



How useful is fluctuating asymmetry in conservation biology: Asymmetry in rare and abundant *Coenonympha* butterflies

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Abstract

It has been suggested that minor, fluctuating differences in size of bilateral traits could validly indicate individual differences in developmental stability. One plausible reason for instability to occur could be lowered population size, which has been suggested to increase fluctuating asymmetry due to inbreeding, for example. We measured seven wing asymmetries of three *Coenonympha* butterfly species in central Sweden. One species is abundant (*C. pamphilus*), one rather common (*C. arcana*), and one rare (*C. hero*). We expected that if fluctuating asymmetry is a reliable indicator of population quality and thus a useful tool for conservation purposes, the most abundant species should show lowest asymmetry and the most endangered, the highest. Contrary to our expectations, the highest wing asymmetry was found in the relatively common species *C. arcana* and the most abundant and rare species did not show significant differences in levels of wing asymmetry. Our results obtained from three *Coenonympha* species hence suggest that the use of fluctuating asymmetry as an indicator of population conservation status may be misleading. Possible increase in asymmetry of small and/or isolated populations of butterflies may be masked by local differences in environmental conditions that could have high impact on bilateral development as well.

Introduction

Fluctuating asymmetry (FA), ‘random departures from anticipated bilateral symmetry’ (Van Valen 1962; Markow 1995), is thought to reflect quality of organisms (Møller & Swaddle 1997, but see for example Clarke 1998; Simmons *et al.* 1999). The idea behind this concept is that developmentally unstable genotypes cannot control their development precisely, and consequently more often develop different phenotypes on the left and right side. Inbreeding and high levels of stress, for example, may induce asymmetry. Thus fluctuating

asymmetry might be a very useful tool in conservation biology (Clarke 1995; Møller & Swaddle 1997), in reflecting various forms of environmental change.

There are, however, conflicting reports on the relationship between quality and asymmetry (reviewed amongst others by Palmer & Strobeck 1986; Parsons 1990; Møller & Swaddle 1997; Clarke 1998; Palmer 1999; Simmons *et al.* 1999). Two recent examples of a relationship between FA and quality are: elevated levels of FA in five tropical bird species living in fragmented habitats, compared to large undisturbed area of rain forest (Lens *et al.* 1999) and increased levels of FA in



threatened butterfly species compared to species with stable populations in Denmark (Poulsen 1996). Two recent examples of no relationship between FA and quality are cactophilic fruitflies, *Drosophila pachea*, that did not have elevated levels of FA under food stress (Hurtado *et al.* 1997), and the rare British butterfly *Plebejus argus* where bottlenecked populations did not have elevated levels of FA (Brookes *et al.* 1997).

In this paper we evaluate asymmetry in wings and eyespots of three *Coenonympha* species. Poulsen (1996) reported that asymmetry in wing length in decreasing Danish butterfly populations tended to be higher than in stable populations. She included among others pairs of *Coenonympha* species and reported elevated levels of FA in decreasing populations. Here we examine whether asymmetry differs between abundant and decreasing Swedish *Coenonympha* species, taking into account the effects of measurement error, trait size and type of asymmetry. We end with a discussion of the usefulness of analysing FA for conservation purposes in butterflies.

Methods

Species

Three species were analysed. The scarce heath (*Coenonympha hero*) is placed on the red list of Swedish butterflies as vulnerable (Ehnström *et al.* 1993).

The number of populations is decreasing because of reforestation of suitable habitat. A reduction in fitness related traits has also been documented in local small and isolated populations (Cassel *et al.* 2001). One of us (AC) sampled one of the remaining populations in central Sweden, east of Arvika (Värmland province) in the summers of 1997 and 1998. This population was one of the larger and consisted of about 200 adults. Total sample size consisted of 25 males and 21 females.

The small heath (*Coenonympha pamphilus*) is one of the most abundant butterfly species in Swedish grasslands. Two of the present authors (JJW and SN) sampled it in the national city-park of Stockholm in the summer of 1997. There are many extensively managed grasslands (predominantly mown once a year) in this park. It has a large population, of several thousand, possibly more than ten thousand individuals, of *Coenonympha pamphilus*. Total sample size consisted of 27 males and 24 females.

