, E. Rojas, J.C. Ryu, and Y.F. Sasaki 2000, Environmental Molecular Mutagenesis 35: 206-221.

Density separation of larvae using polyethylene glycol.

Khurana, Sukant. National Centre for Biological Sciences, Bangalore 560065, India. Present address: Section of Neurobiology, School of Biological Sciences, University of Texas at Austin, Austin, Texas 78712, USA. sukant@mail.utexas.edu

Abstract

Large populations of larvae, required for many behavioral assays are difficult to be obtained by individual brush picking from the growth medium. Polyethylene glycol was found as a suitable replacement of sucrose, for density separation. Polyethylene glycol is a non-physiological agent and was found to have no adverse effect on olfactory response of the larvae.

Introduction

The ability to test large populations of *Drosophila* sets it apart from many other genetically amenable systems to study behavior. Testing statistically large populations allows for isolation of even subtle behavioral phenotypes. Large populations of larvae cannot be taken out of bottle, as can imago, by thumping the bottle. When large populations of larvae are required, individual picking of larvae by a brush is not practical. Here, an earlier method of separation of larvae is evaluated and an alternative method using polyethylene glycol solution is suggested.

Experimental Procedures

Synchronous cultures of larvae were reared on standard cornmeal yeast medium at 24°C and

maintained on a 12 h day/night cycle. Standard procedures were followed in handling cultures (Ashburner and Thompson, 1978; Roberts, 1988).

To collect early third instar larvae, the cornmeal medium bottles having larvae of the appropriate age were briefly filled with tap water. With a thick paint brush the sides and the top of cornmeal medium were gently disturbed. The contents of the bottle were upturned on a fine sieve. The larvae, along with the debris sticking to them, were transferred with a brush to a beaker filled with 30% Polyethylene Glycol solution (carbowax 6000). After five minutes the cornmeal debris settled at the bottom of the beaker, while larvae floated up. The top contents of the beaker, having the larvae, were gently poured on a sieve. For half a minute gently running tap water was allowed to flow over the sieve. The larvae, free of debris, were transferred to a Petri plate having a very thin layer of *Drosophila* Ringers - Agar (2%). For sucrose separation exactly same procedure was followed, except that sucrose was used instead of polyethylene glycol.

The olfactory response of larvae was measured using the olfactory assay described by Heimbeck *et al*. (1999). The numbers of larvae in odor and control zone were counted from the images obtained by Intel image capture. Counting from photographs eliminated the timing error in measurement. The response index was defined as:

(larvae in odor zone – larvae in control zone)/(total larvae in odor and control zones).

Liquid paraffin was used as a diluent for all the odorants tested. All the fine reagents used were procured from Sigma Aldrich. Errors were calculated as standard deviation of mean of means. Student's t test was used to calculate significance values

Results and Discussion

Large numbers of larvae have been separated in earlier studies by density separation employing 3M NaCl (Roberts, 1988) and 20%w/v sucrose (Nothiger, 1970) solution. Separation using 3M NaCl (Roberts, 1988), is known to have much adverse effect than sucrose on behavior and is not employed by people studying olfaction and gustation. This is due to high osmolarity of salt solution. Larvae are usually waited for 15 to 30 minutes after sucrose separation, to give them time to recover from the shock. Thus experiments cannot be conducted immediately. Moreover sucrose solution separation can act as food, biasing results in a reward conditioning experiment. Hence it became necessary to look for alternatives of sucrose solution separation.

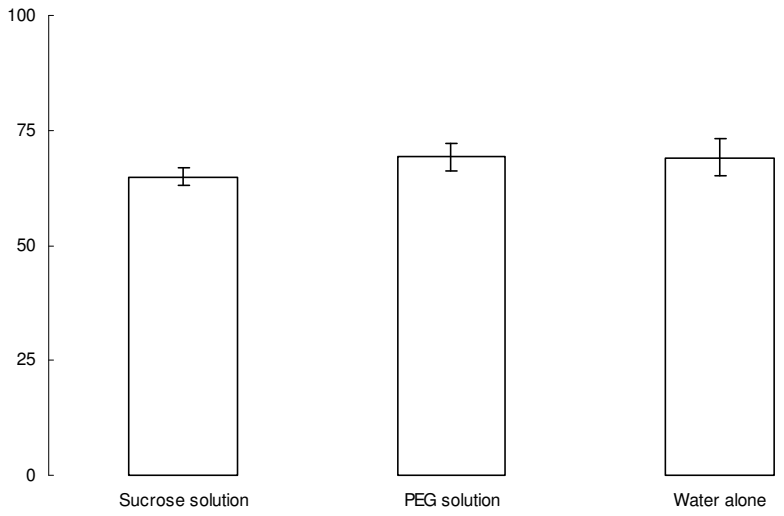

Figure 1. Response of larvae to 10^{-4} Ethyl acetate after different methods of separation from cornmeal medium. The response was tested after 30 minutes of waiting in Ringers–agar (2%). N = 10 independent experiments for each data point.

Varying concentrations of polyethylene glycol were tried to obtain optimal separation of larvae from the cornmeal medium debris. In 20% and weaker solutions of polyethylene glycol most

larvae stayed at the bottom of the beaker, along with debris. In 35% and stronger solutions the debris also started to float up, along with larvae. 28% to 30% polyethylene glycol solution was found to be suitable to recover almost all larvae, without any debris sticking on them. The same method can be employed to separate any instar of larvae from debris. The different instars of larvae did not show clear cut differences in density for this method to be employed for separating larvae of one stage from a mixed population.

