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Genetic constraints at the metamorphic boundary: Morphological development in the wood frog, *Rana sylvatica*

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Abstract

Organisms with complex life-cycles often experience very different environments in different phases of their life. Genes expressed in more than one phase could potentially create a conflict or constraint on evolutionary change if the pattern of selection on those genes were different in the different phases. The potential importance of this type of constraint across metamorphosis in frogs was assessed by measuring the genetic correlation between several morphological traits in both larval and juvenile *Rana sylvatica*. Genetic correlations within a stage tended to be moderately high and significant whereas correlations across stages were low and not significant. Errors on the genetic parameters make it impossible to prove that there are no genetic constraints across metamorphosis in this population of frogs, but the results are consistent with the hypothesis that gene expression and developmental regulation are partitioned separately before and after metamorphosis.

Introduction

Complex life-cycles are characterized by "an abrupt ontogenetic change in an individual's morphology, physiology, and behavior, usually associated with a change in habitat" (Wilbur, 1980, p. 67). Prime examples of organisms with complex life-cycles are holometabolous insects and amphibians, although almost all organisms undergo some change in morphology and habitat during their development (Werner and Gilliam, 1982). One consequence of possessing a complex life-cycle is that the same individual must live in two or more very different

environments. It may be difficult, however, for an individual to be well adapted to both phases of its life-cycle.

Constraints or trade-offs caused by physiological and functional interrelationships within an organism might place limitations on the amount of change that an organism can undergo during its development. In frogs, for example, the same individual develops from an egg into an aquatic tadpole, which then metamorphoses into a terrestrial adult form. The functional requirements of the larval phase of the frog life-cycle are likely to be very different from those of the adult phase. The question remains whether the morphological features required to fulfill these functional requirements are developmentally compatible across the two stages (Wassersug, 1989). Many metamorphic organisms, such as holometabolous insects, do not appear to be as constrained by this limitation because adult structures are derived somewhat independently from larval ones (Fristrom, 1981). In amphibians, however, there is no obvious independence of larval and adult morphology and no period of developmental quiescence equivalent to pupation in insects. Amphibians, then, serve as an extreme example of the potential conflict inherent in ontogenetic evolution.

One method of assessing the importance of a developmental constraint is by measuring the degree of genetic correlation between two traits (Cheverud, 1984; Maynard Smith et al., 1985). The genetic correlation between two traits can have a considerable impact on short-term evolutionary dynamics because selection acting on one trait can cause a correlated response to selection in the other trait (Dickerson, 1955; Falconer, 1981). This relationship is described by Lande's (1979) equation for the expected amount of phenotypic change after one generation of selection:

$$\Delta\bar{\mathbf{z}} = \mathbf{G}\beta,$$

where $\bar{\mathbf{z}}$ is a vector of phenotypic trait means, $\mathbf{G}$ is a genetic variance-covariance matrix, and β is a vector of selection gradients (Lande, 1979; Lande and Arnold, 1983). In ontogenetic studies, the phenotypic expression at each age is considered to be a separate character, so $\mathbf{G}$ includes genetic covariances both within and between ages (Lande, 1982; Cheverud et al., 1983). In the context of complex life-cycles, then, the genetic correlations of most interest will be those that involve traits on either side of the boundary between life-cycle phases, such as metamorphosis in amphibians. Large genetic correlations between traits expressed in larvae and adults would mean that selection acting in one phase of the life-cycle would cause a correlated response to selection in the other phase. If selection acts in opposing ways during each phase, the further evolution of those traits might therefore be constrained (Dickerson, 1955; Charlesworth, 1990).

This paper investigates the importance of constraints across stages of the life cycle in a population of wood frogs (*Rana sylvatica*) via the estimation of the genetic correlations among several morphological characters. Correlations both within and between ages were measured, with a particular focus on correlations between traits expressed on either side of the metamorphic boundary.