The pearly heath (*Coenonympha arcania*) is still rather abundant in Sweden. PTR sampled it near Lövsta (Uppland province near Uppsala) in the summer of 1998. Population size is probably a few hundred individuals. Total sample size consisted of 26 males and 11 females.

Traits and measurements

A total of seven fore and hind wing traits were measured (Figure 1). These were: area of fore and hind wing,

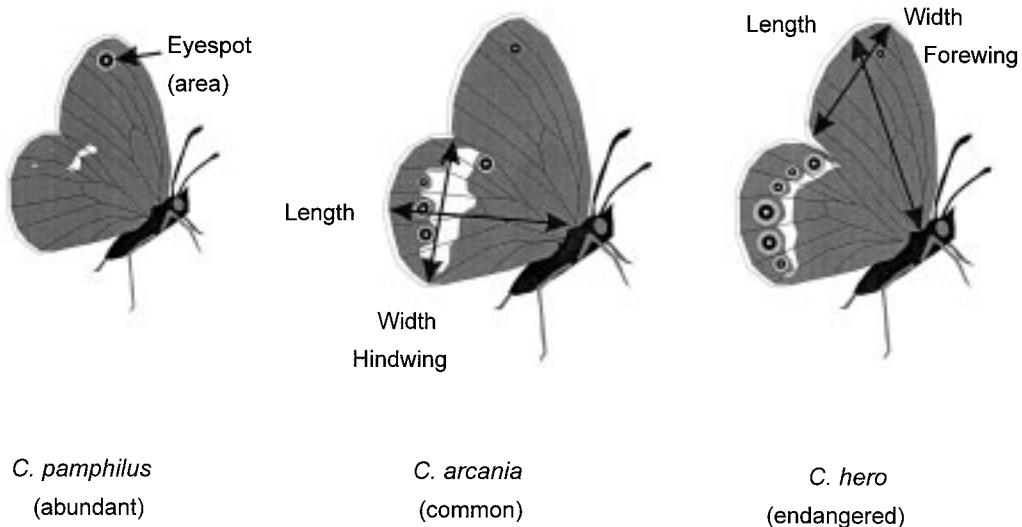


Figure 1. Schematic representation of the three species analysed and measurements performed. Besides measurements shown, wing area for both fore and hind wing were measured as well.



length of fore and hind wing, width of fore and hind wing, and total area of the eyespot on the ventral side of the fore wing. The fringe of hairs at the edge of the wings was excluded from all measurements since it was worn off in some individuals. Several width and length measurements were tried, and the following were chosen that gave the smallest error. The length of the fore wing was measured from the wing base to where vein M_1 (nomenclature, see Chinery 1978) reached the edge of the wing. The length of the hind wing was measured from the angle at the edge just above the base of the wing to where vein Cu_{1a} reached the edge. The width was measured from the edge below the vein 1A to vein R_{3+4} in the fore wing, and from vein 2A to M_1 in the hind wing. The eyespot measured was the only eyespot that was present in all individuals in all three species.

Measurements were made with an image analyser. An image analyser consists of a video camera coupled to a computer, and allows for very precise measurements of butterfly wings (Windig 1991). The image analyser used here consisted of a JVC TK 5066E Camera and a Leica image analysis system. Measurements were performed semi-automatically with the help of a program written by JJW in the Qwin image analysis software (produced by Leica). Wings were detached from the body before measurement, by carefully cutting their connection to the body at the root of the wings. The program automatically thresholded a wing from the background for the area measurements (= number of pixels converted to mm^2). Start and end-points for width and length were indicated manually on the monitor. Both were more than 300 pixels in length so that pixellation error (warned for in Palmer 1994) was not large. Area of eyespots were measured by first thresholding black (white) colour from the rest of the image, and then manually indicating which black and white part or parts made up the eyespot. Measurement results were sent to a spreadsheet without the person operating the program (one of the authors, PTR) being able to inspect them.

