

Developmental instability as phenodeviance in a secondary sexual trait increases sharply with thermal stress

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Abstract

We test for effects of thermal stress applied to pupal flies from Noumea (New Caledonia) and Taipei (Taiwan) on developmental instability (DI) in the male sex comb of *Drosophila bipectinata*, as well as on pre-adult survivorship and adult body size. The temperature treatments were Low (25 °C), High (29 °C) and Variable (18 h at 29 °C, 6 h at 34 °C). Although the Variable treatment reduced survivorship and body size, absolute comb size and fluctuating asymmetry generally were invariant across treatments. In contrast, comb phenodeviance increased with stress in both populations. Phenodeviance in one comb segment (C2) increased sharply with stress, whereas phenodeviance in a second major segment (C1) also increased with stress but only in Noumea flies. A major conclusion is that phenodeviations induced in a secondary sexual trait reflect the developmental environment that also damages fitness components, a foundation stone of the hypothesis that expressions of DI reveal phenotypic quality in sexual selection.

Introduction

Developmental instability (DI) refers to small, random perturbations or accidents during development (Waddington, 1957). These minor perturbations are believed to arise from the stochastic molecular and cellular processes common to developmental systems of all traits of organisms (McAdams & Arkin, 1999; Klingenberg, 2003; Graham *et al.*, 2010). Morphological measures of DI include both fluctuating asymmetry (FA) and phenodeviance, with FA being by far the most commonly employed measure of DI in ecology and evolution (Møller & Swaddle, 1997; Polak, 2003; Van Dongen, 2006). FA refers to subtle deviations from perfect symmetry in otherwise bilaterally symmetrical traits, and because the two sides of such traits are the products of the same genetic background, FA is widely assumed to be the result of perturbations to the normal course of development (Van Valen, 1962; Palmer & Strobeck, 1986; Zakharov, 1992; Klingenberg, 2003).

Phenodeviance, in turn, refers to the expression of minor morphological abnormalities (MMAs) arising from

developmental accidents (Rasmuson, 1960), and in some fields, such as human clinical genetics and ecotoxicology, the incidence of phenodeviance is used to predict particular fitness outcomes (Graham *et al.*, 1993a; Hoyme, 1993; Jones, 2006). By definition, phenodeviants occur at relatively low frequency in the population and thus may be a relatively less sensitive diagnostic measure of DI than asymmetry (Møller & Swaddle, 1997; Jones, 2006). Nevertheless, phenodeviance can increase to considerable levels in populations exposed to environmental stress factors such as pollution (Warwick, 1985; Graham *et al.*, 1993b; Diggins & Stewart, 1998), and studies have shown that phenodeviant classes in a population may express greater levels of FA than non-phenodeviant groups (Bailit *et al.*, 1970; Leary *et al.*, 1984; Groenendijk *et al.*, 1998; Polak & Taylor, 2007), supporting the idea that FA and phenodeviance tap a common property of the organism (Zakharov, 1992; cf. Van Dongen *et al.*, 2009).

Because DI is expected to reflect the capacity of individuals to cope with stress during development, such as in the form of nutrient deprivation, parasites, temperature extremes, ultraviolet radiation, and so on (Palmer & Strobeck, 1986; Møller, 2006), FA and phenodeviance are expected to predict components of individual performance later in life. This outcome is expected because stress, which we define as any agent that exerts

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potentially negative fitness effects on the organism (Hoffmann & Parsons, 1991), can affect a wide range of molecular and physiological functions within the body. Thus, on the basis of the expectation that stress simultaneously can impair developmental stability and components of health and vigour, the DI-sexual selection hypothesis predicts that developmental stability, as relatively low FA and phenodeviance, should represent useful markers of phenotypic quality to females and rival males (Møller & Pomiankowski, 1993).

Indeed, the role of FA in sexual selection has been the topic of a great deal of research (Markow, 1987; Møller, 1990; Thornhill, 1992; Møller & Swaddle, 1997; Tomkins & Simmons, 2003; Van Dongen, 2006; Polak, 2008). Whereas generalizations about the role of DI in sexual selection are weak overall (Tomkins & Simmons, 2003; Polak, 2008), this literature has revealed that in at least some species the most symmetrical males in the population have a mating advantage, either because they are more attractive to females or because they are better competitors than asymmetrical males (Møller & Pomiankowski, 1993). In contrast, phenodeviance in sexual selection has received far less attention than FA, although some evidence for the importance of phenodeviance in sexual selection does exist. For example, in barn swallows (*Hirundo rustica*) studied at Chernobyl, Ukraine, males with phenodeviant ('brushy and transparent') outermost tail feathers, reproduced later in the breeding season, suggesting that females may be avoiding aberrant phenotypes in this population (Møller, 1993).

In a recent study, Polak & Taylor (2007) demonstrated that both phenodeviance and asymmetry in the male sex comb of *Drosophila bipectinata* were independently and negatively correlated with the probability of copulation in a natural population in Noumea, New Caledonia. Interestingly, phenodeviance and FA in only one of the major comb segments (segment C1, see below) were found to be under sexual selection. These effects, which were consistent across days, occurred despite the finding that phenodeviance and FA were only weakly heritable (Polak & Taylor, 2007). Thus, this previous study points to the potential for environmental factors in creating covariation between DI and phenotypic quality. We envision that such covariation could arise if flies emerging from a high-stress microhabitat were more developmentally unstable and of reduced phenotypic quality relative to flies emerging from a low stress microhabitat co-expressing low DI and superior quality (Simmons *et al.*, 1995; Polak & Starmer, 2005; and see Grafen, 1988, pp. 459–460). Heterogeneity in environmental stress during development in this way could be fuelling the sexual selection for developmental stability detected among adults in New Caledonia (Polak & Taylor, 2007).

