

## Effects of parental radiation exposure on developmental instability in grasshoppers

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transgenerational genomic instability.

### Abstract

Mutagenic and epigenetic effects of environmental stressors and their transgenerational consequences are of interest to evolutionary biologists because they can amplify natural genetic variation. We studied the effect of parental exposure to radioactive contamination on offspring development in lesser marsh grasshopper *Chorthippus albomarginatus*. We used a geometric morphometric approach to measure fluctuating asymmetry (FA), wing shape and wing size. We measured time to sexual maturity to check whether parental exposure to radiation influenced offspring developmental trajectory and tested effects of radiation on hatching success and parental fecundity. Wings were larger in early maturing individuals born to parents from high radiation sites compared to early maturing individuals from low radiation sites. As time to sexual maturity increased, wing size decreased but more sharply in individuals from high radiation sites. Radiation exposure did not significantly affect FA or shape in wings nor did it significantly affect hatching success and fecundity. Overall, parental radiation exposure can adversely affect offspring development and fitness depending on developmental trajectories although the cause of this effect remains unclear. We suggest more direct measures of fitness and the inclusion of replication in future studies to help further our understanding of the relationship between developmental instability, fitness and environmental stress.

### Introduction

Genomic instability in the form of chromosomal irregularities or genetic mutations can occur because of environmental stressors such as viruses (Wu *et al.*, 2010), plant toxins (Eizirik & Kisby, 1995), mutagenic chemicals (Mishra *et al.*, 2009) and radiation exposure (Dubrova, 2003). This instability occurs in mitotically dividing cells and interferes with the function of tissues and organs (Wong *et al.*, 2003). Transgenerational genomic instability results from genomic instability transferred from the parental germline to the somatic cells of offspring, leading to the destabilization of developmental pathways (Natarajan, 2006). Exposure to external stressors and transgenerational genomic instability, which increases sensitivity of the progeny genome to mutagens

(Hoyes *et al.*, 2001), can have developmental and fitness consequences for individuals inhabiting stressful environments.

Exposure to radiation was one of the first external factors observed to induce transgenerational genomic instability in mammalian models (Sinclair, 1964; Nomura, 1982; Lyon & Renshaw, 1988; Luke *et al.*, 1997; Morgan, 1999). Additionally, transgenerational effects from radiation exposure have also been demonstrated in insects, particularly in the form of inherited sterilization (North & Holt, 1968, 1969; Bloem *et al.*, 1999; Carpenter *et al.*, 2009). Ionizing radiation generates reactive oxygen species (ROS) either by the oxidation of water or secondarily by producing reactive intermediates (Rugo *et al.*, 2002). Accumulation of ROS can result in oxidative stress, defined as the imbalance between ROS generation and the antioxidant defences that neutralize them (Monaghan *et al.*, 2009), which can cause an increase in mutation rates (Riley, 1994). More recently, radioactivity has been linked to inhibition of standard epigenetic

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processes such as methylation (Koturbash *et al.*, 2006), histone modification (Rogakou *et al.*, 1998) and RNA-associated silencing (Jaenisch & Bird, 2003), which may result in genomic instability (Kovalchuk & Baulch, 2008). These mutagenic and epigenetic effects of ionizing radiation and their consequences across generations could lead to amplification of natural genetic variation and provide a source of heritable phenotypic variation (Vasileva *et al.*, 1997; Johannes *et al.*, 2009). Thus, radiation effects may serve as a model for understanding the mechanism producing phenotypic evolution of a population in a stressed environment (Badyaev, 2005).

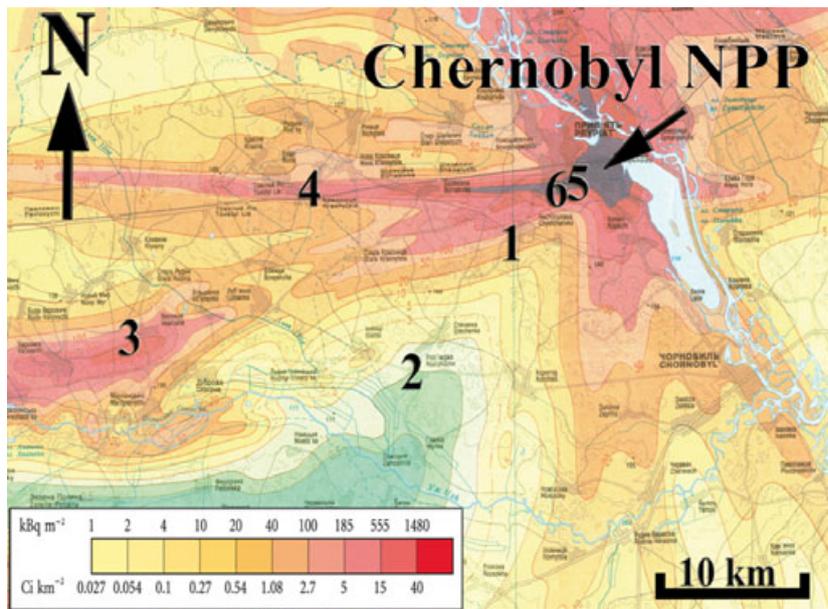
The effects of chronic radiation exposure on growth, development and reproduction have historically been the subject of numerous studies (Marshall, 1962; Brown, 1964; Woodwell, 1967; Woodwell & Rebeck, 1967; Geras'kin *et al.*, 2007). These studies have shown adverse effects on the fitness, longevity and health of living organisms (Zaire *et al.*, 1997; Chen *et al.*, 2001; Nicastro *et al.*, 2002; Møller *et al.*, 2005; Ryabokon & Goncharova, 2006; Veremeyeva *et al.*, 2010). Human populations exposed to high radionuclide contamination have shown increased rates of germline instability (Dubrova *et al.*, 1998), transmissible, clastogenic factors from exposed cells that damage unexposed cells (i.e. bystander effects) (Marozik *et al.*, 2007) and elevated chromosome alterations (Neronova *et al.*, 2003). Recently, Aghajanyan & Suskov (2009, 2010) demonstrated increased levels of abnormal cells and chromosomes in children born after the Chernobyl accident to irradiated fathers (i.e. liquidators) and non-irradiated mothers. These aberrations were analogous to the cytogenetic changes observed in children living in areas contaminated with radionuclides (Aghajanyan & Suskov, 2009, 2010). These results are consistent with previous findings of children of Chernobyl liquidators conceived after the accident showing high genomic DNA mutation levels that decreased with elapsed time between exposure and conception (Weinberg *et al.*, 2001). These studies imply transgenerational consequences of low-dose radiation exposure, although the degree of the effect remains unclear (Slebos *et al.*, 2004; Furitsu *et al.*, 2005).

