

ECOLOGICAL AND DEVELOPMENTAL CONTEXT OF NATURAL SELECTION: MATERNAL EFFECTS AND THERMALLY INDUCED PLASTICITY IN THE FROG *BOMBINA ORIENTALIS*

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Abstract.—Variation in fitness generated by differences in functional performance can often be traced to morphological variation among individuals within natural populations. However, morphological variation itself is strongly influenced by environmental factors (e.g., temperature) and maternal effects (e.g., variation in egg size). Understanding the full ecological context of individual variation and natural selection therefore requires an integrated view of how the interaction between the environment and development structures differences in morphology, performance, and fitness. Here we use naturally occurring environmental and maternal variation in the frog *Bombina orientalis* in South Korea to show that ovum size, average temperature, and variance in temperature during the early developmental period affect body sizes, shapes, locomotor performance, and ultimately the probability of an individual surviving interspecific predation in predictable but nonadditive ways. Specifically, environmental variability can significantly change the relationship between maternal investment in offspring and offspring fitness so that increased maternal investment can actually negatively affect offspring over a broad range of environments. Integrating environmental variation and developmental processes into traditional approaches of studying phenotypic variation and natural selection is likely to provide a more complete picture of the ecological context of evolutionary change.

Key words.—Amphibian, eco-devo, Korea, norm of reaction, ovum size, tadpole.

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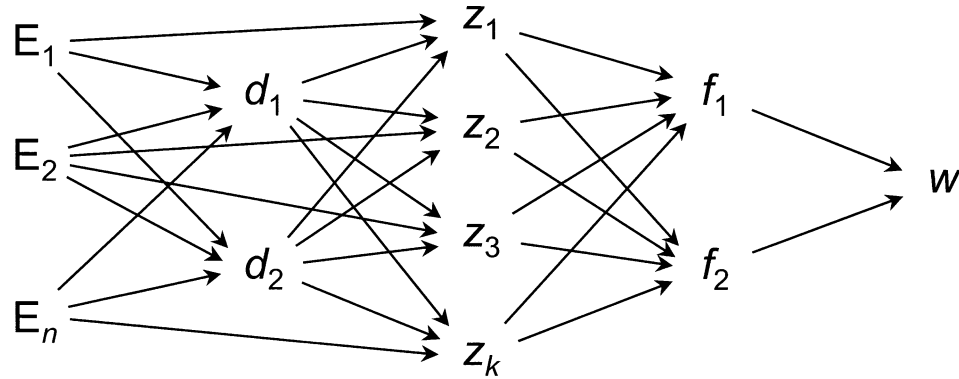
The environment mediates the effects of natural selection in two ways. First, biotic and abiotic features of the environment generate the functional context within which variation in individual fitness is generated. Second, environmental conditions throughout development have an important influence on the generation of the phenotypes that are themselves subject to selection. Although there are well-studied instances of adaptive phenotypic plasticity (reviews in West-Eberhardt 2003; Doughty and Reznick 2004), most studies that have actually tried to quantify the influence of environmental variation on natural selection have done so by concentrating either on the relationship between phenotype and fitness or between environment and phenotype (but see Stratton 1994). Development stands firmly at the center of the integration of these two approaches. In this study, we examine the interface between these two domains of environmental influence (the immediate and developmental) in a suite of correlated traits including developmental rate and morphological differentiation at the hatching stage in the fire-bellied toad, *Bombina orientalis*, in a natural population in South Korea.

