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SEPARATING DEVELOPMENTAL AND ENVIRONMENTAL EFFECTS ON FLUCTUATING ASYMMETRY IN *LYTHRUM SALICARIA* AND *PENTHORUM SEDOIDES*

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Environmental stress can disrupt developmental processes on biological, physiological, and chemical levels and thereby affect the symmetry of a trait. For this reason, fluctuating asymmetry is often proposed as a measure of stress encountered by an individual. One problem is that asymmetry may have multiple causes, including developmental noise and genetic background, and genetic differences may interact with any physiological stress imposed by the environment. The main objective of this research was to determine whether developmental noise and genetic stress can be separated from environmental effects on leaf asymmetry. The experiments were conducted on two wetland plants, *Lythrum salicaria* (purple loosestrife) and *Penthorum sedoides* (ditch stonecrop). Replicates of different genotypes were measured when young and after they matured, with the latter group grown under two nutrient treatments. The largest and healthiest leaf of each plant was measured for length, width, and differences in width between the left and right sides (measuring from the central vein at the widest point). Nutrient enrichment increased leaf asymmetry, while age reduced asymmetry in *L. salicaria*. However, leaf asymmetry changed only as a consequence of development in *P. sedoides* and decreased. Genotype did not affect asymmetry in either species.

Keywords: genetic variation, leaf asymmetry, nutrient stress, ontogeny, size, wetland plants.

Introduction

Developmental stability is the ability of an organism under varying environmental conditions and genetic backgrounds to develop an "ideal" form (Schmalhauzen 1949; Zakharov 1992). Developmental stability involves a collection of processes that buffer disruptions that would affect symmetry (Palmer 1994). These disruptions can take place on a cellular, physiological, or biochemical level (Palmer 1996; Polak 2003). Both genetic and environmental perturbations cause variation in symmetry. Different genotypes within a species or identical genotypes that face differing environmental conditions may vary in developmental stability (Møller and Swaddle 1997). The most commonly used index of developmental stability is fluctuating asymmetry (FA; Møller and Swaddle 1997). This asymmetry in bilaterally symmetrical organisms is usually described as distributions in measurements of right side minus left ($R - L$).

An important assumption in studies involving FA as a measure of developmental stability is that the subtle asymmetries should not have a heritable basis (Leamy 2003). If variation in FA among individuals possesses a genetic and an environmental component, then FA might not be a predictable measure of developmental stability and therefore of environmental stress (Palmer 1994). There has been considerable debate on the significance of a genetic contribution to the asymmetry of a phenotype, which ranges between two extremes. Møller and Thornhill (1997) found significant heritability estimates that favor the idea of a substantial genetic contribution, while Leamy (1997) regards the genetic component as negligible. Woods

et al. (1999) have shown that heritability may be trait specific. Most often, the assumption that FA does not have a genetic component has been made (Leamy 2003), but few studies have actually tested whether this assumption holds before analyzing environmental causes of FA. For this reason, an ideal study for testing the sensitivity of FA as an indicator of environmental stress should distinguish between genetic and environmental effects (Sinclair and Hoffmann 2003).

In addition to the unknown genetic contribution to FA, the process of development alone can be taxing on an organism. Rapid growth results in high energy expenditure; however, energy may be limited in early life stages of an organism. In young fish, growth consumes a large portion of the available energy (Pederson 1997), and little information is known as to the degree to which asymmetry changes because of age. Swaddle and Witter (1997) suggest six hypotheses to explain how ontogeny can affect FA: (1) directional external cues, in which asymmetries are biased to one side of the organism; (2) the coin-toss hypothesis, which suggests that morphogenesis is made of independent developmental units: as randomly assigned developmental units increase, asymmetry decreases; (3) magnification of asymmetry, in which random asymmetries early in development are magnified during growth and development; (4) accumulation of accidents, in which developmental noise accumulates over time: asymmetry increases with the time it takes to develop a trait; (5) persistent asymmetries, in which size and direction of asymmetry are determined early on and are not altered among growth stages; and (6) compensational growth, which implies a regulatory feedback between left and right sides that reduces asymmetry with age.

