Oecologia (2007) 151:593–604
DOI 10.1007/s00442-006-0611-0

POPULATION ECOLOGY

Environmental stress increases skeletal fluctuating asymmetry in the moor frog *Rana arvalis*

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Received: 3 February 2006 / Accepted: 2 November 2006 / Published online: 29 November 2006

Abstract Whether fluctuating asymmetry (FA) provides a useful metric indicator of the degree of environmental stress experienced by populations is still a contentious issue. We investigated whether the degree of FA in skeletal elements is useful in elucidating the degree of environmental stress experienced by frog populations, and further, tested the proposition that a trait's sensitivity to stress—as reflected in the degree of FA—is related to the degree of directional selection experienced by the given trait. We compared the degree of FA in four bilateral skeletal elements of male and female moor frogs (*Rana arvalis*) originating from low (acidified) and neutral pH populations. While the degree of uncorrected FA was unrelated to the degree of acidity, the growth rate and age of the individuals, the size-corrected FA was significantly higher in low than in neutral pH populations and decreased with individual ages and growth rates. In addition, both measures of FA were significantly higher in males and in particular in traits presumably under high sexual selection as indicated by the degree of sexual size dimorphism. All in all, the results indicate that individuals from acidified localities are smaller, younger and exhibit a significantly higher degree of FA than individuals from neutral pH populations. These results constitute the first assessment of FA in amphibians and suggest that the degree of FA in skeletal traits can be a useful indicator of the degree of environmental stress experienced by amphibian populations.

Keywords Amphibians · Acidification · Developmental stability · FA · Environmental stress

Introduction

Most, if not all, ecosystems are suffering to some extent from the effects of human activities. One environmental stressor that has had a strong impact on freshwater ecosystems in the northern hemisphere and Fennoscandia in particular is the acidification of watercourses as a consequence of acid rain (Bertills and Hanneberg 1995). Due to acidification, many local fish populations in Scandinavia have become extinct (e.g., Hesthagen et al. 1999), and amphibian populations have also suffered to some, often-unknown degree (Böhmer and Rahmann 1990). Nevertheless, there are also indications that some frog populations have actually been able to adapt to acidity in their environment: e.g., moor

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Communicated by Roland Brandl.

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frogs (*Rana arvalis* Nilsson) from certain heavily acidified areas in Sweden. Apparently, they have evolved a better tolerance of low pH than populations from less acidified areas (Räsänen et al. 2003a, b; Merilä et al. 2004). However, frogs from acidified localities appear to suffer from decreased post-metamorphic growth, but in general, little is known about the impact of acidity on the adult life histories and population dynamics of amphibians. Such information could come from carefully controlled experiments and/or from field observations in a range of populations that differ in their degree of acidification. In the latter case, effects of reduced pH could be studied on a range of morphological traits as biomarkers. Traditionally, measures like size, weight and condition have been applied, but also, fluctuating asymmetry (FA) has been put forward as a biomarker of stress. However, the latter has been the subject of many debates in the literature, and its usefulness as a biomarker is questionable.

Fluctuating asymmetry (small random deviations from perfect symmetry, Møller and Swaddle 1997) is a controversial indicator of stress. On the one hand, a wealth of studies show an increase in FA with stress, while many others fail to find any response (reviews in: Leung and Forbes 1996; Møller and Swaddle 1997; Møller and Alatalo 1999; Lens et al. 2001). Although very simple in principle, the general use of FA as a biomonitoring tool is hampered by the lack of knowledge about the factors that predict if and when an association can be expected. Some attempts have been made trying to identify factors that predict the strength of the association between FA and stress. In a meta-analysis, Lens et al. (2001) compared effect sizes of FA-stress associations in a conservation context and found higher effect sizes (1) in aquatic versus terrestrial habitats, (2) when FA was estimated at the population level versus the individual level and (3) in meristic versus metric traits. There were also differences among species groups, with plants yielding the highest effects sizes, while reptiles and fish showed the lowest estimates. It has also been put forward, yet rarely tested, that the selection history of traits (sexual selection, trait functionality and thus strength of stabilising selection) may determine the sensitivity to the effects of stress (e.g., Karvonen et al. 2003). Different types of stress have been shown to interact in sometimes unpredictable ways, which complicates matters even more (see Van Dongen 2006 for examples). Furthermore, the statistical analysis of FA is very cumbersome and complex and has been the source of much discussion (see Van Dongen 2006 for recent review). In addition, how to deal with other forms of asymmetry, directional asymmetry (DA) and antisymmetry in particular, has been the subject of many debates. On the one hand, methods correcting for the presence of these forms of asymmetry should be used with great care (Van Dongen 2006), while on the other hand some suggestions have been found that DA and antisymmetry might increase with stress as well, hereby acting as a biomonitorring tool itself (Graham et al. 1993; Lens and Van Dongen 2000). Last but not least, the use of FA at the individual level has been questioned since in many cases single-trait FA only poorly reflects the underlying process of interest, namely developmental instability (DI) (Whitlock 1996; Van Dongen 2006). Taking these problems all together has led to a general abandoning of studies of FA, in spite of the fact that in some cases it turned out to be a very useful and sensitive biomarker (e.g., Lens et al. 2001). We therefore argue that further research on the suitability of FA (and DA and antisymmetry as well) in an ecological context is very relevant, provided that the above-mentioned problems and unanswered questions are studied in great detail.