Conditions in areas such as Chernobyl may serve as a natural laboratory for studying the effects of transgenerational genomic instability on fitness in populations under chronic radiation exposure (Møller & Mousseau, 2006). On 26 April 1986, Reactor Four of the Chernobyl nuclear plant exploded during a test of the reactor's ability to generate power during an unplanned shut down. The resulting explosion released between  $9.53 \times 10^3$  petabecquerel (PBq) and  $1.25 \times 10^4$  PBq of radionuclides into the atmosphere (Konstantinov & Gonzalez, 1989; European Union, 1998; Chernobyl Forum, 2005). Radioactive fallout dispersed across large portions of the Soviet Union and Europe, particularly Ukraine, Belarus and Russia (Shestopalov *et al.*, 2003). Most of the radionuclides dissipated within days, but

Caesium-137, Strontium-90 and Plutonium-239, among others, still persist in the environment to this day (Yablokov *et al.*, 2009).

Previous ecological studies in Chernobyl have demonstrated fitness and developmental consequences because of radiation exposure in a variety of vertebrate and invertebrate species. Barn swallows (*Hirundo rustica*) in the Chernobyl area have shown increased mutation rates (Ellegren *et al.*, 1997; Møller *et al.*, 2010), increased frequency of partial albinism (Ellegren *et al.*, 1997; Møller & Mousseau, 2001), sperm abnormalities (Møller *et al.*, 2005, Møller *et al.*, 2008a,b; Bonisoli-Alquati *et al.*, 2011), increased DNA damage and oxidative stress (Bonisoli-Alquati *et al.*, 2010a,b), reduced levels of circulating and stored antioxidants (Møller *et al.*, 2005), elevated levels of fluctuating asymmetry (FA) in tail length (Møller, 1993a,b) and smaller brains (Møller *et al.*, 2011) in relation to radiation exposure. A study on stag beetles (*Lucanus cervus*) in Chernobyl found that males in highly contaminated sites had significantly elevated levels of FA in secondary sexual characters compared to males from control sites, which affected mating status as a larger portion of asymmetric than symmetric males were unmated (Møller, 2002). Additionally, studies of human populations in areas such as the Semipalatinsk Nuclear Test Site and the Techa River in Southern Urals, Russia, where radioactive waste is dumped, showed similar increases in mutation rates in the germline as a result of long-term radiation exposure (Dubrova *et al.*, 2002; Dubrova, 2006). Consistently, a decline in populations of both vertebrate and invertebrate species has been shown in radioactively contaminated areas in Chernobyl (Galván *et al.*, 2011; Møller & Mousseau, 2007a,b, 2009, 2011a,b; Mousseau & Møller, 2011). This is particularly apparent in invertebrate species that live or lay eggs in the topsoil where most of the radiation is currently located, suggesting a negative effect of direct exposure to radiation (Møller & Mousseau, 2009).

The aim of this study was to assess the effect of parental radiation exposure on offspring development under common garden conditions. We expected to see a comparable reduction in fitness across differing radiation levels reflected in delayed development and reduced body size at reproductive maturity in offspring derived from high radiation sites. In addition, it could be expected that disruption of development would be buffered by longer developmental times (Shykoff & Møller, 1999). Therefore, the effect of parental exposure to radioactive contamination is predicted to depend on the developmental trajectory, as indexed by time to sexual maturity. We used variation in wing shape and size and FA as proxies for the magnitude of developmental instability among offspring (Palmer & Strobeck, 1986, 2003; Klingenberg & McIntyre, 1998). FA reflects the inability of the individual to undergo identical development of bilateral traits under a wide range of environmental and genetic stressors (Møller, 1993a,b;



**Fig. 1** Locations of field collection sites and background radiation around Chernobyl. Partly developed from Shestopalov (1996). The collection site numbers are reported in Table 1.

Swaddle *et al.*, 1994; Graham *et al.*, 2010). Additionally, a negative correlation between degree of FA and fitness components has been observed in many species (Møller 1992, Møller, 2002; Nosil & Reimchen, 2001; Mateos *et al.*, 2008; St-Amour *et al.*, 2010), suggesting that FA may be a reliable measure of phenotypic quality (Leary & Allendorf, 1989; Parsons, 1990; Møller & Thornhill, 1998), although its ability to reliably reflect fitness has been criticized in the literature (Palmer, 1999; - but see Thornhill *et al.*, 1999; Leung *et al.*, 2003; Van Dongen, 2006; Floate & Coghlin, 2010). We predict that the offspring of parents from relatively high radiation sites would show high levels of asymmetry in bilateral traits compared to offspring from low radiation sites, as long as radiation did not cause increased rates of selective mortality of asymmetric individuals, measured as the number of nymphs that failed to reach adulthood in the irradiated and nonirradiated groups (Floate & Fox, 2000).