The Ecological and Developmental Context of Morphology, Performance, and Fitness

Within evolutionary ecology there have been three prominent and largely parallel approaches to linking environmental variation to phenotypic variation and natural selection. Each of these approaches relies to some degree on functional response curves. First, the role of the environment in generating phenotypic plasticity has been examined through the use of reaction norms in which the phenotype is seen to vary as a functional response to environmental variation (Scheiner

1993; Schlichting and Pigliucci 1998). Second, functional morphologists and physiologists have examined the relationship between morphological variation and organismal performance using performance curves (Huey and Kingsolver 1989). Third, the relationship between phenotypic variation and individual fitness has been analyzed using fitness surfaces (Lande and Arnold 1983; Schluter 1988; Phillips and Arnold 1989). Arnold (1983) showed how the latter two perspectives could be tightly linked to one another by inserting an analysis of performance into the relationship between phenotype and fitness. The need to expand this perspective through the inclusion of environmental variation is clear (Kingsolver and Gomulkiewicz 2003; Kingsolver and Huey 2003). Significant progress along these lines has been made through the development of methods that analyze patterns of natural selection on the norm of reaction as a whole (Weis and Gorman 1990; Scheiner and Callahan 1999). Another way that this can be accomplished is by including the influence of environmental variation on the generation of phenotypic variation in the same framework as the performance/fitness relationship (Fig. 1).

A developmental perspective that links environmental inputs to phenotypic outcomes (Travis et al. 1999) allows us begin to understand how the interaction of environmental variation with developmental processes structures the context within which natural selection operates (Fig. 1). This analysis can then be combined with information about genetic processes to explore the consequences of this variation for evolutionary change and adaptation (Lande 1979; Ghalambor et al. 2003). This integrated perspective allows for any developmental response to environmental effects to be analyzed directly within the context of the fitness effects to which they give rise. The arrows connecting different elements in the



Environment \rightarrow Development \rightarrow Morphology \rightarrow Performance \rightarrow Fitness

FIG. 1. A path diagram illustrating how the causal connections between morphology (or phenotype, in general), performance, and fitness can be expanded by the addition of environmental variation and developmental processes (e.g., levels of gene expression or rates of differentiation). Each level in the path is assumed to causally lead to variation in the levels above (forward arrows). For clarity, not all possible connections are shown. In particular, we also use causal paths that bypass intermediate levels (e.g., effects of morphology on fitness that are not solely accounted for by differences in performance). Two-headed arrows describing patterns of correlation among traits at a given level are also omitted.

path diagram displayed in Figure 1 are in fact partial regression coefficients that one would normally obtain from a functional analysis at any particular level of causation (e.g., norm of reaction or fitness surface)—they are simply brought together here into a single coherent picture. An additional advantage to a unified approach is that many potential sources of systematic error (i.e., bias), such as shared environmental influences, can be accounted for within the context of the overall analysis (Scheiner et al. 2002).

Plasticity and Selection in Amphibians

Here, we parse the environment influencing early development in *B. orientalis* along two primary axes: abiotic thermal effects and the maternal effect of ovum size. The thermal environment has long been known to have a profound influence on many aspects of larval development (e.g., Bradford 1990). We show that, in addition to the constant temperature effects that tend to predominate in laboratory studies, diel and seasonal variation plays a critical role in morphological development in the natural environment (see also Shine and Elphick 2001; Ashmore and Janzen 2003; Podrabsky and Somero 2004). In addition, ovum size, besides being a life-history trait per se, can be thought of as an environmental influence on the development of an embryo that interacts with subsequent environmental and genetic effects to which the embryo is exposed as it develops (Kaplan 1998; see also Einum and Fleming 2002; Laugen et al. 2002; Pakkasmaa et al. 2003). Furthermore, thermally varying environments (and other environmental factors) can interact with ovum size both additively and nonadditively to drive potentially complex developmental patterns (Parichy and Kaplan 1992a,b; and see Berven and Chadra 1988; Fox and Mousseau 1996; Moran and Emler 2001; Loman 2002).

