EVOLUTION & DEVELOPMENT 5:3, 231–239 (2003)

Heat shock in the developmentally sensitive period of butterfly eyespots fails to increase fluctuating asymmetry

Casper J. Breuker* and Paul M. Brakefield

Evolutionary Biology, Institute of Evolutionary and Ecological Sciences, Leiden University, 2300 RA Leiden, The Netherlands
*Author for correspondence (e-mail: breuker@rulsfb.leidenuniv.nl)

SUMMARY Fluctuating asymmetry (FA) is considered to provide a means of evaluating developmental stability and to reflect an individual's quality or the stress experienced during development. Stress is predicted to increase the phenotypic variation of both FA and trait size. In this study we examined the effect of a particular heat shock on both FA and size of eyespots in the butterfly, *Bicyclus anynana.* We also examined whether those eyespots thought to be involved in partner choice and sexual selection were particularly sensitive to stress. We applied a heat shock of 39.5°C for 3 h before, during, and after a sensitive period in eyespot development. We examined the FA, variation in FA, size, and variation in size of five eyespots, two on the dorsal forewing (sexually selected traits), two on the ventral forewing, and one on the ventral hindwing (nonsexually selected traits). For each sex and treatment, the heat shock did not result in significant changes in mean trait size and FA nor in the variation of size and FA. There were no differences in the response to the heat shock between sexually and nonsexually selected traits. We discuss how the increased production of heat shock proteins, including HSP60, may have stabilized development and how this might explain the results.

Introduction

A developing organism is likely to encounter forms of stress of environmental and genetic origin (Parsons 1990). Fluctuating asymmetry (FA) describes small nondirectional deviations from bilateral symmetry. FA is thought to provide a means of evaluating developmental stability or the capacity of individuals to buffer development against the disruptive effects of developmental stress (Van Valen 1962; Palmer and Strobeck 1986, 1992; Palmer 1994; Markow 1995). If there are no a priori reasons to assume significant differences between individuals in developmental stability, then FA may be used as a reliable indicator of stress (Leary and Allendorf 1989). Results from several studies have been inconsistent, however. FA does not always increase under stress, and if so, it seems to do so in a trait-specific way (Bjorksten et al. 2000; Clarke et al. 2000). In this study we examined the effect of a particular heat shock on both FA and size of eyespots in the butterfly, *Bicyclus anynana*. We also examined whether those eyespots thought to be involved in partner choice and sexual selection were particularly sensitive to the shock.

It is hypothesized that the development of sexual ornaments is weakly stabilized due to their presumed recent history of directional selection (experimental evidence reviewed by Møller and Swaddle 1997). Furthermore, The development of these traits are proposed to be highly condition dependent and thereby more likely to show higher levels of FA and a greater variability in trait size and FA (Møller 1990). It thus follows that sexual traits are more likely to respond to stress than homologous nonsexual traits (Hunt and Simmons 1997; Woods et al. 1999; Bjorksten et al. 2000). However, the results of six studies reported by Bjorksten et al. (2000) testing these hypotheses are also inconsistent.

The wings of the tropical butterfly *B. anynana* have eyespots on both their ventral and dorsal surfaces. Each eyespot consists of concentric rings of different color centered around a white "pupil." The development of these eyespots is progressively specified in four stages (Brakefield et al. 1996; Brunetti et al. 2001) (Fig. 1A). In the first stage, during the last larval instar, the transcription factor *Distal-less* (*Dll*) is expressed in a prepattern in the developing wing disks. In the second stage, organizing groups of cells called foci are established in the particular areas of the larval wing disk destined to become the pupil areas of each adult eyespot. The expression of *Dll* and other developmental genes is upregulated in the cells of an eyespot focus. In the third stage, covering the first 24 h after pupation, an information gradient is established around each focus apparently by focal signaling. This is then interpreted by surrounding cells to determine which color pigments will be produced later, just before adult eclosion (fourth stage). Brunetti et al. (2001) showed

© BLACKWELL PUBLISHING, INC.

Fig. 1. (A) Schematic diagram of the development of *Bicyclus anynana* with respect to eyespot formation at 27°C. Squares indicate the timing of single applications of a heat shock of 39.5°C for 3 h and therefore correspond to the five treatments. Time 0 is the onset of pupation, and –24 indicates the onset of the prepupal stage. (B) *Bicyclus anynana* wings with the five study eyespots as measured for each treatment and for each sex. WL, an index of wing length, was measured as the distance between the white pupils of the two dorsal eyespots (interfocal distance). DF1, anterior eyespot of the dorsal forewing; DF2, posterior eyespot of the dorsal forewing; VF1, anterior eyespot of the ventral forewing; VF2, posterior eyespot of the ventral forewing; VHW, ventral hindwing.

that developmental genes are also expressed at this stage in a pattern that reflects the color rings in an adult eyespot. The period of focal signaling and pattern determination represents a sensitive period for environmental stress (Brakefield 1997). We thus have a system for which the developmental mechanisms are well known, and we are able to examine how stress during the sensitive period affects FA of the adult phenotype. Each eyespot is formed by the same developmental pathway. However, although the dorsal forewing eyespots are secondary sexual traits, the ventral eyespots play a clear role as anti-predator devices (Brakefield and Breuker 1996; Breuker and Brakefield 2002).

We examine in this study whether FA is a reliable indicator of stress by observing if FA and the variation in FA of eyespots in *B. anynana* increase as a result of a specific known stress. Bjorksten et al. (2000) proposed that trait size

may be a more reliable indicator of stress than FA. We therefore also tested this alternative and examined whether trait size would change and variation in trait size would increase as a result of stress and compared the trait size results with the FA results. In addition, we asked whether sexually selected traits are particularly sensitive to stress by comparing the responses to stress in dorsal and ventral eyespots. Brakefield (1997) found that eyespot FA only reflected a period of cold temperature stress when it coincided with the period of pattern determination. We therefore applied a heat stress of 39.5°C for 3 h before, during, and after the proposed sensitive period for eyespot development and compared the responses with the stress between the different treatments. We predicted that the responses would be most pronounced when the shock was applied during the sensitive period of eyespot development.

