Temperature shock during development fails to increase the fluctuating asymmetry of a sexual trait in stalk-eyed flies

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The fluctuating asymmetry (FA) of bilateral traits is claimed to be a general indicator of environmental stress. Exaggerated sexual ornaments are thought to show elevated levels of FA and a greater response to stress than other traits. Previous work with stalk-eyed flies (*Cyrtodiopsis dalmanni*) has shown that the FA of the sexual trait (male eye stalks), wing length and wing width were unaffected by a continually applied food stress. Here we tested whether a transient stress (24-h heat shock at 31 °C during development) affected the FA of these traits. A second experiment tested the combined stresses of transient heat shock at 31 °C with continuous exposure to desiccation. In each experiment, temperature shock reduced the trait size, confirming that the treatments were stressful. However, stress had no effect on the FA of individual traits or the FA summed across all traits. Exposure to the combined stresses significantly elevated mortality and reduced trait size compared to the single-stress regime. However, FA did not differ significantly between flies from the two experiments. We found no evidence that FA in sexual and non-sexual traits reflects transient stress during the development of *C. dalmanni*.

Keywords: fluctuating asymmetry; stalk-eyed flies; environmental stress

1. INTRODUCTION

One of the most widespread claims about fluctuating asymmetry (FA), i.e. non-directional deviations from bilateral symmetry, is that it is a sensitive and reliable indicator of environmental stress (Leary & Allendorf 1989; Parsons 1990, 1992; Clarke 1995). However, the evidence from experimental studies for such a role for FA is inconsistent. Some studies have detected an increase in FA under environmental stress (Parsons 1961, 1962; Møller 1992; Polak 1993; Freebairn *et al.* 1996; Brakefield 1997; Hunt & Simmons 1997; Imasheva *et al.* 1997; Blankenhorn *et al.* 1998; Roy & Stanton 1999; Woods *et al.* 1999) while others have not (Beardmore 1960; Campbell & Emlen 1996; Arnqvist & Thornhill 1998; David *et al.* 1998; Hochwender & Fritz 1999). Where stress increases FA it usually does so in a trait-specific way (Møller 1992; Brakefield 1997; Hunt & Simmons 1997; Imasheva *et al.* 1997; Blankenhorn *et al.* 1998; Roy & Stanton 1999; Woods *et al.* 1999), but the FA of traits within an individual is generally uncorrelated (Clarke 1998; Woods *et al.* 1999), suggesting that FA is not a general indicator of organism-wide developmental stability.

The lack of consistency in FA responses may reflect variation between trait types in their susceptibility to environmental stress (Woods *et al.* 1999). Sexually selected ornamental traits have been claimed to have special characteristics that will make their FA unusually responsive to environmental stress (Møller 1990; Møller & Pomiankowski 1993; Watson & Thornhill 1994). Sexual traits are usually costly to produce and show a high degree of condition dependence (Andersson 1994). The FA of sexual traits may therefore be more sensitive to

environmental and genetic stress than the FA of other traits. Møller & Pomiankowski (1993) argued that sexually selected traits should have greater sensitivity to environmental stress during development due to their recent history of strong directional selection, which is predicted to lead to a reduction in canalization. To date, five experimental studies have compared the effect of environmental stress on a sexually selected trait versus other traits (reviewed in Bjorksten *et al.* 2000b). Two detected an increase in the FA of the sexual trait under stress but not other traits (Møller 1992; Hunt & Simmons 1997) and three found no effect of stress on the FA of any traits (David *et al.* 1998; Roy & Stanton 1999; Bjorksten *et al.* 2000a).

Most studies have used continuous stresses. However, organisms may adjust their development so as to decrease the impact of continuous stresses, perhaps by adjusting their overall body size or resource allocation to particular traits. If there are reductions in size and changes in allometric relationships between traits, these may limit the loss of developmental stability and any change to patterns of FA.

In order to evaluate this possibility we investigated the effect of transient temperature shocks on the size and asymmetry of sexual and non-sexual traits in stalk-eyed flies (*Cyrtodiopsis dalmanni*). Our previous studies with continuous food stress showed no effect on FA in either sexual or non-sexual traits (David *et al.* 1998; Bjorksten *et al.* 2000a). In our primary experiment, flies were stressed by transient exposure to high temperature (31 °C) for 24 h at one of three developmental stages between the late larval and early pupal period. A further experiment tested whether a more severe stress regime combining transient temperature shock with continuous exposure to desiccation caused additional responses in FA.