Over many decades amphibians at all stages of their life cycle have proven to be useful models for studying the sensitivity of vertebrate development to environmental pertur-

bation that is natural (e.g., Zweifel 1955) or, more recently, engendered by human disturbance (Blaustein and Johnson 2003). The response of embryos to the effects of a variety of environmental factors such as temperature and temperature variation (Parichy and Kaplan 1995), oxygen availability (Bradford and Seymour 1988; Bradford 1990; Mills and Barnhart 1999; Seymour et al. 2000), predation risk (e.g., Sih and Moore 1993; Laurila et al. 2002a; Van Buskirk 2002; Warkentin 2002; Johnson et al. 2003; Relyea 2004), and pond drying (e.g., Laurila et al. 2002b; Rose 2005) provides an ideal system for investigating the coupling of environmental variation with morphological variation. At the same time, studies have demonstrated the fundamental role of both abiotic (Brown and Taylor 1995; Parichy and Kaplan 1995; Watkins 1996, 2000; Arendt 2003) and biotic (e.g., Van Buskirk and McCollum 2000a; Relyea 2002) influences on larval performance, although the integration of pre- and post-hatching developmental events is only now beginning to be explored (e.g., Warkentin 1999; Moran and Emler 2001; Anderson and Petranksa 2003). Each of these studies (and indeed many more than can be cited) has contributed to a view of amphibian larval development as a dynamic process in which environmental context and developmental response can be tightly integrated, with obvious morphological and functional consequences.

Here, the functional consequences of environmental, developmental, and morphological variation are explored by extending our previous work on the morphology-predation susceptibility relationship that we have demonstrated in the field (Kaplan 1992) and the morphology-sprint speed performance relationship that we demonstrated in the laboratory (Parichy and Kaplan 1995; see also Huey 1980; Brown and Taylor 1995; Watkins 1996, 2000; Arendt 2003; Fitzpatrick et al. 2003) with a synthetic study of much of the full relationship in the field. In the end, nonadditive interactions of the environment with morphological development are shown

to have an important influence on larval swimming performance, and ultimately, fitness.

MATERIALS AND METHODS

We investigate the role of environmental variation during development by testing for the interaction of egg size effects and postfertilization temperature effects on offspring development, morphology, performance, and survivorship in a temporally repeated multifactorial field experiment. We focus on components of the life cycle and fitness that includes events only through early larval life. Selection during other stages of development (e.g., metamorphosis, overwintering survival, mating success) will obviously also be critical to structuring lifetime reproductive success (e.g., Altwegg and Reyer 2003). Ovum diameter was treated as a two-level fixed effect (large and small eggs), and embryos were blocked spatially and temporally across numerous natural ponds on eight and 19 occasions between May 27 and June 30 in 1993 and 2000, respectively. This design resulted in matched large and small embryos being exposed to 182 unique thermal environments.

Field Setting

Experiments were conducted at a site established in 1986 near the city of Tong-hae in the Province of Kangwon-do, in the Republic of Korea (for a full description see Kaplan 1987, 1992). Briefly, males migrate from adjacent woodlands to a quickly moving stream ($\sim 17^{\circ}\text{C}$) in early May. Females arrive beginning in mid-May and remain for two or three days while ovipositing in small, stagnant, granite depressions adjacent to the streambed. Different females arrive at the site each night through early July. Earlier, in late March the brown frog, *Rana dybowskii*, breeds in the same ponds. By the time *Bombina* arrive these ranid larvae, which prey on the *Bombina* larvae, are already approximately 20 mm in length and continue to grow as larvae through the month of June.

Temperature variation.—At this location oviposition takes place in approximately 20 different temporary to permanent ponds of variable size, averaging 1 m in diameter and 25 cm in depth. None were more than 10 m from the stream and the most distant ponds were 50 m apart. Temperature variation was monitored in each of the ponds every 4 h from 0800 to 2000 h throughout the course of these two experiments. Midnight and 0400 h temperatures were interpolated. Cloudy, rainy weather resulted in cooler average pond temperatures and lower daily variation in temperature within each pond. Sunny weather resulted in overall warmer average temperatures and higher daily variation in temperature within each pond. In addition, the among-pond variability is much greater during warmer years than cooler years as a result of trees shading some ponds at different times of the day.