Materials and Methods

Experimental animals and heat shock

The *B. anynana* butterflies used in this experiment were derived from a pure breeding laboratory stock, which was established from a founder stock of about 80 gravid females collected in Malawi in 1988 (Brakefield et al. 1996). Adult population size has always been maintained at over 400 butterflies.

Two replicate experiments were carried out consecutively but under the same conditions. In both replicates approximately 900 larvae were raised without food stress to butterflies on young maize plants, in a climate room at 27°C, 12:12-h light:dark, and high relative humidity. The very first and last animals to pupate during each day were not used. The larvae were collected individually approximately 48 h before applying the heat treatment, thereby minimizing the risk of confounding the effects of the temperature shock with those of any handling effect. The collected animals were then placed in small plastic pots and about 24 h before pupation assigned at random to the different experimental groups. The heat shocks (3 h at 39.5 ± 0.2°C) were applied at either the prepupal stage (i.e., approximately 12 h before pupation) or at 1 h (early sensitive period), 14 h (mid-sensitive period), 25 h (just outside the sensitive period), or 62 h (late larval, well out of sensitive period) after pupation (Fig. 1A). A highly accurate Snijders™ climate cabinet (Snijders Scientific BV, Tilburg, The Netherlands) was used (12:12-h light:dark, relative humidity ± 80%, same as rearing conditions). Control pupae were maintained at 27°C. These constitute the six different treatments. This temperature shock did not result in increased mortality as compared with the controls, excluding any possibility of differential mortality (results not shown). Exposing the pupae for 2 or more hours to a temperature of 39.5°C significantly increases mortality above background levels. For this reason the heat shock is likely to be a developmental stress.

We used a pilot experiment to determine that this shock increased the production of HSP60, thus indicating that the animals had been exposed to an extreme temperature, which might be regarded as a stress. The production of other heat shock proteins (Hsps), like the commonly investigated HSP70 and HSP90, was also examined, but the commercially available antibodies were ineffective in our samples. To visualize the production of Hsps, a sample of heat-shocked animals of the five treatments were either immediately frozen at −80°C after the heat shock or given a recovery time of 13 h and then frozen at −80°C (cf. Fittinghoff and Riddiford 1990). HSP60 production was visualized using Western blotting (protocols are described by Lundebye et al. 1995). We used mouse anti-HSP60 (LK1) monoclonal antibody (StressGen Biotechnologies Corp., Victoria, British Columbia, Canada) as the primary antibody. We used anti-mouse IgC (DAKO A/S, Glostrup, Denmark) as the secondary antibodies. Before gel loading, samples were standardized for total protein content by means of a bicinchononic acid (BCA) assay (Pierce Biochemicals, Rockford, IL, USA) according to the manufacturer's instructions.

Measurements

Measurements on the size (in mm^2) of the black ring of five eyespots (Fig. 1B) were carried out using a WILD binocular microscope (Wild, Heerbrugg, Switzerland) attached to a digitizing tablet (10×). The five eyespots are the anterior eyespot of the dorsal (DF1) and ventral forewing (VF1), the posterior eyespot of the dorsal (DF2) and ventral (VF2) forewing, and the largest eyespot of the ventral hindwing (VHW) The sexual traits are the two dorsal eyespots (DF1 and DF2). Size was determined by the interfocal distance (also labeled wing length, WL). This is the distance between the centers (foci) of the dorsal eyespots. Each trait was measured twice for each individual, with repeated measurements made 5 days apart. Individuals were measured at random with respect to experimental group, without knowledge of their treatment and without reference to earlier measurements. Repeatabilities of the measurements were very high (99.3–99.8%). There was no significant heterogeneity in measurement error between the two replicate experiments and across populations, traits, and sexes (using the methods described by Van Dongen et al. 1999a). Furthermore, it was ascertained that there were no systematic errors, for example, due to possible nonperpendicular measurements stemming from the use of a binocular microscope.

Statistical analyses

The FA analyses were carried out according to Palmer and Strobeck (2003). The sequential Bonferroni procedure was applied to each set of related tests to avoid making type I errors. The results of both experimental replicates did not differ significantly from each other, and the data were pooled. Males and females were analyzed separately. Using Grubb's test statistic (t_G; Palmer and Strobeck 2003), 105 of 1547 (6.8%) animals were considered to be outliers and were discarded from the analyses. In general, the asymmetry value for one or more of the five traits of these outliers differed by more than 3 standard deviations from the mean signed asymmetry value.

Phenotypic variation in individual FA (see index below) and trait size was estimated by means of the coefficient of variation (CV_{FA} and trait size $CV = \sqrt{(VAR/mean^2)}$; Gangestad and Thornhill 1999). For each treatment, FA did not significantly correlate with trait size or size of the animal (i.e., WL). Part of the FA variation could, however, be explained by size differences among traits but not among treatments. We therefore divided FA1 (the absolute differences between the sides) by the mean trait size of all treatments together. This is FA19, which resembles FA3 for the multisample case (Palmer and Strobeck 2003). This standardization enables the comparison of traits differing significantly in size. FA19 values were transformed before applying parametric tests that assume normality because they are half-normally distributed. For each sex, we applied Box-Cox power