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Figure 1. Experiment 1: transient temperature shock. The effect of temperature shock on (a) male and (b) female trait size and unsigned FA of (i) eye stalk length, (ii) wing length, and (iii) wing width in two populations (G and L). Error bars show standard errors. Trait size was significantly decreased by stress (asterisk), but FA did not differ significantly between treatments for any of the traits. Shaded bars, FA; filled diamonds, size.

2. MATERIAL AND METHODS

(a) Stocks

Two laboratory populations ('L' and 'G') were used in these experiments. They were founded in 1993 from independent collections made at two locations in Malaysia. They had been maintained in population cages of at least 200 individuals per cage at 25 °C under a 12 L:12 D cycle on ground corn medium.

(b) Experimental design

Batches of ten females were allowed to lay eggs over a two-week period for 5 h intervals on damp filter paper that had been folded to form a series of parallel ridges. Groups of 12 eggs, taken from all areas of the filter paper, were placed in 400 ml plastic pots, with cotton wool and filter paper dampened with 80 ml of water in order to maintain humidity. Pots were misted daily with water for the first 4 days in order to prevent desiccation of the eggs and newly hatched larvae.

Each pot contained 30 ml of pureed sweetcorn food medium to provide a non-limiting density (David *et al.* 1998) and was assigned randomly to three experimental treatments and one control treatment and for culture at 25 °C. The sex ratio of the emerged adults did not differ significantly from 1:1 for any treatment.

(c) Experiment 1: transient temperature shock

The stress used was 24 h exposure to 31 °C. Previous experiments had shown that constant development at 31 °C was lethal but that a 24 h exposure caused a large reduction in eye stalk length if applied around pupation. The temperature shocks were applied ten (late larval), 12 (early pupal) or 14 (mid-pupal) days after hatching. The shock was administered by moving experimental cultures to a growth cabinet at 31 ± 0.5 °C for 24 h. Control flies were maintained continuously at 25 °C. Eclosing adults were collected daily and frozen for later measurement. Forty-one pots were set up per treatment and the number of adults collected varied from 87 to 130.

Figure 1. (continued).

Table 1. Effects on trait size and FA in experiment 1 for eye stalk length (EL), wing length (WL) and wing width (WW)

(Mean squares denoted by an asterisk are significant at $p < 0.05$ following sequential Bonferroni correction.)

trait	population	source of variation	male		female	
			d.f.	mean square	d.f.	mean square
EL	G	stress	3	4.2850*	3	0.2546*
		residual	176	0.9902	208	0.0182
	L	stress	3	23.2381*	3	0.1710*
		residual	183	0.7445	192	0.0365
WL	G	stress	3	4.1933*	3	0.1600
		residual	166	1.2006	182	0.8143
	L	stress	3	14.3866*	3	2.1866
		residual	191	1.3039	175	0.8344
WW	G	stress	3	0.0005	3	0.0004
		residual	160	0.0003	168	0.0002
	L	stress	3	0.0061*	3	0.0003
		residual	193	0.0005	176	0.0002

Figure 2. Experiment 2: transient temperature shock combined with desiccation. The effect of temperature shock on (a) male and (b) female trait size and unsigned FA. Trait size was significantly decreased by stress (asterisk), but FA did not differ significantly between treatments for any of the traits. Other details are as in figure 1. Shaded bars, FA; filled diamonds, size.

(d) Experiment 2: temperature shock combined with desiccation

We decreased the humidity by reducing the amount of water from 80 to 60 ml and omitting the daily misting with water. The 24-h temperature shock was applied as in experiment 1. Therefore, the day 10, 12, and 14 treatments experienced temperature shock and desiccation, while the control group experienced desiccation only. Sixty-eight pots were set up and the number of individuals collected varied from 31 to 41.