Ideally, we would account for the influence of this variation by integrating the instantaneous effects of temperature at each point in development over the entire temperature regime experienced by each individual, for instance, by using the known effects of different fixed temperatures on patterns of growth (e.g., Kingsolver and Gomulkiewicz 2003). In reality, however, this is a highly multidimensional, multistate problem, because we need to know not only the effect of a tem-

perature at a given developmental period, but also the effects of shifts among different temperature states at different developmental stages, as well as the carry-over effects of previously experienced temperatures.

The overall influence of thermal variability (diel variability) was therefore approximated using the coefficient of variation (CV; Kaplan 1989) in temperature experienced by an individual during the developmental period under study. We used the CV rather than variance per se to account for the positive correlation between mean temperature and variation in temperature. Full analysis of the data on the raw variance scale does not change the qualitative results and indeed has little influence on the quantitative estimates. While the CV in temperature captures average differences in variation during incubation, it does not allow for an evaluation of unique changes in thermal variation during a single incubation period. We expect ontogenetic changes in thermal tolerances and reaction norms during this period, but fully quantifying these effects will require further study.

Ovum size variation and detailed experimental design.—The experimental design was modeled after Kaplan (1992). Amplectant pairs of *B. orientalis* were collected and allowed to deposit eggs overnight in inflated 5-L polyethylene bags containing 500 ml of stream water and 4.0 L of air. The following morning adults were released, eggs were counted, and five eggs from each clutch were measured to the nearest 0.01 mm with an 8 \times 30 monocular scope fitted with an opticle reticle (Macroscope 25, RF Interscience, New York). Ovum size was estimated from developmental stage via methods determined by King and Kaplan (1997), who also described egg measuring techniques in greater detail.

This procedure was repeated on eight occasions in 1993 and 19 occasions in 2000. Both experiments were similar in having two levels of egg size (large and small). In 1993 the egg size treatment was generated by pooling the embryos of 25% of the top ranked sibships (never fewer than five and up to 20) that produced the largest eggs and 25% of the lowest ranked sibships that produced the smallest eggs in one night. Egg number collected per sibship averaged approximately 105. In 2000, the design was modified to increase temporal blocking. As soon as two sibships, one with large eggs (>2.18 mm) and one with small eggs (<2.05 mm), were identified they were used for the egg size treatment effect (42 sibships total). The benefits of increased temporal blocking (and therefore a greater exploration of natural thermal variation) that was achieved in this manner was felt to be worth the cost of potentially introducing additional among-sibship variation. Some of the egg size effect will therefore be confounded with among-sibship differences, to the extent that the egg size effects and sibship effects are correlated (e.g., if females who produce large eggs also tend to produce offspring who grow faster for genetic reasons in addition to the egg size effect). Previous work with this species has demonstrated, however, that egg size is most strongly influenced by the recent environmental history that a female experiences (Kaplan and King 1997).

To prevent intra- and interspecific oophagy (Marshall et al. 1990), 30 embryos at the midblastula stage for each level of egg size were put into two clear, colorless, polyethylene bags each containing 500 ml of stream water, inflated with

4 L of air, and left floating in ponds. We were thus able to cross the egg size fixed effect with numerous thermal regimes that varied as a result of pond characteristics and weather.

Embryos were monitored and the time when 50% of each group of 30 hatched was recorded. Hatchlings ($n = 5$ in 1993; $n = 3$ in 2000) were randomly selected from the group and photographed for subsequent measurement of length from the snout to the posterior margin of the vent (SVL), tail length, and maximum height of the tail (e.g., Van Buskirk and McCollum 2000b). Newly hatched larvae were also staged via standard Gosner series (Gosner 1960). At hatching, *B. orientalis* larvae are between Gosner stages 19 and 21. They are capable of motion but retain nearly all their original yolk within the gut and are therefore neither streamlined nor feeding. Prior work indicated that the modal stage at hatching is Gosner stage 21 (corresponding to clarity of the whole cornea) and that the time of hatching is not influenced by confinement in polyethylene bags under these conditions (Parichy and Kaplan 1995). In this study Gosner stage 20 to 21 was divided into five substages (20.00, 20.25, 20.50, 20.75, and 21.00) corresponding to the proportion of the cornea that had become transparent (for a discussion of heterochrony of external morphological characters in the staging of amphibian hatchlings and larvae, see Gollman and Gollman 1999).