Table 1. Descriptive statistics for FA in the six treatments for each sex

		(R + L)/2		(R − L)					FA1					
Sample	Trait	Mean	SE	Mean	SE	*n*	Skew	Kurtosis	Mean	SE	CV_{FA}	Ms_m	(σ^2_i)	*df*
Fem Control	DF1	1.17	0.029	0.012	0.013	249	0.07	−0.05	0.17	0.008	73	0.00190	0.0149	219
	DF2	6.32	0.100	0.071	0.034	249	0.01	−0.03	0.46	0.019	66	0.00095	0.0031	186
	VF1	1.00	0.018	−0.013	0.008	249	−0.17	0.07	0.11	0.005	69	0.00194	0.0079	196
	VF2	6.41	0.081	0.080	0.031	249	−0.09	0.16	0.41	0.019	71	0.00062	0.0027	198
	VHW	3.15	0.043	0.025	0.019	249	−0.02	0.22	0.25	0.011	70	0.00010	0.0013	230
Fem PP	DF1	1.20	0.064	0.015	0.037	45	0.16	0.08	0.19	0.022	76	0.00204	0.0205	40
	DF2	6.83	0.299	−0.065	0.108	45	−0.32	0.59	0.56	0.070	84	0.00084	0.0062	39
	VF1	0.94	0.035	0.023	0.023	45	−0.08	1.69	0.11	0.016	92	0.00186	0.0112	37
	VF2	6.16	0.185[DA]	0.197	0.066	45	−0.07	−0.49	0.40	0.040	67	0.00041	0.0022	37
	VHW	2.91	0.087[DA]	0.151	0.043	45	−0.03	−0.67	0.28	0.026	63	0.00011	0.0012	40
Fem 1 h	DF1	1.28	0.050	0.004	0.025	113	*0.62	0.12	0.22	0.015	73	0.00315	0.0235	98
	DF2	6.39	0.160	0.010	0.058	113	0.52	1.10	0.49	0.037	80	0.00103	0.0042	88
	VF1	0.94	0.029	−0.008	0.012	113	−0.01	0.06	0.10	0.007	72	0.00188	0.0074	88
	VF2	6.69	0.113	0.111	0.043	113	0.09	0.48	0.38	0.027	76	0.00070	0.0022	82
	VHW	2.99	0.066	−0.035	0.024	113	0.12	−0.21	0.22	0.013	62	0.00010	0.0010	101
Fem 14 h	DF1	1.04	0.048	0.009	0.021	65	−0.06	−0.61	0.14	0.012	66	0.00188	0.0093	53
	DF2	6.39	0.198	−0.090	0.102	65	−0.25	0.58	0.65	0.064	79	0.00096	0.0080	57
	VF1	0.95	0.035	−0.016	0.018	65	0.49	0.18	0.12	0.011	72	0.00207	0.0104	53
	VF2	6.30	0.167	−0.095	0.065	65	−0.14	−0.03	0.44	0.038	70	0.00070	0.0030	51
	VHW	3.05	0.067	0.001	0.034	65	0.39	0.21	0.23	0.020	70	0.00010	0.0007	55
Fem 25 h	DF1	1.24	0.040	0.002	0.017	121	0.11	0.04	0.16	0.010	72	0.00249	0.0115	97
	DF2	6.09	0.154	0.093	0.063	121	0.28	0.29	0.57	0.038	72	0.00097	0.0055	101
	VF1	0.98	0.023	−0.009	0.011	121	0.21	0.19	0.10	0.006	67	0.00262	0.0066	82
	VF2	6.16	0.104	0.046	0.044	121	−0.03	−0.16	0.41	0.025	68	0.00073	0.0026	91
	VHW	2.90	0.054	0.026	0.023	121	0.07	0.34	0.22	0.014	69	0.00010	0.0010	109
Fem 62 h	DF1	1.05	0.055	−0.052	0.031	45	−0.25	0.16	0.17	0.019	72	0.00103	0.0152	41
	DF2	5.95	0.244	−0.154	0.086	45	−0.27	0.02	0.47	0.056	80	0.00053	0.0039	39
	VF1	0.95	0.036	−0.014	0.019	45	0.49	−0.08	0.11	0.011	66	0.00218	0.0079	34
	VF2	6.47	0.170	−0.036	0.063	45	−0.15	0.33	0.36	0.035	64	0.00059	0.0019	33
	VHW	3.21	0.104	−0.058	0.036	45	−0.42	−0.25	0.21	0.021	68	0.00031	0.0007	30
Male Control	DF1	0.92	0.025	−0.012	0.012	304	0.08	−0.09	0.17	0.007	73	0.00230	0.0228	274
	DF2	3.77	0.063	0.043	0.029	304	0.19	0.23	0.41	0.017	73	0.00190	0.0078	239
	VF1	0.96	0.014	−0.002	0.007	304	−0.15	0.15	0.11	0.004	70	0.00204	0.0080	237
	VF2	4.71	0.046	0.031	0.025	304	−0.02	0.16	0.36	0.014	71	0.00074	0.0036	248
	VHW	2.51	0.025	0.007	0.014	304	−0.02	−0.05	0.21	0.008	69	0.00010	0.0013	281
Male PP	DF1	1.08	0.061	−0.033	0.028	42	−0.50	0.66	0.14	0.019	88	0.00231	0.0181	36
	DF2	4.33	0.198	0.051	0.077	42	0.19	*2.01	0.39	0.051	84	0.00230	0.0075	31
	VF1	0.89	0.038	−0.015	0.019	42	0.02	−0.14	0.10	0.011	72	0.00173	0.0073	33
	VF2	4.40	0.126	0.071	0.051	42	0.30	0.19	0.28	0.030	69	0.00060	0.0021	31
	VHW	2.30	0.074	0.052	0.036	42	0.10	−0.64	0.21	0.019	60	0.00011	0.0007	36
Male 1 h	DF1	0.96	0.038	0.009	0.017	116	0.34	0.15	0.15	0.010	75	0.00352	0.0174	94
	DF2	4.04	0.114	−0.077	0.050	116	−0.39	0.24	0.44	0.031	77	0.00215	0.0091	92
	VF1	0.94	0.025	0.004	0.013	116	−0.11	−0.19	0.12	0.007	66	0.00238	0.0100	91
	VF2	5.02	0.083	0.047	0.037	116	−0.05	0.00	0.34	0.021	67	0.00095	0.0029	84
	VHW	2.48	0.047	−0.039	0.022	116	−0.24	0.63	0.20	0.014	74	0.00010	0.0008	102
Male 14 h	DF1	0.85	0.039	−0.024	0.017	123	−0.08	0.01	0.15	0.011	77	0.00240	0.0193	108
	DF2	3.48	0.116	−0.083	0.041	123	0.07	−0.12	0.38	0.025	74	0.00164	0.0065	96
	VF1	0.90	0.023	0.012	0.012	123	−0.17	0.17	0.11	0.007	76	0.00206	0.0086	97
	VF2	4.79	0.080	−0.035	0.040	123	0.06	0.19	0.37	0.023	70	0.00070	0.0039	103
	VHW	2.60	0.044	−0.043	0.020	123	−0.18	−0.20	0.20	0.012	65	0.00020	0.0009	97
Male 25 h	DF1	0.98	0.039	0.013	0.017	142	0.10	0.03	0.16	0.010	74	0.00270	0.0210	124
	DF2	3.76	0.102	−0.001	0.037	142	0.06	−0.02	0.37	0.022	73	0.00221	0.0058	98
	VF1	0.98	0.024	0.002	0.011	142	0.41	0.31	0.11	0.007	73	0.00256	0.0082	105
	VF2	5.00	0.082	0.024	0.034	142	0.17	0.25	0.32	0.021	76	0.00072	0.0031	113
	VHW	2.56	0.052	0.020	0.021	142	0.17	0.30	0.21	0.013	72	0.00010	0.0009	126
Male 62 h	DF1	0.92	0.043	0.007	0.025	77	−0.09	−0.33	0.18	0.014	70	0.00189	0.0266	71
	DF2	3.82	0.136	−0.043	0.050	77	−0.05	−0.12	0.36	0.027	66	0.00112	0.0060	63
	VF1	0.97	0.026	0.034	0.016	77	−0.08	−0.06	0.12	0.010	73	0.00202	0.0096	62
	VF2	4.77	0.094	0.048	0.038	77	−0.10	−0.43	0.29	0.020	61	0.00097	0.0019	47
	VHW	2.62	0.063	−0.029	0.026	77	−0.07	0.18	0.20	0.014	64	0.00018	0.0007	59