In the present study, we tested for effects of thermal stress experimentally applied to pupal flies from Noumea on the expression of FA and phenodeviance in the sex

comb, and for comparison, we also examined FA responses in sternopleural bristles, a nonsecondary sexual trait. The intent of the various temperature treatments was to create variation in levels of environmental stress; the experiment was not designed to assess the relative contributions of different forms of thermal stress, say mean vs. variance in temperature, on developmental outcomes. We also tested a prediction at the core of the DI-sexual selection hypothesis, that stress can simultaneously damage both DI and other fitness-related traits. We incorporated into the study a second geographic population of *D. bipectinata*, from Taipei (Taiwan), which differs from New Caledonian flies in the average size of the sex comb (being smaller in Taiwan) and in the relationship between DI and sexual selection. As mentioned above, FA and phenodeviance in C1 were significantly negatively and consistently (across days) associated with copulation probability in New Caledonia, whereas no such relationships were detected in Taiwan (M. Polak & Y. Hsu, unpublished data). Thus, we also evaluated the prediction that DI specifically in C1 would be relatively more sensitive to stress in the population from Noumea compared to Taipei.

Materials and methods

Source populations and fly culture

Two geographic populations of *D. bipectinata* Duda (Diptera: Drosophilidae) were used in this study. Flies were captured by aspirating copulating pairs directly from the surface of fruit substrates in the field. One population was initiated from Noumea, New Caledonia, with 100 nonvirgin females and an approximately equal number of males collected between 8 and 12 January 2006 at a site approximately 80 m from La Baie de L'Anse Vata. A second population was initiated in Taiwan with 280 nonvirgin females and an approximately equal number of males at the Center of Academic Activities, Academia Sinica, Taipei, collected between 15 and 26 September 2006. The climatic conditions are such at both localities that fly populations persist throughout the year; Taipei has an oceanic subtropical climate whereas Noumea has a temperate oceanic climate (Logan & Cole, 2001; Chen & Chen, 2003). Collections at both sites coincided with a relatively wet time of the year.

For 2–3 generations after initiation, each population was mass-cultured in 45-mL polystyrene vials containing 2.0 g Instant *Drosophila* medium (Carolina Biological, Burlington, NC, USA), 6.6 mL water, and 1.5-mL banana-Brewer's live yeast slurry applied to the surface of the medium. In all subsequent generations, flies were mass-cultured in 8–10 240-mL milk bottles containing 12.0 g Instant *Drosophila* Medium, 50.0 mL water, and 5.0 mL banana-yeast slurry, held at 25 ± 1 °C and a 12 : 12 h light/dark cycle.

Temperature treatments

Temperature treatments were administered in November 2006. Virgin flies were harvested from culture bottles within 6 h of emergence and aged in single-sex holding vials containing a cornmeal-agar substrate and live yeast. When flies were 5- to 6-days old, 50 flies of each sex were combined into each of four culture bottles with fresh medium. Females were allowed to lay eggs for 48 h, after which time bottles were emptied of all flies. Five days later, a pupation substrate (consisting of loosely rolled-up sterile tissue paper) was inserted into each bottle. Sixteen hours later, the tissue paper with newly formed larvae was extracted from each bottle, carefully cut into three approximately equal-sized pieces without contacting the pupae, and each piece was gently placed into a separate glass bottle containing three large cotton balls moistened with 5.0 mL of deionized water. Bottles with the pupae were then sealed with cotton and parafilm perforated with 10 small holes using a sterile probe to permit gas exchange. Bottles were then immediately allocated randomly to one of three incubators, comprising the following temperature treatments: Low (constant 25 °C); High (constant 29 °C); and Variable (cycling between 18 h at 29 °C and 6 h at 34 °C). The bottles containing pupae were maintained in their respective incubators until all adults emerged. All flies that emerged were harvested and immediately preserved in 70% ethanol, for later processing (see next section). When emergences ceased, all the pupae from each bottle were carefully teased free from the tissue paper and individually examined in a drop of water on a depression slide under a stereomicroscope to determine whether the pupal case was empty (meaning that the fly had survived through development and successfully emerged as an adult) or contained a dead fly. Pupal mortality was scored on a binary scale, as dead (0) or not (1).

Phenotypic traits

Males were extracted from alcohol, hydrated in water, and their forelegs carefully pulled free from the body and placed onto double-sided transparent tape on a microscope slide against a white background. To estimate body size, thorax length was measured as the linear distance from the end of the scutellum to the front edge of the thorax using an ocular micrometer of an Olympus SZX12 stereomicroscope. The number of teeth in each of the comb segments on the right and the left forelegs were counted. TC1 and TC2 refer to the average tooth number between forelegs in C1 and C2 (the two major comb segments), respectively. Total comb size (CTOT) refers to the sum of TC1 and TC2, that is the average number of teeth in the sex comb as a whole.

Fluctuating asymmetry was calculated as the absolute difference in trait size between the right and left members of a given trait ($|R-L|$). FAC1 and FAC2 refer

to FA in traits C1 and C2, respectively. FACTOT refers to FA in total sex comb teeth, calculated as $|total\ teeth\ on\ right - total\ teeth\ on\ left|$. Measurement error in tooth counts has been assessed previously and is negligible (Polak *et al.*, 2004). All counts of sex comb teeth (and sternopleural bristle number, see below) in the present study and in the previous study in which measurement error was assessed were performed by the same researcher (MP).