For sprint speed performance trials, each of the measured and staged larvae was then sprinted three times while recorded on videotape, from which the shortest time it took a larva to move out 3 cm from its starting point was recorded as maximum sprint speed (for details, see Parichy and Kaplan 1995). In 1993, temperature during sprint measurement averaged 21.2°C (SD = 3.30, $n = 912$) and was positively related to sprint speed. However, this source of temperature variation during sprinting (while obviously directly influencing sprint speed and not to be disregarded) had no effect on the estimates of other contributing variables to speed; for simplification it is therefore not considered further. For selection trials, the larvae were individually placed in 1 L of stream water in bags along with five individual larvae (about 20 mm in total length) of *R. dybowskii*, and after 24 h the presence or absence of the hatchling was recorded. In 1993, the temperature during this 24-h period averaged 20.5°C (SD = 2.18, $n = 878$). There was no significant influence of this temperature on the probability of surviving predation, nor did including this factor in the analysis interact with any of the other contributors to survival; therefore, it too is not included in the remaining presentation of the analysis. Although any enclosure experiment is necessarily artificial (Skelly and Kiesecker 2001), observations over eight field seasons including the two described here have shown that variance in densities of *Bombina* ova and larvae, as well as that of predatory tadpoles, readily encompass the densities used here. In addition, observations of unimpeded predation of eggs and larvae have consistently shown this tadpole predator to be the most prevalent cause of mortality of *Bombina* in these ponds up to 24 h post-hatching (R. H. Kaplan, pers. obs.). In addition, predator densities were high enough in most cases that litter debris offered limited refugia for tadpole prey.

Although no significant bag effects were detected, any analyses involving the egg size and temperature effects were

conducted on the overall pond and treatment averages in a conservative fashion to avoid any potential problems from pseudoreplication. (Analyses of the full dataset yielded nearly identical estimates and significance levels.) This approach resulted in 187 unique pond-egg size combinations in 1993 and 177 in 2000. Because each individual was tested separately in the selection trials, all of the individual responses were used in the selection analysis. A total of 940 larvae were subjected to selection via predation in 1993 and a total of 632 in 2000.

Statistical Methodology

The conceptual framework that separates environmental, developmental, morphological, performance, and fitness effects into individual components can be achieved analytically using multiple regression analysis within the context of path analysis (Arnold 1983; Lande and Arnold 1983). This approach relies strongly on the assumption that most of the interesting relationships among the variables under investigation can be captured using a linear model. Although developmental processes are frequently based on nonlinear processes, the majority of the relationships studied here are in fact remarkably linear. As might be predicted, the relationship between temperature and time to hatching showed the most nonlinearity. However, inverting time to hatching to generate a developmental rate linearized the relationship between this developmental parameter and temperature, with the added benefit of normalizing the residuals. Similarly, sprint speed was log transformed. Scrutiny of the residuals of the fitted data for nonnormality and heteroscedasticity revealed that no other transformations were necessary. The linear nature of this transformation helps to minimize the potential trap of overinterpreting a change in the mean of a response variable with changing variances of predictor variables via Jensen's inequality (Ruel and Ayres 1999). In addition to these transformations, we directly address the influence of environmental variation by combining both a regression-based approach (Inouye 2001, 2005) in the case of temperature variation and a multilevel fixed-factor approach (Benedetti-Cecchi 2003, 2005) in the case of ovum size variation within the context of path analysis.

