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Acid stress increases pelvic spine asymmetry in juvenile three-spined sticklebacks

D. Mazzi* and T. C. M. Bakker†

Institute of Zoology, University of Bern, Wohlenstrasse 50a, CH-3032 Hinterkappelen, Switzerland

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In the evaluation of the effect of acidic water on the symmetrical development of the pelvic spines in juvenile three-spined sticklebacks *Gasterosteus aculeatus*, directional rather than fluctuating asymmetry was found, with left spines being, on average, longer than the right. Fish that were exposed for a period of *c.* 2 months to acidic pH levels grew significantly more asymmetrical pelvic spines than their control full-siblings held in unmanipulated water. Average spine size was not affected by the experimental treatment.

Key words: directional asymmetry; environmental stress; fluctuating asymmetry; *Gasterosteus aculeatus*; pH.

INTRODUCTION

Developmental stability refers to the ability of an organism to withstand genetic and environmental perturbations encountered during development, so as to produce an *a priori* defined optimum phenotype (Zakharov, 1989). Fluctuating asymmetry (FA), the most commonly used measure of developmental stability, is defined as small random deviations from bilateral symmetry in a morphological character for which differences between the right and left sides are normally distributed around a mean of zero in a population (Van Valen, 1962; Palmer & Strobeck, 1986). The impairing effects of environmental (e.g. temperature and pollutants) and genetic (e.g. inbreeding and chromosomal anomalies) stresses on developmental stability have been established in a wide range of organisms (Parsons, 1990; Møller & Swaddle, 1997). Environmental stresses are factors which, when present in shortage or in excess, are likely to lower the fitness of organisms. Several such environmental agents are known to induce substantial alterations of morphological characters in fishes (Tåning, 1952), one of them being water acidity. Reduced pH (i.e. increased acidity) has severe effects on fishes, ranging from physiological disorders to the impoverishment or even the extinction of entire populations (Witters, 1998). In spite of the growing impact of anthropogenic acidification of ground and surface waters, only a few studies have investigated the relationship between FA and depressed pH levels in fishes. FA was found to be higher in centrarchid fish (*Micropterus* and *Lepomis*)

*Author to whom correspondence should be addressed. Tel.: +41 31 6319158; fax: +41 31 6319141; email: dominique.mazzi@esh.unibe.ch

†Present address: Institut für Evolutionsbiologie und Ökologie, University of Bonn, An der Immenburg 1, D-53121 Bonn, Germany.

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collected from a naturally acidic pond heavily contaminated by mercury than in samples from several heated, heated and polluted, and unheated locations (Ames *et al.*, 1979). Jagoe & Haines' (1985) comparison of FA levels in white sucker *Catostomus commersoni* (Lacépède), lake chub *Couesis plumbeus* (Agassiz) and brook trout *Salvelinus fontinalis* (Mitchill) inhabiting acidified and unacidified lakes of north-eastern U.S.A. revealed that, although a few anatomical characters in each species were more asymmetric in acidified lakes, asymmetries of most characters were unrelated to lake pH. Wiener & Rago (1987) report that three bilateral meristic characters in bluegills *Lepomis macrochirus* (Rafinesque) from 11 clear-water lakes in northern Wisconsin were either unrelated or only weakly related to pH. Given the weak associations between asymmetry in single morphological traits, or composites thereof, and water pH, these studies point to the conclusion that FA may not be a suitable indicator of pH-related stress. Yet, the available evidence for the postulated link between environmental stress by acidification and increased FA stems from correlational approaches, and may thus be confounded by uncontrolled factors. In order to infer causality, stresses must be applied in a controlled and systematic way. In the present study the effect of a 2 months exposure to acidic water on the development of pelvic spines in juvenile three-spined sticklebacks *Gasterosteus aculeatus* L. was experimentally investigated to test the prediction that fish exposed to acid stress would exhibit elevated levels of asymmetry.

MATERIALS AND METHODS

EXPERIMENTAL SUBJECTS AND EXPERIMENTAL CONDITIONS

The experimental subjects were laboratory-bred offspring of fish from a large anadromous Dutch population, obtained by mating sexually mature wild fish (collected during the 1998 spring migration on the island of Texel, The Netherlands) at random. Egg clutches were removed from the male's nest 1 h after fertilization and placed in aerated plastic beakers filled with water of 17° C from a well (*c.* pH 8) replenished twice a day. Following hatching, in May 1998, each full-sib group was distributed evenly between two randomly assigned 10 l aquaria under summer conditions (16L : 8D, water from a well at 16–19° C, *c.* pH 8). Group densities were reduced and equalized at regular intervals by indiscriminately removing a given number of the fish with a small net, in order to standardize the effects of social interactions during rearing. Fry were fed for the first 3 days on infusoria cultures, then switched to a diet of live *Artemia* nauplii, and later of frozen *Artemia* and chironomid larvae, and live *Tubifex* worms. Fish were fed daily to satiation.