## Materials and methods

### Study species and field procedures

Studies on the effects of irradiation on insect reproduction and development have shown Orthopterans to be particularly radiosensitive (Willard & Cherry, 1975; Bakri *et al.*, 2005). The brown form of the lesser marsh grasshopper (*Chorthippus albomarginatus* (Acrididae: Orthoptera)) is a medium-sized grasshopper that has undergone a northward range expansion within the last 10–15 years (Bellman, 1985; Vedenina & von Helversen, 2003). It is found in agricultural areas in dry and wet soil throughout Europe from the southern half of Scandinavia and Finland to Spain and Italy (Bellman, 1985) along

coastal sand dunes, rivers and roadside verges. The species has a univoltine life cycle with an obligate egg diapause stage for overwintering. Nymphs typically emerge between May and June and develop through 4–5 nymphal instars. Adults occur from July to October.

We field collected *C. albomarginatus* from six different locations in the Chernobyl area during 20–26 September 2009, across a range of radioactive contamination levels by a factor of 1670 (Fig. 1; Table 1). The six locations were Chystohalivka (0.03  $\mu\text{Sv h}^{-1}$ ), Rozizhdzhe (0.1  $\mu\text{Sv h}^{-1}$ ), Vesniane (5.4  $\mu\text{Sv h}^{-1}$ ), Krasnytsia (10.72  $\mu\text{Sv h}^{-1}$ ), and two sites within the Red Forest (35.31 and 50.06  $\mu\text{Sv h}^{-1}$ , respectively). Background radiation levels were measured using a hand-held dosimeter (Inspector; SE International, Summertown, TN, USA). Although we did not measure the dose received by individuals, we expect it to be highly correlated with background radiation levels measured at the collection sites owing to the short dispersal

**Table 1** Number of families and average number of individuals included in our study in each collection site. See Material and Methods for more details.

Site	Radiation ( $\mu\text{Sv h}^{-1}$ )	No. of families	Average number of F1 progeny per family (minimum–maximum)
Chystohalivka (1)	0.03	10	10.8 (0–16)
Rozizhdzhe (2)	0.1	29	9.3 (0–17)
Vesniane (3)	5.4	38	11.7 (5–20)
Krasnytsia (4)	10.72	21	14 (4–26)
Red Forest 1(5)	35.31	21	12.6 (5–27)
Red Forest 2 (6)	50.06	4	16.5 (7–23)

distance of our model species compared to the scale at which radioactive contamination levels vary (Fig. 1). Individuals were collected from each collection site using a standard insect sweep net. Females were placed individually in Styrofoam cages (height *ca.* 12 cm, diameter *ca.* 8 cm) by collection site and provided with sterilized sand for ovipositing and fresh lettuce. Plastic cups (height *ca.* 10 cm, diameter *ca.* 8 cm) were used to enclose the top of the cages. Following removal from breeding cages, the number of egg pods per female was counted and they were placed in vials containing moistened vermiculite to allow for the completion of prediapause embryonic development.

Upon returning to the laboratory, we placed the egg pods in a 4 °C refrigerator to break diapause. Egg pods were removed from the refrigerator following a 3-month cold treatment and stored at room temperature (24 °C) to induce post-diapause embryonic development. Egg pods were monitored twice daily, and newly emerged nymphs were transferred to 9 cm × 9 cm × 8 cm plastic cages. Cage density averaged nine nymphs per cage (range: 2–18 nymphs) over the duration of nymphal development. Families with large numbers of nymphs (> 10) were divided into two cages. Cages were changed and fresh food provided twice weekly. Nymphs were fed organic lettuce, carrots and wheat germ and provided with a strip of unbleached paper towel for cover. We used carbon dioxide (CO<sub>2</sub>) to transfer nymphs to clean cages with minimum mortality. The few individuals that died because of accidental mishandling during the rearing process were excluded from the analysis. Nymph cages were transferred to an incubator set at a constant temperature and photoperiod (28 °C, 15:9 h LD) to complete development.

### Life history estimates

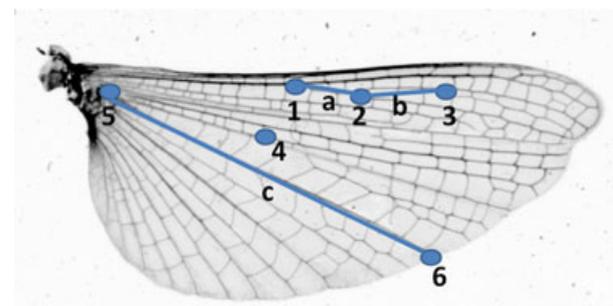
We measured time to sexual maturity (time to maturity hereafter) as the number of days from nymphal hatch to final ecdysis. We also recorded body mass at sexual maturity (body mass hereafter) and the pronotum length (accuracy 0.1 mm) as measures of adult body size. Nymphs were monitored daily for final eclosion (maturity). Adults were removed from nymphal feeding cages and weighed with a Sartorius Research electronic balance (Model Number: R160P) (0.001 g) within *ca.* 12 h after emergence. We then placed adults in individual cages with organic lettuce (~3.0 g) and a carrot slice (~9.0 g). We transferred adults to clean cages and provided fresh food twice weekly to maintain a clean environment. Following death, adults were removed from cages and stored in 95% ethanol for subsequent measurements.

### Wing measurements

The forewings and hind wings were removed from the thorax and wet-mounted on glass microscope slides with

distilled water and covered with a coverslip. Slides were allowed to dry overnight at room temperature (24 °C), and then, coverslips were sealed to the slide with clear nail polish.