Materials and methods

Breeding design and husbandry

Male and female adult *Rana sylvatica* were collected on March 25–27, 1989, from a pond in the Indiana Dunes National Lakeshore (Porter County, Indiana, USA). The collected frogs were immediately stored in a refrigerator at 4 °C for several days. This procedure does not affect fertility when used for short periods of time (Smith-Gill and Berven, 1980). Starting March 28, seven males were crossed with four females by stripping the eggs from each female into sperm suspensions made from the testes of each male (DiBerardino, 1967). In order to minimize any possible effects of egg order in the oviduct, females where rotated haphazardly among the sperm suspensions while the eggs were being deposited. A separate set of similar crosses using different males and females was made every other day for the next six days, so that four independent mating blocks of the crosses were generated. Because of infertility problems with some of the frogs, however, not all of the crosses were successful. The final design, shown in Table 1, consisted of

Table 1. Breeding design.

Sires	Dams													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	X	X	X											
2	X	X	X											
3	X	X	X											
4	X	X	X											
5	X	X	X											
6	X	X	X											
7				X	X	X								
8				X	X	X								
9				X	X	X								
10				X	X	X								
11				X	X	X								
12				X	X	X								
13				X	X	X								
14							X	X	X	X				
15							X	X	X	X				
16							X	X	X	X				
17							X	X	X	X				
18							X	X	X	X				
19											X	X	X	X
20											X	X	X	X
21											X	X	X	X
22											X	X	X	X
23											X	X	X	X
24											X	X	X	X

twenty-four sires and fourteen dams, yielding eighty-three half-sib families, twenty-four paternal half-sib families, fourteen maternal half-sib families, and a total of 830 larvae.

Full-sib embryos were raised together until hatching, at which point ten individuals from each set of full-sibs were placed separately in 600 ml of aged tap water. Embryos, larvae and juveniles where raised at 18 °C, which corresponds to the mid point of the temperature range experienced during larval development in the natural population (Phillips, 1991). Within each treatment, full-sib larvae were distributed as evenly as possible across six laboratory shelves. The developing larvae were fed boiled spinach *ad lib.*, and their water was changed weekly. Five weeks after fertilization, each tadpole was photographed in lateral view. After reaching metamorphosis, frogs were individually housed in one liter plastic containers and fed *Drosophila ad lib.* Three weeks after metamorphosis, each juvenile was anesthetized using MS-222, and photographed in dorsal view.

Measurements were taken on each individual by projecting negatives of the photographic images of the larval and juvenile frogs onto a Hipad digitizing tablet. A millimeter rule was photographed along side each specimen, and correction for photographic distortion was accomplished by re-calibrating the digitizing distances for each individual. Snout-vent (SV) length, tail length, and leg length were measured for each individual by recording the coordinates of these morphological landmarks using the digitizing tablet and an IBM personal computer. The lengths were then calculated as the linear distance between the landmarks. The morphological measurements were transformed to their natural logarithms before analysis to better satisfy the assumption of homoscedasticity (Sokal and Rohlf, 1981).

Statistical analysis

The genetic and environmental components of the phenotypic variance were estimated from the breeding design using the model,

$$Y_{krijl} = \mu + \text{Block}_k + \text{Shelf}_r + \text{Sire(Block)}_{i(k)} + \text{Dam(Block)}_{j(k)} + (\text{Sire} \times \text{Dam}\{\text{Block}\})_{ij(k)} + \text{residual}_{krijl},$$

where Y is the value for an individual measurement, μ is the overall mean, and $A(B)$ means that factor A is nested within factor B. Only the main effect of shelving block was included in the model because mortality created too many empty cells in the full factorial design. These models were analyzed using both univariate and multivariate analysis of variance (ANOVA and MANOVA) as implemented in the General Linear Models Procedure of SAS (SAS Institute Inc., 1985). All sources of variance were treated as random effects. The particular structure of this nested ANOVA precluded using simple F-ratios for significance tests of the mating block effect. Therefore, tests involving mating block were based on quasi F-ratios calculated from the sums and differences of the error mean squares (Tab. 2; Lindman, 1974). Quasi F-ratios for the MANOVAs were constructed using sums

and differences of the error sums-of-squares-and-cross-products matrices. The MANOVA test was then calculated using the greatest root (maximum F) of the product of the inverse of the error matrix and the hypothesis matrix (Harris, 1975). The expected mean squares that serve as the basis for these tests is given in Table 2. Type III sums of squares were used throughout the analysis.