(e) Measurements

Measurements were made with a video camera mounted on a monocular microscope using the image analysis programme NIH image (version 1.55). Three traits, eye stalk length, wing length and wing width, were measured for FA and size using the landmarks described in David *et al.* (1998). All measurements were made 'blind' by a single person (T.B.). The left and right sides of a trait were measured during the same session.

Each trait was measured twice for each individual, with repeated measurements made several days apart.

(f) Tests for directional asymmetry, anti-symmetry and measurement error

The distributions of signed asymmetries (left minus right) for each trait, sex, environment and population were tested for normality (Shapiro–Wilks W) and a mean of zero (single sample t -test) in order to detect the presence of anti-symmetry or directional asymmetry (Palmer 1994).

We used the method of David *et al.* (1999) for distinguishing between FA and measurement error. The standard approach of Palmer (1994) is inappropriate when left and right do not have independent measurement errors. Owing to the large sample size, our measurements were taken in more than one session so that left and right were potentially subject to session bias. In such circumstances it is prudent to analyse the signed difference of left minus right ($L - R$) with a one-way ANOVA (David *et al.*

Figure 2. (continued).

1999), which partitions the total variance into variance between individuals (given by the mean square between individuals, MS_{SA}) and variance between replicates within individuals (given by the mean square within individuals, MS_{err}). The F -ratio of these variances measures the observed FA relative to the measurement error. An estimate of population FA, which is analogous to the FA_{10} of Palmer (1994) but free of both measurement error and session bias, was then calculated as $(MS_{SA} - MS_{err})/2r$, where r is the number of replicates. Separate one-way ANOVAs were performed for each population, trait and sex. Sequential Bonferroni corrections were used in order to correct for multiple comparisons within traits.

(g) Size dependence of FA

Since unsigned asymmetry has a half-normal distribution, the data were transformed for all traits using $|L - R|^{0.4}$ (Swaddle *et al.* 1994) before using parametric tests on the normalized distributions. Scaling of unsigned FA with trait size may lead to apparent differences in FA between samples if the mean size or size variance differs between samples. In order to test for size dependence of FA we regressed the $|L - R|^{0.4}$

transformed unsigned FA on trait size for each sex, trait and population.

(h) Effect of stress: experiment 1

We tested whether there was an effect of stress by measuring decreases in trait size for eye stalk length, wing length and wing width. As the size distributions for each trait were skewed, the data were normalized using the Box-Cox transformation $((X^4 + 1)/4)$. The sexes differed significantly in size, so each trait was analysed by one-way ANOVAs for each sex and population separately. Where the ANOVA showed a significant 'stress' effect, we used Dunnett's test in order to compare each treatment to the control.

As trait sizes differed between the sexes, we analysed the effect of stress on FA in males and females separately by one-way ANOVAs. Tests were only performed where a significant stress effect was detected on trait size and only those treatments that differed from the control were included.

We also created a combined FA index by summing FA over the three traits. The data were standardized by subtracting the sample mean from each data point for unsigned FA (Bennett &

Table 2. Effects on trait size and FA in experiment 2 for eye stalk length (EL), wing length (WL) and wing width (WW)

(Mean squares denoted by an asterisk are significant at $p < 0.05$ following sequential Bonferroni correction.)

trait	population	source of variation	male		female	
			d.f.	mean square	d.f.	mean square
EL	G	stress	3	0.8591	3	0.0239*
		residual	62	2.5400	69	0.1667
	L	stress	3	6.3366*	3	0.0733
		residual	91	0.9158	58	0.0279
WL	G	stress	3	1.5901	3	3.1833*
		residual	56	1.7567	52	0.5123
	L	stress	3	5.7400	3	1.2633
		residual	90	1.6342	56	0.9058
WW	G	stress	3	4.3018	3	5.1463
		residual	53	5.6603	41	2.4390
	L	stress	3	0.0018	3	5.2692
		residual	89	0.0006	52	1.9231

Hoffmann 1998). This centres the unsigned FA distribution around zero. Combined FA was analysed using separate two-way ANOVAs for males and females, with the factors 'population' and 'stress'.

Finally, we tested the claim that exaggerated ornamental traits have higher FA than other traits. Since the individual trait ANOVAs showed no significant effect of stress on FA in males (see below) and preliminary analysis showed no differences between populations, we pooled absolute unsigned FA values across stress categories and used one-way ANOVA for comparing the level of FA between traits for males. In further ANOVAs we compared the levels of relative FA (unsigned FA divided by the trait mean) because the traits differ considerably in size.