From each of 17 full-sib groups, 14 randomly chosen young three-spined sticklebacks were used for the experiment. Fish of a given sib group came from one of the two rearing tanks. At the beginning of the experiment fish were 14 weeks of age. Half the number of fish from each sib group were randomly assigned to a low-pH experimental group, while the other half served as a control group. The paired groups of seven fish each were placed in small aerated plastic aquaria (11 × 18 cm, 9 cm water level). Grey opaque partitions prevented visual interactions between neighbouring tanks. The tanks were illuminated for 16 h day^{-1} by an 18 W fluorescent tube, mounted 16 cm above the water surface. The tanks were distributed in randomized blocks (Hurlbert, 1984), whereby each block consisted of the two aquaria with paired sib group halves. Each treatment was randomly assigned to one tank in each block, so as to reduce the probability of chance segregation of treatments and eliminate pre-existing room-gradients (Hurlbert, 1984). For 7 weeks, twice a week (every 3 or 4 days) water pH in the experimental tanks was reduced to a value of 6·0 by carefully adding 10–12 droplets of hydrochloric acid (technical HCl, 32%

concentrated) to the water, while gently stirring with a plastic stick. The control groups were handled in an identical fashion, except that water from a well (*c*. pH 8) was added instead of hydrochloric acid. Once a week (before every second treatment) about two thirds of the water in each tank was exchanged and food left over and faeces were removed. During the experiment, fish were fed daily *ad libitum* with frozen chironomid larvae.

Shortly before every treatment, the pH of the water was measured with a temperature-compensated pH-meter (ORION 210A, relative accuracy ± 0·02 units) and the water temperature (accuracy ± 0·1° C) in the experimental and control tanks. The water pH was recorded again immediately after each treatment. The pH value measured shortly after the treatments was always exactly 6·0 in experimental groups and between 8·0 and 8·2 (median 8·1) in control groups. The mean pH value measured shortly before each treatment ranged from 7·3 to 7·6 (median 7·5) in experimental groups and from 8·1 to 8·3 (median 8·2) in control groups. The difference in pH values between the experimental and control groups was highly significant both before and after the treatments (Wilcoxon signed-rank test, $n=17$, $T=76{\cdot}5$, $P<0{\cdot}0001$ in both cases), as was the difference before and after the treatments within groups (Wilcoxon signed-rank test, $n=17$, $T=76{\cdot}5$, $P<0{\cdot}0001$ and $n=17$, $T=52{\cdot}5$, $P<0{\cdot}0001$, for experimental and control groups, respectively). The mean temperature recorded shortly before the treatments ranged from 14·1 to 16·3° C in experimental groups (median 15·0° C), and from 14·2 to 16.3° C (median 15·0° C) in control groups. The difference in temperature between the experimental and control groups was not statistically significant (Wilcoxon signed-rank test, $n=17$, $T=8{\cdot}0$, $P>0{\cdot}6$).

The reduction of the water pH to a value of 6 is within the range of natural variation, as three-spined sticklebacks are known to inhabit waters with a pH as low as 3·5 (Giles, 1983). Just after the acid was dropped into the water the fish often tended to rise towards the water surface, but they resumed normal behaviour within a few seconds. During the test procedure 13 fish died overall, nine belonging to four different experimental groups, and four belonging to four different control groups. The mortality did not differ significantly between the experimental and control groups (Wilcoxon signed-rank test, $n=17$, $T=3{\cdot}5$, $P=0{\cdot}5$). In order to prevent the confounding effect of different growth conditions due to different densities, only groups in which no mortality occurred ($n=11$) were considered for analyses.

Before the start and after the end of the experiment the fish were deprived of food for 1 day and their standard length (L_S) and body mass (W) were measured to calculate their condition factor (K), $K=100W\,L_S^{-2{\cdot}65}$ where the exponent is the slope of the regression of log(W) on log(L_S) at the start of the experiment (Bolger & Connolly, 1989).

At the end of the experiment fish were killed in 0·1% 2-phenoxy-ethanol. The pelvic girdle, with the attached spines, was removed from the body and dried in air for several days.