Slides were scanned face down with an Epson V300 Photo Color Scanner set at a resolution of 2400 dpi. Because measurement precision is important for the analysis of FA (Palmer & Strobeck, 1986, 2003), on a subsample of 20 individuals we scanned wings three times to assess scanning error using a Procrustes ANOVA with wings as the main effect, number of scans as the error effect and identity of specimens as the individual effect (Klingenberg, 2011). All landmarks were located at wing–vein intersections and termination points (Fig. 2). Most importantly, landmarks were anatomically homologous among all individuals, thus fulfilling the criterion for Type I landmarks (Bookstein, 1991). Landmarks were digitized on the left and right hind wing using TpsDig2.16 software (Rohlf, 2005). All wings within the sample were measured three times to allow assessment of digitizing error with a Procrustes ANOVA with wings as the side effect, digitizing as the error effect and ID as the individual effect (Klingenberg, 2011). Individuals were digitized in random order, blind to information on parental exposure to radiation. The Procrustes ANOVA found the between sides variation to far exceed the measurement error because of the digitizing ( $F_{72} = 12.09$ ,  $P < 0.0001$ ,  $r^2 = 0.97$ ) and scanning technique ( $F_{72} = 17.20$ ,  $P < 0.0001$ ,  $r^2 = 0.99$ ) (Klingenberg, 2011). Additionally, we ran an analysis of error because of our mounting technique by wet-mounting 54 wings on glass microscope slides and covering them with a coverslip before scanning. We repeated the process three times and then measured the length of the radial vein (pixels) twice for each wing using TpsDig2.16 software (Rohlf, 2005). We used a one-way ANOVA with vein length as our response variable and individual as our independent variable to assess the repeatability of our measurements. Measurement error because of mounting was very low for both the left wing ( $F_{26} = 699.23$ ,  $P < 0.0001$ ,  $r^2 = 0.99$ ) and right wing ( $F_{26} = 245.86$ ,  $P < 0.0001$ ,



**Fig. 2** Hind wing of lesser marsh grasshopper (*Chorthippus albomarginatus*) showing landmark positions on wing veins (numbers) and vein lengths (letters) selected for fluctuating asymmetry measurements with image analysis.

$r^2 = 0.99$ ) with repeatability in excess of 99.43%. In the final analyses, we used mean landmark coordinates calculated by averaging the three measurements per landmark for each individual.

### Statistical analysis

Only the individuals for whom both wings could be measured were included in the final analysis. Our analysis included 454 individuals (223 males and 231 females). We extracted shape and size information from the raw data with the software MorphoJ (Klingenberg, 2011) using a full Procrustes fit that controlled for wing orientation, position and size (Klingenberg & McIntyre, 1998; Klingenberg *et al.*, 2002). We ran a Procrustes ANOVA to obtain mean individual values of centroid size as our measure of size variation and two measures of shape: Procrustes distance and Mahalanobis distance. Procrustes distance is the absolute measure of shape that treats all aspects of shape variation equally (Klingenberg & Monteiro, 2005). Mahalanobis distance is a measure of shape relative to the variation in the total sample, with emphasis on degree of invariance (Klingenberg & Monteiro, 2005). Thus, this measure can be interpreted as the degree to which shape is unusual within the population of individuals being analysed (Dryden & Mardia, 1998; Klingenberg & Monteiro, 2005; Breuker *et al.*, 2006). Centroid size is the square root of the sum of squared distances of a set of landmarks or the centre of mass of the landmarks. It is the size measure used to scale a configuration of landmarks so they can be fitted in a frame of space, ultimately removing translation, size differences and rotation from shape analysis (Rohlf, 1999; Klingenberg & Monteiro, 2005). To further investigate shape variation, we used the software tpsRelw (Rohlf, 2003) to calculate a matrix of shape variables, called partial warp scores, which are coefficients indicating the position of an individual relative to the consensus shape, and further reduced them by a relative warps analysis (Zelditch *et al.*, 2004). Relative warps analysis is a principal component analysis of the partial warps and uniform components (Zelditch *et al.*, 2004). The calculated relative warp scores (RWS) were subsequently used as dependent variables in a MANOVA (Soto *et al.*, 2008).

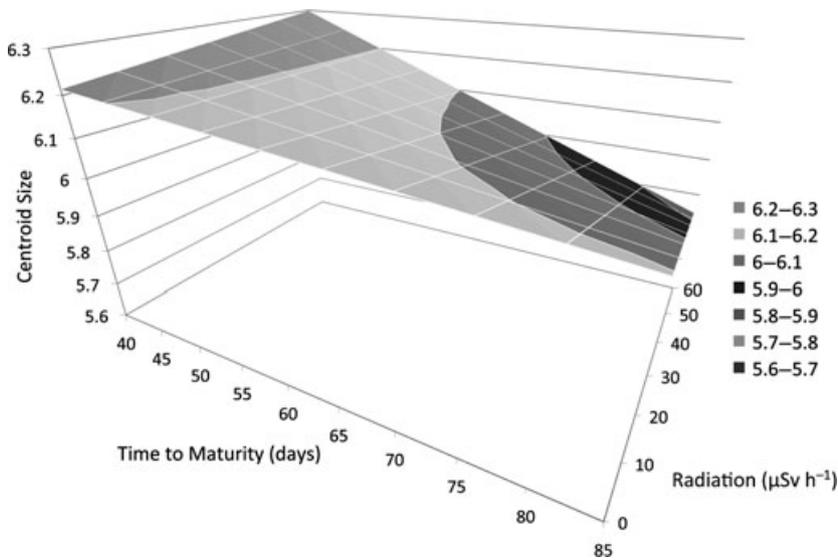
The possibility of detecting a relationship between FA and environmental stress is usually weak when testing only one trait (Leung *et al.*, 2000). Thus, we obtained three measures of FA for our analysis. We defined wing size FA as the absolute difference between the right centroid size value and the left centroid size value (R-L) (Takahashi *et al.*, 2011). We also defined wing venation FA as the absolute difference between the Euclidean distances in Procrustes coordinates between landmarks on the right and left radial and one of the anal wing veins (Smith *et al.*, 1997; Takahashi *et al.*, 2011). We confirmed that there was no significant directional asymmetry by performing a *t*-test on the signed right minus left trait

values (all  $P > 0.06$ ) and confirmed the absence of antisymmetry with tests of normality and visual inspection of data (all kurtosis  $> 1.00$ ) (Palmer & Strobeck, 1986, 2003). We also confirmed no significant size dependence of FA by regressing the unsigned, absolute difference of the right and left trait values on trait size (all  $P > 0.46$ ) (Palmer & Strobeck, 1986, 2003). The data were inspected for statistically significant outliers, defined as being greater than three standard deviations from the mean. Five individuals presenting significant aberrant values for life history traits, shape or size were excluded. We used the Kolmogorov–Smirnov test to check the distribution of data. All measures of FA, Procrustes distance and Mahalanobis distance were not normally distributed, and we therefore log-transformed them before analyses, although the log transformation did not normalize the data.