As an environmental stress, nutrient variation may also affect developmental stability (Lappalainen et al. 2000; Black-Samuelsson and Andersson 2003). Deficiencies in nutrients

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such as nitrogen, phosphorous, and potassium lead to slower growth, alterations in shoot and root biomass, and changes in leaf coloration and development (Bottrill et al. 1970; Sultan and Bazzaz 1993; Yeh et al. 2000). However, any deviation from normal nutrient availability, whether increase or decrease, may result in decreased developmental stability (Lappalainen et al. 2000). The total FA often measured in stressed environments comprises the additive or synergistic effects of FA due to age (development) and FA due to stress, whether environmental or genetic. To get a true measure of FA due to stress alone, measurements should also account for ontogenetic effects of FA when comparing performance in different environments (fig. 1). FA due to development alone makes the assumption that growth occurs under optimal conditions and that the organism is taxed in the process of growth.

The objective of this study is to partition the contributions of development, environment, and genotype to FA. Our test was run on two wetland plant species, *Lythrum salicaria* and *Penthorum sedoides*, under two nutrient conditions. We investigated the effect of development (age) on leaf length, width, and FA in the two species, in addition to assessing responses to variable nutrient conditions and the possible additive or synergistic effects of age and nutrient variation. A test for genetic contributions to FA was included by conducting these tests among clones of multiple genotypes in both *L. salicaria* and *P. sedoides*.

Material and Methods

Lythrum salicaria (purple loosestrife) is an invasive species nonnative to North America that originated in Eurasia and was recorded from Canada and New England as long ago as 1814 (Mal et al. 1992). It is a perennial, dicot shrub found in marshes, sedge meadows, bogs, riverbanks, and roadside ditches. *Penthorum sedoides* (ditch stonecrop) is a native species of the eastern and midwestern United States. It is a perennial, dicot herb found in stream banks, roadside ditches, and

other low, wet places. Therefore, each species can be found to cover a variety of habitats and is exposed to diverse stresses. The leaves of both are bilaterally symmetrical, allowing a straightforward means of measuring FA (fig. 2).

Thirty genotypes of each species were germinated in an indoor facility; each genotype was cloned to produce six identical plants that were grown indoors for 8–9 wk before being moved outdoors. These 360 plants were randomly numbered and placed in numerical order in an outdoor experimental area on the Cleveland State University campus (fig. 3). Six clones of each genotype were selected and prescribed one of two nutrient treatments. One treatment group received no nutrients, and the other group received the commercial fertilizer Miracle-Gro in the proportion of one tablespoon per gallon of water, with 375 mL of the fertilizer solution added to each plant per treatment. Fertilizer treatments began when the clones were 11 wk of age, and treatments were repeated every 2 wk, at ages 13, 15, and 17 wk.

At week 11, before nutrient treatment, the largest healthy leaf of each plant was sampled and pressed in a herbarium for later measurement. Ten weeks later, the largest healthy leaf from each plant was collected and pressed. The width of each sampled leaf was measured with digital calipers (Mitutoyo Absolute Digimatic CD-6"CS) at the widest point of the lamina. The widths of the left and right halves were measured from the midrib to the leaf margin, and the length of the lamina was also measured (fig. 2). All measurements were repeated for accuracy; the second set of measurements was taken on different dates and in a different order to reduce bias. The average of the two measurements was used in all analyses.

