Procrustean analysis of fluctuating asymmetry in the bulb mite *Rhizoglyphus robini* Claparede (Astigmata: Acaridae)

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We studied fluctuating asymmetry (FA) in two generations of the bulb mite *Rhizoglyphus robini*. We used Procrustes analyses, which allow the comparison of dimensionless shapes of body sides. We found little (<4%) directional asymmetry in either sex. Of the two morphs occurring in this species, fighters, which possess a thickened third pair of legs, exhibited higher FA than did scramblers, the morph with unmodified legs; this may reflect the costliness of the fighter developmental pathway. There was a negative relationship between FA and female fecundity, but the regression slope of mid-offspring on mid-parent FA was not significantly greater than zero. We propose that heritability estimates can be biased downwards if highly asymmetric individuals produce fewer viable offspring. However, we found no significant association between parental FA and the proportion of viable embryos in their broods. Furthermore, we hypothesized that parental FA might indicate the presence of largely recessive mutations deleterious to developmental homeostasis that would cause increased embryo mortality under inbreeding. However, we found no significant association between FA of parents that were mated to their full sibs and the proportion of viable embryos in their inbred progeny.   © 2003 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2003, **80**, 499–505.

ADDITIONAL KEYWORDS: developmental stability – fitness – heritability – male dimorphism – positional asymmetry.

INTRODUCTION

Fluctuating asymmetry (FA), directionally random deviations from bilateral symmetry (Van Valen, 1962; Palmer & Strobeck, 1986), is often used as a measure of the ability of individuals to undergo normal development in spite of environmental or genetic stress (Møller & Swaddle, 1997). Part of the appeal of FA is that it may reveal an important component of the heritable genetic quality of individuals and thus play an important role in mate choice (Møller, 1990; Thornhill & Sauer, 1992; Thornhill, 1992a,b; Watson & Thornhill, 1994). However, the utility of FA as a measure of developmental homeostasis has been questioned (e.g. Bjorksten, Fowler & Pomiankowski, 2000b), mainly on the basis of an alleged lack of correlations among the FAs of different traits within individuals. Polak *et al.*

(2003) conducted a meta-analysis of data on within-individual FA, however, and found a significant positive relationship. Heritabilities of single trait FAs do appear to be very low (below 5%, reviewed by Gangestad & Thornhill, 1999; Van Dongen, 2000; see also Bjorksten *et al.*, 2000a; Bryden & Heath, 2000; Chapman & Goulson, 2000).

Existing studies of FA have used mainly meristic or linear traits, each of which have been analysed separately. However, because of the random nature of deviations from bilateral asymmetry, aggregate measures including several traits can be much more powerful measures of developmental stability (Gangestad & Thornhill, 1999; Leung, Forbes & Houle, 2000; Gangestad & Thornhill, 2003). Moreover, as recently shown by Polak & Starmer (2001), positional fluctuating asymmetry (PFA) can be a much more sensitive measure of developmental stability than comparing linear sizes of traits on both sides of the body. They propose that the reason for superiority of PFA may be that it

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reflects the ability to harmonize interconnected developmental pathways (Polak & Starmer, 2001).

A multitrait approach that provides information on PFA through comparison of shapes of left and right sides of the body can be implemented with Procrustes analysis (Bookstein, 1991; Smith, Crespi & Bookstein, 1997; Klingenberg & McIntyre, 1998). Such methods compare size-independent shapes based on several landmarks, and have already proved to be more sensitive in detecting significant associations between FA and genetic stress than are traditional methods (Auffray *et al.*, 1996). We applied Procrustes analysis to our study of the heritability of FA and the association of FA with female fecundity in the bulb mite *Rhizoglyphus robini* (Astigmata: Acaridae).

Besides employing single or individually analysed meristic or linear traits, we suggest a new potential cause for the underestimation of the heritability of FA. If high FA is caused by genes that seriously decrease developmental stability, then progeny (or other relatives) of highly asymmetric individuals that inherit these genes may show relatively higher embryo mortality or lower survival to adulthood compared with progeny of more symmetric individuals. Consequently, the mean FA of progenies of the most asymmetric parents would be lower than expected given the parental genotypic values, because a proportion of offspring with the lowest stability would never develop to measurement age. This would bias the coefficient of regression of offspring on parents downward. To test for this effect, we looked for an association between parental FA and embryo mortality in their offspring. To further test the hypothesis that FA may reveal the presence in individuals of partially recessive mutations deleterious to developmental homeostasis, we related FA of sib-mated parents to the embryonic mortality in their inbred broods.