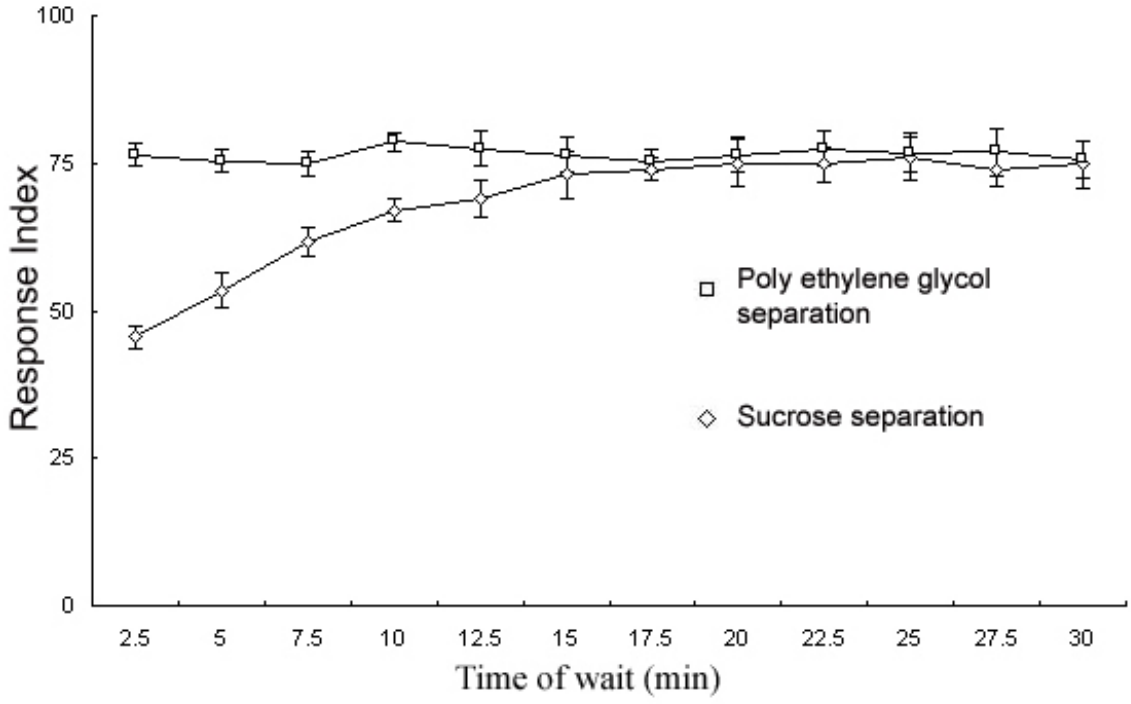

Figure 2. Recovery of response after sucrose (open diamonds) and polyethylene glycol (open squares) separation to 10^{-3} ethyl acetate. Polyethylene glycol separation did not induce any reduction in olfactory response, while a significant reduction in response was observed after sucrose separation. N = 8 independent experiments for each data point.

To verify if polyethylene glycol causes any adverse effect on olfactory response, controls with sucrose separation (Nothiger, 1970) and water washing alone were tested from the same batch of larvae. The water washed set of larvae were individually picked by a brush and washed many times. All the larvae were kept waiting in Ringers agar (2%) for 30 minutes before testing the olfactory response to 10^{-4} ethyl acetate. The responses of larvae, separated by polyethylene glycol solution were found to be no different from ones separated by other means (Figure 1).

To test if separation using polyethylene glycol solution can reduce the time of wait required after sucrose separation, olfactory response of larvae were measured after different time periods of waiting on a thin layer of Ringers-agar (2%). There was no reduction in response to 10^{-3} ethyl acetate after separation from polyethylene glycol solution, while a drastic reduction in olfactory response was observed after using sucrose separation (Figure 2). This is because of the low molar concentration of PEG in solution compared to sucrose. The other possible reason for this phenomenon could be because Polyethylene glycol is not a physiologically active agent like sucrose.

Thus polyethylene glycol was established as a suitable substitute for sucrose in density separation of larvae.

Acknowledgments: Without the help of Professor Obaid Siddiqi, this work would not have been possible.

References: Ashburner, M., and J.N. Thompson, jr. 1978, *In*: *The Genetics and Biology of* Drosophila, volume 2a (Ashburner, M., and T.R.F. Wright, eds.), pp. 1-109. Academic Press, New York; Nothiger, R., 1970, Dros. Inf. Serv. 44: 177; Roberts, D.B., 1998, Drosophila: *A Practical Approach*, 2nd edition. Oxford University Press, Oxford.

Male courtship intensity: a new method of *Drosophila* courtship measurement.

Ruedi, Elizabeth A.,[1] Yaa N. Owusu, and Kevin Dixon. [1]Program in Ecology and Evolutionary Biology, University of Illinois at Urbana-Champaign, Urbana, Illinois, 61801.

Male *Drosophila* exhibit complex courtship behavior that has been extensively documented (Hall, 1994). These behaviors are sequentially ordered, and include orienting toward the female, chasing her, tapping her abdomen, using wing vibrations to produce a courtship song, licking her genitalia, attempting copulation, and copulation (Hall, 1994; Sokolowski, 2001; Emmons and Lipton, 2003). These behaviors are induced by chemosensory cues, and assist with species recognition and assessment of the sex and receptivity of the partner (Hall, 1994; Emmons and Lipton, 2003). Each behavior therefore plays a fundamental role in successful copulation (*e.g*., Tauber and Eberl, 2001). Analysis of these complex behaviors in a model organism such as *Drosophila melanogaster* is also an asset for disciplines such as behavioral genetics and quantitative genetics.