Statistical analysis. To determine the treatment and genetic effects on leaf morphology and specifically asymmetry, FA was calculated as $|\log L - \log R|$ to account for size differences in FA (Graham et al. 2003). The FA data were Box-Cox transformed using the equation $(|\log L - \log R| + 0.00005)^{0.33}$ (Freeman et al. 1999) because using the absolute value of $(L - R)$ results in a half-normal distribution (Freeman et al. 2003). Hereafter, the Box-Cox–transformed FA is referred to as BCFA.
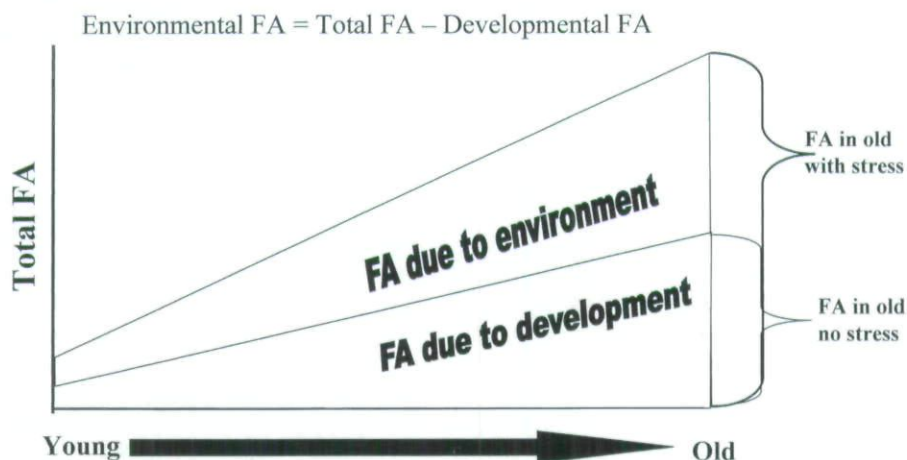

Fig. 1 Possible additive and synergistic effects of age and stress on fluctuating asymmetry (FA) of an organism. FA due to development alone assumes development under optimal conditions. FA due to environment refers to the increase in asymmetry due to additional environmental stress. Genetic background is assumed to be unimportant.
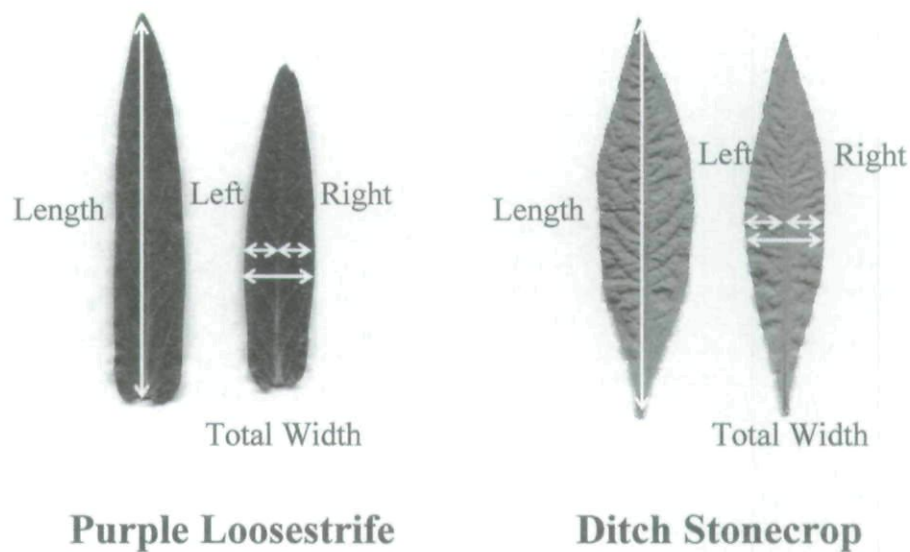
Fig. 2 Example of leaf measurements of length, width, and asymmetry in both purple loosestrife and ditch stonecrop.

For each species, two-way ANOVAs were run to test for the effects of genotype, treatment, and genotype by treatment interaction on leaf length, leaf width, and BCFA. Random effects (genotype) and the fixed (treatment) effect were tested against the genotype by treatment interaction mean square. To determine the effects of development (age), a one-sample *t*-test was run on the difference between old leaves and young leaves for length, width, and BCFA in both fertilized and unfertilized plants. To discern the nutrient effects on the three characters while accounting for effects of age, a two-sample

t-test was run on the differences between old and young leaves, grouping them by nutrient treatments. All statistical analyses were carried out using SYSTAT 8.0 (SPSS, Chicago).