The aim of this study is twofold. First, we compare a suite of morphological traits—including FA, between acidified and non-acidified locations. Second, we address a number of specific questions investigating the usefulness of FA in skeletal traits of moor frogs as a biomarker of the effects of acidity. More specifically, we addressed the following questions: (1) Are there differences in the levels of FA between acidified and non-acidified populations, and if so, (2) could FA be used as an indicator of environmental stress in amphibians? Given that amphibians grow throughout their life, we also asked (3) whether the levels of FA are size and age related. Differences in size may either hide or amplify the differences in FA among populations or associations with other covariates, but a priori expectations are difficult to formulate. However, all else being equal, we expected to find the degrees of FA to be inversely related to age due to the pruning effect of natural selection. Next, (4) we compared the sensitivity of FA to acidity among traits under different degrees of sexual selection. Levels of FA in traits under sexual selection can be expected to be more sensitive to stress than those not, although this hypothesis has only rarely been tested explicitly (but see, e.g., Björksten et al. 2000). We (5) investigate the usefulness of FA as a measure of individual levels of developmental instability by investigating trait correlations in FA and estimating the amount of between-individual variation in the underlying DI. The application of the concept of DI at the individual level clearly requires that it is an organism-wide property that shows sufficient between-individual variation. Finally, (6) we also explore whether DA or antisymmetry differs among acidified and non-acidified lakes. Although a couple of earlier FA studies in amphibians exist (Lamb et al. 1990; Ryan et al. 1995; Lauck 2006), only one of them has been conducted in an environmental-stress context (Lauck 2006). Hence, this study is one of the very few studies that clearly explores the usefulness of FA as a biomarker in amphibians. In doing so, we not only provide a general framework that can be followed by ecologists in any field to explore the use of FA, but also demonstrate that in this particular case, both at the population and individual level, FA appears to exhibit high potential as a biomarker in moor frogs in relation to acidity stress.

Materials and methods

Study species and populations

The moor frog is a small ranid with a broad distribution in northern and Eastern Europe and Asia (Ischenko 1997; Kuzmin 1999). In Sweden, it occurs up to 66°N, and breeds in small lakes, ponds and bogs in agricultural and forested areas in low-altitude areas (Elmberg 1984; Gislén and Kauri 1959). It is known to be relatively tolerant to acidity (Leuven et al. 1986) and can show local adaptation to acidity (Räsänen et al. 2003a; Merilä et al. 2004). Moor frogs are typical explosive spring breeders, and breeding starts in late March or early April when the water temperature has reached about +10°C (Kuzmin 1999). Four *R. arvalis* populations (Table 1) were included in this study. Two of them (Lilla Brödhållartjärn and Tottatjärn) were low pH (pH ≈4) localities, and two others were neutral pH (pH ≈7) populations (Häggedal and Lindrågen).

Preparation of skeletons and measurements

The material for this study was collected from 1998–2001 by sampling temporarily and spatially homogenous samples of breeding adults from breeding aggregations for the purposes of other studies, which required killing the individuals. In total, 237 individuals of both sexes were included in this study (Table 1).

Table 1 Descriptive information about study populations and samples

Locality	Coordinates	pH	Sample size (*n*)		
			Males	Females	Total
Häggedal	59°51″N 17°14″E	6.7	31	30	61
Lilla Brödhållartjärn	59°37″N 13°58″E	4.6	30	30	60
Lindrågen	59°28″N 13°31″E	6.5	30	26	56
Tottatjärn	57°36″N 12°34″E	4.0	30	30	60

From each individual, we noted the sex, measured the snout-vent-length (SVL) from the nose tip to the end of the urostyle with a digital calliper to the nearest 0.1 mm and recorded the fresh body weight (with an electronic balance to nearest 0.1 g). For the assessment of FA in morphological traits, we chose to quantify it from weights of bilateral bones as weights are likely to be less prone to measurement error than linear measurements (Karvonen et al. 2003). To prepare skeletons, all individuals were skinned and cleaned by dermestid beetles. To be sure that all soft tissues were carefully removed, the bones were rinsed for 4 h in 1% solution of Neutras (Novozymes A/S), after which they were rinsed in water. After the cleaning, the side of five bone elements (viz. radio-ulna, humerus, tibio-fibula, femur and ilium) was identified and marked on the bones with different colours on the different sides. Ilium in anurans is a part of very specialised structure, the caudopelvic mechanism, which is central to their locomotion, as are also the femur and tibio-fibula. The radio-ulna and humerus are bones in the arms and are—among other things—used by males for holding the females during pre-copulation and copulation. These two bones are also presumably under strong directional sexual selection as males in most amphibians have a proportionally longer radio-ulna and humerus than females and because there are direct demonstrations of selection (i.e., improved mating success) favouring males with longer arms (Hedengren 1987; Höglund and Säterberg 1989).

All bone elements were individually weighed with a microbalance (AND ER-60A) to the nearest 0.0001 g. To increase the accuracy of the weight estimates, weightings were made three times for each bone, except for the radio-ulna, which was weighted five times due to its small size, and hence, there was relatively low accuracy of weightings. All weightings were made blindly with respect to the origin of samples, and repeated measurements of the same individuals and bones were temporarily separated. The bone elements were handled with forceps to avoid increasing the variability in weights due to attached fat and moisture from fingers.