To quantify measurement error all individuals were measured twice. When part(s) of a wing was damaged this wing was not analysed for a trait that could have been influenced by the damage. This resulted in only 7 females of *C. arcana* that could be analysed for fore wing length. The repeated measurement included frame grabbing. To minimise influence of variation in illumination, light intensity was standardised at the start of each session by measuring the same wing until it reached a standard value. Care was taken to ensure that the directional asymmetry found in some traits was not

the consequence of a slight bias in the system. Change in the order left and right wings were measured did not influence the asymmetry, nor did the position of the wings in the image, or whether wings were measured on the dorsal or ventral side. When wings were damaged the damage was either corrected manually, e.g. a small part missing at the edge of the wing was drawn on the screen with the mouse when the original contour of the wing was clear, or in most cases the wing was discarded for that measurement.

Statistical analysis

For a better overview and to reduce the number of statistical tests, we used principal component analysis (PCA) to transform wing size measurements into two traits. PCA combines, based on correlation coefficients, traits into new traits (principal components) so that the first principal component (PC1) describes most of the variation originally separately present in the old traits. Each following principal component describes the largest part of variation that is not correlated to previous principal components. PCA was run on a data set of the three species and two sexes combined. Wing areas were log-transformed before PCA, to obtain normally distributed variables. PCA resulted in the first two components describing over 95% of the variation. If for an individual a measurement was missing for one trait only, a dummy measurement was filled in for this measurement with the help of multiple regression, before the PCA was performed. If more than one measurement was missing, individuals were discarded. Results were hardly influenced by the inclusion of dummy measurements. Only the statistical power would have been lower, and not enough females of *C. arcana* would have had PCA values for analysis.

Differences in trait size between species and sexes were analysed by a two-way ANOVA. Reliability of FA-measurements and the possibility of the presence of directional asymmetry (DA) was evaluated using a repeated measurements ANOVA (Palmer 1994) for each species separately. In such an ANOVA, individuals and sides and their interaction are the factors evaluated. Significance of the side factor indicates presence of DA, significance of the side * individual factor indicates that FA is significantly larger than measurement error (Appendix A). DA was further analysed by evaluating the differences between species and sexes in a two way ANOVA on the signed difference between left and right ($L - R$). We calculated kurtosis of the frequency distribution of $L - R$ to see whether



it was platykurtic (an indication of anti-symmetry) or leptokurtic (indicates a mixture of several normal distributions with different variances). The possibility of anti-symmetry was further evaluated by inspection of graphs of (L – R) vs. trait size (following Rowe *et al.* 1997).

FA was analysed by calculating the variance of L – R (FA4 of Palmer 1994, Appendix A) and the mean of the unsigned difference between left and right, |L – R| (FA1 of Palmer 1994). Bartlett's test for homogeneity of variance of L – R, the most powerful test according to Palmer (1994) was used to evaluate the significance of differences in FA across species. Measurement error can influence all asymmetry indices. From the repeated measurements ANOVA, its magnitude can be estimated and used to correct FA4, by subtracting the variance due to measurement error from the variance of L – R giving the index FA10 of Palmer (1994). Differences between sexes were evaluated by an *F*-test using the ratio of FA10 of males and females. We evaluated the relationship between FA and trait size by calculating the correlation between |L – R| and trait size. Trait size effects were corrected for by taking the residuals of the regression of |L – R| on trait size (Leung 1998; Windig 1998). FA4 is not influenced by DA, so no correction for DA was needed.

Results

Trait size

Scores for the first axis (PC1) in the PCA for size measurements are remarkably similar for all traits (ranging from 0.39 to 0.42). PC1 is thus clearly a measurement of overall size and it accounts for 93.6% of all variation in size traits. Hind wing measurements had negative scores for PC2 (length –0.316, width –0.216 and area –0.181) and fore wing measurements had positive scores, except for length (–0.141). Fore wing width had the highest score (0.862), area the lowest (0.107). PC2 can thus be seen as an index of shape where individuals with relatively broad wings and relatively large fore wings have high values, and individuals with more pointed wings and relatively small fore wings have negative values. It accounts for 3.7% of total variation in size traits. *C. pamphilus* is the smallest of the three, *C. arcana* is slightly larger than *C. hero*. Both *C. arcana* and *C. hero* have small eyespots, *C. hero* slightly smaller than *C. arcana*. Eyespots of *C. pamphilus* are about five times as large as the eyespots of the other two species. Females are significantly larger than males and

Table 1. ANOVA of trait size for sex and species. *** = $P < 0.0001$.