Results

FA (measured as BCFA) did not have a genetic component in either *Lythrum salicaria* or *Penthorum sedoides*. Asymmetry levels were similar and not significantly different among
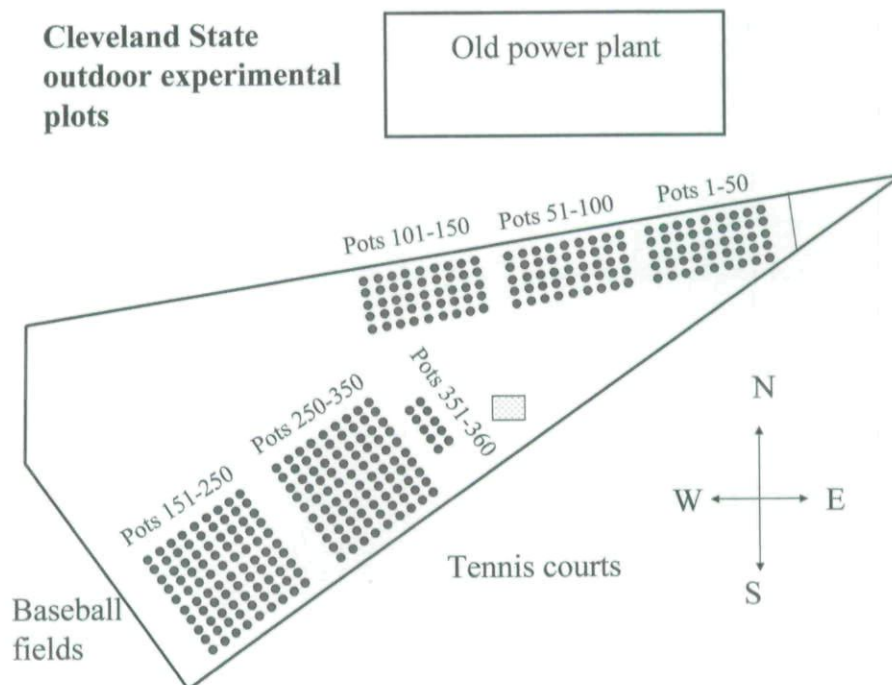

Fig. 3 Diagram of experimental plot area, demonstrating position of randomly numbered pots.

randomly chosen genotypes of both species under both nutrient conditions, producing no significant main effects. Furthermore, there were no significant interactions between genotype and treatment for this trait in either species. However, leaf size varied genetically. In *L. salicaria*, size of leaves combined across young leaves, old leaves without nutrient treatment, and old leaves with nutrient treatment differed significantly by genotype for leaf length ($F_{29, 58} = 3.08$, $P < 0.001$) and leaf width ($F_{29, 58} = 4.02$, $P < 0.001$). The interaction between genotype and treatment was also significant for both size traits ($F_{58, 260} = 2.1$, $P < 0.001$ for length; $F_{58, 260} = 1.7$, $P < 0.01$ for width). In contrast, size of leaves in *P. sedoides* did not differ significantly among genotypes as a main effect for length ($F_{29, 58} = 1.26$, not significant) or width ($F_{29, 58} = 1.21$, not significant), but there was interaction effect between genotype and treatment for width ($F_{58, 231} = 1.50$, $P < 0.05$).

Leaf size in both species was significantly affected by treatment. In *L. salicaria*, nutrient addition increased leaf length but not leaf width (table 1). In *P. sedoides*, both leaf length and leaf width increased because of nutrient treatment when age was taken into account (table 1).

In contrast to the lack of genotypic effects on FA, a significant effect of treatment occurred for BCFA in *L. salicaria* ($F_{2, 58} = 5.9$, $P < 0.01$) and *P. sedoides* ($F_{2, 58} = 8.4$, $P < 0.001$). BCFA in *L. salicaria* was significantly larger for young leaves than for older leaves without fertilizer. However, when fertilizer was added, the difference in asymmetry between young leaves and old leaves was no longer significant (table 1). BCFA in *P. sedoides* was also significantly larger in young leaves than in old leaves whether plants received added nutrients or not (table 1).