The traditional quantitative measurement of male *Drosophila* courtship behavior is the courtship index (CI), which is the proportion of total time observed that a male performs any courtship behavior (*e.g*., Gaines *et al.,* 2000; Orgad *et al.,* 2000). This measurement indicates the amount of time and energy that a male fly invests in pursuing a potential partner and can be very informative in making comparisons between different groups of flies. However, the CI measurement provides no information about the specific behaviors that the male is performing or the pattern of behavior seen over the course of the trial. To specifically address these types of questions, we have created an ordinal courtship intensity scale in which the lowest scores reflect behavior not usually associated with courtship, and the highest scores reflect the behaviors that immediately precede copulation, as well as copulation itself (Table 1). This scale could be particularly informative in situations comparing a male that spends the majority of a behavior trial following the female but not advancing his courtship to a male that performs wing vibrations for only a few minutes; the first male would receive a higher CI score, but that would not reflect the general intensity of his courtship. We used this scale to measure the male courtship intensity of two different populations that would presumably differ in courtship behavior (outbred vs. inbred) in two

Table 1. Male Courtship Intensity Scale

1.	Motionless
2.	Moving
3.	Orienting toward female
4.	Following/chasing female
5.	Wing vibrations
6.	Licking genitalia
7.	Attempted copulation
8.	Copulation

* Note: Tapping not included because it cannot be accurately recorded by the naked eye.

different mating situations to examine the effectiveness of this measurement.

We analyzed the differences in male mating behavior of two populations of *D. melanogaster*, Ives and Raleigh isogenic line 83 (R83). The Ives population is a large, randomly mating stock adapted to laboratory conditions. The R83 population is one of a set of third chromosome isogenic lines created in Dr. Trudy Mackay's laboratory and obtained from Dr. Jeff Leips. All chromosomes except the third are derived from the completely inbred Samarkand stock (De Luca *et al.,* 2003). Considering the extent of inbreeding in the R83 stock, we hypothesized that the R83 population would have significantly lower male courtship intensity than the Ives outbred population.

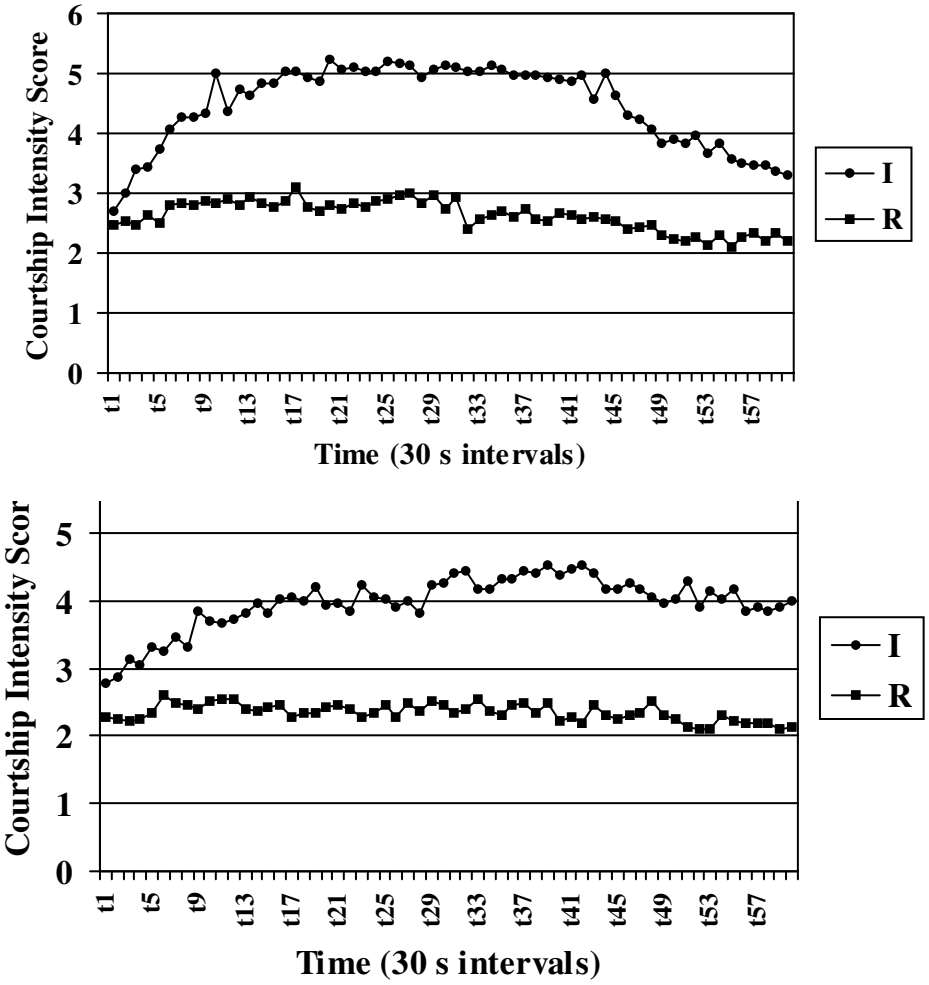

Figure 1. Male courtship intensity of Ives (I) and R83 (R) populations. a) Non-competitive mating trials, b) Competitive mating trials.

We also wanted to determine if this method of measurement could detect differences in courtship behavior in different mating situations. We placed 32 Ives and 31 R83 males in non-competitive situations (1 male:1 female), and 32 Ives and 30 R83 males in competitive mating situations (2 male:1 female). The females and competitor males used in these experiments were from our *ebony* stock (*e/e* mutation on Ives background), which is a large, randomly mating population.