We used a linear mixed model approach to test for differences in FA, wing shape and wing size according to radiation applying PROC MIXED in SAS 9.1 (SAS Institute, Cary, NC, USA). We separately tested three response variables (Procrustes distance, Mahalanobis distance and centroid size) one at a time as a function of sex (as fixed factors), pronotum length, radiation level and time to maturity (as covariates). In addition, we included in the model the interaction between radiation and sex, radiation and time to maturity and sex and time to maturity. We also included identity of the collection site and identity of the family of origin (nested within the collection site) as random effects to test the effect of both shared genes and developmental environment on the response variable. We used the Wald test ( $z$ ) as the test of significance for our random effects. This is equivalent to the *F* test used to test significance of our fixed effects (SAS Institute Inc., 2008). We approximated degrees of freedom using the Satterthwaite approximation.

We also used a linear mixed model approach to test whether within-family mortality predicted wing shape variation and FA within the family, while controlling for nymphal density, as indexed by the total number of individuals per family. We tested average Procrustes distance per family, average Mahalanobis distance per family and average measures of shape variation per family, one at a time, as a function of radiation level and family mortality. We also included in the model the interaction between radiation level and mortality per family.

Additionally, we tested the independent effect of radiation on time to maturity, family mortality, family hatching success (measured as the number of individuals that hatched over total number of eggs produced) and fecundity (measured as the total number of egg pods produced) following the same linear mixed model approach. Because nymphal density varied during the rearing process, we also tested for independent effects of density on shape variation and FA within the family. For all linear mixed models, we applied an Akaike information criterion (AIC) approach for model selection,



**Fig. 3** Relationship between centroid size, parental radiation exposure and time to maturity. Results for both males and females were combined because they showed a similar pattern. A surface was interpolated based on the coefficients for the effects of radiation, time to maturity and the interaction between the two in the best-fit model. The coefficients of the model are reported in Table 2.

sequentially removing the least significant terms, starting from the interactions. We removed a term from the model only if removal did not increase the AIC values by more than 2.00 (Burnham & Anderson, 1998).

For the MANOVA, we applied PROC MIXED in SAS 9.1 and tested the relative warp scores as functions of the same fixed factors, covariates and random effects applied in the linear mixed model approach. We also tested the average relative warp scores per family as a function of radiation level, family mortality estimated as the percentage of a family that died and the interaction between radiation and mortality to determine whether family mortality predicted shape variation within the family.

## Results

### Wing size

Radiation effects on wing size depended on whether the individual was a male or female (Table 2), with wing size increasing more with radiation levels in females (coefficient =  $9.37 \times 10^{-3}$  ( $3.51 \times 10^{-3}$  SE),  $t_{291} = 2.67$ ,  $P < 0.01$ ) compared to males (coefficient =  $1.07 \times 10^{-2}$  ( $3.65 \times 10^{-3}$  SE),  $t_{299} = 2.94$ ,  $P < 0.01$ ). In addition,

males showed an increase in wing size with longer time to maturity (coefficient =  $5.34 \times 10^{-3}$  ( $1.54 \times 10^{-3}$  SE),  $t_{253} = 3.47$ ,  $P < 0.01$ ), whereas time to maturity did not significantly affect wing size in females (coefficient =  $-1.85 \times 10^{-3}$  ( $2.02 \times 10^{-3}$  SE),  $t_{363} = -0.92$ ,  $P = 0.36$ ). Overall, the wing size response to radiation depended on the time at which the individual reached maturity (Fig. 3). Individuals that matured early had larger wings with increasing radiation levels, whereas wing size in late maturing individuals decreased with increasing radiation levels (Table 2).

In addition, neither collection site ( $z > 0.41$ ,  $P > 0.26$ ) nor family ( $z > 1.26$ ,  $P > 0.10$ ) had a significant effect on wing size. Thus, the shared genes and environment were not significantly associated with variation in wing size among our experimental individuals.

### Wing shape

We first analysed wing shape, as indexed by either Procrustes distance (Table 3) or Mahalanobis distance (Table 4) as a function of radiation, sex, pronotum length and time to maturity. Radiation and time to maturity did not have joint or independent effects on any measure of

**Table 2** Results for linear mixed model for wing size variation as measured by centroid size. Statistically significant relationships ( $P < 0.05$ ) are marked in bold.

Final model	Coefficient	SE	d.f.	<i>F</i>	<i>P</i>
Pronotum	$7.11 \times 10^{-3}$	$2.66 \times 10^{-3}$	1, 414	7.14	<b>&lt; 0.001</b>
Radiation	$9.37 \times 10^{-3}$	$3.51 \times 10^{-3}$	1, 291	7.95	<b>0.01</b>
Time to maturity	$-1.85 \times 10^{-3}$	$2.02 \times 10^{-3}$	1, 363	1.37	0.24
Sex			1, 404	11.43	<b>&lt; 0.001</b>
Time to maturity × radiation			1, 319	7.44	<b>0.01</b>
Radiation × sex			1, 401	4.41	<b>0.04</b>
Time to maturity × sex			1, 404	12.92	<b>&lt; 0.001</b>

**Table 3** Linear mixed models for wing shape as measured by Procrustes distance. Dropped terms are reported in the order with which they were removed from the model, with their associated statistics at removal.