Although path analysis is, in principle, capable of being used to reveal many different relationships among a set of variables (Kingsolver and Schemske 1991; Scheiner et al. 2000), here we have constrained the direction of causation to follow the path outlined in Figure 1, which follows a multiple regression analysis (or general linear model analysis in the case of egg size) performed at each level of the hierarchy. The path coefficients were calculated as the standardized partial regression coefficients resulting from a multiple regression analysis of each variable at a higher level on the set of all causal factors at all additional lower levels (implemented using StatView; Abacus Concepts 1992). Only coefficients that are significant at $P < 0.05$ are presented here. The overall relationship between environment and survivorship is visualized using a bivariate cubic spline (Schluter and Nychka 1994) as implemented in SAS (smoothing parameter $\lambda = 0.5$; SAS Institute 2000). Quadratic and higher-order polynomial response surfaces (Phillips and Arnold

1989) provided qualitatively similar results. Note that this is one of the largest selection studies to have been conducted in a vertebrate and is also distinguished by being fully repeated over two years (Kingsolver et al. 2001). The experimental design provides for sufficient power to detect even fairly small selection coefficients (Hersch and Phillips 2004).

RESULTS

Environment

Thermal patterns.—The 1993 breeding period was cooler and thermally less variable than the 2000 season (Fig. 2). Because oviposition took place almost each night during these periods, average incubation temperatures and the CV in temperature over a particular embryonic incubation period varied greatly within a season, even within a single pond. In 1993, the hottest pond in which embryos were naturally oviposited had an overall mean of 22.4°C (SD = 4.42, $n = 169$) with a CV of 19.7% (Fig. 2A). This changed to an overall mean of 23.4°C (SD = 5.02, $n = 169$) and a CV of 21.4% in 2000 (Fig. 2A). In 1993, the coldest pond in which embryos were naturally oviposited had an overall mean of 16.9°C (SD = 1.94, $n = 169$) with a CV of 11.5% in 1993 (Fig. 2A). This changed to a warmer mean of 17.1°C (SD = 1.98, $n = 169$) with a CV of 11.6% in 2000 (Fig. 2A). While these are average values for whole seasons, each paired grouping of large and small embryos was exposed to a unique thermal environment ($n = 182$; Fig. 2B).

Ovum size treatment (maternal effect).—In 1993 ovum size averaged 2.08 mm (SD = 0.097, $n = 292$ females) and in 2000 it averaged 2.15 mm (SD = 0.100, $n = 128$). (The average within-clutch variation for both years was similar at 0.058 SD and 0.059 SD, respectively.) In 1993 the large egg size treatment was 2.20 mm (SD = 0.036, $n = 8$) and the small egg size treatment was 1.94 mm (SD = 0.047, $n = 8$). In 2000 the large egg size treatment was 2.25 mm (SD = 0.063, $n = 18$) and the small egg size treatment was 2.01 mm (SD = 0.083, $n = 18$; see also Kaplan and King 1997). The difference between large and small ovum size treatments amounted to approximately a 35% difference in volume in each of the years in addition to a 10% difference in volume between years. Volume differences have been shown to be highly correlated with differences in dry mass and energy content (e.g., Kaplan 1980; Komoroski et al. 1998).

Development

The simultaneous occurrence of differentiation and growth makes the two processes difficult to measure separately. To simplify, we restricted our analysis to one ecologically relevant moment in the life cycle, the time to and developmental stage at the transition from an immobile, encapsulated embryo to a mobile, free-swimming larva (i.e., hatchling). We first treated each developmental and morphological trait from a univariate perspective and then incorporated layers of multivariate complexity as appropriate.

Time to hatching and rate to hatch.—The time to hatching (also called incubation time) was the primary datum, and its reciprocal, the rate to hatch, was used in most of the following analysis (see Materials and Methods). The time to hatching