Calculations of each variable, except FA10, are based on the average of the two replicate measurements. CV_{FA}, coefficient of variation in FA; MS_m, measurement error mean square; σ^2_i, nondirectional asymmetry (= FA10); DA, directional asymmetry; Fem, female; con, control; PP, prepupal stage. For trait names see text.

* $P < 0.05$.

transformations (Leamy 1999; Van Dongen et al. 1999b; Fuller and Houle 2003) of the form $(FA19 + 0.000001)^{\lambda}$ to achieve normality (females, $\lambda = 0.24$; males, $\lambda = 0.32$). CFA19 is the sum of transformed FA19 values of each eyespot per individual and was used to test for differences in FA between the treatments combining information from various traits (CFA19 is comparable with both CFA1 and CFA2 of Leung et al. 2000). This method increases the likelihood of detecting a pattern in FA differences related to the heat treatment (Leung et al. 2000).

To test for departures from ideal FA, distributions of the signed differences between left and right were tested for departures from normality and a mean of 0, using kurtosis, skewness, and a *t*-test (Palmer 1994). Of 60 tests, there was only one significant kurtosis, one significant skew, and two traits showed DA (two sexes, five traits, and six treatments; Table 1). When DA was present, the mean signed difference was subtracted from the individual signed difference between the sides before calculating the FA19 values.

Factors used in the different analyses of variance (ANOVAs) are replicate measurements, populations, sides, traits, individuals, and samples. A two-factor ANOVA following Palmer (1994), with sides as a fixed effect and individuals as a random effect, using replicate measures enabled us to calculate FA10 (between-sides variance after partitioning out ME) because the side × individual interaction was substantially greater than measurement error variance in all ANOVAs ($P << 0.001$). These values were compared with each other using F tests. The appropriate degrees of freedom were calculated using the formula provided by Palmer (1994). Furthermore, modified Levene's tests for heterogeneity of variances were carried out to investigate the variability in levels of individual FA, trait FA, and treatment FA (Palmer 1994).

Significance is at the 0.05 level. All analyses were carried out with the statistical software package MINITAB 12.22 (MINITAB Inc., State College, PA, USA).

Results

Heat shock protein production

The production of HSP60 was visualized by means of Western blotting, an example of which can be found in Figure 2A. All heat-shocked animals produced similar bands. Measurements of the areas of the bands of four such gels showed a significant increase in the production of HSP60 between controls and heat-shocked animals, whether allowed a recovery or not (Kruskal-Wallis: $H = 17.28$, $df = 1$, $P << 0.001$). The increase in HSP60 was somewhat greater in animals allowed to recover for 13 h from the heat shock (Fig. 2B; three replicates per treatment, Kruskal-Wallis: $H = 21.77$, $df = 1$, $P << 0.001$). There were no significant differences between the temperature treatments (Kruskal-Wallis $P >> 0.90$). The production of Hsps starts during a stress but usually increases after the stress has been removed to buffer development (Feder and Krebs 1997). We conclude that it is highly probable that the heat shock has been a stress during development for the butterflies.

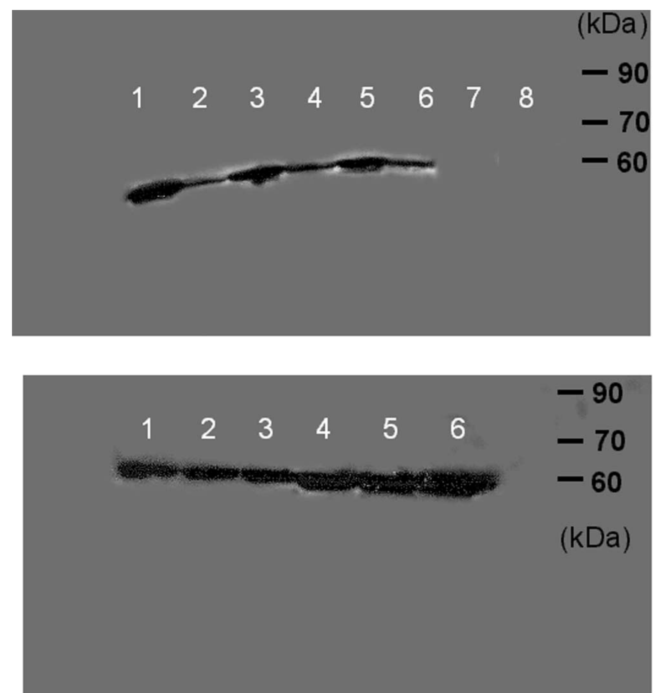

Fig. 2. Western blots showing HSP60 production in *Bicyclus anynana*. (A) Western blot comparing HSP60 production between heat-stressed animals (3 h at 39.5°C) and controls. Lanes: 1, 1 h; 2, control; 3, 14 h; 4, control; 5, 25 h; 6, control. (B) Western blot comparing HSP60 production between heat-stressed 1-h-old animals that were not allowed to recover (lanes 1–3) and those that were allowed to recover for 13 h (lanes 4–6).