For each male, all instances of MMAs in C1 and C2 of the sex comb (Polak & Taylor, 2007) were recorded. A MMA (which we use synonymously with 'phenodeviant') occurred as either a misplaced tooth (arising either behind or in front of the main row) or a break in a row of teeth (appearing as a gap between any two teeth of a size greater than or equal to the basal diameter of an individual tooth). A normal segment is one in which the teeth tightly line up in a straight row.

The two sets of bristles on the sternopleuron of males were also counted. Anterior bristles (S1) occur in an oblique row from the forecoxa towards the midline, whereas the transverse bristles (S2) encircle the thorax from the sternopleural bristles to the sternal bristle. TS1 and TS2 refer to the average number of anterior and transverse sternopleural bristles between sides, respectively. STOT is the sum of TS1 and TS2. Asymmetry for each sternopleural bristle type was calculated as for the sex combs. FAS1 and FAS2 refer to FA in S1 and S2, respectively. FASTOT refers to FA calculated as $|total\ sternopleurals\ on\ right - total\ sternopleurals\ on\ left|$. Measurement error in sternopleural bristle counts was assessed previously and is negligible (Polak, 1997). All counts of sternopleural bristles were conducted by the same investigator (MP) here and in Polak (1997).

Thus, males that emerged from the temperature treatments were characterized with respect to (i) thorax length, (ii) size (as tooth number), FA and phenodeviance of the sex comb, and (iii) number and FA of sternopleural bristles.

Analyses

Asymmetry diagnostics

For traits exhibiting ideal FA, distributions of signed right-minus-left (R-L) values should be approximately normal or leptokurtic, and the mean should be zero (Palmer & Strobeck, 1986). To check for ideal FA, mean, skewness and kurtosis for signed R-L values were calculated (Sokal & Rohlf, 1995) for both comb segments (C1 and C2) and sternopleural bristle types (S1 and S2), separately for the Noumea and Taipei populations.

Population and temperature effects

Logistic regression tested for effects of source population (Noumea and Taipei), experimental temperature treatment (Low, High and Variable), and the interaction between these terms, on pupal mortality (as 0 or 1) with

trait size included as a covariate. For thorax length, analysis of variance (ANOVA) tested for the effects also of these terms.

Multivariate analysis of covariance (MANCOVA) assessed effects of population and treatment on size and FA of phenotypic traits, separately; two MANCOVAs were conducted, each with four dependent variables. Overall differences among populations and treatments were assessed using Roy's greatest root statistics (Scheiner, 1993). In the event the MANCOVA produced significant differences, 'protected' univariate analyses of variance (ANOVAs) were conducted as *post hoc* procedures, to identify which groups differed from each other (Scheiner, 1993). For all univariate ANOVAs on FA and size traits, interactions between the covariate (i.e. thorax length) and population, and between the covariate and temperature, were nonsignificant ($P > 0.05$), confirming the equality of slopes assumption of these analyses (Neter *et al.*, 1990).

Logistic regression models were constructed to analyse incidence of phenodeviance (present or not) in the comb as a whole, as well as in C1 and C2, separately. For each model, the effects of population, temperature treatment and their interaction were evaluated. For any given model, the appropriate measure of trait size was entered as a continuous covariate.

The actual number of MMAs in the comb as a whole was also analysed. Because the data were best described by a gamma Poisson distribution ($P = 0.63$), and hence were strongly non-normal (Wilk–Shapiro $W = 0.68$, $P < 0.00001$), a one-way Kruskal–Wallis ANOVA tested for equality of mean ranks between populations and temperature treatments.

Results

Asymmetry diagnostics

The distribution of signed FA (R–L) values for each of the four traits (comb segments C1 and C2, and sternopleural bristles S1 and S2) were examined separately by population. None of the means were significantly different from zero (P s ranged from 0.085 to 0.73), and no significant skewness was detected (P s ranged from ≈ 0.2 to > 0.9) in any of the eight distributions. Signed FA in segment C2 in the Noumea population, and sternopleural bristle trait S2 in the Taipei population, exhibited significant leptokurtosis ($k = 1.026$ and 0.825 , P s < 0.005 and 0.02 , respectively), but such departure from normality is expected because it is consistent with the existence of individual differences in DI in the population (Gangestad & Thornhill, 1999; Van Dongen, 2006).

Pupal mortality and adult body size

Logistic regression revealed marked differences in pupal mortality across temperature treatments ($\chi^2 = 141.17$,

d.f. = 2, $P < 0.0001$), being sharply higher at the Variable temperature than at the other two treatments (Fig. 1). Although pupal mortality did not differ between the two populations ($\chi^2 = 0.37$, d.f. = 1, $P = 0.54$), there was a significant population \times temperature interaction ($\chi^2 = 16.2$, d.f. = 2, $P = 0.0003$), reflecting a different pattern of expression of mortality in the two populations (Fig. 1). For Noumea flies, there was a progressive increase in mortality from the Low through High to Variable temperatures, with mortality at the Variable temperature being 10-fold greater than at the Low temperature. For Taipei flies, whereas mortality dipped slightly from the Low to High temperatures, it then also increased, but only five-fold, at the Variable temperature.

Factorial ANOVA on thorax length revealed significant effects of population ($F_{1, 401} = 18.0$, $P < 0.0001$), temperature ($F_{2, 401} = 86.35$, $P < 0.0001$), and the population \times temperature interaction ($F_{2, 401} = 9.87$, $P < 0.0001$). For both populations, thorax length was relatively stable between Low and High temperatures, but then decreased sharply at the Variable temperature (Fig. 2). The decrease was more pronounced in the Taipei population, reflecting the significant population \times temperature interaction.