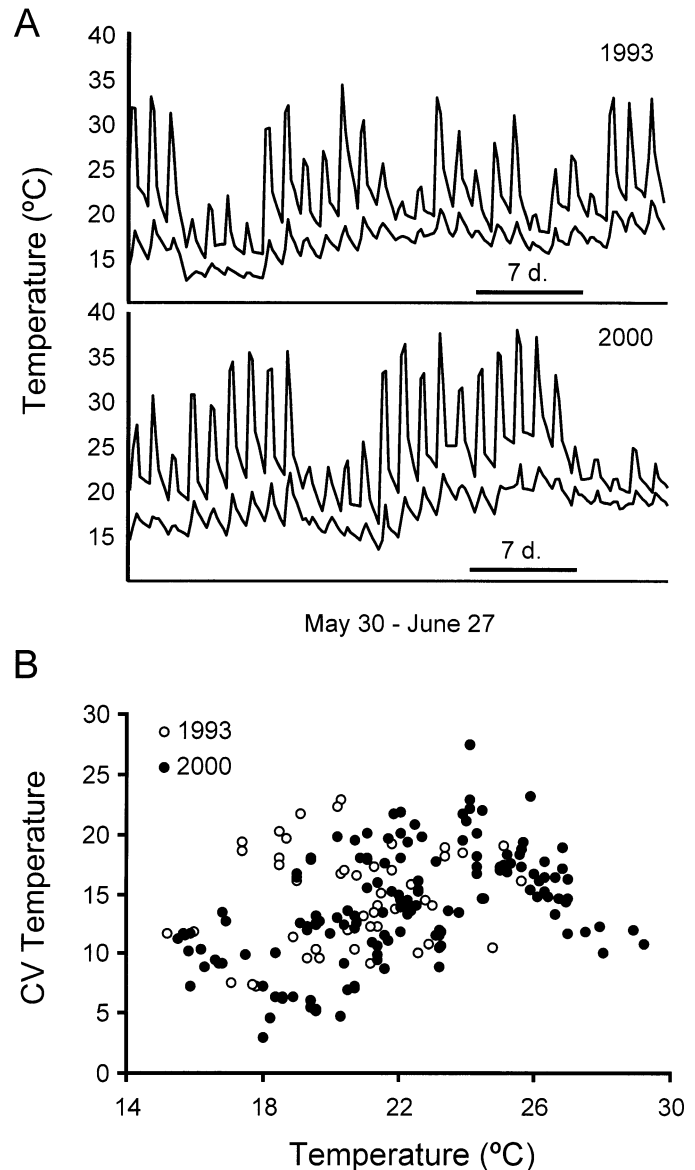


FIG. 2. (A) Diurnal fluctuations in temperature. The upper panel shows changes in temperature in the hottest and coldest ponds in the study in 1993. The lower panels show data for the same two ponds in 2000. Data were taken every 4 h beginning at 0800 h (the leading low point in each cycle) and interpolated for the 2400 and 0400 h time slots. (B) Temperature and coefficient of variation (CV) in temperature combinations. Each point shows the average value for the mean temperature during an incubation period for a specific set of matched large and small embryos. The open circles show data for the 1993 season and the closed circles show data for the 2000 season.

varied considerably, ranging between 2.2 and 8.6 days (Fig. 3), with a small difference in the averages between the two years (Table 1; $F = 4.57$, $df = 1$, 364 , $P < 0.05$). The time it took for an embryo to hatch was highly influenced by the thermal environment to which it was exposed. Average incubation temperature had a strong positive effect on the rate to hatch (with Q_{10} [the ratio of the rate difference over 10°C] ~ 3.2 over the full range of naturally occurring average temperatures). There was a significant, small difference in this