Causal components of variance were calculated from the observational components of variance generated by the MIVQUE option of the SAS Varcomp Procedure (SAS Institute Inc., 1985). The MIVQUE method was used because of computational feasibility (see below), but estimates based on Type-I sums of squares and maximum-likelihood methods yielded similar results. It is possible for the variance components to be negative because they are calculated from the difference of expected mean-squares (Knapp et al., 1989). Negative estimates were retained because excluding them biases the genetic estimates and disrupts the distribution of jackknifed pseudovalues (see below).

The additive genetic and dominance variances were estimated as four times the sire, and sire-by-dam components of variance, respectively (Cockerham, 1956; Becker, 1984). Similarly, the maternal variance was measured as the difference between the dam and sire components of variance (Willham, 1963). Heritability was calculated as the ratio of additive genetic to total phenotypic variation (Wright, 1921; Falconer, 1981). Standard errors on estimates of these genetic parameters were calculated by jackknifing the variance component estimates across sires, dams, or full-sib families, where appropriate for the parameter being measured (Arvesen and Schmitz, 1970; Knapp et al., 1989). For example, the additive genetic variance was jackknifed by repeatedly re-estimating the sire variance component after sequentially removing all offspring sired by a particular male from the analysis (see also Blouin, 1992). One advantage of the jackknife procedure is that it allows the calculation of standard errors on parameters derived from complex, unbalanced designs such as that used in this experiment. However, standard errors obtained using a jackknife tend to be larger than parametric estimates, so the significance of the genetic parameters was evaluated using both a t-test on the jackknifed genetic variance component estimates and the analysis of variance of the sire and dam effects described above.

The covariance components were estimated using the variance of the sum of two traits. For example, for traits X and Y, the covariance component between X and Y can be obtained from the among-sire variance components by

$$\mathrm{Cov}(X, Y) = [\mathrm{Var}(X + Y) - \mathrm{Var}(X) - \mathrm{Var}(Y)]/2$$

(Kempthorne, 1957). The genetic correlation between X and Y is then defined as

$$r_g = \mathrm{Cov}(X, Y)/\sqrt{\mathrm{Var}(X)\,\mathrm{Var}(Y)}$$

(Falconer, 1981). Phenotypic correlations were similarly calculated from the components of total phenotypic variance and covariance rather than the correlation between individuals in order to eliminate the potential bias that the family-structure of the data might cause. Standard errors for the phenotypic and genetic correlations

Table 2. Expected mean squares for the ANOVA. Expected mean squares are based on a balanced design. Indented sources of variance are nested within the factor above them. The variance components (σ^2) associated with each source of variance are identified by the subscripted letters. The coefficients the variance components (k_i) vary according to the sample sizes within each cell (Lindman, 1974). MANOVA results give Roy's greatest root for each stage tested separately.

Source	d.f.	Expected mean square	*F*-ratio	Larval stage	Juvenile stage
A. Shelf	5	$\sigma^2_W + k_1\sigma^2_{S/I}$	A/F	0.07*	0.04
B. Mating block	3	$\sigma^2_W + k_2\sigma^2_{S\times D} + k_3\sigma^2_D + k_4\sigma^2_S + k_5\sigma^2_B$	B/(C + D − E)	5.09	0.88
C. Sire	20	$\sigma^2_W + k_6\sigma^2_{S\times D} + k_7\sigma^2_S$	C/E	1.95*	4.17*
D. Dam	10	$\sigma^2_W + k_8\sigma^2_{S\times D} + k_9\sigma^2_D$	D/E	3.82*	2.44*
E. Sire × dam	49	$\sigma^2_W + k_{10}\sigma^2_{S\times D}$	E/F	0.22*	0.26*
F. Within family	563†	σ^2_W			
Maternal effect			D/C	3.69*	1.19

† Degrees of freedom for the larval stage. Degrees of freedom the juvenile stage was 402.
* Significant at $p < 0.01$ by the sequential Bonferroni method adjusted for the entire table.

were calculated by jackknifing the variance component estimates across sires (Arvesen and Schmitz, 1970; Knapp et al., 1989). Maternal and dominance correlations are not presented because none of the variance estimates for the underlying traits were significantly different from zero (see below).