The age of the individuals was determined by using standard skeletochronological methods (e.g., Castanet et al. 1977) as described in Hemelaar (1985). This method is based on the cyclic growth pattern of amphibian bones, where a line of arrested growth becomes visible as a ring in the cross-section of the bone (Kleinenberg and Smirina 1969) after each growth period (one per year in temperate latitudes).

By counting these annual rings, the age of the individual can bedetermined in a rather straightforward manner.

Statistical analyses

General morphology and population structure

The body weight, SVL, age and growth rate (defined as SVL divided by age) were compared between the acid and neutral locations (factor abbreviated as ACID) using nested ANOVA's where location (nested in ACID) and the sex × location interaction were added as random effects. Standard error and degrees of freedom of tests of the fixed effects were approximated using the Kenward–Rogers procedure (see also below).

Fluctuating asymmetry

The statistical analysis of asymmetry data has received a lot of attention in the literature for a long time. A recent review of the current state of the art can be found in Palmer and Strobeck (2003) and Van Dongen (2006). Three important issues need to be considered when using asymmetry as a measure of developmental instability: measurement error, other forms of asymmetry and the degree of association between-individual levels of asymmetry and the underlying process of developmental instability (Van Dongen 2006). Since levels of asymmetry are usually small, repeated measurements are required to separate real asymmetry from measurement error. We achieved this by obtaining individual asymmetry values for each trait from mixed regression models as described in Van Dongen et al. (1999) and Van Dongen (2000). These estimates are corrected for measurement error (ME). In addition, these statistical models allow one to test and correct for directional asymmetry (DA), a form of asymmetry that may confound the estimation of DI (Van Dongen 2006). By testing the interaction between side and location, we explored if levels of DA differ between the study areas. Significance levels of FA relative to ME (likelihood ratio test) and of DA (F-test) are provided in Table 2. Data from the ilium were excluded from further analyses because we could not detect any FA with the current measurement techniques and their degree of error (Table 2). Data from the humerus and radius were included in the subsequent analyses in spite of their significant directional component (Table 2). The individual asymmetry values are corrected for this apparent DA by subtracting the average degree of asymmetry from all individual values. We hereby explicitly assume that for these traits, the mean degree of asymmetry reflects the optimal state, like perfect symmetry does for the traits without any directional component (Van Dongen 2006). This may be incorrect, however, and we explicitly test for differences in observed patterns in FA among the different traits (see below).

Table 2 Overview of results from mixed regression analyses on repeated measurements of the left and right side of five bones

Trait	σ^{2a}_{FA}	σ^2_{ME}	DA[b]		SDI (%)[c]
			df	*F*	
Femur	**0.18****	0.33	1,289	2.29	18.5
Tibio-fibula	**0.28*****	0.51	1,292	0.14	21.4
Humerus[d]	**0.36*****	0.08	1,289	7.63**	49.5
Radio-ulna[d]	**0.03*****	0.03	1,281	24.0***	38.3
Ilium[e]	0	0.41	1,296	23.8***	9.0

*$P<0.05$, **$P<0.01$, ***$P<0.001$

[a] Significance of the variance component reflecting true asymmetry was based on one-tailed likelihood ratio tests (Van Dongen et al. 1999)

[b] Significance of the fixed side effect in the mixed regression models

[c] *SDI* sexual dimorphism index, i.e., proportional difference in bone weight (difference/mean male weight) is used as an indication of the degree of sexual selection. In all traits, males had heavier bones than females (all $P<0.0001$)

[d] Included in analyses after correction for DA

[e] Excluded from further analyses

Individual and trait-specific FA values were obtained as the empirical Bayes estimates of the random slopes of the mixed regression models (see Van Dongen et al. 1999 and Van Dongen 2000 for details). The distributions of these signed asymmetries will be examined for the presence of antisymmetry, which is reflected by a negative kurtosis. To examine to what extent the unobservable DI is a property of the individual or trait specific, we examined correlations in signed and unsigned FA between-traits. Positive correlations in the signed asymmetry would suggest common developmental pathways and may confound correlations in the unsigned asymmetry. Relatively strong positive correlations in the unsigned asymmetry (in the absence of correlations in the signed FA) indicate that levels of DI are individual rather than trait specific. However, it has been suggested that individual FA values only crudely reflect the underlying developmental instability, hereby resulting in a downward bias of between-trait correlations in the unsigned FA (Whitlock 1996, 1998; Van Dongen 2006). Patterns in FA can be transformed into patterns in developmental instability using the so-called hypothetical repeatability (R) (see Whitlock 1998 for details). Values of *R* can be interpreted as normal repeatability, in the sense that it reflects the repeatability of the unsigned FA, assuming that a trait would develop more than once under exactly the same environmental conditions (Van Dongen 2006).