We collected male and female virgins from stock vials no more than four hours post-eclosion. These virgins were separated by sex and type, and were housed on standard cornmeal media in an

incubator at 25ºC with a 12h:12h light:dark cycle. The experiments began once the virgin males were at least two days old, though most males were five to seven days old. Behavior trials took place in an 8-dram glass vial filled 2/3 full with media, allowing for an approximately 1" deep mating arena. In the non-competitive trials, the focal Ives and R83 males were individually aspirated into a vial before the mating trials began. In the competitive trials, the focal males were added to the vial along with an *ebony* competitor. In both situations, the *ebony* females were first individually aspirated into a regular stock vial, and then transferred into the behavioral vial by placing the two vials together and tapping.

During the behavior trials, the behavior of the focal male was recorded every 30 seconds by instantaneous scan sampling (Martin and Bateson, 1993) for a total of 30 minutes. At each scan, the focal male was observed and the number of the courtship intensity scale corresponding to his behavior was recorded. Once the 30-minute trial was completed, the flies were lightly gassed and the females were removed to a new stock vial for housing overnight (the males were left in the behavior vials). The trials were repeated two more times in the next two days using the same males (always in the same mating conditions as the first trial) and females (females were randomly assigned to new males each day). Thus we had information on virgin males and males when they had accomplished successful copulation. We analyzed the data using repeated measures ANOVA in JMP (SAS Institute, 2003), and included the effect of period (day) and male type in the model as well as the effect of individual subject and time (changes in male behavior over the course of one trial).

The differences in courtship intensity between the two groups of males were significant in both mating situations (Table 2). As expected, the Ives outbred population had higher courtship intensity than the R83 population (Figure 1). In both mating situations, the effect of time*subject was significant, which indicates that the mating pattern of each individual male is different (which is not surprising considering the influence of female behavior on male response). In competitive conditions, the effect of time*type was significant. Therefore, this method of courtship measurement shows promise for examining the general behavioral patterns of groups of males over the course of the trial.

Table 2. Repeated Measures ANOVA for Non-competitive and Competitive Trials

	Non-Competitive Trials		Competitive Trials	
Effect	F(numDF,denDF)	P-value	F(numDF,denDF)	P-value
Period (Day)	2.04(1,112)	0.1561	3.19(1,103)	0.0771
Subject	1.32(60,112)	0.1045	1.11(59,103)	0.3236
Type	52.45(1,112)	<0.0001***	39.73(1,103)	<0.0001***
Time	1.25(59,54)	0.2042	0.95(59.45)	0.5813
Time*Type	1.45(59,54)	0.0860	2.18(59,45)	0.0037**
Time*Subject	1.07(3540,2944.3)	0.0306*	1.07(3481,2538.3)	0.0428*

The courtship intensity scale has proven to be a valid method of measurement for male courtship behavior in these circumstances. It provides different information than the courtship index, allowing the observer to examine the intensity of the courtship instead of the total time spend courting, as well as the pattern of courtship intensity over time. This measurement should be useful in many different disciplines, and in a model organism such as *Drosophila melanogaster* can provide information about behavior that can be used for quantitative genetic analysis or gene expression analysis.

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Do it in the dark.

Lerach, Stephanie L. Department of Biochemistry, Biophysics, and Molecular Biology, Iowa State University, Ames, IA 50011.

Drosophila researchers have often encountered specific phenotypic pairings that are troublesome to mate. It has been observed, for example, that a wild-type female fly will preferentially mate with a wild-type male over a male fly with yellow body color (Sturtevant, 1915). This dilemma can be particularly frustrating, because mutant body color phenotypes, such as yellow or ebony, are frequently included as markers on balancer chromosomes. In one instance, matings of wild-type females with yellow-bodied, curly-winged males consistently gave no progeny. Each individual strain is reasonably healthy in stock, but examination of eggs collected from their combined mating showed a lack of fertilization. If wild-type female flies were possibly rejecting potential mates due to their differing physical appearance, it seemed plausible that they may be more receptive in the dark. It can be difficult to keep incubator environments in complete darkness, so a less technological alternative was attempted. Phenotypic combinations that repeatedly showed unsuccessful mating under normal mating conditions were placed in bottles covered with aluminum foil. Ample progeny were produced. This technique has been utilized in the lab for several different types of previously problematic mating combinations with great success.

References: Sturtevant, A.H., 1915, J. of Animal Behaviour 5: 351-366.

Evaluation of an injectable method for embryonic RNA interference (RNAi) using the *Drosophila beltless* gene as an example.

Dzitoyeva, Svetlana, Alan Gutnov, and Hari Manev. The Psychiatric Institute, Department of Psychiatry, University of Illinois at Chicago, 1601 West Taylor Street, MC912, Chicago, IL 60612, USA. E-mail: sdzitoyeva@psych.uic.edu

Introduction

Since the discovery of the RNA interference (RNAi) mechanism of gene silencing, RNAi has became a popular tool in the field of functional genomics (Grant and Wilkinson, 2003). In RNAi,

targeted down-regulation/silencing of gene expression (Fire *et al*., 1998) is triggered by double-stranded RNAs (dsRNAs) that are cleaved into small interfering RNAs (siRNAs) by a dsRNA-specific enzymes. Ultimately, the siRNAs guide the destruction of a cognate mRNA. Specific properties of *Drosophila* cell membranes make the use of in vitro RNAi relatively easy. Thus, a "dsRNA" approach in *Drosophila* cell cultures is frequently used to study individual genes or to conduct extensive gene screenings (Boutros *et al*., 2003, Lum *et al.*, 2003). In vivo injection of dsRNA into adult Drosophila is a powerful method for triggering RNAi and for inducing gene silencing that does not interfere with developmental mechanisms (Dzitoyeva *et al*., 2001, 2003a,b; Goto *et al*., 2003).