Differences in FA between the treatments

The 105 outliers of 1547 animals (6.8%) were distributed randomly across sexes, traits, and treatments. Thus, the heat stress did not result in more developmental abnormalities (chi-square analysis with frequency distribution of outliers per treatment across sexes and traits: $\chi^2 = 1.89$, $df = 5$, $P = 0.87$).

A mere 14 of the 150 possible pair-wise comparisons (comparing treatments per trait and per sex) using FA10, from which ME has been partitioned out (Palmer 1994), were significant (Fig. 3). These significant results are due to a relatively low FA10 value of DF2 in the control females (four significant comparisons, nr 1 in Fig. 3A), high FA10 value of DF1 in the 1-h females (four significant comparisons, nr 2 in Fig. 3A), and high FA10 value of VHW in the control males (four significant comparisons, nr 3 in Fig. 3B; see also Table 1). Taking into account only the sign of the comparison (a+ for higher FA, a− for lower FA) across traits and samples (stressed vs. nonstressed), there were no significant patterns in FA (all possible one-proportion tests, $P > 0.05$). One-way ANOVA results using CFA19 and the factor treatment show the same result for each sex (females: $F_{5,632} = 0.63$, $P = 0.68$;

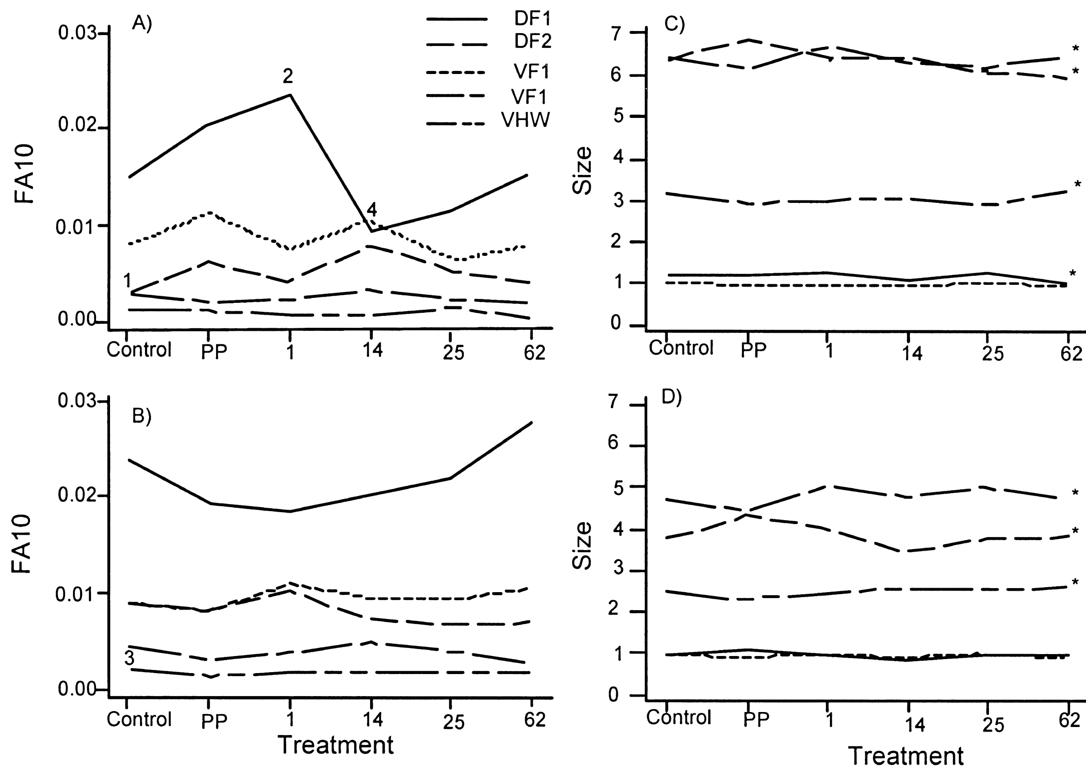

Fig. 3. FA10 values per trait per treatment, (A) females and (B) males. Points are joined up to facilitate reading. Numbers 1–4 refer to relatively extreme FA10 values: 1, low FA10 value of DF2 in the control females; 2, high FA10 value of DF1 in the 1-h females; 3, high FA10 value of VHW in the control males; and 4, FA10 values of DF1, DF2, and VF1 not significantly different in the 14-h females. Size (mean (R + L/2)) per treatment for each sex, (C) females and (D) males. Asterisks (*) indicate significant ANOVA results ($P < 0.05$) testing for size differences between the six treatments. PP, prepupal stage. For trait names see text.

males: $F_{5,798} = 1.31, P = 0.26$). Combining the information on the sexual traits only (CFA19 $_{sexual}$) yields the same result (females: $F_{5,632} = 0.94, P = 0.45$; males: $F_{5,798} = 1.32, P = 0.26$). Thus, there is no indication that the applied stress resulted in differences in FA of the eyespots between control (non-stressed) animals and animals at any stage of eyespot development or that there were any differences between dorsal and ventral eyespots in their response to the stress.

Differences in FA among traits

The five eyespots differ significantly from each other in FA (120 F-test comparisons between trait within each sample and sex, $P << 0.001$). The lines indicating levels of FA in Figure 3 do not cross each other, with one exception (nr 4 in Fig. 3A), as FA10 values of DF1, DF2, and DF1 are not significantly different in the 14-h females. The first dorsal eyespot (DF1) has clearly the highest FA in each sex. The average (box-cox transformed) FA19 values of the sexual traits (DF1 and DF2) is significantly higher than that of the non-sexual traits for each sex (ANOVA results combining data from treatments, females: $F_{1,1274} = 105.5$, $P << 0.001$; males: $F_{1,1274} = 53.6$, $P << 0.001$).

Besides F tests, it is also possible to use a modified Levene's test for heterogeneity of variances with individuals as a random effect and trait as a fixed effect, per treatment and sex (Palmer 1994). The F values for both these effects were consistently highly significant ($P << 0.001$), indicating that traits differed significantly in the stability of their development and that the level of developmental stability varied significantly among individuals.

In general, within each sex the FA of a particular eyespot correlates poorly with the FA of another eyespot (120 possible regression results: R^2 between 0.0% and 6.3%). This analysis includes traits expected to be highly correlated, such as the two sexual traits (DF1 and DF2) (R^2 between 0.1% and 4.5%). Most associations are, however, positive (90/120: one-proportion test $P << 0.001$).