Final model	Coefficient	SE	d.f.	<i>F</i>	<i>P</i>	AIC
Pronotum	$-3.50 \times 10^{-4}$	$1.37 \times 10^{-2}$	1, 411	0.01	0.98	764.0
Time to maturity	$-7.60 \times 10^{-3}$	$5.09 \times 10^{-3}$	1, 201	2.23	0.14	
Radiation	$-1.40 \times 10^{-3}$	$2.14 \times 10^{-3}$	1, 9.1	0.43	0.53	
Sex			1, 415	2.39	0.12	
Dropped terms						
Radiation × sex			1, 411	0.11	0.74	791.1
Time to maturity × sex			1, 410	1.08	0.30	781.7
Time to maturity × radiation			1, 248	2.98	0.09	775.5

**Table 4** Linear mixed models for wing shape as measured by Mahalanobis distance. Statistically significant relationships ( $P < 0.05$ ) are marked in bold. Dropped terms are reported in the order with which they were removed from the model, with their associated statistics at removal.

Final model	Coefficient	SE	d.f.	<i>F</i>	<i>P</i>	AIC
Time to maturity	$-5.07 \times 10^{-3}$	$4.57 \times 10^{-3}$	1, 248	1.23	0.27	654.20
Sex			1, 416	5.17	<b>0.02</b>	
Radiation	$-8.90 \times 10^{-4}$	$1.96 \times 10^{-3}$	1, 10.6	0.20	0.66	
Pronotum	$-2.48 \times 10^{-3}$	$1.20 \times 10^{-2}$	1, 417	0.04	0.84	
Dropped terms						
Days to maturity × sex			1, 407	0.48	0.49	683.00
Radiation × sex			1, 407	0.59	0.44	675.90
Days to maturity × radiation			1, 305	2.17	0.14	666.60

**Table 5** Results for multivariate mixed model for wing shape variation as measured by relative warp scores. Statistically significant relationships ( $P < 0.05$ ) are marked in bold. Dropped terms are reported in the order with which they were removed from the model, with their associated statistics at removal.

Final model	Coefficient	SE	d.f.	<i>F</i>	<i>P</i>	AIC
Pronotum	$7.00 \times 10^{-4}$	$4.02 \times 10^{-4}$	1, 3300	3.04	0.08	-10029.9
Radiation	$2.00 \times 10^{-5}$	$5.70 \times 10^{-5}$	1, 3300	0.17	0.68	
Time to maturity	$2.82 \times 10^{-4}$	$5.70 \times 10^{-5}$	1, 3300	2.77	0.10	
Sex			2, 3300	3.06	<b>0.05</b>	
Dropped terms						
Radiation × sex			1, 3297	0.07	0.79	-10025.1
Time to maturity × radiation			1, 3298	0.58	0.45	-10027.0
Time to maturity × sex			1, 3299	0.55	0.46	-10028.5

wing shape (Tables 3 and 4). Males and females did not differ significantly in wing shape, nor did they differ significantly in how time to maturity was related to wing shape (Tables 3 and 4). Time to maturity was not significantly associated with shape of wings (Tables 3 and 4).

Males and females differed significantly in wing shape, as indexed by RWS, but sex did not have an interaction effect with radiation or time to maturity on wing shape. Radiation and time to maturity did not have interaction or independent relationships with wing shape (Table 5).

The collection site was not significantly related to wing shape when measured as Procrustes distance ( $z > 0.00$ ,  $P > 0.22$ ) or Mahalanobis distance ( $z > 0.07$ ,  $P > 0.23$ ).

The relationship between collection site and wing shape variation when measured as relative warp scores was negligible. Additionally, the effect of family on shape was not significant when measured as Procrustes distance ( $z > 0.10$ ,  $P > 0.22$ ), Mahalanobis distance ( $z > 0.86$ ,  $P > 0.19$ ) or relative warp scores ( $z > 0.27$ ,  $P > 0.39$ ).

Radiation and family mortality were not jointly related to average shape as indexed by average Procrustes distance (coefficient =  $4.25 \times 10^{-6}$  ( $1.20 \times 10^{-5}$  SE),  $F_{97.5} = 0.13$ ,  $P = 0.72$ ), average Mahalanobis distance (coefficient =  $6.30 \times 10^{-5}$  ( $3.22 \times 10^{-4}$  SE),  $F_{95.3} = 0.04$ ,  $P = 0.84$ ) or average relative warp scores (coefficient =  $-3.05 \times 10^{-6}$  ( $6.33 \times 10^{-6}$  SE),  $F_{299} = 0.23$ ,  $P = 0.63$ ), nor was radiation significantly independently related to any measures of shape variation ( $P > 0.55$ ). Average

**Table 6** Results for linear mixed model for wing shape fluctuating asymmetry as measured by distances between landmarks on the radial (LM1–LM2, LM2–LM3) and anal (LM5–LM6) veins. Akaike information criterion (AIC) model selection approach removes least significant terms starting with interactions. Dropped terms are reported in the order with which they were removed from the model, with their associated statistics at removal.