TRAIT SIZE AND ASYMMETRY

The length of the pelvic spine on left and right sides was measured with a digital calliper to the nearest 0·01 mm. Samples were coded with respect to both family group and treatment. Owing to the random and independent nature of measurement error, and since FAs are usually very small relative to trait size (Møller & Pomiankowski, 1993), within-subjects repeats are necessary to estimate the contribution of measurement error to the variation in asymmetry data (Palmer & Strobeck, 1986; Swaddle *et al*., 1994). All spines were measured twice, the first spine to be measured being randomly determined. The callipers were adjusted to the spine (from the basis at the anterior end of the pelvic plate to the tip) by eye with the display facing away from the investigator. The callipers were set to zero before every measurement. For analysis, each spine's mean length was computed from its two measurements, which halves the measurement error variance (David *et al*., 1999). A possible relationship between the magnitude of FA and average character size was tested for by regressing the residual absolute asymmetry on the residual average trait size (both corrected for sib group effects). As no evidence for size-dependence was found ($r^2=0{\cdot}005$, $n=71$, $F_{1,69}=0{\cdot}36$, $P>0{\cdot}5$ and $r^2=0{\cdot}005$, $n=69$,

$F_{1,67}=0{\cdot}35$, $P>0{\cdot}5$, for experimental and control groups, respectively), asymmetry was not scaled by trait size (Palmer, 1994). Ten fish (four belonging to four different experimental groups and six belonging to five different control groups) with damaged spines, for unknown reasons, and four that were damaged by the investigator during handling (two out of two different experimental groups and two out of one control group) were excluded from the analyses. Therefore, a total of 71 experimental fish and 69 control fish (11 groups each) were used in the final analysis.

STATISTICAL ANALYSIS

Asymmetry was analysed following an approach modified from Leamy (1984) and recommended by Palmer & Strobeck (1986). A two-way, mixed-model analysis of variance was performed for the repeated measurements of each side, with the factors entered as side (left or right; fixed) and individual (random). The interaction term in this analysis provides information about the magnitude of measurement error relative to true asymmetry, while the side term allows the detection of directional asymmetry (i.e. the trait value on one side is consistently larger than its counterpart). The distributions of the signed asymmetry were checked for departures from normality, indicating antisymmetry (i.e. asymmetry is the norm but the side with the larger trait value varies). The experimental and control groups were tested separately.

The effect of the experimental treatment on the response variables was analysed using two-way ANOVA models, with sib group (random) and treatment (fixed) as factors. Whether the recorded variables met the assumptions of parametric statistics was checked by using the Shapiro–Wilk W test to check normality and Bartlett's test to check variance homogeneity. Given P-values are two-tailed throughout. Analyses were performed using JMP IN (Sall & Lehmann, 1996) statistical package.

RESULTS

TYPE OF ASYMMETRY AND MEASUREMENT ERROR

As demonstrated by the significance of the side term in the mixed-model ANOVA (Table I), both experimental and control groups exhibited a significant degree of directional asymmetry, having, on average, longer left spines than right. Normality of the signed asymmetry distributions indicated no antisymmetry ($n=71$, $W=0{\cdot}97$, $P=0{\cdot}28$ and $n=69$, $W=0{\cdot}98$, $P=0{\cdot}55$, for experimental and control groups, respectively). The between-individual variation in asymmetry was far greater than measurement error, as indicated by a highly significant individual × side interaction (Table I).

TRAIT SIZE AND TRAIT ASYMMETRY

Fish that were exposed to low pH levels grew significantly more asymmetrical pelvic spines than their control counterparts reared in unmanipulated water (two-way ANOVA, $F_{10,128}=0{\cdot}85$, $P=0{\cdot}58$ for the effect of sib group, $F_{1,128}=11{\cdot}85$, $P=0{\cdot}0008$ for the effect of treatment; Fig. 1). In contrast, no significant difference was found in average (left and right) spine length per individual between fish in the acid treatment and their control sibs, while controlling for between-group differences (two-way ANOVA, $F_{10,128}=9{\cdot}51$, $P<0{\cdot}0001$ for the effect of sib group, $F_{1,128}=2{\cdot}48$, $P=0{\cdot}12$ for the effect of treatment).