Differences in size between the treatments

There were no significant size (WL) differences between the different treatments; only females were significantly larger than males (nested ANOVA: $F_{1,1430}$ sex = 2507.0, $P << 0.001$; $F_{1,1430}$ treatment (sex) = 1.70, $P = 0.076$). Size (average (R+L)/2) of each eyespot correlated significantly ($P <$

0.05) with WL within each treatment for the females (positive relationships, R^2 between 4.3% and 34.8%) but only in males for VF2 and VHW (positive relationships, R^2 between 5.3% and 35.9% and nonsignificantly between 0.0% and 9.4% for the other traits). To examine the effect of the heat stress on eyespot size, we therefore corrected the trait values by dividing by WL when there was a significant association.

The size of each eyespot did not change as a result of the stress in either sex (Fig. 3, C and D, and Table 1). Although differences in eyespot size existed between treatments (7/10 ANOVAs testing for size differences were significant, $P < 0.05$), there is no consistent relation to stage of development. For each eyespot and sex, the differences between the control animals and the heat-stressed animals were never significant (Tukey pair-wise comparisons, $P > 0.05$). The heat stress applied in the middle of the proposed sensitive period for eyespot development (14 h) led to a reduction in eyespot size in 7 of 10 cases compared with the controls. This, however, was not significant (one-proportion test, $P = 0.344$). Furthermore, there is no indication that the size of the two dorsal eyespots (DF1 and DF2) responded differently to the stress than the ventral eyespots (Fig. 3, C and D).

FA and trait size variability among individuals

The results of the modified Levene's test given above indicated significant variability among individuals in levels of FA. However, this variability did not change as a result of the applied stress as shown by the CV_{FA} values for each sex (Table 1; using all eyespots Kruskal-Wallis, females: H = 2.08, $df = 5$, $P = 0.84$; males: H = 5.08, $df = 5$, $P = 0.41$; using only dorsal eyespots Kruskal-Wallis, females: H = 4.92, $df = 5$, $P = 0.43$; males: H = 10.23, $df = 5$, $P = 0.07$). The dorsal eyespots tended to be more variable in FA than the ventral ones (Kruskal-Wallis tests using CV_{FA} values of all treatments, females: H = 8.96, $df = 4$, $P = 0.062$; males: H = 9.85, $df = 4$, $P = 0.043$). The mean CV_{FA} of the dorsal eyespots is 75.4 in the males and 74.4 in the females, and the mean CV_{FA} of the ventral eyespots is 69.5 in the males and 69.9 in the females (Table 1). These results indicate that eyespots closely related to sexual selection show higher levels of variability for FA than eyespots associated with other aspects of fitness, such as survival, but that these levels did not change significantly as a result of the stress.

Similar results were obtained when examining the CV of trait size. Again, there are no significant differences between treatments in males or females either when taking all five eyespots into account (Kruskal-Wallis, females: H = 0.37, $df = 5$, $P = 0.996$; males: H = 1.02, $df = 5$, $P = 0.96$) or only the two dorsal eyespots (Kruskal-Wallis, females: H = 0.62, $df = 5$, $P = 0.987$; males: H = 2.62, $df = 5$, $P = 0.76$). The trait size CV of the dorsal eyespots was significantly higher than that of the ventral eyespots in each sex (Kruskal-Wallis using trait size CV values of all treatments, females: H = 25.6, $df = 4$, $P << 0.001$; males: H = 26.6, $df = 4$, $P << 0.001$). The average trait size CV for the dorsal eyespots was 37.9 for males and 32.1 for females. These values are consistent with the CV values of traits under sexual selection (Gangestad and Thornhill 1999). The average trait size CV of the ventral eyespots is much lower, 22.0 for males and 22.7 for females.

Discussion

Numerous studies have reported that the adult eyespot pattern of various species of Nymphalinae and Lycaenidae butterflies may show aberrations due to temperature extremes during wing pattern development (reviewed by Brakefield 1984). The absence of any effect of heat shock on the frequency of developmental abnormalities in this study suggests that some earlier interpretations of increases in such "aberrations" in this context require further analysis.

Eyespot FA and CV_{FA} did not increase as a result of the heat shock, but neither did trait size change nor the variation in trait size increase. The timing of the heat shock had no effect on developmental instability. In particular, heat shocks applied within what we can assume from our knowledge of developmental mechanisms to be the sensitive period in eyespot formation do not induce any additional FA. This compares closely with our findings in a previous study in which we applied a heat stress of 39.5°C for 5 h during the sensitive period of eyespot development to examine the effects of a selection for higher developmental stability (Breuker and Brakefield, unpublished results). It is contradictory, however, with the results of Brakefield (1997), who found a response in FA to a temperature shock during the sensitive period. However, he used a cold shock of 3°C for a much longer period of 24 h. This seems to indicate that a cold shock may be a more appropriate stress (Zijlstra et al. 2001). Studies applying a temperature stress to Lepidoptera have mostly used low temperatures (Yocum et al. 1991; Denlinger et al. 1992; Kim and Kim 1997; Lewthwaite et al. 1998).

The pupae exposed to a short heat shock did produce significantly more Hsps such as HSP60, which induce thermotolerance and stabilize development (Morimoto et al. 1997; Feder and Hofmann 1999). Hsps may, however, also have negative effects on development and fitness. Their synthesis, maintenance, and degradation could consume large portions of the (developing) cell's energy and amino acid reserves and preoccupy the machinery of protein synthesis. Large amounts of Hsps may further interfere with ongoing processes in the cell. Overexpression of Hsps may even be deleterious (Feder et al. 1995; Krebs and Feder 1997, 1998; Feder and Hofmann 1999). In other words, if the stress is severe enough the Hsps themselves may turn against the cells they are designed to protect and thus lower survival and de-

velopmental stability, and hence in theory increase FA. The Hsps that were identified belonged to the HSP60 family. These proteins stabilize preexisting proteins under stress conditions such as a heat shock and provide a refuge for proteins to fold to their normal confirmations, which protects them from reactive elements in the cytoplasm (Langer et al. 1992; Martin et al. 1992). The finding that animals that were allowed to recover from the heat shock for 13 h showed even higher levels of HSP60 suggests that production started during the heat shock and continued for some time after the shock to repair sustained damage and stabilize further development. This lends support to the idea that the shock had been a stress.