	Sex		Species		Sex*Species	
	F	P	F	P	F	P
PC1 (size)	70.16	****	159.93	****	4.677	0.0098
PC2 (shape)	216.93	****	66.26	****	0.749	0.47
Eyespot	15.57	0.0001	187.59	****	0.033	0.97

have larger eyespots (Table 1). Differences between sexes are similar for all three species except for size (PC1, significant interaction term, Table 1). In *C. hero* the difference between males and females was smaller than in the other two species.

Reliability and type of asymmetry

For all traits FA is significantly larger than measurement error both in males and females, as indicated by the repeated measures ANOVA's (Table 2). The ANOVA's indicated directional asymmetry (DA) in four instances, three of them in *C. arcana*. After Bonferroni correction for multiple comparisons only PC2 (shape) in males of *C. arcana* had significant DA. Despite the difference in significance between species there are no significant differences in DA of PC2 between species ($P = 0.099$) or sexes ($P = 0.89$) when tested with a two way ANOVA for L – R. All three species have on average larger PC2 values (= relatively larger and broader fore wings) on the left than on the right, but only for *C. arcana* the difference between left and right is so large that it is significantly different from zero.

There is no relationship between trait size (mean of left and right side) and asymmetry for PC1 (size) and PC2 (shape) (all $r < 0.10$, $P > 0.12$, Figure 2). For the eyespots, however, a clear relationship exists between trait size and absolute difference between left and right side ($r = 0.32$, $P < 0.0001$, Figure 2). The relationship is, however, only linear after log-transformation of both |L – R| and trait size and the correlation coefficient is then considerably higher ($r = 0.586$, $P < 0.0001$). To remove the effect of trait-size, the residuals of |L – R| to the regression line calculated after log-transformation (Figure 2) will be used further on.

Difference between sexes and species

There are no significant differences in FA between sexes after the Bonferroni correction (Table 3). FA



Table 2. Reliability of FA measurements. Repeated measures ANOVA with side and individuals as factors. *F*- and *P*-values for side factor under directional asymmetry (DA), for side by individual interaction under fluctuating asymmetry (FA). All *P*-values for FA significant after Bonferroni-correction, only value in bold for DA significant after Bonferroni-correction.

Trait	Species	Directional asymmetry				Fluctuating asymmetry				Kurtosis	
		Males		Females		Males		Females		Males	Females
		F	P	F	P	F	P	F	P		
PC1 (size)	<i>pamphilus</i>	1.32	0.26	0.00	0.99	19.92	****	20.30	****	-0.82	-1.04
	<i>arcania</i>	5.34	0.031	4.40	0.081	10.93	****	13.07	****	-0.34	-0.13
	<i>hero</i>	0.00	0.95	0.81	0.39	72.57	****	58.52	****	0.81	1.69
PC2 (shape)	<i>pamphilus</i>	3.67	0.066	1.60	0.22	13.25	****	30.19	****	0.77	1.90
	<i>arcania</i>	53.577	****	0.01	0.92	4.96	****	8.88	****	0.34	0.52
	<i>hero</i>	0.56	0.46	2.51	0.13	22.41	****	43.72	****	1.74	3.12
Black ring	<i>pamphilus</i>	5.46	0.027	0.12	0.73	3.14	0.0002	4.09	****	0.16	3.26
	<i>arcania</i>	3.90	0.060	8.97	0.012	3.96	****	8.88	****	0.62	0.62
	<i>hero</i>	0.00	0.93	0.10	0.71	10.81	****	50.26	****	2.53	-0.35