METHODS

Rhizoglyphus robini infests subterranean structures of plants, and is commonly found on bulbs of onions, garlic and other members of the Liliaceae. They also infest stored food products (Diaz *et al.*, 2000). Stored-product Acaridae have probably long evolved in synanthropic habitats (O'Connor, 1979), therefore laboratory conditions are likely to be similar to their natural environment. Like other acarid mites, *Rhizoglyphus* is diploid and lacks parthenogenesis (Oliver, 1971, 1977). The sex ratio at emergence of adults is near unity (Gerson, Capua & Thorens, 1983).

The two male morphs occurring in this species, heteromorphic males with thickened legs and homeomorphic ones with unmodified legs, differ in aggressiveness. Heteromorphs attack and kill other males irrespective of their morph (Radwan *et al.*, 2000). We

shall refer to these as 'fighters', and to non-killing homeomorphic males as 'scramblers'. The mites used in this study came from a stock culture derived from a colony of about 100 individuals found on onions from a garden near Cracow, Poland, in 1998. They were kept in the laboratory as a large population (>1000 individuals) at 22–26°C, >90% humidity, and fed a 3 : 1 mixture of powdered yeast and wheat germ. The average generation time under these conditions is about 2 weeks. Thus, the mites were reared in the laboratory for about 50 generations before the commencement of this research.

During experiments, individual and paired mites were kept in 50-μL eppendorf vials filled to 1/3 height with solidified, moist plaster-of-Paris darkened with 10% powdered charcoal for better visualization of the whitish-coloured mites. Vials were closed with nonabsorbent cotton wool. Food was provided ad libitum and temperature maintained at 22°C.

The experiment started with the isolation of quiescent larvae (first instar) from our stock culture; the quiescent stage precedes each molt and lasts a few hours, so all larvae were approximately of equal age. Upon reaching adulthood, males and females were paired randomly, and we recorded the number of eggs laid by females over the subsequent 6 days. Female oviposition rate does not change significantly over the egg-laying period of about 3 weeks (Konior, Radwan & Kołodziejczyk, 2001). Therefore the 6-day recording period is representative of a female's lifetime egg output.

On day 7 we mounted parental adults on slides in Berlese medium (50 mL distilled water, 50 g chloral hydrate, 20 mL glycerine, 30 g gum Arabic; Hughes, 1976). Eggs were kept under standard conditions and we recorded the number of unhatched eggs 8 days after the removal of parents, by which time all viable embryos should have hatched.

Daughters obtained from each family were first paired with their brothers for 3 days. We collected eggs from these matings for estimation of embryo mortality as described for the parental generation. The brothers were mounted on slides on day 3, and the daughters were re-assigned a random unrelated male for further matings. Eggs laid by females during days 3–8 were counted to assess the fecundity of female offspring.

For each mounted individual we measured idiosoma length (i.e. the whole body without mouthparts) and FA, the Procrustes distance between shapes of left and right body sides based on several landmarks.

PROCRUSTES ANALYSIS

In a preliminary survey of many potential morphological landmarks, we selected those that could be readily

identified on most mounted specimens. These were positioned at sharp edges of hard structures on the ventral side of the body. Some landmarks in the posterior part of the body were excluded, because obscuring artefacts from the specimen fixation and mounting process tended to accumulate in this region. In the end, five landmarks on the ventral side of males, and four on females, were selected that could be unambiguously identified on most mounted specimens; the additional landmark for males was located on the sclerotized penis base (Fig. 1).We then found landmark coordinates under 200× magnification using Image-1 AT software (Universal Imaging Corporation). Unfortunately, about 30% of mounted mites were impossible to measure because our mounts did not clear well enough to locate all landmarks. A subset of 30 individuals of each sex was selected for repeated measurements, out of which 22 males and 18 females were measurable. They were measured twice on two differ-
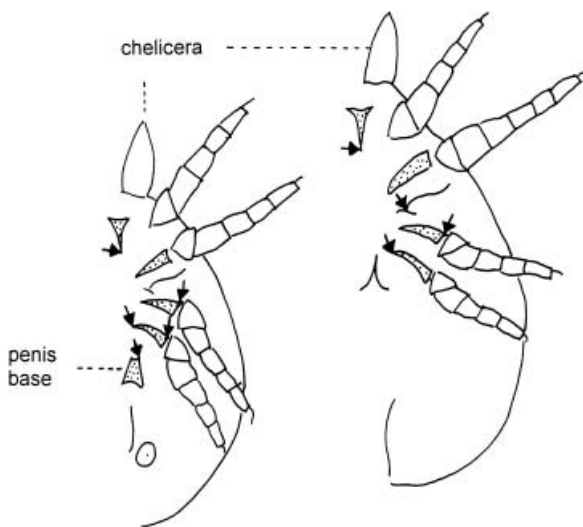