Comparisons of asymmetry values are based on the unsigned FA (absolute value of the random slopes), which are corrected for measurement error and degree of DA (above). It has been questioned whether or not these asymmetry values should be corrected for trait size. We performed all analyses with and without correction for trait weight, both after log-transformation to assure the approximate normality of the residuals of the statistical models. Asymmetry was compared between acid and neutral populations using a repeated-measures ANOVA model. We mainly followed the guidelines given in Verbeke and Molenberghs (2000) for model building and interpretation. Individual and trait-specific transformed asymmetry values were used as a dependent variable. Thus, four observations for each individual were used in a single analysis, which we accounted for by explicitly incorporating the correlation structure in our analysis. We used a compound symmetry structure with heterogeneous variance as this model had the lowest value of the Bayesian Information Criterion (Littell et al. 2002). Standard errors and degrees of freedom of tests for fixed effects were approximated by the Kenward and Rogers procedure to avoid a downward bias of the confidence intervals and to liberal inference due to the low number of repeats (i.e., only four localities) (Littell et al. 2002). Savin et al. (2003) recently showed that the Kenward Roger method results in appropriate confidence intervals and coverage probabilities. As fixed effects, ACID, age, growth rate and sex were added, as well as trait and its interaction with all previous fixed effects. As random effect, location nested within ACID was added. Pairwise differences in levels of asymmetry were compared among traits using the least square means, and significance levels were adjusted for multiple comparisons using the Tukey–Kramer method.

Multivariate approach

In order to investigate the relative magnitude by which the different variables analysed above differed among populations and what the effect of acidity is, we performed a canonical discriminant analysis on mean individual FA (both corrected and uncorrected for the individual trait weight), age, SVL, growth rate, body weight and sex (added as an indicator variable with the value zero for females and one for males). Both univariate and multivariate results are presented. Results are summarised graphically by combining the means of the different locations for the first two canonical variables and the degree of correlations of the different variables with the canonical variables in a single plot (a so-called biplot). All analyses were performed in SAS version 8.02 (Fig. 1).

Results

Sexual dimorphism, general morphology and age structure

All five bone elements were heavier in males than females ($P<0.0001$ in all cases; Table 2; Fig. 2). This sexual dimorphism was strongest in the arms, i.e., humerus and radio-ulna, as indicated by the proportional differences in the weights (Table 2).

In the acidified locations, the frogs were smaller as indicated by their significantly lower body weight and SVL. The significant two-way interactions indicated that the size difference was smaller in females compared to males (Table 3). No differences in mean age and growth rate were observed (Table 3).

Fluctuating asymmetry

Trait-specific asymmetry values were significantly correlated for both the signed and the unsigned FA (Table 4). The correlations in the signed FA were strongest within arm and leg traits, respectively, indicating a common developmental pathway (Table 4). The hypothetical repeatabilities of the different traits ranged between 0.29 and 0.48. Using these values to transform the correlations in the unsigned FA into correlations in DI led to correlation coefficients between 0.40 and 0.99 (Table 4). However, these values should be interpreted with caution, because there were also correlations in the signed FA values (Table 4), indicating that these traits did not represent independent growth events.

The patterns in FA differed strongly between the size-corrected and uncorrected asymmetry values (Table 5). While the uncorrected asymmetry differed between males and females, with males being more asymmetrical, FA corrected for trait weight showed additional differences. Size-corrected FA was higher in acid locations, appeared to be negatively associated with age (slope $=-0.47$; SE $=0.18$) and differed among traits (Fig. 2; Table 5). The size-corrected degree of asymmetry was highest in the radio-ulna (all $P_{adj}<0.0001$ for pairwise comparisons with this bone), followed by the humerus, which differed significantly from both the femur ($t_{326}=2.78$, $P_{adj}=0.03$) and the tibio-fibula ($t_{306}=4.13$, $P_{adj}=0.0003$). The size-corrected FA levels did not differ between the latter two ($t_{304}=0.96$, $P_{adj}=0.77$).