Trait size

MANCOVA, with thorax length as covariate, revealed significant overall effects of population (Table 1) on the four size traits, which persisted in a separate model without the thorax length covariate ($F_{4, 393} = 70.14$, $P < 0.0001$). Follow-up univariate ANOVAs revealed that, for all traits, Noumea flies had significantly larger trait values than Taipei flies (Table 2): comb size segments C1 and C2 were 7.6% and 14.7% greater, whereas the number of anterior bristles and transverse bristles

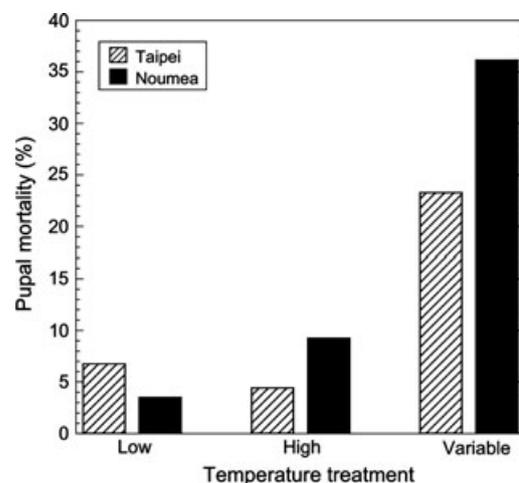


Fig. 1 Pupal mortality (%) in the Noumea and Taipei populations across temperature treatments.

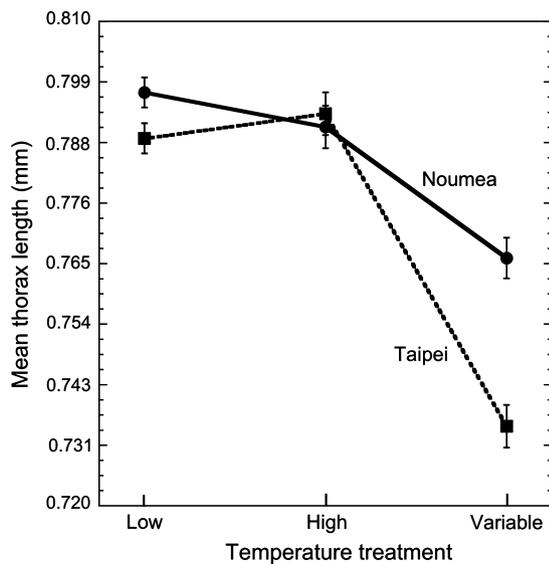


Fig. 2 Mean thorax length (mm) of males in the Noumea and Taipei populations across temperature treatments. Error bars represent ± 1 SE.

Table 1 Results of MANCOVAs of the effects of thorax length, population, temperature treatment and the population-by-temperature interaction, on size and FA traits. Significant effects are in bold.

Response traits/Factor	Roy's greatest root statistic	F	d.f.	P
Sizes (TC1, TC2, TS1, TS2)				
Thorax length	0.0813	7.95	4, 391	< 0.0001
Population	0.641	62.63	4, 391	< 0.0001
Temperature	0.0509	4.98	4, 392	0.0006
Population \times Temperature	0.00979	0.96	4, 392	0.43
FAs (FAC1, FAC2, FAS1, FAS2)				
Thorax length	0.00331	0.32	4, 391	0.86
Population	0.0330	3.22	4, 391	0.013
Temperature	0.0138	1.36	4, 392	0.25
Population \times Temperature	0.0312	3.05	4, 392	0.017

FA, fluctuating asymmetry.

were 10.7% and 6.8% greater, respectively. Thorax length was significantly positively related to three of the four size traits [slope (SE), C1: 6.16 (1.38), $P < 0.0001$; C2: 3.27 (1.37), $P = 0.017$; S1: 0.71 (1.51), $P = 0.64$; S2: 3.24 (1.37), $P = 0.018$].

The MANCOVA also revealed significant overall effects of temperature on the four size traits (Table 1), with three of the four traits responding significantly to the temperature treatments as indicated by follow-up ANCOVAs (Table 3). The pattern of expression of the four size traits across treatments was qualitatively similar, with all traits tending to increase in size with tempera-

Table 2 Least-squares mean (± 1 SE) size and fluctuating asymmetry (FA) of sex comb and sternopleural bristle traits of two geographic populations of *Drosophila bipectinata*. F and P values are from follow-up ANCOVAs to the multivariate analyses in Table 1. ANCOVAs for overall trait size and FA are also presented. Significant effects are in bold.

Trait	Population		F (d.f.'s)	P
	Taipei	Noumea		
Size				
TC1	5.671 \pm 0.0610	6.103 \pm 0.0584	25.79 (1, 400)	< 0.0001
TC2	7.165 \pm 0.0604	8.216 \pm 0.0578	155.43 (1, 400)	< 0.0001
TS1	4.877 \pm 0.0665	5.401 \pm 0.0646	31.45 (1, 394)	0.0001
TS2	7.136 \pm 0.0604	7.622 \pm 0.0587	32.83 (1, 394)	0.0001
CTOT	12.837 \pm 0.0903	14.319 \pm 0.0865	138.34 (1, 400)	< 0.0001
STOT	12.013 \pm 0.0973	13.024 \pm 0.0946	54.54 (1, 394)	0.0001
Fluctuating asymmetry				
FAC1	0.958 \pm 0.0619	0.891 \pm 0.0604	0.57 (1, 400)	0.45
FAC2	0.731 \pm 0.0583	0.892 \pm 0.0569	3.79 (1, 400)	0.052
FAS1	0.710 \pm 0.0525	0.746 \pm 0.0528	0.22 (1, 394)	0.64
FAS2	0.691 \pm 0.0559	0.866 \pm 0.0562	4.65 (1, 394)	0.032
FACTOT	1.712 \pm 0.0913	1.757 \pm 0.0897	0.11 (1, 400)	0.74
FASTOT	1.405 \pm 0.0801	1.607 \pm 0.0809	2.94 (1, 394)	0.087

ture stress (Table 3). When the multivariate analysis was run without the body size covariate, the treatment effect persisted but it was considerably weakened ($F_{4, 393} = 2.95$, $P = 0.020$), and when univariate analyses were performed without the thorax length covariate, none of the size traits differed significantly between temperature treatments (Table 3).