(i) *Effect of stress: experiment 2*

The effects on trait and FA values were tested in one-way ANOVAs for each population, sex and trait where stress showed a significant effect on trait size. Mortality and trait means were compared between experiments in order to test for effects of combined stresses.

3. RESULTS

(a) *Repeatability and measurement error of FA*

The trait size measurements had high repeatability ($r > 0.9$ and $p < 0.0001$ for all traits). The asymmetry measurements were also highly repeatable ($r > 0.72$ and $p < 0.0001$ for all traits).

Signed asymmetry ($L - R$) values were subjected to one-way ANOVAs for each trait, sex and population. In all cases the F -ratios of the variance between individuals to that between replicates within individuals were highly significant ($p < 0.01$), indicating that our measure of FA was not obscured by measurement error. The percentage of variation attributable to FA was high (mean $\pm$ s.d., eye stalk length = $50 \pm 21\%$, wing length = $65 \pm 21\%$ and wing width = $47 \pm 21\%$).

(b) *Directional asymmetry, anti-symmetry and size dependence of FA*

Signed asymmetry was normally distributed in all samples (Shapiro–Wilks W), indicating FA rather than

anti-symmetry. Single-sample t -tests showed that the distribution means did not differ significantly from zero, indicating FA rather than directional asymmetry. The coefficients from regressions of $|L - R|^{0.4}$ transformed, unsigned FA on trait size were never significantly different from zero, indicating that there is no relationship between trait size and degree of FA for any of the traits.

(c) *Effect of stress: experiment 1*

(i) *Trait size*

The effects of stress on trait size are shown in figure 1 and summarized in table 1. Stress treatment had significant effects on eye stalk length for both sexes in both the G and L populations. In males, comparisons of treatments with the controls showed that eye stalk length was reduced by temperature shock on day 12. Females also had smaller eye stalks on day 12 and an even greater reduction when shocked on day 10.

The effects of stress on wing traits varied across populations and sexes. Stress reduced wing traits in males but not in females (table 1). Wing traits in L males' wing length and wing width were most strongly affected by shocks on day 10 whilst G males responded significantly to shocks on days 10 and 14.

(ii) *Individual trait FA*

Tests for an effect of temperature shock on FA were restricted to treatments where a significant effect on trait size had been demonstrated. Individuals stressed on day 12 did not differ from the controls for eye stalk FA in G males ($t_{90} = -2.097$ and $p = 0.039$). Eye stalk FA in L males also showed no difference between the controls and males stressed on days 10 and 12 ($F_{2,148} = 0.935$ and $p = 0.395$). There were also no effects of stress on eye stalk FA on days 10 or 12 in G females ($F_{2,143} = 0.509$ and $p = 0.602$) or L females ($F_{2,161} = 0.085$ and $p = 0.919$).

There were no effects of stress on wing length in males on days 10 and 14 in the G population ($F_{2,124} = 0.213$ and $p = 0.808$) or on day 10 in the L population ($t_{80} = -0.381$ and $p = 0.704$). In addition, the wing width FA of L males showed no significant differences between controls and flies stressed on days 10 or 12 ($F_{2,151} = 0.928$ and $p = 0.398$).

(iii) *Composite FA*

We found no evidence to support the notion that combining FA over several traits can reveal underlying subtle responses to stress. G males had higher composite FA than females ($F_{1,266}=12.47$ and $p=0.0005$) but stress ($F_{3,266}=2.11$ and $p=0.099$) and the sex–stress interaction ($F_{3,266}=1.16$ and $p=0.326$) were non-significant. L males had higher composite FA ($F_{1,410}=17.469$ and $p<0.0001$) but stress ($F_{3,410}=1.235$ and $p=0.297$) and the sex–stress interaction ($F_{3,410}=0.325$ and $p=0.808$) were non-significant.