PHYSICAL CONDITION

At the beginning of the experiment sib groups significantly differed in their physical condition, for genetical and or environmental reasons. However, paired

TABLE I. Type of asymmetry and reliability of asymmetry measurements

Treatment	Individual				Side				Individual × Side			
	MS	d.f.	*F*	*P*	MS	d.f.	*F*	*P*	MS	d.f.	*F*	*P*
Control	0·551	68,138	755·192	<0·0001	0·025	1,138	34·361	<0·0001	0·008	68,138	11·098	<0·0001
Treatment	0·656	70,142	1369·596	<0·0001	0·185	1,142	385·424	<0·0001	0·014	70,142	29·707	<0·0001

Significance of the 'Individual' term indicates that the differences between individuals are larger than the measurement error. Significance of the 'Side' term indicates the presence of directional asymmetry. Significance of the 'Individual × Side' term indicates that the asymmetry is larger than the measurement error.

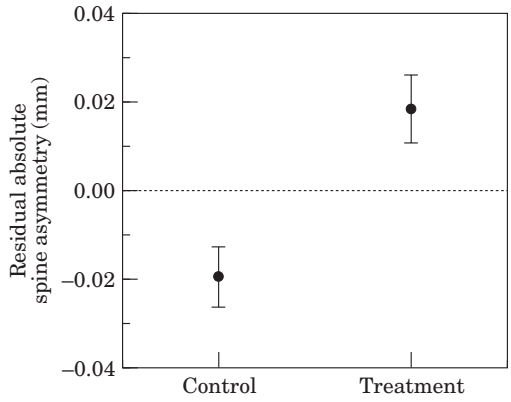

FIG. 1. Mean (± S.E.) absolute pelvic spine asymmetry (corrected for family effects by taking the residuals of an ANOVA on absolute asymmetry values, with sib-group entered as a factor) for unmanipulated water control groups and low pH treatment groups.

sibs assigned to the experimental and control groups did not differ significantly in their condition (two-way ANOVA, $F_{10,142}=5{\cdot}04$, $P<0{\cdot}0001$ for the effect of sib group, $F_{1,142}=1{\cdot}49$, $P=0{\cdot}22$ for the effect of treatment). At the end of the experiment sib groups still differed significantly in their condition, while fish assigned to experimental and control groups did not (two-way ANOVA, $F_{10,142}=8{\cdot}62$, $P<0{\cdot}0001$ for the effect of sib group, $F_{1,142}=1{\cdot}00$, $P=0{\cdot}32$ for the effect of treatment). Neither changes in condition factor, growth length, nor mass gain were significantly affected by the experimental treatment, while controlling for sib group effects (all $P>0{\cdot}8$).

SENSITIVITY TO ENVIRONMENTAL STRESS

In order to control for between-group differences in spine symmetry, a measure of stress-sensitivity was computed by subtracting from each individual's asymmetry value in the experimental groups, the mean asymmetry value for the corresponding control group. The magnitude of this difference gives an indication of the group-specific capability of buffering the harmful effect of developmental perturbations. Sib groups significantly differed in their susceptibility to stressful conditions (one-way ANOVA, $F_{10,61}=2{\cdot}13$, $P=0{\cdot}036$), thus suggesting genetic variation in the magnitude of the stress response.

DISCUSSION

Juvenile three-spined sticklebacks that were reared for a period of 7 weeks in acidic water grew significantly more asymmetrical pelvic spines than their control siblings held in unmanipulated water. In contrast, the mean of left and right spine length was not significantly affected by the experimental treatment.

The pH that experimental groups were subjected to varied more than that of control groups. The present study does not, therefore, disentangle the effect of low pH and that of periodic changes of pH. Low pH and or pH fluctuations may interact with the metabolism of calcium (McDonald *et al.*, 1983), which in turn affects pelvic structure in sticklebacks (Giles, 1983). Although the overall

investment into spine growth was not significantly affected by the experimental treatment, the control over the redistribution of resources between body sides appears more seriously impaired in fish exposed to acid stress.

Many previous studies have failed to test for the type of asymmetry that is expressed (Rowe *et al.*, 1997). In published datasets, the assumptions of FA are often unwarranted and characters may, rather, exhibit directional asymmetry or antisymmetry (Rowe *et al.*, 1997). Although spine asymmetry in the present study was directional rather than fluctuating, asymmetry was analysed with regard to the sensitivity to the applied environmental stress. Directional asymmetry (DA) in pelvic spines may impair balance, swimming performance, or their functionality as an effective defence mechanism, and thus ought to be under strong selective pressure for symmetry. It cannot be conclusively ruled out that measurement error has led to a systematic overestimation of left spine lengths and or underestimation of right spine lengths. Yet, such a consistent error source would not explain why the experimental groups were more strongly affected than the control groups, given that the investigator was ignorant with respect to treatment at the time measurements were taken.