The two component parts of each trait type (the sex comb and sternopleurals) were summed to form CTOT and STOT, respectively, in each case reflecting overall trait size. Differences among temperature treatments for overall sex comb size and overall sternopleural bristle number were significant (Table 3), increasing in size with temperature stress. Overall comb size and sternopleural bristle number increased 3.5% and 4.3%, respectively, from Low to Variable temperature treatments. When these analyses were conducted without the thorax length covariate, the temperature effect persisted only for STOT, that is overall sternopleural bristle number (Table 3).

Fluctuating asymmetry

MANCOVA, with body size as covariate, revealed a significant overall effect of population on the four asymmetry traits (Table 1), with FA values being higher in the Noumea population (Table 2). In contrast to the analysis of trait size, the overall effect of temperature was not significant (Table 1). However, the population-by-treatment interaction was significant, warranting an examination of temperature effects separately by population. For Noumea, the overall treatment effect was not significant ($F_{4, 195} = 1.87$, $P = 0.12$), whereas for Taipei, the effect was close to significant ($F_{4, 194} = 2.32$,

Table 3 Least-squares mean (± 1 SE) size of sex comb and sternopleural bristle traits across the three temperature treatments. Trait sizes have been corrected (top panel) or uncorrected (bottom panel) by thorax length. Significant treatment effects are in bold.

Trait	Temperature treatment			<i>F</i> (d.f.'s)	<i>P</i>
	Low	High	Variable		
Thorax length-corrected					
TC1	5.835 \pm 0.0582	5.740 \pm 0.0810	6.087 \pm 0.0896	3.93 (2, 400)	0.020
TC2	7.552 \pm 0.0576	7.752 \pm 0.0802	7.767 \pm 0.0888	3.08 (2, 400)	0.047
TS1	5.026 \pm 0.0636	5.121 \pm 0.0882	5.272 \pm 0.0988	2.02 (2, 394)	0.13
TS2	7.231 \pm 0.0577	7.399 \pm 0.080	7.506 \pm 0.0897	3.57 (2, 394)	0.029
CTOT	13.387 \pm 0.0862	13.492 \pm 0.120	13.854 \pm 0.133	3.89 (2, 400)	0.021
STOT	12.257 \pm 0.0931	12.520 \pm 0.129	12.778 \pm 0.145	4.52 (2, 394)	0.011
Uncorrected by thorax length					
TC1	5.906 \pm 0.0575	5.805 \pm 0.0817	5.884 \pm 0.0798	0.52 (2, 401)	0.60
TC2	7.588 \pm 0.0559	7.785 \pm 0.0794	7.664 \pm 0.0746	2.05 (2, 401)	0.13
TS1	5.030 \pm 0.0613	5.125 \pm 0.0867	5.259 \pm 0.0863	2.36 (2, 395)	0.096
TS2	7.270 \pm 0.0561	7.435 \pm 0.0793	7.394 \pm 0.0790	1.72 (2, 395)	0.18
CTOT	13.494 \pm 0.0853	13.590 \pm 0.121	13.547 \pm 0.118	0.221 (2, 401)	0.80
STOT	12.300 \pm 0.0901	12.560 \pm 0.127	12.653 \pm 0.127	3.036 (2, 394)	0.049

$P = 0.058$). Follow-up univariate ANCOVAs indicated that FA in transverse sternopleural bristles differed significantly among temperature treatments in the Taipei population, being highest at the variable treatment (Table 4). This significant effect was not an artefact of the trait size covariate, as the effect persisted without the covariate in the model ($F_{2, 197} = 5.02$, $P = 0.0075$). When the remaining univariate analyses were conducted without their respective covariates, the nonsignificant effect of temperature persisted in all cases (P s > 0.05).

Phenodeviance

In the analysis examining the incidence of phenodeviance in the sex comb as a whole, multiple logistic

regression revealed significant effects of the temperature treatment ($\chi^2 = 13.77$, d.f. = 2, $P = 0.001$), with the probability of phenodeviance increasing with temperature stress (Fig. 3). Phenodeviance in the comb as a whole also increased with the comb size covariate ($\chi^2 = 25.30$, d.f. = 1, $P < 0.0001$), whereas the effects of population ($\chi^2 = 1.75$, d.f. = 1, $P = 0.19$) and the treatment \times population interaction ($\chi^2 = 0.102$, d.f. = 2, $P = 0.95$) were not significant.

When C1 phenodeviance was examined separately, logistic regression revealed that the population and temperature effects were not significant (Table 5). However, because the interaction between these terms was close to significant ($P = 0.067$, Table 5), a further breakdown of these effects was conducted, so that the effects of

Table 4 Least-squares mean (± 1 SE) fluctuating asymmetry (FA) from univariate ANCOVAs on sex comb and sternopleural bristle traits across the three temperature treatments. The covariate for a given FA trait was its respective measure of trait size. Significant effects are in bold.