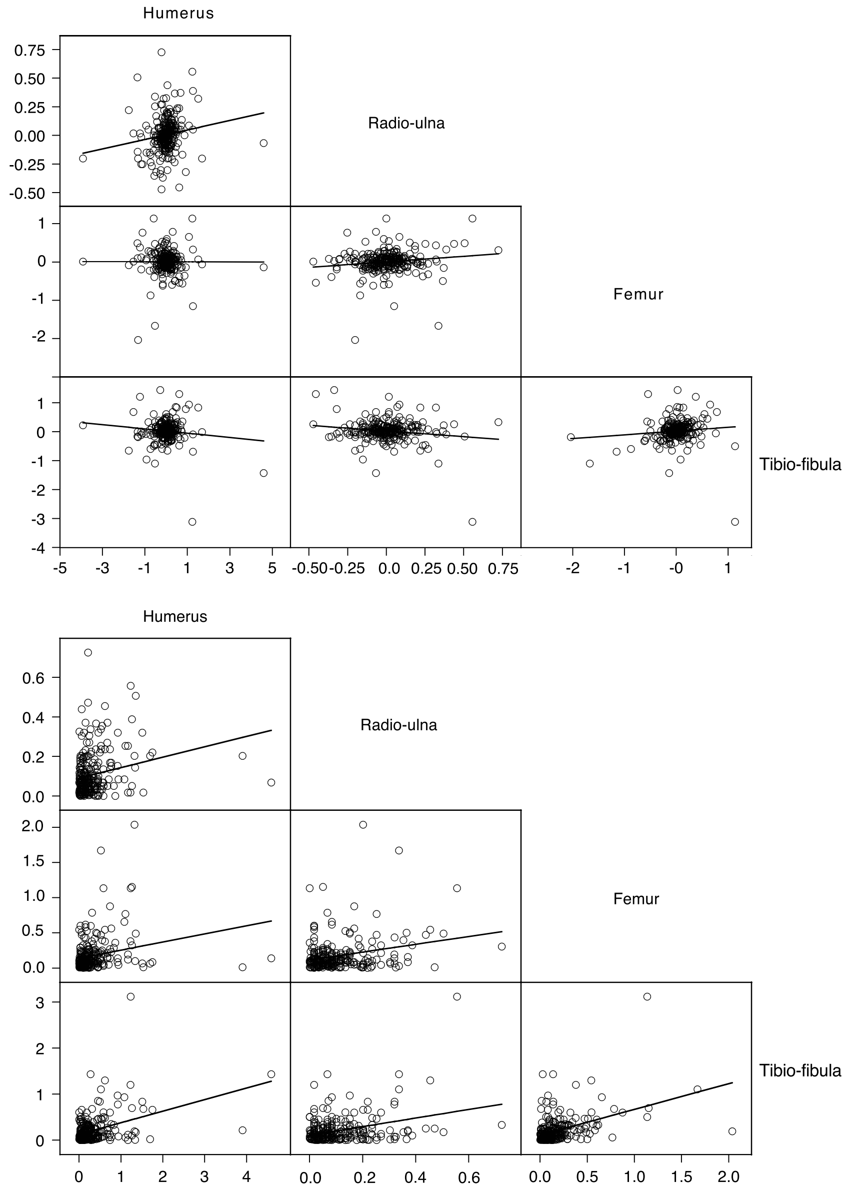

Fig. 1 Between-trait correlations in signed (*top panel*) and unsigned (*lower panel*) asymmetries. Total sample size equalled 237

Table 3 Overview of tests comparing morphology and population structure between acidified and non-acidified locations

Factor	Body weight	SVL	Growth rate	Age
Acidity	$F_{1,2}$=45.7*	$F_{1,196}$=132***	$F_{1,2}$=2.76	$F_{1,2}$=16.8
Sex	$F_{1,193}$=3.69	$F_{1,196}$=7.84**	$F_{1,2}$=0.66	$F_{1,3}$=2.46
Acid × sex	$F_{1,193}$=6.18**	$F_{1,196}$=5.92*	$F_{1,2}$=0.08	$F_{1,2}$=0.28

Location (nested within acidity) and sex × location were added as random factors such that standard errors and the number of degrees of freedom could be approximated appropriately using the Kenward–Roger method

*P<0.05, **P<0.01, ***P<0.001

Multivariate approach

Two canonical variables significantly differed among the four populations capturing 97% of the between-population variation (canonical variable 1: eigenvalue =1.02 (84%), $F_{21,509}$=8.77, P<0.0001; canonical variable 2: eigenvalue=0.16 (13%), $F_{12,356}$=2.84, P=0.001; canonical variable 3: eigenvalue =0.03 (3%), $F_{5,179}$ =1.13, P=0.35). According to univariate analyses, significant differences between-populations were observed for SVL ($F_{3,183}$=38.8, P<0.0001), growth rate ($F_{3,183}$=3.20, P=0.03), age ($F_{3,183}$=11.5, P<0.0001), body weight ($F_{3,183}$=57.1, P<0.0001) and mean size-corrected asymmetry ($F_{3,183}$=5.80, P=0.001). There were no significant differences in mean uncorrected asymmetry ($F_{3,183}$=0.59, P=0.62). A plot of both the means for the two significant canonical variables and the correlation between the observed data and these two canonical variables showed that differences due to acidity are mainly described by the first canonical variable, while

Table 4 Between-trait correlations in signed and unsigned asymmetry above and below the diagonal, respectively

	R	Radio-ulna	Humerus	Tibio-fibula	Femur
Radio-ulna	0.29	–	**0.25*****	0.05	**0.12***
Humerus	0.47	**0.25***** (0.68)	–	0.07	−0.06
Tibio-fibula	0.48	**0.17**** (0.46)	**0.29***** (0.61)	–	**0.22*****
Femur	0.43	**0.14*** (0.40)	**0.31***** (0.69)	**0.45*******(0.99)**	–

The first column indicates the hypothetical repeatability (*R*) of the unsigned FA. Transformed correlations in the unsigned asymmetries are indicated within brackets. Total sample size *n*=237

Table 5 Significance tests of fixed effects of the repeated measures ANOVAs analysing asymmetry (both corrected and uncorrected for individual trait weight) in relation to acidity, age, growth rate and sex

Factor	Uncorrected asymmetry			Corrected asymmetry		
	Df	*F*	*P*	*Df*	*F*	*P*
Acidity	1,2	0.84	0.46	**1,184**	**16.5**	**0.0001**
Age	1,193	0.01	0.93	**1,184**	**6.79**	**0.01**
Growth rate	1,193	0.01	0.93	1,185	3.06	0.08
Sex	**1,324**	**94.6**	**0.0001**	**1,334**	**92.1**	**0.0001**
Trait	3,350	0.19	0.90	**3,350**	**43.3**	**0.0001**
Trait × acidity	3,345	0.87	0.46	3,345	0.83	0.48
Trait × age	3,347	0.38	0.77	3,346	0.56	0.64
Trait × growth rate	3,344	0.73	0.54	3,343	1.09	0.35
Trait × sex	3,373	1.92	0.13	3,370	1.90	0.13
Acidity × sex	3,343	1.37	0.22	3,344	1.31	0.25

df degrees of freedom

other geographical effects are captured by the second one (Fig. 3). More specifically, populations 'Lilla Brödhållartjärn' and 'Lindrågen' differ in acidity, but are located very close to each other geographically. They also appear to have similar values for the second canonical variable. Given the high eigenvalue of the first canonical variable, this shows that most of the observed morphological differences are due to the acidity. The first canonical variable showed the highest correlation with body weigh, SVL and mean size-corrected FA. Thus, these three morphological characteristics appear to be influenced by acidity, where more acid environments lead to smaller, lighter and more asymmetric frogs (Fig. 3). The geographical variation appeared to be mainly determined by differences in age and growth rate (Fig. 3).