Figure 1. Positions of landmarks (arrows) on the ventral side of the male (left) and female (right), located on edges of sclerotized epimera or penis base in the male. Schematic outline of one side of the body is shown.

ent days. The coordinates for the right side and mirror images of the left side were than entered into the GRF-NS software (Slice, 1994) to obtain coordinates of optimally superimposed landmark configurations of left and right sides scaled to a centroid size of 1. The data from repeated measurements were entered into Procrustes ANOVA according to the procedure described by Klingenberg & McIntyre (1998). This is equivalent to a two-way ANOVA with individual as a random factor, the method of analysis recommended by Palmer & Strobeck (1986). In short, optimally superimposed coordinates are entered into the standard ANOVA and then sums-of-squares for each effect are summed over landmarks. Degrees of freedom for the mean square calculation are obtained by multiplying standard degrees of freedom by the number of landmarks minus four degrees lost during superimposition (Klingenberg & McIntyre, 1998). In both sexes, FA could be discerned from measurement error, as indicated by highly significant side × individual interactions. However, directional asymmetry was not significant (Table 1).

Our measures of FA, namely, Procrustes distances, were calculated as square roots of sums-of-squared differences between coordinates of all landmarks on the left and right sides of the body (see details in Klingenberg & McIntyre, 1998). The Procrustes distance is equivalent to the absolute left–right difference with overall size controlled by the prior standardization of all coordinate sets to the same centroid size. The directional component of asymmetry was calculated as a square root of the sum (over landmarks) of squared mean differences between landmark locations for left and right sides (Smith *et al.*, 1997; Klingenberg & McIntyre, 1998).

Because about 30% of mounted mites were impossible to measure, we had unequal family sizes for parent-offspring regression. We therefore calculated weighting factors for family averages based on family sizes, and calculated the slope of regression and associated standard errors using an iterative procedure derived by Kempthorne & Tandon (1953) as described in Lynch & Walsh (1998).

Table 1. Results of Procrustes ANOVA with individual as a random effect

	Males				Females			
	MS × 1000	d.f.	F	P	MS	d.f.	F	P
Side	0.166	6	0.748	0.612	1.115	4	0.231	0.999
Individual	1.396	126	24.255	<0.001	49.525	68	956.982	<0.001
Interaction	0.222	126	3.852	<0.001	5.003	68	96.681	<0.001
Error	0.057	268		0.052	144			

RESULTS

Examinations of scatter plots of vectors corresponding to right minus left differences for each landmark revealed no distinct clumping in either sex, i.e. there was no indication of antisymmetry (Debat *et al.*, 2001). The directional component of asymmetry in the whole sample was small, accounting for 3.4% of female asymmetry and 3.7% of male asymmetry, confirming the conclusion from a subset of twice-measured individuals in which we found no significant directional component.

Fighters did not differ from scramblers in size (respective means $\pm$ SD in μm were 623.7 $\pm$ 33.8 and 619.6 $\pm$ 41.9, $t_{177} = 0.515$, $P = 0.607$ in the parental generation, and 652.1 $\pm$ 32.8 and 662.5 $\pm$ 29.2, $t_{189} = -1.29$, $P = 0.198$ in F_1). The male FA was not significantly correlated with size (parental fighters: $r_{116} = -0.13$, $P = 0.164$, scramblers: $r_{11} = 0.338$, $P = 0.311$; F_1 fighters: $r_{140} = -0.13$, $P = 0.122$, scramblers: $r_{15} = -0.21$, $P = 0.458$). Slopes of FA on size did not differ between morphs (parental generation: $F_{1,120} = 1.91$, $P = 0.168$; F_1: $F_{1,151} = 0.11$, $P = 0.738$).

In both generations, FA was higher for fighters than it was for scramblers (respective means $\pm$ SD were -3.09 ± 0.35 and -3.29 ± 0.34, $t_{125} = 2.21$, $P = 0.034$ in the parental generation, and -3.06 ± 0.35 and -3.27 ± 0.37, $t_{153} = 2.25$, $P = 0.026$ in F_1), even though no landmarks measured on males were positioned on the legs. Therefore, when calculating the mean FA for parents and offspring, we standardized FA for males by subtracting means and dividing by SD for respective morphs/sexes (Lynch & Walsh, 1998).