Population/Trait	Temperature treatment			<i>F</i> (d.f.'s)	<i>P</i>
	Low	High	Variable		
New Caledonia					
FAC1	0.801 \pm 0.0800	1.005 \pm 0.114	0.946 \pm 0.109	1.25 (2, 203)	0.29
FAC2	0.807 \pm 0.0830	0.936 \pm 0.119	0.963 \pm 0.113	0.77 (2, 203)	0.47
FAS1	0.825 \pm 0.0718	0.602 \pm 0.101	0.890 \pm 0.101	2.38 (2, 197)	0.099
FAS2	0.865 \pm 0.0775	0.951 \pm 0.110	0.843 \pm 0.108	0.29 (2, 197)	0.75
FACTOT	1.293 \pm 0.110	1.300 \pm 0.156	1.546 \pm 0.149	1.03 (2, 203)	0.36
FASTOT	1.688 \pm 0.110	1.555 \pm 0.155	1.734 \pm 0.154	0.37 (2, 197)	0.69
Taipei					
FAC1	1.007 \pm 0.081	0.909 \pm 0.115	0.876 \pm 0.115	0.51 (2, 196)	0.60
FAC2	0.601 \pm 0.0683	0.838 \pm 0.0969	0.720 \pm 0.0962	2.04 (2, 196)	0.13
FAS1	0.669 \pm 0.0671	0.772 \pm 0.0948	0.609 \pm 0.0948	0.76 (2, 196)	0.47
FAS2	0.666 \pm 0.0697	0.456 \pm 0.0984	0.892 \pm 0.0986	4.93 (2, 196)	0.0081
FACTOT	1.216 \pm 0.0969	1.130 \pm 0.137	1.078 \pm 0.137	0.37 (2, 196)	0.69
FASTOT	1.340 \pm 0.0986	1.224 \pm 0.139	1.495 \pm 0.139	0.96 (2, 196)	0.38

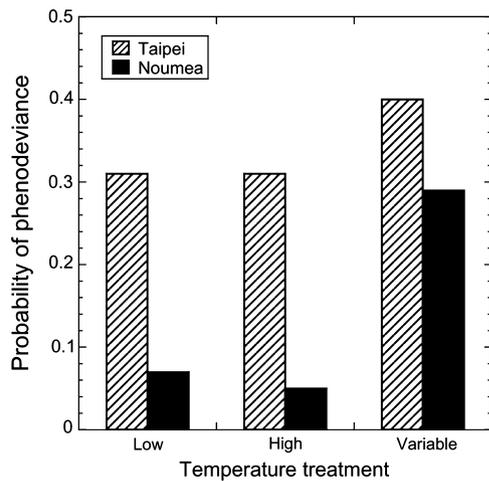


Fig. 3 Probability of phenodeviance across temperature treatments separately in comb segments C1 and C2.

temperature were examined separately by population. Incidence of C1 phenodeviance in the Taipei population did not differ across temperature treatments ($\chi^2 = 1.15$, d.f. = 2, $P = 0.56$), but for the Noumea population, the effect of temperature treatment was significant ($\chi^2 = 7.05$, d.f. = 1, $P < 0.030$), being highest at the Variable temperature (Fig. 4).

For C2 phenodeviance, the temperature effect was strongly significant (Table 5). C2 phenodeviance was similar in Low and High temperatures, but it increased sharply in the Variable treatment; in the Taipei population, C2 phenodeviance rose 17-fold, whereas in the Noumea population it rose by a factor of 3 (Fig. 4). This differential response is reflected in the significant population-by-temperature treatment interaction (Table 5). Phenodeviance levels for either segment did not differ significantly between the two populations (Table 5), consistent with the analysis of phenodeviance in the comb as a whole reported above.

Table 5 Results of logistic regression of the effects of trait size, population and temperature treatment on the incidence of phenodeviance in comb segments C1 and C2 separately. Significant effects are in bold.

Response trait/Factor	d.f.	χ^2	P
C1 phenodeviance			
C1 size (TC1)	1	87.23	< 0.0001
Population	1	1.14	0.29
Temperature	2	1.89	0.39
Population \times Temperature	2	5.42	0.067
C2 phenodeviance			
C2 size (TC2)	1	3.18	0.075
Population	1	0.37	0.54
Temperature	2	33.11	< 0.0001
Population \times Temperature	2	6.44	0.040

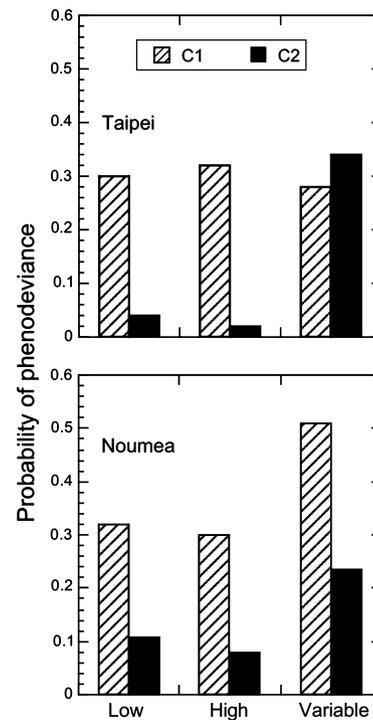


Fig. 4 Probability of phenodeviance across temperature treatments in comb segments C1 and C2, separately by population.