There are thus two, not mutually exclusive, possible explanations for the observed FA and trait size patterns: (a) Both FA and the alternative, trait size, are not reliable indicators of stress (supported by many studies reviewed by Bjorksten et al 2000) but Hsps may be (Buchanan 2000), and (b) the amounts of Hsps produced were sufficient to stabilize development of the shocked pupae but not to stop normal development and thus increase mortality and developmental instability (cf. Feder and Hofmann 1999; Rutherford 2000; Klingenberg 2003).

The FA and CV_{FA} of the dorsal forewing eyespots is higher than the ventral (nonsexual) eyespots, and males show higher FA for these traits than females. This is consistent with the hypothesis that the development of sexual ornaments is weakly canalized due to their recent history of directional selection. Sexually selected traits are furthermore proposed to be highly condition dependent and thereby more likely to show higher FA. The CV variation for the size of the dorsal eyespots is between 30 and 40, which is indicative of a trait under (very) strong directional selection (Gangestad and Thornhill 1999). The dorsal eyespots did not respond to heat shock any differently than ventral eyespots. If, however, the developing individuals were capable of successfully reducing the impact of the heat shock by means of Hsps, we cannot reject or confirm our hypothesis that a potential stress increases the level of FA (Bjorksten et al. 2000).

This study shows that for a specific trait where it is known at which stage of development a potentially (severe) stress is most likely to affect developmental precision, we nevertheless do not find a response in terms of increased FA in this trait. The presence of increased amounts of Hsps may not only reflect the impact of a stress but may also indicate the mechanism by which (developing) organisms buffer themselves against its harmful effects. The results demonstrate how unwise it is to assume any given relationship between environmental stress and FA. Even though the applied stress was designed to increase FA, this was not observed. However, an earlier experimental study of the same eyespot pattern, using a different stress but applied at a similar stage of development, did yield some increase of eyespot FA (Brakefield 1997). This illustrates how the effect on FA is likely to be highly dependent on the precise nature of the stress. Furthermore, it emphasizes that use of the FA of any set of traits as an index of exposure to environmental stress can be misleading without robust examination of the assumptions concerning the underlying developmental mechanisms. The chosen traits may simply lack sensitivity during ontogeny to a particular stress such that no effect on FA is detected even with exposure to stress.

Acknowledgments

We thank E. Schlatmann, B. de Winter, and colleagues for rearing maize; J. G. Sørensen, M. Christensen, and D. Anderson for technical assistance and advice in Western blotting; and V. Loeschcke for hospitality. We are also grateful to two anonymous reviewers for their constructive comments. C. J. Breuker was funded by a NWO grant 33.360–P.

REFERENCES

Bjorksten, T. A., Fowler, K., and Pomiankowski, A. 2000. What does sexual trait FA tell us about stress? *Trends Ecol. Evol.* 15: 163–166.

Brakefield, P. M. 1984. The ecological genetics of quantitative characters of *Maniola jurtina* and other butterflies. In R. I. Vane-Wright and P. R. Ackery (eds.). *The Biology of Butterflies*. Academic Press, London, pp. 167–190.

Brakefield, P. M. 1997. Phenotypic plasticity and fluctuating asymmetry as responses to environmental stress in the butterfly *Bicyclus anynana*. In R. Bijlsma and V. Loeschcke (eds.). *Environmental Stress, Adaptation And Evolution.* Birkäuser, Basel, pp. 65–78.

Brakefield, P. M., and Breuker, C. J. 1996. The genetical basis of fluctuating asymmetry for developmentally integrated traits in a butterfly eyespot pattern. *Proc. R. Soc. Lond. B* 263: 1557–1563.

Brakefield, P. M., Gates, J., Keys, D., Kesbeke, F., Wijngaarden, P. J., Monteiro, A., French, V., and Carroll, S. B. 1996. Development, plasticity and evolution of butterfly eyespot patterns. *Nature* 384: 236–242.

Breuker, C. J. & Brakefield, P. M. 2002. Symmetry of dorsal forewing eyespots does not predict mating success in the butterfly *Bicyclus anynana*. *Proc. R. Soc. Lond. B 269*: 1233–1239.

Brunetti, C. R., Selegue, J. E., Monteiro, A., French, V., Brakefield, P. M., and Carroll, S. B. 2001. The generation and diversification of butterfly eyespot color patterns. *Curr. Biol.* 11: 1578–1585.

Buchanan, K. L. 2000. Stress and the evolution of condition-dependent signals. *Trends Ecol. Evol.* 15: 156–160.

Clarke, G. M., Yen, J. L., and McKenzie, J. A. 2000. Wings and bristles: character specificity of the asymmetry phenotype in insecticide-resistant strains of *Lucilia cuprina*. *Proc. R. Soc. Lond. B* 267: 1815–1818.

Denlinger, D. L., Lee, J., R.E., Yocum, G. D., and Kukal, O. 1992. Role of chilling in the acquisition of cold tolerance and the capacitation to express stress proteins in diapausing pharate larvae of the gypsy moth, *Lymantria dispar*. *Arch. Insect Biochem. Physiol.* 21: 271–280.

Feder, M. E., and Hofmann, G. E. 1999. Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annu. Rev. Physiol.* 61: 243–282.

Feder, M. E., and Krebs, R. A. 1997. Ecological and evolutionary physiology of heat shock proteins and the stress response in *Drosophila*: complementary insights from genetic engineering and natural variation. In R. Bijlsma and V. Loeschcke (eds.). *Environmental Stress, Adaptation And Evolution*. Birkäuser, Basel, pp. 155–173.

Feder, M. E., Parsell, D. A., and Lindquist, S. L. 1995. The stress response and stress proteins. In J. L. Lemasters and C. Oliver (eds.). *Cell Biology of Trauma*. CRC Press, Boca Raton, FL, pp. 177–191.

Fittinghoff, C. M., and Riddiford, L. M. 1990. Heat sensitivity and protein synthesis during heat-shock in the tobacco hornworm, *Manduca sexta*. *J. Comp. Physiol.* 160: 349–356.