Directional asymmetry and antisymmetry

In order to compare levels of directional asymmetry between acidified and non-acidified lakes, we tested for the significance of the side × ACID interaction in the mixed regression model used to separate real asymmetry from measurement error and to obtain the signed FA values corrected for DA. Location (nested within the indicator variable ACID) and its interaction with side were added as random effect to avoid problems of pseudo replication, and degrees of freedom were approximated using the Kenward Rogers method (see also above). The side × ACID interaction was not statistically significant in any trait after correction for multiple testing (femur $F_{1,2}$=0.52, *P*=0.54; tibio-fibula $F_{1,229}$=5.94, *P*=0.015; humerus $F_{1,2}$=0.14, *P*=0.75; radio-ulna $F_{1,2}$=0.07, *P*=0.82). There were indications of a difference in the degree of DA between acidified and non-acidified lakes for the tibio-fibula, yet the difference was opposite to the expectation. Parameter estimates showed that the degree of DA was on average larger in the control areas for this trait. None of the traits showed negative kurtosis values, indicating that there was no antisymmetry.

Discussion

Our results corroborate the earlier studies indicating that low pH constitutes a strong environmental stressor that influences development and growth of amphibian embryos and tadpoles (e.g., Pierce 1985; Böhmer and Rahmann 1990; Räsänen et al. 2003a, b). More importantly, our results indicate that the stress effects induced by low pH are also visible at later (adult) life stages once the individuals have changed from aquatic (larvae) to mostly terrestrial (adults) life. These effects were very clear in both trait means and FA, suggesting that FA per se can be a useful indicator of the degree of environmental stress experienced by amphibians. Increased FA among individuals exposed to acid stress is known from fishes (Jagoe and Haines 1985; Vøllestad and Hindar 2001), but the results of this study