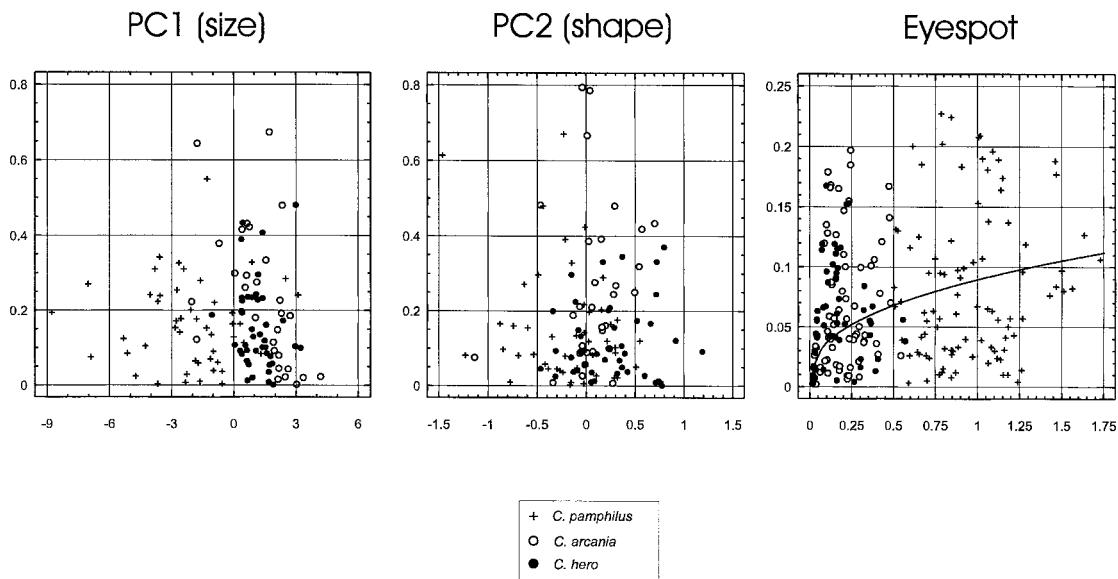


Figure 2. Asymmetry vs. trait size. Asymmetry (FA) = absolute value of difference between left and right side. Trait size (TS) = mean of left and right side. For eyespot there is a clear relationship between TS and FA. Plotted line is regression line of $\log(\text{FA})$ on $\log(\text{TS})$, $r = 0.59$. Residuals of regression (vertical distance to plotted line) are used for measure of FA corrected for trait size.

differs significantly between species for males for all three traits, and for females for the eyespots (Table 3). *C. arcania* has the highest asymmetry in all significant cases. The difference in FA between *C. pamphilus* and *C. hero* tends to be small, and FA is not consistently smaller for one of the species.

Discussion

There are clearly differences in levels of FA between the three *Coenonympha* species sampled in this study.

They are, however, not in the expected direction. It is not the vulnerable *C. hero* that has the highest levels, but *C. arcania*. This result casts doubt on the reliability of FA as an indicator of the quality of populations, and thus on the usefulness of FA in conservation biology. On the other hand the stresses on the populations that we sampled may have been different from what we expected, based on the assumption of population size reflecting quality.

There is no doubt that *C. hero* is threatened in Sweden. Populations in southern Sweden have become extinct, and the remaining central Swedish populations



Table 3. Size of fluctuating asymmetry and difference between sexes and species. Different indices are used to describe FA (numbering follows Palmer 1994, L = value on left side, R = value on right side TS = trait size = $L + R/2$). FA1 = mean $|L - R|$, FA3 = mean $|L - R|/\text{mean TS}$, FA4 = Variance $L - R$, FA10 = Variance $|L - R|/2$ corrected for measurement error, estimated from repeated measures ANOVA (Table 2). Twice the value is given for comparison with FA4. Residuals = mean of the residuals of FA1 on TS (Figure 2). Significance of difference was tested with Bartlett's test for homogeneity of variance (PC1 and PC2) or ANOVA on residuals (eyespot). Significance of sex difference was tested by an F-test using the ratio of male and female FA10 (PC1 and PC2) or ANOVA on residuals (eyespot). Significant P-values after Bonferroni correction are in bold. *** = $P < 0.0001$.