On the other hand, RNAi offers advantages when used selectively to understand the developmental role of genes because when mutated, many developmentally-important genes cause early lethality. An example of utilizing RNAi in finding phenotypes related to a novel gene is given by our recent study that characterized the *Drosophila beltless* gene (Dzitoyeva *et al.*, 2003b). According to computational analysis, the beltless protein belongs to a family of acyltransferases and is crucial for oogenesis. This is demonstrated by adult injectable RNAi and for embryogenesis, as shown by embryonic injectable RNAi. We named the gene "beltless" based on cuticular abnormalities induced by embryonic RNAi.

Since injecting embryos could cause mechanical damage that might interfere with morphological phenotypes, the question arises of whether an injectable embryonic dsRNA-induced RNAi produces only gene-silencing-specific phenotypes. Furthermore, the efficiency of embryonic injectable RNAi has not been established precisely, i.e., what is the proportion of injected embryos displaying such specific phenotypes? To answer these questions, we analyzed embryos injected with various double-stranded RNAs, one *Drosophila*-specific, *i.e*., the beltless dsRNA, and another Drosophila-unrelated control dsRNA. As an additional control, we also injected embryos with a buffer solution without dsRNA.

Materials and Methods

dsRNA synthesis

We targeted the 22-nucleotide long region 2-23 of the *blt* gene (CG5652) and the 22nt of the human 5-lypoxygenase (5-LOX) cDNA sequence (a *Drosophila*-unrelated gene). Eight 39-mer DNA oligonucleotides with an attached T7 RNA polymerase promoter sequence were synthesized (Integrated DNA Technology, Inc.); these oligonucleotides represented both sense and antisense strands. The sequences of the sense and antisense DNA oligonucleotides corresponding to the *blt* and 5-LOX genes used for the *in vitro* transcription reaction are shown in Table 1; only one match was found in the *Drosophila* genome database pattern search analyses for the *blt* gene and no match for 5-LOX sequences.

Both oligonucleotides were designed to produce dsRNA probes with a 3'overhanging 2UU nt. Equal amounts of oligonucleotides were annealed to form a double-stranded template by heating at 80˚-85˚C for 5 min and cooling on ice. The *in vitro* transcription reaction (30 µl volume) for the synthesis of the 22nt RNA run of transcripts contained 0.1 µg of a template, 500 µM each CTP, GTP, ATP, and UTP, 1× transcription buffer (Tris-HCl, pH 7.5, 10 mM dithiothreitol, 1% bovine serum albumin), 20 U of RNAse inhibitor, and 50 U T7 RNA polymerase (Gibco BRL). The reactions were carried out at 37ºC for 1 h. The RNA molecules were annealed together in heat denaturing conditions (65˚-70˚C for 5 min) and placed on ice. The quality of both RNA and DNA oligonucleotides was analyzed on 6% NuSive agarose gel (Sigma).

Injections

Canton S female flies were placed in removable vials on agar/grape juice plates. Syncytial blastoderm stage embryos were collected every 30-40 min from the plates, placed on a small piece of filter paper, and washed thoroughly with distilled water to remove traces of food. Thereafter, the embryos were aligned on a strip of double-sided adhesive tape attached to a glass slide, air-dried for 5 min, and covered with mineral oil. Injection of embryos was performed using the Nanoliter 2000 injector (World Precision Instruments) set at 69 nl. Embryos were injected with *blt* dsRNA, 5-LOX dsRNA, or with the injection buffer (0.1 mM sodium phosphate, pH6.8, 5 mM KCl). We noticed earlier that the remaining chorion significantly increases the survival rate of the embryos. Thus, the chorion was left intact to the end of experiments. The injected embryos were staged under mineral oil at 25°C in a humidified chamber and examined 15-20 hr later. These were 356 embryos injected with *blt* dsRNA, 381 with 5-LOX dsRNA, and 330 were injected with injection buffer only. The concentration of double-stranded RNAs for both genes, *blt* and 5-LOX, was 100ng/µl.

Cuticle preparation

Embryos were manually dechorionated, covered with mineral oil on a glass slide, and flattened with a cover slip. They were steam-fixed on top of boiling water for 1 min, the cover slips were slowly removed and the mineral oil was replaced with a mounting solution (glycerol/NaCl). The vitteline membrane and all debris were manually removed and covered with a cover slip. The preparations were examined under a microscope and photographed.

Table 1. List of DNA oligonucleotides used for in vitro double-stranded RNA synthesis.

T7 promoter **(bold characters)** and targeted cDNA sequences (22 nt)	
Sense *blt*	**5'-TAATACGACTCACTAT**ATGCGCCGATCGCCTGGTGTTT-3'
Antisense *blt*	5'-AAACACCAGGCGATCGGCGCA**TATAGTGAGTCGTATTA-3'**
Sense *blt*	**5'-TAATACGACTCACTAT**AAAACGCGGCTAGCGGACCACA-3'
Antisense *blt*	5'-TGTGGTCCGCTAGCCGCGTTT**TATAGTGAGTCGTATTA-3'**
Sense *5-LOX*	5'-**TAATACGACTCACTATAT**TCATGCACATGTTCCAGTCTT-3'
Antisense *5-LOX*	5'-AAGACTGGAACATGTGCATGAA**TATAGTGAGTCGTATTA**-3'
Sense *5-LOX*	5'**-TAATACGACTCACTAT**AAAGTACGTGTACAAGGTCAGAA-3'
Antisense *5-LOX*	5'-TTCTGACCTTGTACACGTACTT**TATAGTGAGTCGTATTA**-3'

Table 2. Summary of the injection results.