The proportion of unhatched eggs in each outbred brood was not significantly correlated with the
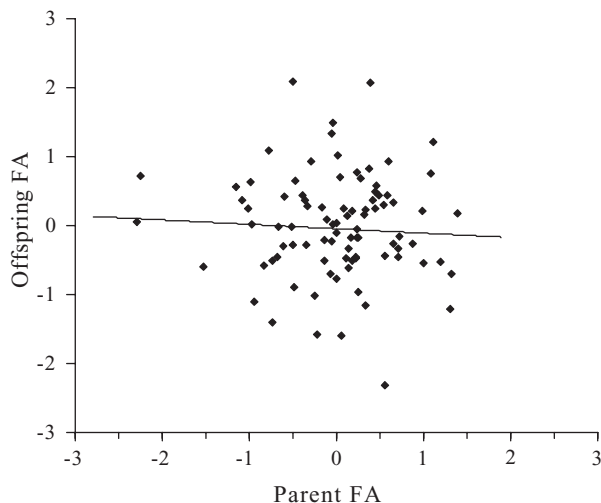
parents' mean FA (Spearman $r_s = -0.106$, $n = 105$, $P = 0.28$). The same was true for embryonic mortality amongst inbred broods obtained from sib-mated F_1 females (Spearman $r_s = -0.075$, $n = 161$, $P = 0.34$).

The slope of the regression of progeny FA on parental FA was -0.050, SE = 0.094 (Fig. 2). The null hypothesis that the slope was less than or equal to zero could not be rejected ($t_{88} = -0.627$, $P = 0.734$). The slope of the regression of daughter fecundity on mother fecundity was 0.135 (SE = 0.091), which was significantly greater than zero at a 0.1 significance level ($t_{156} = 1.486$, $P = 0.069$), giving a heritability estimate of 0.27.

Female size was negatively associated with FA in both the parental and F_1 generations ($r_{170} = -0.15$, $P = 0.043$ and $r_{162} = -0.13$, $P = 0.090$, respectively). Combining P-values of the tests for the two generations (Sokal & Rohlf, 1981) confirmed that the association was significant ($\chi^2 = 11.11$, d.f. = 4, $P = 0.025$). Multiple regression revealed a significant association between female size and fecundity, and between FA and fecundity in the F_1 generation (Table 2, Fig. 3). Combining P-values of the tests for the two generations (Sokal & Rohlf, 1981) confirmed that the negative association between female FA and fecundity was significant ($\chi^2 = 12.47$, d.f. = 4, $P = 0.014$).

DISCUSSION

The utility of FA as a measure of developmental stability and its heritability are subjects of much debate (e.g. Houle, 1997; Markow & Clarke, 1997; Møller & Thornhill, 1997a,b; Palmer & Strobeck, 1997; Whitlock & Fowler, 1997; Gangestad & Thornhill, 1999; Bjorksten *et al.*, 2000a; Bjorksten *et al.*, 2000b; Leung *et al.*, 2000; Møller, 2000; Van Dongen & Lens, 2000), stressing the need for empirical studies that use more sensitive measures of developmental stability, such as those based on FA of several traits (Gangestad & Thornhill, 1999; Leung *et al.*, 2000), or that employ

Figure 2. Association between mean parent and mean offspring fluctuating asymmetry (FA).

Table 2. Results of multiple regression with female fecundity as a dependent variable, for parental generation (P) and F_1

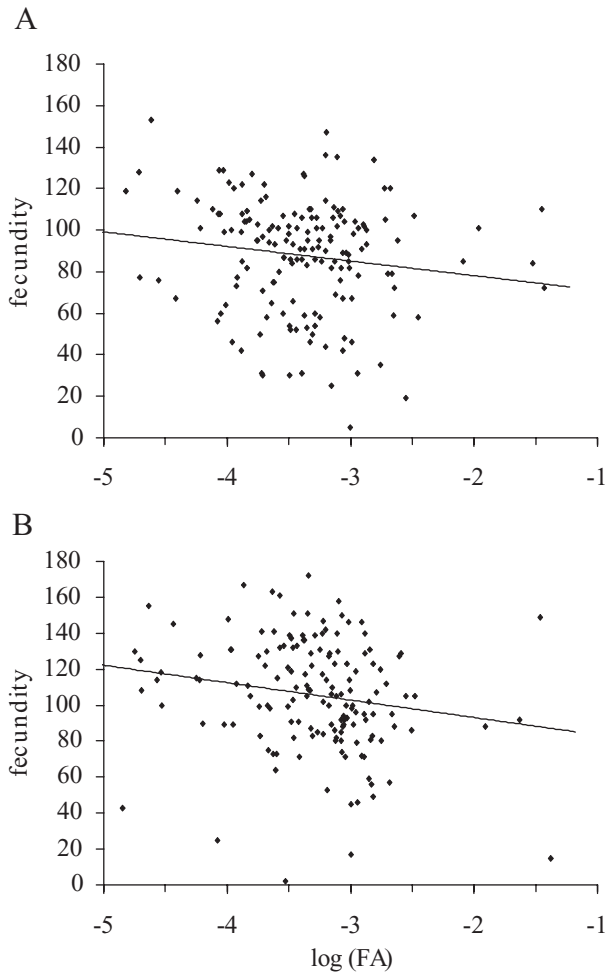|	β	t_{157}	P
P			
Size	0.18	2.36	0.019
FA	−0.12	−1.64	0.103
F$_1$			
Size	0.17	2.24	0.026
FA	−0.21	−2.65	0.009