DA regularly occurs in the gonads of birds and other vertebrates (Møller, 1994) and various other vertebrate characters (Alvarez, 1995). Left-biased directionality of pelvic girdle expression is known to occur in three-spined sticklebacks, usually in association with pelvic reduction (Bell *et al.*, 1985; Reimchen, 1997). Kraak (1997) argued that DA might be more widespread than hitherto suspected, but often overlooked. While FA has been widely accepted as a measure of developmental stability, owing to its presumed exclusive environmental basis, the use of DA has not been recommended because of its assumed genetic component (Leary & Allendorf, 1989). Although many studies report low heritabilities of FA (Whitlock & Fowler, 1997), some estimates have been significantly different from zero (Scheiner *et al.*, 1991), and FA has been shown to respond to directional selection (Reeve, 1960), suggesting a heritable basis of FA. Møller & Thornhill's (1997) meta-analysis of published heritability estimates revealed that the overall mean effect size of heritabilities of individual FA significantly differed from zero, thus indicating a significant additive genetic component to developmental stability (but see also Leamy, 1997; Pomiankowski, 1997; Whitlock & Fowler, 1997). In contrast to FA, a heritable basis for DA has often been assumed and sometimes detected (Leamy *et al.*, 1997). However, the level of genetic variation for DA can be very low, and the observed phenotypic variation in DA overwhelmingly environmental in its origin (Leamy, 1999; Leamy *et al.*, 2000). In the light of these findings, and given that the significant genetic variation for FA in at least some characters has not precluded its use, it has been argued that DA as well could serve as a predictor of developmental stability (Graham *et al.*, 1993).

Besides environmental stress, genomic stress is hypothesized to affect developmental stability (Mitton & Grant, 1984). The underlying idea is that more heterozygous individuals are better at suppressing developmental accidents than relatively homozygous ones (Mitton, 1993). Numerous studies have reported an association between superior developmental stability and heterozygosity, both at the individual (Leary *et al.*, 1983, 1984, 1985) and at the population level (Soulé,

1979; Kat, 1982; Biémont, 1983; Mitton & Grant, 1984). It remains debatable, whether this is a consequence of heterozygous advantage, of the increased expression of deleterious alleles in less heterozygous individuals, or of a combination of both (Mitton & Grant, 1984). In spite of the relatively small sample size, the reported between-group differences in stress-sensitivity suggest that some genotypes were better than others at buffering themselves against the disruptive effect of depressed pH. These differences can hardly be ascribed to differences in heterozygosity, since the experimental subjects were first-generation, laboratory-reared, outbred offspring of individuals from a large and genetically diverse population (unpubl. data). However, it is conceivable that the observed between-group differences in stress-sensitivity have a genetic basis, if, for example, particular genetic backgrounds offer an advantage in terms of metabolic requirements needed to counteract the detrimental effect of developmental ' noise '.

Owing to the small dimensions of juvenile three-spined sticklebacks *c.* 5 months of age, only pelvic spine length could be reliably measured. It has been argued that a single character may not provide a good indicator for the magnitude of stress imposed on an individual's development, and that composite indices containing information from a number of characters would provide more accurate information (Leary & Allendorf, 1989). Correlations between different character FAs measured within the same individual are often weak, leading to the opinion that different traits are affected by different stresses or have different periods of vulnerability to developmental perturbations (Van Valen, 1962; Møller & Swaddle, 1997). In contrast, Gangestad & Thornhill (1999) proposed a generalized model of organism-wide asymmetry and stated that the individual differences affecting the asymmetry of any one character are largely shared across other traits in the organism rather than trait-specific. The above mentioned relationship between heterozygosity and developmental stability measured in terms of individual FA is consistent with a model of generalized asymmetry (Leary & Allendorf, 1989; Parsons, 1990). According to this situation, FA measures of a single character may be representative for any other trait and thus help tapping the underlying developmental imprecision.

Several authors have promoted the use of measures of developmental stability as a monitor of environmental stress (Clarke, 1995; Sommer, 1996), as they can provide more accurate information about the magnitude of disturbance than customary estimates of population size, survival or fecundity (Clarke, 1992). The present study suggests that morphological asymmetries could indeed serve as a so-called ' early warning system ' (Clarke, 1995), allowing remedial action to be undertaken before the onset of relevant environmental deterioration. In practice, however, the use of morphological asymmetries as a diagnostic tool in conservation biology will require, for example, that they are a proven surrogate measure of fitness, that damaged, injured or regenerating traits can be reliably discerned from intact traits, and that the most asymmetric individuals in a population do not suffer from selective mortality.

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