Discussion

Both growth and nutritional stress may affect leaf FA independent of genetic background. In *Lythrum salicaria*, nutrient enhancement appeared to be a contributor to FA as leaves matured, while age had an inverse effect. This development, however, was the main factor contributing to reduced FA in *Penthorum sedoides*, because the addition of fertilizer did not influence leaf asymmetry. In neither species did genetic background contribute to variation in asymmetry directly or as an interactive factor with age and nutrition.

Age

Age (survivorship/growth) has been directly related to developmental stability (Møller and Swaddle 1997), where the trend is for less asymmetry with age in animals (Naugler and Leech 1994; Ueno 1994; Swaddle and Witter 1997). The animals that survive may be those least affected, and analysis of adults may bias estimates of FA. By contrast, plants may be more tolerant of developmental errors and therefore express greater asymmetry in older leaves (Freeman et al. 1994). However, of the few studies that have investigated the relationship between age and developmental stability in plants, size-corrected leaf asymmetry decreased with age in leaves (Pélabon et al. 2006), which is in accord with the diminished FA we observed, except where *L. salicaria* was grown in a high-nutrient environment. These faster-growing, larger leaves expressed asymmetry levels similar to those of young leaves.

Two of Swaddle and Witter's (1997, p. 329) "nonmutually exclusive hypotheses" are the most likely candidates to explain the development of leaf FA: the compensational-growth hypothesis and the coin-toss hypothesis. During juvenile stages, under optimal growth conditions, plants have a faster growth rate that decreases with time (Koelewijn 2004). Rapid growth may influence developmental stability, and as a result, there may be trade-offs between growth and the plants' ability to correct errors in development (Pélabon et al. 2006). Because plant growth occurs by adding new cells to older ones and by cell expansion, asymmetries would accumulate with age if regulatory feedback mechanisms were not present (Freeman et al. 2003). A study conducted on *Cucurbita pepo* L. (pumpkin) concluded that leaves are subject to feedback mechanisms (Freeman et al. 2003). Covering half the leaf with foil to perturb growth resulted in leaf asymmetries, but these asymmetries gradually declined over time. Randomly assigned developmental units, as in the coin-toss hypothesis, seem less

Table 1

Fertilizer Treatment Effects on *Lythrum salicaria* and *Penthorum sedoides*

	ΔLength (mm)		ΔWidth (mm)		ΔBCFA	
	Mean ± 1 SE	P	Mean ± 1 SE	P	Mean ± 1 SE	P
L. salicaria :						
Δ (old − young), unfertilized	20.65 ± 2.53	<.0001	1.61 ± .48	<.01	−.038 ± .014	<.01
Δ (old − young), fertilized	33.57 ± 2.46	<.0001	2.14 ± .57	<.001	−.004 ± .015	ns
Comparison of Δ for fertilizer treatments	12.92 ± 3.55	<.001	.52 ± .74	ns	.035 ± .02	ns
P. sedoides :						
Δ (old − young), unfertilized	12.20 ± 3.04	<.001	7.49 ± .84	<.0001	−.047 ± .013	<.01
Δ (old − young), fertilized	24.52 ± 3.39	<.0001	11.52 ± .94	<.0001	−.027 ± .012	<.05
Comparison of Δ for fertilizer treatments	12.33 ± 4.86	<.05	4.04 ± 1.34	<.01	.021 ± .01	ns

Note.   Mean change in length, width, and BCFA (Box-Cox–transformed fluctuating asymmetry) between old and young plants and for the effect of fertilizer treatments, accounting for the effect of age, in *L. salicaria* and *P. sedoides*. Tests for significance of Δ (old − young) unfertilized and Δ (old − young) fertilized used one-sample *t*-tests. Comparison of Δ for fertilizer treatments used a two-sample *t*-test. ns = not significant.

likely to correct large asymmetries. The directional-external-cues hypothesis states that asymmetry will be consistently biased toward the same side of the organism, with no prediction as to the magnitude of asymmetry. We did not examine that bias in this experiment.