	Injection buffer	5-LOX dsRNA	blt dsRNA
Number of embryos injected	330	381	350
Number of embryos died	26 (8%)	73 (19%)	65 (19%)
Embryos with cuticular abnormalities	0	0	186 (65%)

Results and Discussion

As shown in Table 2, we found that dsRNA-injected embryos died at a slightly higher rate than the buffer injected ones. Thus, 65 embryos (19%) from 350 injected with *blt* dsRNA and 73 embryos (19%) from 381 injected with 5-LOX died during the procedure. On the other hand, out of 330 buffer-injected embryos only 26 (8%) died.

We analyzed the surviving embryos for the presence or absence of cuticular abnormalities. We found that out of 285 remaining *blt* dsRNA-injected embryos, 186 (*i.e.*, 65%) developed a number of cuticular abnormalities, whereas none of the remaining embryos injected with 5-LOX dsRNA or with the injection buffer displayed any visible abnormalities (Table 2). This finding points

out the specificity of the RNAi-induced phenotype and suggests an efficiency of about 65%. As observed earlier (Dzitoyeva *et al.*, 2003b), the RNAi-altered cuticular phenotypes in *blt* dsRNA-injected embryos ranged from partially differentiated cuticle to misdirected and only partially-formed denticle belts (Figure 1).

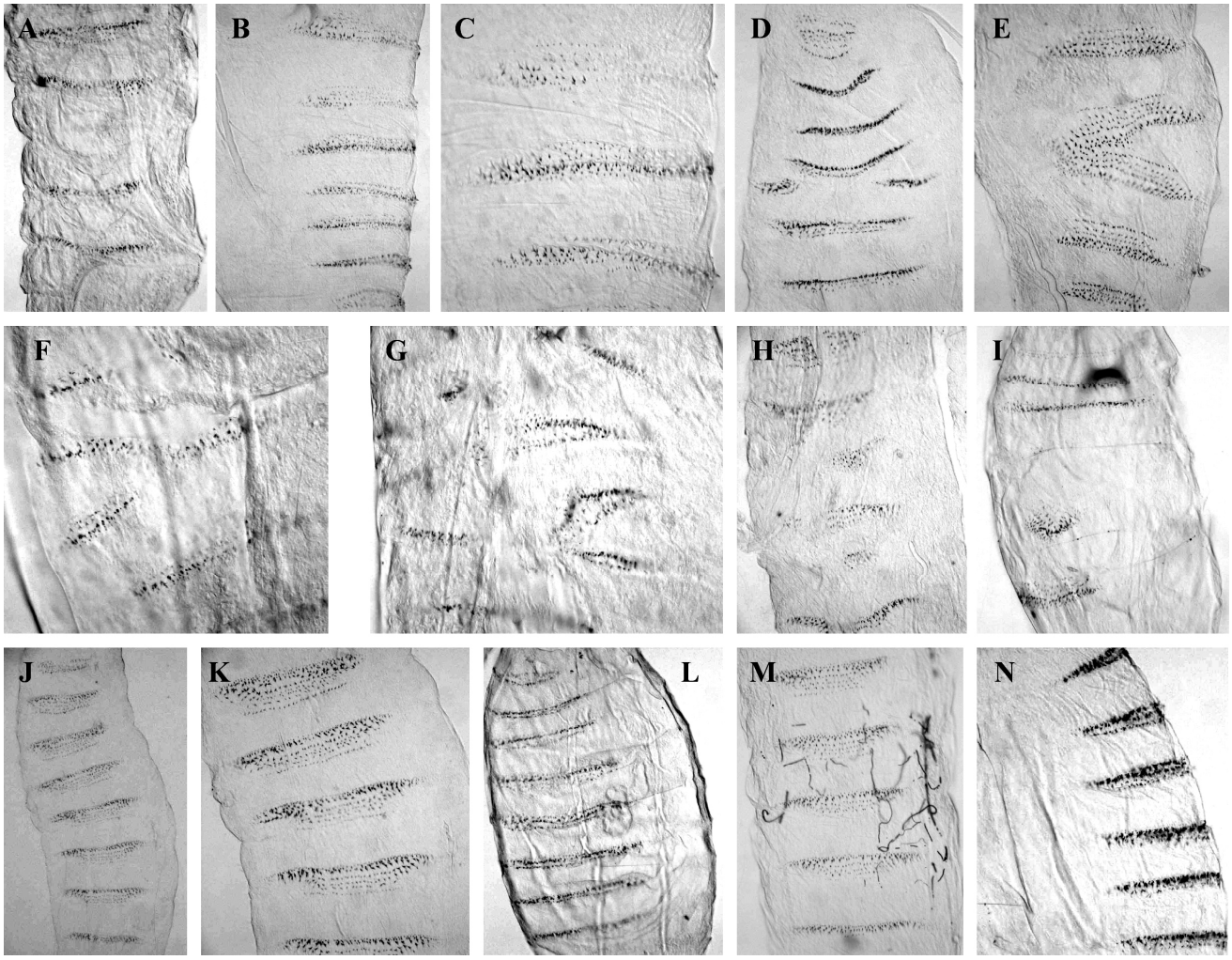

Figure 1. Examples of cuticular phenotypes of the injected embryos. A-I: blt dsRNA; J-L: 5-LOX dsRNA; M & N: buffer injections. Only blt dsRNA caused cuticilar abnormalities: a dentical belt is missing (A-C), it is only partially formed (D), misdirected and fused (E-G), and underdeveloped or not developed (H, I). No visible defects are observed in the cuticle of embryos injected with 5-LOX dsRNA (J-L) and buffer only (M, N).