Phenodeviance was also analysed as the total number of abnormalities within the sex comb as a whole. Kruskal–Wallis ANOVA revealed significant differences among temperature treatments ($\chi^2 = 17.55$, d.f. = 2, $P = 0.0002$); distributions of MMAs by temperature treatment are presented in Fig. 5. Whereas mean ranks of MMAs did not differ between Low and High temperature treatments ($\chi^2 = 0.011$, d.f. = 1, $P = 0.92$), mean ranks of MMAs in the Variable treatment was significantly greater than both the Low ($\chi^2 = 14.80$, d.f. = 1, $P = 0.0001$) and High ($\chi^2 = 11.46$, d.f. = 1, $P = 0.0007$) treatments (Fig. 5). In contrast, mean ranks of MMAs did not differ between populations ($\chi^2 = 1.26$, d.f. = 1, $P = 0.26$).

Discussion

Previous work on the Noumea population revealed how minor phenodeviations and FA in the sex comb are the targets of sexual selection in the field (Polak & Taylor, 2007). Here we have focused on the environmental sensitivity of these same traits. This focus was motivated by the fact that in *D. bipunctinata*, which is one of a few species in which sexual selection has been shown to act on DI in a secondary sexual trait (Tomkins & Simmons, 2003; Polak, 2008), the genetic and phenotypic correlations between trait size and DI within populations are typically positive (Polak & Taylor, 2007). This pattern of

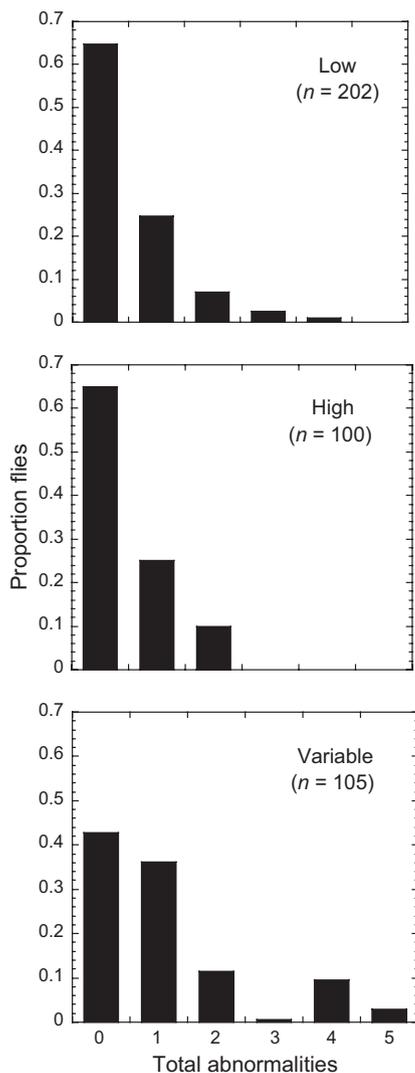


Fig. 5 Proportion flies across category of increasing phenodeviance in the comb as a whole, with the categories ranging from 0 to 5 total abnormalities.

association is contrary to the DI-sexual selection hypothesis, which predicts negative covariation between trait size and DI in secondary sexual traits because both *large* trait size and *low* DI are expected to be features of the highest (genetic) quality males in the population (Møller, 1990; Møller & Pomiankowski, 1993; Møller & Cuervo, 2003). We therefore investigated how the environment might generate variation in the expression of trait DI and size in two geographic isolates (genotypes) of the focal species.

The effects of temperature on developing flies transiting their pupal stage were dramatic in showing that the Variable temperature treatment (cycling between 29 and 34 °C) had a stronger negative influence on physiological functions compared to the constant 25 and 29 °C

treatments. Perhaps the most straightforward manifestations of these damaging effects are those on pupal mortality and adult body size. Because declines in body size often have negative fitness consequences (e.g. Partridge *et al.*, 1987; Markow & Ricker, 1992; Bangham *et al.*, 2002; Polak *et al.*, 2004), the responses of both these fitness components provide a good barometer of the levels of developmental stress that flies experienced in our experimental treatments.

Whereas there was an overall marked increase in pupal mortality at the Variable temperature, Noumea flies were significantly more sensitive to thermal stress: Noumea flies suffered greater mortality relative to Taipei flies in both the High and the Variable temperature treatments. There are several possible explanations for these differential responses. One is that the Noumea flies are more developmentally unstable as revealed by higher FA levels in C2 and transverse sternopleural bristles. Thus, if this elevated DI translates to increased physiological and survival costs under stress conditions (McKenzie, 2003), this could explain the differential mortality we observed. Alternatively, the greater sensitivity of the Noumea population could also at least in part be the result of local adaptation to climatic conditions. Noumea has a temperate oceanic climate, receiving on average just over 1000 mm of precipitation annually (Logan & Cole, 2001; climatemp.info, 2010). The average temperature is 22.7 °C, with a monthly average maximum temperature of 30 °C and minimum of 16 °C (climatemp.info, 2010). Taipei on the other hand has a maritime subtropical climate, receiving over 2000 mm of rain annually (climatemp.info, 2010; Ellicott, 2010). Whereas it has a slightly cooler average monthly temperature (21.6 °C) than Noumea, the average maximum temperature in Taipei is 33 °C and the minimum is 12 °C (climatemp.info, 2010). Thus, flies in Taipei experience a more extreme thermal environment (although one that is also more humid), potentially explaining why flies from this region showed a reduced mortality relative to Noumea flies under high thermal stress in this study. Testing this idea more rigorously, however, would require a survey of a greater number of populations throughout *D. bipunctinata*'s range, as performed by Coyne *et al.* (1983) working with *Drosophila pseudoobscura*. These authors surveyed seven different populations of *D. pseudoobscura* and found differences among populations in pupal mortality in response to temperature in the laboratory. Mortality generally correlated with climatic conditions with populations derived from hotter habitats tending to be more heat resistant, providing evidence for the hypothesis that differences in temperature sensitivity are adaptations to local climatic conditions (Coyne *et al.*, 1983).