Nutritional Stress

The initial impetus for our study was the possibility of using FA as a measure of environmental stress. Variation in nutrient availability could lead to an increase in plant asymmetry either because of stresses on growth from insufficient nutrients or because of toxic levels of nutrients following enrichment. Studies on sweet flag (*Acornus calamus* L.) demonstrated that moderate increases in nitrogen positively affect growth, but, at high-nitrogen treatments, negative effects on growth presented themselves (Vojtíšková et al. 2004). Nutrient addition had a significant positive effect on leaf size in both *L. salicaria* and *P. sedoides* but did not significantly alter leaf asymmetry in older leaves of *P. sedoides* and had only a very weak effect on *L. salicaria* leaf asymmetry. In one of the previous studies on the effects of nutrients on leaf asymmetry, Black-Samuelsson and Andersson (2003) investigated FA in maple (*Acer plantanoides*) and birch (*Betula pendula*) in low- and high-nutrient treatments. In both trees, nutrient addition positively affected leaf size and resulted in increased mean absolute asymmetry. Nevertheless, when leaf asymmetry was adjusted to account for size differences, the effects of nutrients were no longer significant. In another study of varying nutrient enrichment, Lappalainen et al. (2000) found an increase in asymmetry after nutrient enrichment in birch. Correspondingly, Andalo et al. (2000) found greater asymmetry in birdfoot trefoil (*Lotus corniculatus*), a grassland species, raised in elevated nitrate and carbon dioxide. Our result for the wetland species *L. salicaria* agreed with those findings. It is possible that the rapid growth associated with nutrient enrichment reduces an organism's ability to correct subtle asymmetries.

Genetic Effects

The potential for a genetic basis of FA remains one of the most contentious issues surrounding the application of this character to studies of plant stress in nature (Leamy and Klingenberg 2005). The variation in leaf length and leaf width among clones of *L. salicaria* or *P. sedoides* clearly demonstrates that the experimental material was genetically variable; however, no genetic basis for FA was found under the growth conditions used. Sultan and Bazzaz (1993) also demonstrated that genotypes of different populations of *Polygonum persicaria* react similarly along a nutrient gradient for leaf area and mean leaf size. Only in treatments of extremely high nutrition

was genetically based variation observed. Likewise, no additive genetic variation of leaf or ramet FA was found in half-sibling families of birch (Wilsey et al. 1998; Wilsey and Saloniemi 1999), and no significant effect of genotype was found for leaf or flower asymmetry in *L. corniculatus* (Andalo et al. 2000). Even in *Drosophila melanogaster*, additive genetic variation for FA in wing length was not found (Scheiner et al. 1991). These results suggest that genetic variation for nutrient-dependent growth characteristics is common; however, FA may not be greatly affected. Nonetheless, Leamy and Klingenberg (2005) hypothesize that FA levels can be influenced by dominance and epistatic interactions among the numerous genes that affect growth, but the presence of buffering systems may prevent deleterious alleles from disrupting development (Rutherford and Lindquist 1998; Milton et al. 2003).

Conclusions

Increased nutrient levels led to slightly higher leaf asymmetry in *L. salicaria*, but for the same treatment, FA was unchanged for *P. sedoides*. It is possible that the degree of nutrient stress imposed on *L. salicaria* and *P. sedoides* was not great enough to elicit a strong response in terms of FA and that a nutritional gradient examining greater extremes of nutrient stress would better serve to describe potential responses in FA. The use of FA as an assessment tool for environmental stresses should be encouraged because under standard growth conditions and with added nutrition, there was no genetic basis for FA. Anthropogenically induced nutrient enrichment is known to negatively affect wetland ecosystems by altering interspecific relationships and species composition in the long term (Pauli et al. 2002; Vojtíšková et al. 2004). FA in *L. salicaria* may therefore be a better short-term indicator of nutrient enrichment in wetland ecosystems; however, the utility of FA in *P. sedoides* needs supplementary investigation. We further suggest that the age of a population being sampled must be considered when using asymmetry as an indicator of environmental stress. The magnitude of FA appears to be species specific rather than genotype specific, and a possible approach for sampling stress in natural populations therefore may be to examine multiple species along an environmental gradient.

Acknowledgments

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