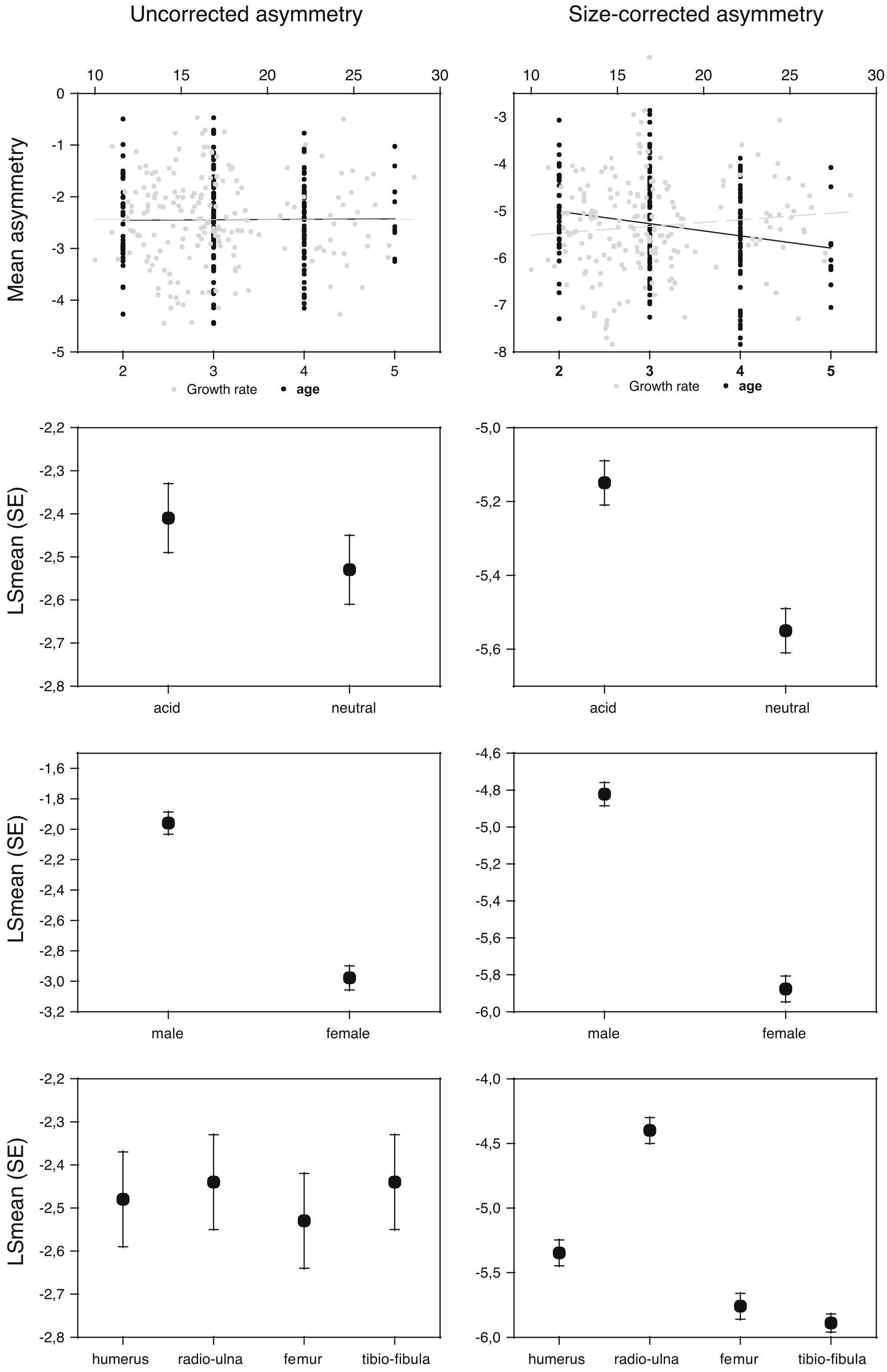

Fig. 2 Overview of patterns in unsigned asymmetry both uncorrected (*left*) and corrected for trait size. See text and Table 4 for details

provide the first test in amphibians. Given the alarming evidence for a global decline of amphibian populations (Alford and Richards 1999; Stuart et al. 2004), our results suggest that FA could provide a useful metric for identifying populations under stress when direct population-size estimates are difficult or impossible to obtain. Indeed, we did not provide any evidence for an association between FA and fitness apart from the decreasing levels of FA with age that suggest that the more symmetric individuals are more likely to grow old. We know that acidity has an important impact on fitness-related components in the earlier developmental

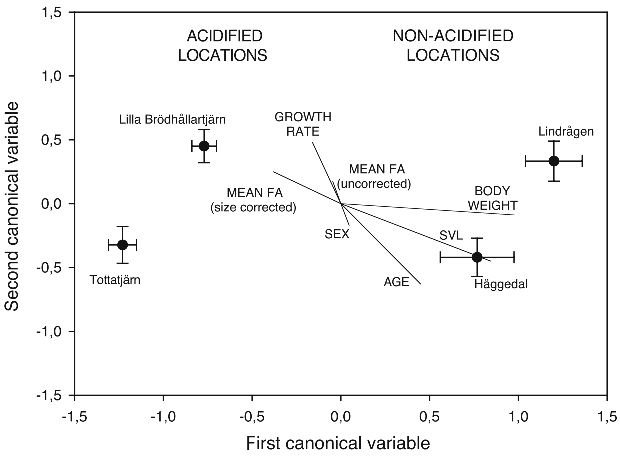

Fig. 3 Graphical summary of multivariate analysis of differences in morphology, age and age structure in the four localities. Mean and standard errors of the first two canonical variables are presented as *symbols*. The degree of correlation between the different variables and these two canonical variables are indicated by vectors

stages (Räsänen et al. 2003a, b; Merilä et al. 2004). What would be of interest, yet impossible to obtain in practice, are long-term data on fitness, size and FA in localities that do and do not increase in their degree of acidification. This would allow explicit testing of the sensitivity of FA as an early warning system and the-evaluation of the possibilities for the evolution of stress resistance. Nevertheless, we do note the need for experimental work that would allow one to pin down the actual mechanism of the effects of acidy on development in the lab.

The four most significant findings of this study with respect to asymmetry where that (1) the degree of size-corrected FA was significantly higher in frog populations living under acid-stress than in those living in non-acidified conditions, (2) that the degree of FA was significantly higher in males than in females, that (3) the patterns were consistent among traits and sexes, and (4) there were no differences in directional asymmetry between acidified and non-acidified lakes. Hence, the results of this study provide interesting insights into the utility of FA as an indicator of environmental stress. It is well known that low pH interferes with calcium metabolism, and in particular, reduces calcium uptake (Packard et al. 1996; Tilgar et al. 2004; Alexander 2002). Since the development of bone structures used in the estimation FA in this study are likely to be sensitive to problems in calcium metabolism and uptake, this provides a possible explanation for increased levels of FA in acid-origin moor frog populations.

In explosively breeding frogs, including the moor frog, males gather in large choruses where females arrive to mate. There is a harsh competition among males for mating, and they use their arms to clasp females. There is evidence both from this (Hedengren 1987; Berglind 1994) and other anuran species (Höglund and Säterberg 1989; Elmberg 1991; Berven 1981; Howard 1981) that arm length is a factor inpositive intrasexual selection: males with longer and stronger arms are better able to monopolize females they have clasped. Hence, the higher degree of FA in the radio-ulna than in other traits could be understood in terms of its poorer developmental buffering attributable to the history of directional selection (Møller and Pomiankowski 1993). On the other hand, the femur and tibio-fibula are important elements in the anuran locomotion mechanism. Hence, these functionally important traits are likely to be under stabilizing natural selection, providing a possible explanation as to why their levels of FA were lower than that in the radio-ulna. Nevertheless, we did not find a significant difference in the effect of acidity on mean FA among the four traits, refuting the hypothesis that traits under directional selection would be more sensitive to stress.