Final model	Coefficient	SE	d.f.	<i>F</i>	<i>P</i>	AIC
<b>Radial vein distance (LM 1–LM 2)</b>						
Time to maturity	$-8.08 \times 10^{-3}$	$1.45 \times 10^{-2}$	1, 336	0.31	0.58	1632.80
Sex			1, 413	0.95	0.33	
Radiation	$-8.38 \times 10^{-1}$	$5.36 \times 10^{-3}$	1, 11.6	2.44	0.14	
Pronotum	$-2.70 \times 10^{-2}$	$3.98 \times 10^{-2}$	1, 414	0.46	0.50	
<b>Dropped terms</b>						
Radiation × sex			1, 411	0.00	0.95	1657.40
Days to maturity × radiation			1, 397	0.05	0.82	1649.90
Days to maturity × sex			1, 413	0.22	0.64	1637.80
<b>Radial vein distance (LM 2–LM 3)</b>						
Time to maturity	$-1.09 \times 10^{-2}$	$1.36 \times 10^{-2}$	1, 207	0.64	0.42	1598.80
Pronotum	$-1.17 \times 10^{-2}$	$3.08 \times 10^{-2}$	1, 415	0.14	0.12	
Radiation	$-7.43 \times 10^{-3}$	$4.63 \times 10^{-3}$	1, 46	2.57	0.70	
<b>Dropped terms</b>						
Radiation × sex			1, 410	0.09	0.77	1625.00
Days to maturity × sex			1, 408	0.14	0.71	1617.60
Days to maturity × radiation			1, 214	0.09	0.76	1612.50
Sex			1, 412	0.00	0.96	1600.20
<b>Anal vein distance (LM 5–LM 6)</b>						
Time to maturity	$1.28 \times 10^{-3}$	$9.15 \times 10^{-3}$	1, 218	0.02	0.89	1266.60
Radiation	$1.64 \times 10^{-3}$	$3.11 \times 10^{-3}$	1, 50.1	0.28	0.60	
Pronotum	$3.96 \times 10^{-2}$	$2.06 \times 10^{-2}$	1, 415	3.69	0.06	
<b>Dropped terms</b>						
Days to maturity × sex			1, 408	0.58	0.45	1294.10
Radiation × sex			1, 411	0.83	0.36	1288.60
Days to maturity × radiation			1, 226	0.77	0.38	1281.20
Sex			1, 412	0.02	0.90	1268.80

shape variation decreased significantly with increasing family mortality when measured as Mahalanobis distance (coefficient =  $-1.19 \times 10^2$  ( $4.79 \times 10^{-3}$  SE),  $F_{97.7} = 6.18$ ,  $P = 0.01$ ), but not as Procrustes distance (coefficient =  $-2.80 \times 10^{-4}$  ( $1.20 \times 10^{-5}$  SE),  $F_{96.5} = 2.64$ ,  $P = 0.11$ ) or relative warp scores (coefficient =  $2.10 \times 10^{-5}$  ( $5.30 \times 10^{-5}$  SE),  $F_{299} = 0.15$ ,  $P = 0.70$ ).

### Wing fluctuating asymmetry

Radiation, sex and time to maturity were not related to any measure of wing FA (Table 6). Collection site ( $z > 0.14$ ,  $P > 0.14$ ) was not significantly related to any measure of wing FA in addition to family not being significantly related to FA.

Average wing FA, as indexed by distance between landmarks on the radial vein, decreased significantly with increasing family mortality (a: coefficient =  $-7.92 \times 10^{-3}$  ( $3.72 \times 10^{-2}$  SE),  $t_{97.9} = -2.13$ ,  $P = 0.04$ ; b: coefficient =  $-1.30 \times 10^{-2}$  ( $4.84 \times 10^{-3}$  SE),  $t_{98} = -2.69$ ,  $P = 0.01$ ) and when indexed by distance between landmarks on the anal vein (c: coefficient =  $-4.95 \times 10^{-3}$  ( $2.13 \times 10^{-3}$  SE),  $t_{98} = -2.32$ ,  $P = 0.02$ ). Radiation was not, independently or jointly, significantly related to any measure of FA.

### Life history

Radiation did not have an effect on time to maturity (coefficient =  $-9.57 \times 10^{-3}$  ( $4.19 \times 10^{-2}$  SE),  $t_{18.3} = -0.23$ ,  $P = 0.82$ ). Family mortality decreased significantly with increasing radiation (coefficient =  $-4.45 \times 10^{-1}$  ( $1.64 \times 10^{-1}$  SE),  $t_{117} = -2.71$ ,  $P < 0.01$ ), whereas family hatching success decreased with increasing radiation but not significantly (coefficient =  $-1.89 \times 10^{-2}$  ( $1.02 \times 10^{-2}$  SE),  $t_{112} = -1.85$ ,  $P = 0.07$ ). Additionally, radiation did not significantly affect fecundity (coefficient =  $9.43 \times 10^{-3}$  ( $9.82 \times 10^{-3}$  SE),  $t_{5.63} = 0.96$ ,  $P = 0.38$ ).

Nymphal density did not have a significant effect on the average wing size ( $F_{93.4} = 0.00$ ,  $P = 0.96$ ), average wing shape as indexed by Procrustes distance ( $F_{99.9} = 1.15$ ,  $P = 0.29$ ), Mahalanobis distance ( $F_{97} = 2.73$ ,  $P = 0.10$ ), relative warp scores ( $F_{700} = 0.02$ ,  $P = 0.90$ ) or any measure of wing FA ( $P > 0.38$ ).

### Discussion

In this study of the transgenerational consequences of environmental stress, we tested for effects of parental radiation exposure on developmental instability of grasshopper offspring. We used three measures of wing shape,

a measure of wing size and various measures of wing FA to test whether the degree of developmental instability was predicted by parental radiation exposure and whether that effect depended on individual sex, body size and time to maturity. We also tested for effects of parental radiation exposure on family mortality, family hatching success and time to maturity in addition to whether mortality and nymphal density predicted developmental instability within families. If early developmental stages include more asymmetric individuals that suffer from differential mortality, this may bias estimates of the relationship between the environmental stressors, such as radiation, and asymmetry (Møller, 1997). Because we found weak, but nonsignificant effects of radiation on fecundity and hatching failure, we can exclude these factors as biasing our asymmetry estimates.