To avoid potentially spurious results caused by the independent analysis of a large number of traits and parameters, a character by character description of the results was limited to those genetic parameters that were determined a priori to be of interest; otherwise, multivariate techniques were used. When multiple comparisons were made, the significance levels were adjusted using the sequential Bonferroni method (Rice, 1989). An initial significance level of 0.05 was used throughout.

Results

Variance components

When all traits are considered simultaneously, there is evidence for the existence of additive genetic variance, as indicated by the significant sire effect (Tab. 2). Breaking down the sire effect individually for each trait, Table 3 shows that there is significant heritability for many of the traits. Individual ANOVAs indicated that each trait at both ages exhibits significant additive genetic variance, whereas the jackknife tests tended to only determine significance for the larger heritabilities (Tab. 3). This discrepancy is probably caused by the greater power of the ANOVA. Accepting only the jackknife analyses is a more conservative approach, but combing these with the ANOVA results suggests, at least, the presence of some additive-genetic variance for most traits.

Table 3. Means and variance components for each trait across temperatures and ages. All measurements are in mm. Variance components are on the loge-scale, and are $\times 10^5$. V_A = additive genetic variance; V_M = maternal variance; V_D = dominance variance; V_E = environmental variance; V_P = total phenotypic variance; h^2 = heritability. Standard errors (in parentheses) based on a jackknife test.

Trait	Mean	V_A	V_M	V_D	V_E	V_P	h^2
Larval stage							
SV length	12.92	220 (141)*	33 (76)	127 (229)	53 (24)	430 (29)	0.51 (0.25)*
Tail length	26.90	187 (75)†*	36 (49)	121 (196)	358 (97)	751 (63)	0.32 (0.15)†*
Leg length	9.06	1021 (949)*	354 (386)	773 (923)	1504 (676)	3652 (318)	0.31 (0.23)*
Juvenile stage							
SV length	11.31	599 (285)†*	−7 (43)	94 (148)	168 (109)	855 (83)	0.73 (0.14)†*
Leg length	28.82	170 (61)†*	−13 (27)	−37 (77)	192 (52)	312 (26)	0.56 (0.22)†*

* Significant by an individual ANOVA test.
† Significant by the jackknife test with no adjustment for multiple comparisons.

The MANOVA results reveal significant maternal effects at the larval stage (Tab. 2), although an analysis of the individual components does not indicate that any single estimate can be bounded away from zero. A similar result was found for the presence of dominance effects. Although estimates of the contribution of dominance variance to the total phenotypic variance reached as high as thirty percent for some traits (Tab. 3), the jackknifed standard errors were too large to bound any of the estimates from zero. MANOVA of all characters found significant dominance effects, as measured by the sire-by-dam interaction (Tab. 2), although no individual ANOVA was significant. Again, there is weak evidence for significant dominance variance in some of the traits, although it is difficult to determine how important dominance might be for any particular trait.

Correlations

In general, correlations among the morphological traits within a given age are large and positive, with the phenotypic and genetic correlation matrices being very similar to one another (Tab. 4). None of the genetic correlations across metamorphosis are significantly different from zero. Genetic correlations between larval and juvenile features (tails and legs) were especially small (average 0.08), although no individual correlation can be shown to be statistically distinguishable from any other correlation. Phenotypic correlations also tend to be much lower across stages than they are within stages, although they are non-zero in almost every case (Tab. 4).

Discussion

In amphibians, metamorphosis is the obvious point at which there might be an ontogenetic change in selection pressure (Wassersug, 1975; Arnold and Wassersug,

Table 4. Phenotypic and genetic correlations. Phenotypic correlations are shown above the diagonal and genetic correlations below the diagonal. Errors are estimated by the jackknife test.