We found that males exhibit a higher degree of FA than females. This has been observed also in a few earlier studies (Jolicoeur 1963; Karvonen et al. 2003), and two general explanations for this pattern have been put forth (Karvonen et al. 2003). First, as the males are usually the larger sex, they may be more prone to nutritional or energetic stress than females (Clutton-Brock et al. 1985; Sheldon et al. 1998). However, as male moor frogs are smaller than females (this study) and as the differences were significant for FA estimates with and without size correction, this explanation can be refuted. Second, the action of steroid hormones may interfere with normal development in males and make them more prone to developmental instability than females (Folstad and Karter 1992; Sheldon et al. 1998). Whatever the proximate explanation, the higher degree of FA in the smaller sex suggests that the sex difference in FA is indeed related to sex per se, rather than to size and to degree of sexual size dimorphism. Therefore, being a male frog appears to be 'stressful' in terms of arm and leg development. However, this presumed type of stress did not appear to interact with the effect of acidity since we found no indications of an acidity-by-sex interaction.

As in a few earlier studies (e.g., Moran et al. 1997; but see Karvonen et al. 2003), we found that the degree of FA decreased with age. This is to be expected if selection prunes out individuals with a high degree of FA. Whether this could be explained by a direct or indirect link between FA and survival probability cannot be answered with the current data. At any rate, FA seems to indicate individual survival to older ages in moor frogs. In addition, the hypothetical repeatabilities for the different traits as well as the between-trait correlations in the unsigned FA were relatively high (even for trait pairs that did not show correlations in the signed FA and thus shared their developmental pathway). These findings suggest that DI is organism wide and supports the presence of an individual asymmetry parameter, and also indicate a high heterogeneity in DI among individuals, both prerequisites for the use of FA at the individual level (Van Dongen 2006).

Our inferences on FA depended strongly on whether size-corrected or size-uncorrected measures were used. FA uncorrected for size differed only between males and females, whereas the size-corrected FA differed also between acid and non-acid populations, as well as between-individuals of different age. These results highlight the point that failure to control for various sources of heterogeneity in FA measurements can lead to erroneous acceptance of the null hypothesis that environmental stress causes no differences in developmental instability. For instance, using uncorrected FA, we did not find any significant differences in either level of FA among neutral and acidified populations or among different traits. Once the size differences were accounted for, the inference suggested otherwise. Such patterns are not really surprising given the fact that frogs differed in size among localities and sexes and that different traits were of different size. Especially under those circumstances, size corrections are preferable. We also favor the use of repeated measures analyses of single-trait asymmetry values instead of composite FA indices. We were able to explicitly incorporate interaction terms in our statistical models, hereby enabling us to explicitly compare patterns across traits and sexes.

We found significant DA in three of the five traits included in this study. Since the asymmetry was quantified by weighing, the observed DA cannot be explained by measurement bias, but must represent true DA. DA is known from frogs and other vertebrates (Blum et al. 1999) and has been sometimes explained as a consequence of handedness (Dill 1977). For instance, several studies have shown a consistent left or right preference in the use of forelimbs in frogs (Malashichev and Nikitina 2002; Bisazza et al. 1997; Borkhvardt and Ivashintsova 1994). The DA found in humerus and tibio fibula in this study could be explained by this handedness. Dill (1977) also showed a leftward jumping preference ability, which was not related to limb size or symmetry. Our study showed DA in the ilium. The ilium in frogs is an important element in the caudiopelvic mechanism, which is unique for anurans and provides their jumping ability (Emerson 1979; Jenkins and Shubin 1998). The DA found in ilium could be an explanation for frogs' sideward jumping. Whatever the proximate explanation for the occurrence of DA in skeletal traits, our results suggest that the reason is biological, rather than methodological, as earlier pointed out by Karvonen et al. (2003). Most importantly, however, the statistical correction for the observed DA appeared to appropriately remove possible confounding by it, since patterns were comparable among traits.

Finally, although we have interpreted the results as being caused by acidity-induced stress, it is worth pointing out that the observed effects could be also caused by reduced food availability in acidified environments. Acidified waters are usually less productive and have often higher concentrations of dissolved humic substances. Because tadpoles are largely herbivorous, any characteristic of a pond that hinders growth of attached algae (such as clarity and nutrients) is likely to have a negative influence on the growth of tadpoles. Therefore, the observed effects of acidity on size and FA could be caused either by acidity per se or its indrect effects on nutrition.

In conclusion, the results of this study support the idea that FA could be a useful tool in identifying amphibian populations and individuals subject to environmental stress. The urgency of developing reliable tools for the estimation of environmental stress in amphibians has never been as acute as now (Stuart et al. 2004). In particular, acidification has been a considerable environmental problem in Northern Europe during the past decades, affecting both aquatic and terrestrial environments, and albeit environmental recovery is in progress (Hasselrot and Hultberg 1984; Lindmark 1984), the results here show that the problems still exist.

Acknowledgments We thank Anssi Laurila, Katja Räsänen and all other people who have helped us in the course of this work. In particular, thanks are due to Peter Mortensen and Jorma Uusitalo at the Swedish Natural History Museum for help in preparation of the skeletons and to Magnus Svensson and Mattias Sterner for doing all the measurements and preparations. The collection was performed with a licence from the Swedish Environmental Protection agency (409-1106-11). Our research was supported by the Swedish Natural Science Research Council, Swedish Forest and Agricultural Science Research Council, Oscar and Lili Lamm Foundation, Nils von Hoffsten Foundation, University of Helsinki Science Foundation and the Academy of Finland.

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