Trait	Sex	Index	Variance of L - R			Species difference Bartlett's test
			pamphilus	arcania	hero	
PC1 (size)	Male	FA1	0.169	0.267	0.186	
		FA4	0.0420	0.0876	0.0581	0.0071
		2*FA10	0.0394	0.0806	0.0501	
	Female	FA1	0.169	0.139	0.145	
		FA4	0.0373	0.0329	0.0340	0.856
		2*FA10	0.0355	0.0296	0.0297	
Sex difference (F-test)			0.82	0.20	0.25	
PC2 (shape)	Male	FA1	0.144	0.286	0.118	
		FA4	0.0327	0.0941	0.0256	****
		2*FA10	0.0304	0.0786	0.0245	
	Female	FA1	0.172	0.236	0.164	
		FA4	0.0612	0.0783	0.0580	0.801
		2*FA10	0.0591	0.0718	0.0570	
Sex difference (F-test)			0.11	0.97	0.051	
Eyespot	Male	FA1	0.0806	0.0423	0.0326	
		FA3	0.89%	3.41%	7.23%	
		Residuals	-0.197	0.248	-0.341	****
		FA4	0.00937	0.00281	0.00327	
		2*FA10	0.00724	0.00220	0.00324	
	Female	FA1	0.0906	0.0948	0.0638	
		FA3	0.86%	3.24%	3.01%	
		Residuals	0.396	0.548	-0.319	****
		FA4	0.0133	0.0075	0.0057	
		2*FA10	0.0083	0.0068	0.0057	
Sex difference (ANOVA)			0.042	0.25	0.74	

are continuously disappearing as a consequence of reforestation. Negative effects of population size on life history traits and genetic variation in the remaining central Swedish populations have been documented (Cassel *et al.* 2001). However, our *C. hero* sample originates from a relatively large population and the *C. arcania* sampling site is in the northern part of its distribution in Sweden. So the difference in population size and stability between *C. hero* and *C. arcania* may be less than we originally thought. However, it is extremely unlikely that the sampled *C. arcania* population is more at risk than the *C. hero* populations, as the levels of FA suggest. It also remains puzzling why *C. hero* and *C. pamphilus* do not show a clear difference in FA, although *C. pamphilus* populations are several orders of magnitude larger than *C. hero* populations.

Another cause of the observed differences may be that the three species were not caught in the same area. Temperature differences, food plant differences, or other environmental influences may have increased FA in the Uppsala area where *C. arcania* was caught. This would mean that such local non-threatening effects may be more important in the determination of FA than the actual status of a population.

There is doubt whether traits displaying DA can be used for analysing developmental instability (Palmer 1994; Palmer & Strobeck 1997). This seems of less concern here since DA was only significant for PC2 (shape) in *C. arcania* and because the DA was small relative to trait size and FA. Despite its small size the DA found here is interesting. DA for different characters of wings has been found for a number of insect species (Smith *et al.* 1997; Klingenberg *et al.* 1998;



Goulson *et al.* 1999; Windig & Nylin 1999). It may be the result of a fundamental process in insect wing development (Klingenberg *et al.* 1998). Windig & Nylin (1999), however, proposed that adaptation might be involved in DA of a satyrine butterfly, *Pararge aegeria*. In *P. aegeria* territorial males show DA, but females or non-territorial males do not, suggesting that DA may be beneficial for territorial flight. Territorial behaviour also occurs in *C. pamphilus* (Wickman 1985), but if and to what extent it occurs for the other two species is not known. Nor is it known exactly how important asymmetry is in relation to flight performance in butterflies in general. A detailed analysis of shape differences of wings and asymmetry between species and in relation to flight behaviour is thus needed.

All the necessary corrections for influences of trait size, measurement error or directional asymmetry were performed here. Except for the influence of trait size, conclusions would have been the same without the corrections. Image analysis is a very precise way to measure traits, and indeed measurement error was relatively low. Correction for measurement error, however, does give more confidence to the results. Trait size has far more influence on the pattern of FA in eyespots. If the correction for trait size had not been performed, *C. pamphilus* would have had the largest amount of FA for eyespots. With the correction for trait size the results for FA on eyespots are in agreement with FA for wing size and shape.