	Larval SV length	Larval tail length	Larval leg length	Juvenile SV length	Juvenile leg length
Larval SV length	–	0.64* ± 0.06	0.61* ± 0.07	0.42* ± 0.08	0.28* ± 0.10
Larval tail length	0.50† ± 0.19	–	0.48* ± 0.08	0.31* ± 0.09	0.17 ± 0.09
Larval leg length	0.54† ± 0.20	0.52† ± 0.22	–	0.29* ± 0.10	0.36* ± 0.09
Juvenile SV length	0.24 ± 0.28	0.21 ± 0.33	0.09 ± 0.40	–	0.71* ± 0.09
Juvenile leg	−0.11 ± 0.32	0.07 ± 0.36	0.18 ± 0.30	0.48† ± 0.22	–

* Significant at $p < 0.05$ by the sequential Bonferroni method adjusted separately for the phenotypic and genetic correlations.

† Significant at $p < 0.05$ when not adjusting for multiple comparisons.

1978; Wilbur, 1980). At metamorphosis, most frogs undergo a transformation from swimming, aquatic herbivores to jumping, terrestrial carnivores (see Lutz [1945] and Elinson [1987] for a discussion of some exceptions). Because of this drastic change in morphology and life-style, the correlations between traits measured on either side of the metamorphic boundary are of particular interest. Results from standard quantitative genetic theory show that selection acting on one trait will result in evolutionary change in other, genetically correlated traits (Lande, 1979; Falconer, 1981). There are no significant genetic correlations across metamorphosis for this population of wood frogs (Tab. 4). Unfortunately, larval mortality somewhat limits the power of the breeding design to detect genetic correlations, and so only correlations greater than about 0.50 would have been deemed significant here. Uncertainty in the magnitude of the genetic correlations therefore precludes any definite statements. However, since all of the genetic correlations across metamorphosis are significantly different from one (Tab. 4), it appears that selection acting in one stage of the life-cycle probably would not greatly influence morphological expression in the other stage.

Blouin (1992) has similarly found little correlation between growth rate between larval and juvenile *Hyla cinerea* (see also Travis et al., 1987). A decline in genetic correlation over time appears to be a general feature of morphological change (Cheverud et al. 1983; Leamy and Cheverud 1984; Riska et al. 1984; Atchley, 1987), and therefore is perhaps not surprising in these metamorphic organisms. Differential gene expression during anuran development is just beginning to be explored (Brown et al., 1995). Results so far show that some loci, such as hemoglobin,

exhibit differential expression in larvae and adults (Hosbach et al., 1983; Hornby et al., 1989; Hranitz and Deihl, 1990; Zorn and Krieg, 1992). There is also evidence for tissue localization of gene expression during development (Hornby et al., 1989). However, we are a long way from understanding the genetic basis for morphological differences between larvae and adults.

Of all metamorphosing animals, amphibians appear unique in that there does not appear to be a clear differentiation between larval and adult features. For example, the adult features of *Drosophila* develop from many larval imaginal discs that remain undifferentiated during the larval phase (Fristrom, 1981). In other insects, however, there can be more whole-scale turnover of larval tissues that more closely resembles the amphibian situation (Locke, 1981). An emerging view of amphibian development suggests, however, that the seeming lack of differentiation between larval and adult forms in amphibians is actually an illusion created by the apparently continuous (temporal) progression of larval to adult development. The pre- and post-metamorphic forms in amphibians may indeed be much more independent from one another than was previously thought (Alberch, 1987; Hanken, 1989). For example, the epibranchial elements in adult *Eurycea* salamanders is derived from undifferentiated mesenchyme rather than the larval epibranchia that are already present (Alberch et al., 1985; Alberch and Gale, 1986). Thus, although there is apparent homology between larval and adult structures, they are actually developmentally independent (Alberch, 1987). Similar processes occur during anuran metamorphosis as the larval intestine, skeleton, and nervous system differentiate into adult features (Fox, 1981; Kollros, 1981; Hanken et al., 1989; Shi and Ishizuya-Oka, 1996). Unfortunately, the lack of significant genetic correlations between larval and adult characters in wood frogs does not necessarily mean that there are few pleiotropic genes affecting these traits. Since genetic correlations are dependent on gene frequencies as well as pleiotropy and linkage, the correlations can be zero despite the existence of pleiotropic genes (Carey, 1988). Thus, small genetic correlations are a necessary, but not sufficient indication of independent developmental gene action. Nonetheless, the results presented here are consistent with the embryological picture described above. Ultimately, the question of genetic independence will need to be addressed through a functional understanding of developmental gene action during metamorphosis.

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