FA = fluctuating asymmetry.

A

B

Figure 3. Association between female fecundity and fluctuating asymmetry (FA) in parents (A) and in F_1 (B).

positional FA rather than FA of linear traits (Polak & Starmer, 2001). Our study utilized Procrustes analysis based on the relative positioning of several landmarks and the results suggest that, in *R. robini*, heritability of developmental stability may indeed be low. The lack of significant heritability of FA can be a consequence of its close association with fitness; such traits typically show low heritability (Mousseau & Roff, 1987; Roff & Mousseau, 1987). At the same time, life-history traits tend to show higher inbreeding depression than do metric traits more distantly associated with fitness (DeRose & Roff, 1999). In this study we did not attempt to estimate the inbreeding depression in FA, but Radwan (2003) found in *R. robini* that six generations of inbreeding significantly increased FA quantified in the same way as in this study.

We hypothesized that heritability of FA can be underestimated if progenies of individuals with high FA contain lower proportions of viable embryos. Our

data did not confirm this hypothesis, but we believe the idea is well worth testing in other taxa.

Genes detrimental to fitness are usually partially recessive (Lynch & Walsh, 1998). Assuming the same is likely to be the case for mutations decreasing developmental stability, such genes may manifest as only small developmental effects such as increased FA. In heterozygotes, more severe effects leading to embryonic mortality may be masked by the presence of dominant alleles that enable normal development. We tested for this scenario by mating F_1 females with their brothers, thus increasing homozygosity, bringing together any FA-related deleterious recessives at the same locus at higher frequencies and, consequently, increasing the likelihood of their full and potentially catastrophic expression in the progenies. However, we did not find the hypothesized association between parental FA and embryonic mortality of their inbred broods. It still may be of interest to examine the possibility that FA reveals deleterious recessives for development in other systems, and our experimental design suggests a way of doing so.

Developmental stability is expected to be associated with other measures of fitness (Møller & Swaddle, 1997), but the association between FA and fitness is debated (Leung & Forbes, 1996; Møller, 1997, 1999a,b; Clarke, 1998; Bourguet, 2000). However, a recent meta-analysis (Møller, 1999b) found positive correlations of FA with fecundity, growth and survival. Our results are consistent with that finding: females with high FA had lower fecundity, and this effect was independent of female size, itself negatively associated with FA and positively with fecundity. In the absence of detectable heritability of FA, this result indicates that FA purely reflects the phenotypic quality of *R. robini* females and that the same environmental factors which elevate FA may also lead to decreased fecundity.

The higher FA that we found in fighter males compared with scramblers is also consistent with FA reflecting higher levels of stress during development. Production of thickened legs appears to be costly in acarids. In the male-dimorphic mite *Sancassania berlesei*, in which differences between male morphs are similar to those in *R. robini,* fighters lose more weight compared with their nymphal weight than do scramblers and, in consequence, are on average smaller compared with scramblers emerging from nymphs of the same weight (Radwan, Unrug & Tomkins, 2002). It therefore seems plausible that fighters experience a higher level of developmental stress that results in higher FA. Adverse effects of developmental stress may result in decreased fitness later in life. Radwan & Bogacz (2000) found that when mortality due to fights between males is experimentally excluded, scramblers live 20% longer than fighters. On the other hand,

fighters have better average survival and mating success in populations in which they are at a frequency high enough to cause substantial mortality, and morph fitness is not frequency-dependent (Radwan & Klimas, 2001). Thus, in *R. robini*, sexually selected fighting ability seems to be traded off against developmental stability and lifespan; this may help to explain the maintenance of genetic variance manifested in high heritability of the male morph in this species (Radwan, 1995).

Using Procrustes analysis we found significant associations of FA with female fitness and male morph. Our study, along with others that have used Procrustes methods or positional asymmetries (e.g. Auffray *et al.*, 1996; Polak & Starmer, 2001), indicate that such measures of FA can be useful indicators of stress and quality of individuals.

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