(iv) *Sexual trait FA*

We compared the FA of sexual and non-sexual traits in males using absolute and relative values. There was a significant effect of trait on the mean FA (absolute FA, $F_{2,1092}=9.902$ and $p<0.0001$ and relative FA, $F_{2,1092}=92.302$ and $p<0.0001$). However, the underlying pattern differed for the two asymmetry measures. For absolute FA, eye stalk FA was significantly higher than wing trait FA (mean $\pm$ s.d., eye stalks = 0.018 ± 0.0008 , wing length = 0.014 ± 0.0006 and wing width = 0.013 ± 0.0006). For relative FA, wing width had the highest FA (0.015 ± 0.0007), eye stalk FA was intermediate (0.009 ± 0.0004) and wing length had the lowest value (0.006 ± 0.0002).

(d) *Effect of combined stress: experiment 2*

The additional factor of desiccation caused the conditions to be more stressful. Mortality was significantly higher in experiment 2 than experiment 1 (88% versus 42%) (Fisher's exact test, $p<0.0001$). The effects of combined stress on the trait and FA values are shown in figure 2 and summarized in table 2. The reduction in trait size was significant in the three treatments: eye stalk length in L males (day 12), eye stalk length in G females (days 12 and 14) and wing length in G females (days 10 and 12). However, FA was not significantly affected by stress in these cases (L male eye stalk, $t_{43}=1.033$ and $p=0.307$, G female eye stalk, $F_{2,43}=1.355$ and $p=0.269$ and G female wing length, $F_{2,43}=1.016$ and $p=0.371$).

4. DISCUSSION

In this study we investigated the effect of short-term temperature shocks on the FA of sexual and non-sexual traits in the stalk-eyed fly *C. dalmanni*. Previous work with *C. dalmanni* using continuous food stresses has shown large reductions in trait size and in eye stalk/body size allometry but no effect on FA (David *et al.* 1998; Bjorksten *et al.* 2000a). These conclusions about FA could be misleading if individuals adjust their development so as to reduce the impact of a continuous stress. Here we have avoided this potential problem by using a short-term transient stress (temperature shock). Brakefield (1997) used this approach in *Bicyclus* butterflies and found that eye spot FA did respond to temperature stress but only if the shock coincided with the developmental stage when the pattern of eye spots was determined. We timed the temperature shock to coincide with development of the sexual trait, the eye stalk. The precise mechanisms and timing of eye stalk development are not yet established but an analysis of eye antenna imaginal disc development

indicates that eye stalk growth occurs throughout the late larval and early pupal periods tested here (I. Hurley, personal communication).

We carried out a pair of relatively large-scale experiments on two independently derived populations. The first experiment subjected flies to 24-h heat shocks and the second examined responses to the combined stresses of heat shock and desiccation. The experimental conditions were stressful as reflected in large reductions in eye stalk and wing size and a large decrease in survival in the second experiment. Despite this, we found no effect of stress on the FA of eye stalks, wing length or wing width in either experiment. Trait FA appeared to be as unresponsive to transient shocks as it was to continuous stress. Our results are contrary to the hypothesis that FA increases under environmental stress.

The notion that sexually selected ornaments are particularly sensitive to stress (Møller & Pomiankowski 1993) is unsupported by our data. FA in male eye stalks was no more sensitive to stress than that of female eye stalks or wing traits. This outcome is identical to our previous findings for continuous food stress (David *et al.* 1998; Bjorksten *et al.* 2000a).

It has also been proposed that sexually selected ornaments have higher average FA than other traits (Møller & Pomiankowski 1993). The absolute FA of male eye stalk length was higher, providing naive support for the prediction. However, given that traits differ in size, a more appropriate comparison can be made using relative FA. This reveals that the relative FA of male wing width is considerably higher than that of male eye stalks and so the prediction remains unsupported.

Some authors (Zhivotovsky 1992; Palmer 1994; Woods *et al.* 1999) have promoted the concept of a composite FA index (summing FA over several traits) as a more reliable indicator of stress. This approach could enhance a stress response signal when individual traits show small but non-significant increases in FA. However, we found no effect of stress on a composite FA measure.

In conclusion, our results are remarkably consistent in failing to support the notion that FA is a sensitive and reliable indicator of stress. The FA of some traits in some species may respond to some stresses. However, this cannot be assumed and needs to be established in detail in each case before FA can be used as an informative measure in this context.

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