Fuller, R. C., and Houle, D. 2003. Inheritance of developmental stability.

In M. Polak (ed.). *Developmental Instability: Causes and Consequences*. Oxford University Press, New York (in press).

Gangestad, S. W., and Thornhill, R. 1999. Individual differences in developmental precision and fluctuating asymmetry: a model and its implications. *J. Evol. Biol.* 12: 402–416.

Hunt, J., and Simmons, L. W. 1997. Patterns of fluctuating asymmetry in beetle horns: an experimental examination of the honest signalling hypothesis. *Behav. Ecol. Sociobiol.* 41: 109–114.

Kim, Y., and Kim, N. 1997. Cold hardiness in *Spodoptera exigua* (Lepidoptera: Noctuidae). *Environ. Entomol.* 26: 1117–1123.

Klingenberg, C. P. 2003. A developmental perspective on developmental instability: theory, models and mechanisms. In M. Polak (ed.). *Developmental Instability: Causes and Consequences*. Oxford University Press, New York (in press).

Krebs, R. A., and Feder, M. E. 1997. Deleterious consequences of Hsp70 overexpression in Drosophila melanogaster larvae. *Cell Stress Chaper.* 2: 60–71.

Krebs, R. A., and Feder, M. E. 1998. Hsp70 and larval thermotolerance in *Drosophila melanogaster*: how much is enough and when is more too much? *J. Insect Physiol.* 44: 1091–1101.

Langer, T., Lu, C., Echols, H., Flanagan, J., Hayer, M., and Hartl, F. U. 1992. Successive action of DnaK, DnaJ and groEL along the pathway of chaperone-mediated protein folding. *Nature* 356: 683–689.

Leamy, L. 1999. Heritability of directional and fluctuating asymmetry for mandibular characters in random bred mice. *J. Evol. Biol.* 12: 146–155.

Leary, R. F., and Allendorf, F. W. 1989. Fluctuating asymmetry as an indicator of stress: implications for conservation biology. *Trends Ecol. Evol.* 4: 214–217.

Leung, B., Forbes, M. R., and Houle, D. 2000. Fluctuating asymmetry as a bioindicator of stress: comparing efficacy of analyses involving multiple traits. *Am. Nat.* 155: 101–115.

Lewthwaite, S. E., Dentener, P. R., Alexander, S. M., Bennett, K. V., Rogers, D. J., Maindonald, J. H., and Connolly, P. G. 1998. High temperature and cold storage treatments to control Indian meal moth, *Plodia interpunctella* (Hubner). *J. stored Prod. Res.* 34: 141–150.

Lundebye, A.-K., Vedel, G. R., Christensen, A. M. K., Kristiansen, K., Hunter, D., and Depledge, M. H. 1995. Improved quantification of stress proteins by Western Blotting. *Anal. Chim. Acta* 311: 109–114.

Markow, T. A. 1995. Evolutionary ecology and developmental instability. *Annu. Rev. Entomol.* 40: 105–120.

Martin, J., Horwich, A. L., and Hartl, F. U. 1992. Prevention of protein denaturation under heat stress by the chaperonin Hsp60. *Science* 258: 995–998.

Møller, A. P. 1990. Fluctuating asymmetry in male sexual ornaments may reliably reveal male quality. *Anim. Behav.* 40: 1185–1187.

Møller, A. P. & Swaddle, J. P. 1997 *Asymmetry, Developmental Stability, and Evolution*. Oxford Series in Ecology and Evolution. Oxford University Press, New York, p. 76.

Morimoto, R. I., Kline, M. P., Bimston, D. N., and Cotto, J. J. 1997. The heat-shock response: regulation and function of heat-shock proteins and molecular chaperones. *Essays Biochem.* 32: 17–29.

Palmer, A. R. 1994. Fluctuating asymmetry analyses: a primer. In T. A. Markow (ed.). *Developmental Instability: Its Origins and Evolutionary Implications*. Kluwer, Dordrecht, pp. 355–364.

Palmer, A. R., and Strobeck, C. 1986. Fluctuating asymmetry: measurement, analysis, patterns. *Annu. Rev. Ecol. Syst.* 17: 391–421.

Palmer, A. R., and Strobeck, C. 1992. Fluctuating asymmetry as a measure of developmental stability: implications of non-normal distributions and power of statistical tests. *Acta Zool. Fenn.* 191: 57–72.

Palmer, A. R., and Strobeck, C. 2003. Fluctuating asymmetry analyses revisited. In M. Polak (ed.). *Developmental Instability: Causes and Consequences*. Oxford University Press, New York (in press).

Parsons, P. A. 1990. Fluctuating asymmetry: an epigenetic measure of stress. *Biol. Rev.* 65: 131–145.

Rutherford, S. L. 2000. From genotype to phenotype: buffering mechanisms and the storage of genetic information. *BioEssays* 22: 1095–1105.

Van Dongen, S., Molenberghs, G., and Matthysen, E. 1999a. The statistical analysis of fluctuating asymmetry: REML estimation of a mixed regression model. *J. Evol. Biol.* 12: 94–102.

Van Dongen, S., Sprengers, E., Lofstedt, C., and Matthysen, E. 1999b. Heritability of tibia fluctuating asymmetry and developmental instability in the winter moth (*Operophtera brumata* L.) (Lepidoptera, Geometridae). *Heredity* 5: 535–542.

Van Valen, L. 1962. A study of fluctuating asymmetry. *Evolution* 16: 125–142.

Woods, R. E., Sgro, C. M., Hercus, M. J., and Hoffmann, A. A. 1999. The association between fluctuating asymmetry, trait variability, trait heritability, and stress: a multiply replicated experiment on combined stresses in *Drosophila melanogaster*. *Evolution* 53: 493–505.

Yocum, G. D., Joplin, K. H., and Denlinger, D. L. 1991. Expression of heat shock proteins in response to high and low temperature extremes in daipausing pharate larvae of the gypsy moth, *Lymantria dispar*. *Arch. Insect Biochem. Physiol.* 18: 239–249.

Zijlstra, W. G., Zwaan, B. J., and Brakefield, P. M. 2001. Bad cold for a butterfly? *Proc. Exp. Appl. Entomol., N.E.V.* 12: 19–23.