In contrast to mortality, body size in *D. bipunctinata* exhibited a reverse response to stress between the populations, decreasing more strongly in the Taipei population than in the Noumea population. This pattern

is expected under the action of developmental selection against small-bodied flies in the Noumea population. If such developmental selection were stronger in our Noumea cultures, as implied by the greater overall mortality we observed in this population, then this could explain the attenuated decrease in body size among the surviving adults relative to the Taipei flies.

Expression of condition-dependent sexual traits is expected to be sensitive to environmental stress (Tomkins *et al.*, 2004). The sex combs in *D. bipectinata* studied here show a significant increase in their relative expression under the most extreme stress level; the *absolute* size of the traits did not change, however. As shown here, the sex comb is a body size-dependent trait, with larger individuals expressing more teeth in the combs (and see Polak *et al.*, 2004). The increase in the relative size of the combs with developmental stress seems most likely to be an artefact of different developmental regulation of comb (and bristle) expression and that of thorax length. If, in comparison with thorax length, the size of the comb is relatively independent of the costs induced by heat stress at the pupal stage, combs would be relatively larger in flies reared under the most stressful conditions, which is indeed what we see. No premating sexual selection on comb size was found in the field in the Noumea (Polak & Taylor, 2007) or Taipei (Polak & Hsu, unpublished data) populations, and here we show that in both these populations comb size is indeed a poor indicator of thermal stress conditions, at least in terms of that experienced by pupal flies.

Overall, the incidence of phenodeviance and the number of MMAs in both parts of the sex comb (C1 and C2) do appear to be environmentally sensitive in generally similar ways in both populations. Therefore, phenodeviance in *D. bipectinata* is reflective of the developmental environments that cause significant fitness decrements in terms of mortality and body size: a foundation stone of the notion that DI is a measure of phenotypic quality in sexual selection (Møller & Pomiankowski, 1993; Watson & Thornhill, 1994; Blanckenhorn *et al.*, 2003; Tomkins & Simmons, 2003). Breaking the effect of stress down into independent analyses of C1 and C2, however, we found heterogeneity in response between the populations. Specifically, environmental stress increased incidence of phenodeviance in C1 in Noumea, whereas this was not the case for the Taipei population where C1 phenodeviance was statistically invariant across temperature treatments. This differential response of C1 is particularly interesting in the light of the fact that C1 phenodeviance is a target of sexual selection in Noumea, but not in Taipei. Our results therefore support the hypothesis that sexual selection for developmental stability can be generated through heterogeneity in exposure to environmental stress (Polak & Starmer, 2005).

If we assume the existence of $G \times E$ interaction for phenodeviance within populations, which is reasonable

in light of the population (genotype) \times treatment interaction reported here, then sexual selection resulting from environmental heterogeneity could be an important mechanism fuelling sexual selection for genetic quality. Under persistent sexual selection of this sort, the Noumea population could be expected to change genetically towards ameliorated C1 phenodeviance. Given especially the notably high levels of DI (i.e. DI enrichment) in Noumea detected previously (Polak & Taylor, 2007), it would be interesting to follow this population in terms of its levels of instability. Yet, although previous work (Polak & Taylor, 2007; Polak & Hsu, unpublished data) failed to detect significant sexual selection on C2 phenodeviance, both populations examined here showed strong increases in C2 phenodeviance in response to thermal stress. Why C2 phenodeviance should escape sexual selection in nature remains unclear.

Similar to phenodeviance, there was a significant interaction between population and treatment on FA, confirming that the sensitivity of DI to thermal stress varies across geographic populations (genotypes). This in itself also shows that genetic differences can affect the way in which developmental stress is manifested as DI. To identify the cause of this significant interaction, temperature effects on FAs were examined separately by population, and we uncovered the following: FA in transverse sternopleural bristles increased significantly in response to temperature in Taipei flies but not in New Caledonia flies. Of further interest is that this analysis also revealed that FA responses to stress were not more pronounced in the sex comb relative to sternopleural bristles, which is contrary to expectation because DI in secondary sexual traits is expected to be differentially sensitive to developmental stress relative to ordinary morphological traits (Møller & Pomiankowski, 1993; Tomkins & Simmons, 2003). Such a lack of relative sensitivity of FA in a secondary sexual trait is not uncommon, however, having been reported previously in a number of species such as stalk-eyed flies (David *et al.*, 1998; Bjorksten *et al.*, 2000, 2001) and medflies (Hasson & Rossler, 2002; and see Tomkins & Simmons, 2003 and Polak, 2008 for general reviews of the subject).

In sum, our results reveal considerable complexity in the relationship between variation in individual secondary sexual traits and the developmental properties of the organisms bearing them, and these complexities go some way towards explaining the patterns of sexual selection seen in nature. A main point of our study is that phenodeviations in a secondary sexual trait provide a more sensitive indicator of exposure to fitness damaging environmental stress during a specific window in development than does FA, and indeed trait size. The significant (but weak) genetic basis to phenodeviations in the sexual trait we examined (Polak & Taylor, 2007), combined with the environmental sensitivity reported here, ought to generate sexual selection against individuals bearing sex comb phenodeviations provided they are

common enough. This has happened for C1 in Noumea but curiously not in Taipei. These differences between populations plausibly arise because phenodeviations in the Taipei population are relatively insensitive to stress; nevertheless, differences in the genetic architecture of DI may also play a role in explaining the differences between populations in sexual selection. The heritability and environmental sensitivity of phenodeviations may provide useful insights into the developmental history of individuals and populations.

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