We also found that wing size decreased relative to body size with longer maturation time and increasing parental radiation exposure. Thus, late maturing individuals born to parents from high radiation sites were smaller than late maturing individuals whose parents came from low radiation sites. Males responded to increasing parental radiation exposure with increasing wing size, whereas wing size decreased, but not significantly, in females. Radiation exposure did not influence wing FA, independently or in combination with any other variable. Previous studies on Orthopteran growth and development show that environmental variation significantly affects the plasticity of adult size and time to eclosion (Moore, 1948; Willott & Hassall, 1998; Finch *et al.*, 2008). Additionally, this effect is greatly influenced by developmental stage. Individuals raised under optimal environmental conditions during early nymphal stages tend to reach adulthood with fewer moults and at a larger size compared to individuals raised under suboptimal conditions regardless of whether conditions are reversed at later nymphal stages (Flanagin *et al.*, 2000; Miller *et al.*, 2001; Taniguchi & Tomioka, 2003). This suggests that the physiological and hormonal events necessary for the timing of eclosion and adult size are already fixed by the later stages (Bradshaw & Johnson, 1995). Thus, if radiation is interfering with these physiological events early in development, it may be reflected in smaller adult size and delayed eclosion. For example, the interaction effect of radiation exposure and longer maturation time resulting in smaller size at maturity may suggest that the offspring's physiological ability to metabolize and allocate nutrients towards growth is developmentally unstable as a result of increasing parental radiation exposure.

In Orthopteran species, body size is significantly correlated with fitness components. For example, Landa (1992) found that late hatching nymphs of *Arphia sulphurea* and *Chortophaga viridifasciata* were smaller, suffered higher overwinter mortality and reached reproductive maturity later compared to controls. Simmons (1995) found in the field cricket (*Gryllus campestris*) that larger males, as measured by their body length, were

more successful in obtaining mates as a result of possessing a larger harp which in turn affected the carrier frequency of the male's courtship song. In studies on bush crickets, larger males tend to produce more ejaculate compared to smaller males (Wedell, 1997). Additionally, larger size has been shown to contribute to an individual's resistance to parasitism (Danyk *et al.*, 2000) and is a determinant of fecundity in females (Fox & Czesak, 2000). Previous studies have demonstrated a physiological trade-off between developmental rate and size at maturity for various grasshopper species in response to geographical and seasonal variation in food quality (Asshoff & Hattenschwiler, 2005; Fielding & Defoliart, 2007; Jannot *et al.*, 2009) that appears to occur at the metabolic level (Behmer & Elias, 1999; Hahn, 2005). Møller & Mousseau (2011a,b) have suggested that the difference in susceptibility to radiation among invertebrates may in part be due to these differences in metabolic rates relative to body size. Species with relatively high metabolic rates may be more vulnerable to radiation effects because of increased demand for antioxidant defence (Møller & Mousseau, 2011a,b). If this is true also at the intra-specific level, faster development may increase susceptibility to oxidative stress and compromise the metabolic capabilities and allocation of resources for further development.

Additionally, poor maternal contribution to eggs as a consequence of parental radiation exposure may be exacerbating developmental selection pressure. During oogenesis, females allocate resources including carbohydrates, proteins, lipids and water to eggs (Wheeler, 1996). Grasshoppers obtain nutrients for oogenesis during adult feeding and insufficient nourishment can adversely affect egg production and subsequent offspring development (Bellman, 1985; Wheeler, 1996; Fox & Czesak, 2000). Egg size has been positively correlated with offspring survivorship and size in unfavourable environments (Braby, 1994; Mousseau & Fox, 1998). Mothers exposed to persistent oxidative stress may incur metabolic constraints that limit the acquisition of resources for eggs and decrease the phenotypic quality of their offspring. Thus, under conditions when mothers are limited in the availability of important nutrients for eggs, as might be the case in radioactively contaminated areas, offspring are more vulnerable to environmental perturbations and may need to compensate for reduced egg resources with longer development time.

The negative trend between family mortality and FA within families suggests that more asymmetric individuals suffered higher mortality rates compared to symmetrical individuals. Considering that offspring are often produced in high numbers, one would expect a decrease in overall FA with increasing mortality as developmentally unstable individuals are removed by selection. This is especially so under chronic environmental stress when developmental selection becomes more intense (Møller, 1997). Increased environmental stress may interfere with

physiological mechanisms involved in maturation. For example, studies on insect ecdysis (i.e. moult) found that failure of ecdysis appears to occur because of disruption of muscle contractions that are involved in working the individual forward out of the old cuticle (Hughes, 1980; Ewer *et al.*, 1997; Park *et al.*, 2003). Irradiation experiments found ecdysis failure to occur owing to abnormal development of abdominal structures responsible for expanding the abdomen and breaking the old cuticle, similar to results found in insects treated with natural or synthetic juvenile hormone analogues (Reynolds, 1980). Under these conditions, adult eclosion failure was found to increase with prolonged exposure to radiation (Beard, 1972; Sharma & Dwivedi, 1997; Faruki *et al.*, 2007). In one study of the tobacco hornworm *Manduca sexta*, duration of development in offspring of irradiated parents was roughly 10% longer and < 60% emerged as adults compared to 90% of controls (Seth & Reynolds, 1993). If families are experiencing increased genomic instability caused by parental radiation exposure, they may experience greater selection pressure because of the increased number of phenodeviants. If such deviance is reflected in physiological, immunological and behavioural function of the individual, insects may be particularly vulnerable to developmental selection pressure from parental radiation exposure because malformed or physiologically deviant individuals would be unable to successfully eclose into adult form.

In conclusion, the effect of parental radiation exposure can adversely affect offspring development and fitness depending on developmental trajectories. However, caution in interpreting results is required. It is not certain whether this effect was because of transgenerational genomic instability or other influences such as maternal contribution to eggs. We must also consider the possibility that the levels of radiation assessed in this study were not sufficient for affecting FA in our model species. To further assess the correlation of FA with fitness, future studies may wish to include more direct fitness parameters such as a female's lifetime contribution to the next generation in addition to life history traits such as escape behaviour, mating success, immunocompetence and maternal investment in egg content. Including more replication in the experimental design so that the repeatability of such measures can be assessed will help further our understanding of the relationship between FA and fitness.

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