Multiple phenotypes, including the normal cuticule found in about 35% of the embryos injected with *blt* dsRNA, could result from a possible variability in the actual delivery of the desired injection volume (nominally set at 69 nl) of experimental solutions (*i.e.*, the amount of dsRNA), and from occasional back leakage from injected embryos. The mortality associated with our procedure appears to be in part due to the injecting procedure itself (about 8% in buffer-injected embryos) and it was also increased further (*i.e.*, doubled) due to dsRNA injection. The latter toxicity might be caused

either by some nonspecific RNAi-related phenomenon or it could be due to trace amounts of chemicals, *e.g*., ethanol, left after preparation and purification of dsRNAs.

In conclusion, we demonstrated that injectable dsRNA-induced embryonic RNAi can be used to produce highly specific developmental phenotypes. From a practical point of view, this methodology is associated with an acceptable rate of lethality (10-20%) and with relatively high rate (about 65%) of efficacy. As demonstrated in our earlier work (Dzitoyeva *et al.*, 2003), the injectable embryonic RNAi can identify novel genes and, as shown by others (Misquitta and Paterson, 1999), it can help in characterizing the role of various genes in developmental mechanisms.

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Two preparations for recording ion channel activity in native rhabdomeral membranes of *Drosophila* retinal photoreceptor cells.

Haab, Joan E.,[1, 3] Cecilia Vergara,[2] Juan Bacigalupo,[2] and Peter M. O'Day [1].
[1]Institute of Neuroscience, and Department of Biology University of Oregon, Eugene OR 97403 [2]Department of Biology, University of Chile, Santiago Chile

Summary: We have developed methodologies for generating cellular and membrane preparations for direct electrophysiological recording from ion channels in rhabdomeral membranes from *Drosophila* retinas. A reduced rhabdomeral membrane preparation yields rhabdomeral clusters, from which we have excised membrane patches and measured single events using patch clamp recording techniques. We have also made a preparation of isolated individual photoreceptor cells for patch clamp recording of ion-currents through cell-attached rhabdomeral membranes.

Within the retinal photoreceptor cells of the *Drosophila* compound eye, electrical responses to light are initiated by the opening of ion channels in the rhabdomere. It has been of great interest to study and characterize light-induced ionic currents and the ion channels through which these currents flow. Direct recording of currents through membrane patches using patch clamp methodologies has offered important insights into the roles of individual ion channels in photoreceptor cell function (Hardie, 1991; Hardie *et al.*, 1991; Chyb, *et al.,* 1999; Haab *et al.*, 2000).

The morphology of the *Drosophila* retina poses problems for recording from the ion channels that initiate the electrical response to light. The compound eye is organized into about 750 ommatidia that appear as hexagonally packed facets (Wolff and Ready, 1993; Ready, [online movie]). Each ommatidium contains eight photoreceptor cells (retinulae) and eleven accessory cells. Hardie (1991) and Ranganathan *et al.* (1991) developed methods for dissociating the retina into cellular clusters, consisting of the eight photoreceptor cells tightly grouped (Figure 1A). Each intact cell cluster is organized in such a way that the rhabdomeres from neighboring cells closely appose

one another, and none is patent to the outer circumference of the cell cluster along its length. These retinal dissociation procedures, which expose the photoreceptor cell membranes to the surrounding bath, were developed for the purpose of making whole cell and cell-attached patch electrical recordings with patch electrodes. The development of this procedure has been of enormous value to the field of invertebrate phototransduction. Use of the procedure in cell-attached patch clamp studies has also led to the discovery of a variety of plasma membrane conductances (*e.g.*, Hardie, 1991; Hardie *et al.*, 1991; Ugarte, 2002).

The intact cell cluster preparations, however, are not useful for patch clamp recording directly from the light-activated channels, which are localized in the rhabdomeral membrane (Niemeyer *et al.*, 1996), because the inward facing rhabdomeral membranes are inaccessible to electrodes approaching from the bathing solution. This problem can be overcome in part by finding disrupted cell clusters, common by-products of the cell cluster preparation, and we have made successful recordings from the rhadomeral regions of cells in these structures (unpublished results). Our rate of successful recordings from the rhabdomeral membranes in these disrupted cell clusters was very low; and so, we sought to develop preparations that exposed large portions of the rhabdomeral membranes. We did this in two ways.

Isolated photoreceptor cell preparation

To record from channels within rhabdomeral membranes of functioning photoreceptor cells using cell-attached patch methodology, we generated preparations of isolated cells (Figure 1C). Our technique offered low yields, however. We dissected several eyes from pupae, just prior to eclosion, into a bubble of dissociation solution (Haab *et al.*, 2000) absent of divalent cations. We shook each eye vigorously, holding the eye with forceps, in a fresh bubble of 150 to 250 µl of dissociation solution and then, with a flattened insect pin, scraped the remaining retinal tissue into the bubble. We then drew the bubble into a glass pipet having an aperture of approximately 100 to 200 µm and a maximum diameter of 1 mm in the region where the bubble would sit. Using the pipet, we triturated the bubble 20 to 100 times, being careful not to draw air into the bubble at any time. The resulting triturated preparation only occasionally yielded dozens of isolated individual cells, from which cell-attached patch recordings could be made (unpublished results). Because of the long and slender shape of the cells and their relative rigidity, they moved easily with slight motions in the solution, making recordings very unstable, and rendering moderately rapid solution exchanges destructive to patch-clamped cells, without a more stable anchor.