The overall conclusion from our FA-measurements of three *Coenonympha* species is that the usefulness of FA in conservation biology is complicated. Possibly,

FA can be useful if it can be measured on animals in the field living in the same area, but we cannot be confident of that, and it anyway limits the applicability of FA in conservation biology. Recently more doubts on the usefulness of FA have appeared in the literature. Vollestad *et al.* (1999) concluded after a meta-analysis of literature reporting on the relationship between heterozygosity and FA that 'at best only a weak association between heterozygosity and FA exists'. Researchers on FA (e.g. Palmer 1999) have repeatedly stressed that negative relationships between FA and quality have to be reported in the literature, although they are at first sight less interesting than positive results. Simmons *et al.* (1999) concluded that reports on the relationship between FA and quality tend to indicate nowadays more often a negative relationship than about 5 to 10 years ago. To summarise, caution must be taken in generalising our findings to other organisms, but they do show that there is no universal relationship between FA and status for conservation.

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Appendix A. Reliability of FA measurements and size of FA (FA4) of traits underlying PC1 and PC2. **** = $P < 0.0001$.

Trait	Species	Directional asymmetry				Fluctuating asymmetry				Kurtosis	
		Males		Females		Males		Females		Males	Females
		F	P	F	P	F	P	F	P		
Area fore	<i>pamphilus</i>	0.89	0.36	0.59	0.46	8.67	****	13.59	****	0.13	0.79
	<i>arcania</i>	4.44	0.046	1.65	0.25	12.70	****	5.74	****	0.02	-0.23
	<i>hero</i>	1.91	0.18	1.59	0.22	27.38	****	64.11	****	2.60	0.98
Area hind	<i>pamphilus</i>	0.62	0.45	0.55	0.47	2.99	0.0003	9.33	****	-0.09	2.27
	<i>arcania</i>	3.86	0.061	3.21	0.12	14.10	****	37.18	****	-0.08	2.30
	<i>hero</i>	0.43	0.52	3.84	0.067	25.41	****	37.28	****	-0.53	0.81
Length fore	<i>pamphilus</i>	3.07	0.095	0.17	0.68	30.90	****	14.46	****	2.02	1.33
	<i>arcania</i>	1.60	0.22	—	—	17.30	****	—	—	-0.04	—
	<i>hero</i>	0.62	0.45	1.42	0.25	34.14	****	24.65	****	0.49	-0.94
Length hind	<i>pamphilus</i>	0.01	0.90	0.64	0.44	26.09	****	17.05	****	4.62	1.47
	<i>arcania</i>	4.00	0.057	2.21	0.18	14.04	****	54.41	****	1.39	-0.66
	<i>hero</i>	0.00	0.98	0.75	0.47	36.71	****	34.73	****	-0.72	-0.30



Appendix A. (Continued.)

Trait	Species	Directional asymmetry				Fluctuating asymmetry				Kurtosis	
		Males		Females		Males		Females		Males	Females
		F	P	F	P	F	P	F	P		
Width fore	<i>pamphilus</i>	0.67	0.43	2.10	0.16	19.23	****	21.07	****	5.73	1.10
	<i>arcania</i>	12.94	0.0011	0.04	0.84	2.99	****	11.22	0.0001	0.70	0.37
	<i>hero</i>	0.05	0.82	0.58	0.46	19.25	****	60.29	****	0.92	4.72
Width hind	<i>pamphilus</i>	21.17	0.0001	4.68	0.042	7.29	****	19.45	****	0.36	1.14
	<i>arcania</i>	1.44	0.24	1.10	0.33	13.95	****	33.74	****	3.58	-0.69
	<i>hero</i>	4.77	0.040	1.55	0.23	31.55	****	25.74	****	0.53	-0.41

Trait	Sex	FA 4 (Variance of L - R)		
		<i>pamphilus</i>	<i>arcania</i>	<i>hero</i>
Area fore	Male	1.40	6.89	5.29
	Female	2.52	3.98	4.49
Area hind	Male	1.45	5.34	4.03
	Female	3.87	5.28	2.87
Length fore	Male	0.0214	0.0407	0.0180
	Female	0.0147	—	0.0164
Length hind	Male	0.0174	0.0165	0.0095
	Female	0.0136	0.0180	0.0097
Width fore	Male	0.0323	0.0953	0.0223
	Female	0.0399	0.0716	0.0570
Width hind	Male	0.0204	0.0451	0.0228
	Female	0.0262	0.0686	0.0108

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