Rhabdomeral cluster preparation

To record directly from channels within rhabdomeral membranes, using excised patch methodology, we developed a procedure to strip away the extra-rhabdomeral plasma membrane, leaving rhabdomeral membranes intact. We dissected eyes from several adult flies into a bubble of ~50 µL bath solution and then transferred the bubble by pipet into a small Eppendorf tube containing ~500 µL bath solution and approximately 10 to 100 µL zirconia/silicone beads of 100 µm diameter (BioSpec Products, Bartlesville, OK, USA). We shook the tube for ~20 sec at 2,500 rpm using a Mini-Bead Beater (BioSpec Products). We then transferred the solution to the recording chamber by pipet, leaving the beads, which had settled at the bottom of the tube. We waited several minutes before recording, to allow cell fragments to settle to the bottom of the dish mounted to the microscope stage. The bath solution was of standard composition (*e.g.*, Hardie, 1991; Bacigalupo *et al.*, 1995), with modifications appropriate to specific experimental questions. This procedure yields many intact, full length *rhabdomeral clusters*, each a cluster of the eight conjoined rhabdomeres (Figure 1D), and many more partial rhabdomeral clusters. The surfaces of the rhabdomeral clusters

appear smooth in scanning electron micrographs (Figure 1E), as they do in the intact photoreceptor cells (Figure 1C) and disrupted cell clusters viewed by differential interference contrast microscopy (Figure 1B). The microvillar microanatomy of the rhadomeres in the clusters is preserved (Figure 1F). The rhabdomeral clusters are very tractable for patch clamp recording. Employing this preparation, we made the first single event recordings from TRP channels in their native membranes (Haab *et al.*, 2000).

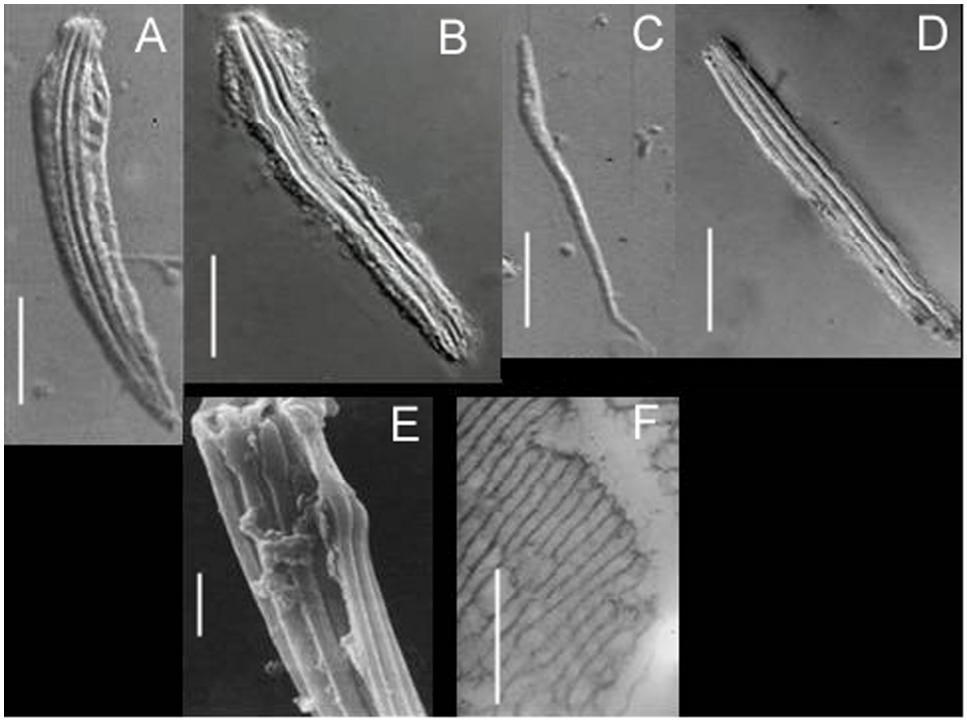

Figure 1. Photoreceptor preparations. A) cell cluster; B) partially disrupted cell cluster; C) isolated single cell; D, E) isolated rhabdomeral clusters; F) rhabdomeral cross section. A-D: Differential contrast interference microscopy; calibration bars = 30 μm; E: Scanning electron micrograph; bar = 5 μm; F: transmission electron micrograph; bar = 0.5 μm.

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Availability of the University of Texas Publications Dealing with *Drosophila*

Marshall R. Wheeler

From 1940 to 1972 many research articles were published by the University Press in the series, "Studies in the Genetics of *Drosophila*" with J.T. Patterson as editor and later (from 1957-1972) with M.R. Wheeler as editor. In 1960 the series title was changed to "Studies in Genetics." There were also a few special issues. Many of these are now out of print (OOP); all known copies of the remaining issues have been made available by Dr. Wheeler.

Some issues were given titles and subtitles, but the Publication Number (*e.g.*, UTP 4213) is the best reference. This is the complete list of all the publications:

1940: UTP 4032 (OOP). 1942: UTP 4213 (OOP). 1942: UTP 4228 (OOP). 1943: UTP 4313, "Drosophilidae of the Southwest" (OOP). 1944: UTP 4445, with "Drosophilidae of Mexico" (OOP). 1947: UTP 4720, "Isolating Mechanisms" (OOP). 1949: UTP 4920 (OOP). 1952: UTP 5204 (25 copies). 1954: UTP 5422 (OOP). 1957: UTP 5721 (45 copies). 1959: UTP 5914, "Biological Contributions." Dr. Patterson's 80th birthday issue (59 copies). 1960: UTP 6014 (16 copies). 1962: UTP 6205 (63 copies). 1966: UTP 6615, Morgan Centennial Issue (28 copies). 1968: UTP 6818 (24 copies). 1969: UTP 6918, W.S. Stone Memorial Issue (12 copies). 1971: UTP 7103 (22 copies). Final volume, 1972: UTP 7213 (29 copies).

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