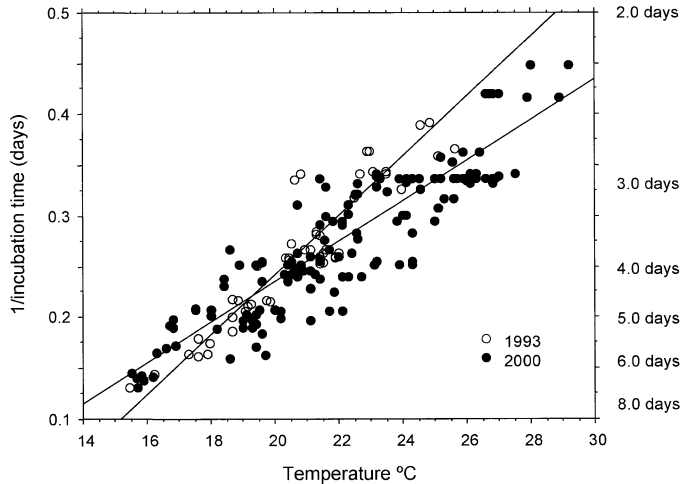


FIG. 3. The influence of average temperature during incubation on the reciprocal of the time to hatching. Each point shows the time at which 50% of the embryos in a group had hatched. Both the rate scale and the reciprocal time scale in days are shown for convenience. All analyses were performed on the rate scale.

relationship between years (Fig. 3; year \times temperature, $F = 71.0$ $df = 1, 361$, $P < 0.01$). The CV in temperature during the incubation period (CV_{temp}) also had a simple positive effect on rate to hatch ($F = 5.6$, $df = 1, 364$, $P < 0.05$), but this belies its effect within and between years due to complex associations between temperature and temperature variation that occurred as a result of weather conditions (Fig. 2B). Thus, in 2000 the simple effect of CV_{temp} on rate to hatch was actually negative regardless of temperature ($F = 20.2$, $df = 1, 173$, $P < 0.01$), but in 1993 CV_{temp} interacted with average temperature ($F = 21.6$, $df = 1, 184$, $P < 0.01$) such that at warmer temperatures that year increasing CV_{temp} resulted in a decrease in rate to hatch, but at cooler average temperatures increasing CV_{temp} increased rate to hatch. Ovum size, however, had no direct effect on incubation time in either year, nor did it interact with thermal characteristics in either year ($P > 0.45$ in all cases).

The sign and magnitude of significant ($P < 0.05$) three main environmental effects and their interactions on rate to hatch (and all the response variables; see below) for each of the two years are shown in path diagrammatic form in Figure 4, with corresponding standardized regression coefficients shown in Table 2. The strong positive effect of temperature

on rate to hatch is indicated by path thickness and solidness that corresponds to its standardized regression coefficients of 0.95 and 0.98 in 1993 and 2000, respectively. The significant, but less strong and negative effect of CV_{temp} on rate to hatch (Table 2) is shown by a thin, dashed path corresponding to standardized regression coefficients of -0.15 and -0.16 in the two years, respectively. As temperature variability during incubation increased, the rate to hatch decreased at a common average. This relationship was complicated by the significant interaction between average temperature and CV_{temp} in 1993, however (Fig. 4, Table 2). In that year, high variability at cooler temperatures increased the rate to hatch, while high variability at warmer temperatures decreased this rate.

Morphology

Stage at hatching (eye development).—Variation in incubation time can result from both variation in differentiation rate and variation in the stage of maturity at which hatching takes place (truncation time; Bradford 1990; Warkentin 1999; Shine and Olsson 2003). In *B. orientalis* we used the transparency of the cornea, which proceeds very clearly in a dorsal to ventral wave, to define the state of differentiation at hatching in the context of the classical Gosner staging series (Gosner 1960; i.e., stage 20.25 characterized an eye with 25% transparency and 20.75 characterized an eye with 75% transparency). All hatching occurred between Gosner stages 19 and 21, with an overall average of 20.4 ($SD = 0.43$, $n = 365$). There was no significant difference among years ($F = 0.96$, $df = 1, 364$, $P > 0.30$; see Table 1).

The simple effect of temperature on stage at hatching varied between the two years, being negative in 1993 and positive in 2000 (temperature \times year interaction $F = 11.6$, $df = 1, 364$, $P < 0.001$). This complex outcome was due primarily to a changing balance of the positive effects of temperature on both the rate to hatching (Fig. 3) and the stage of eye development at the moment of hatching, countered by a negative effect of truncation of the incubation period on eye development (Table 2). Similarly, the simple effect of CV_{temp} on stage at hatching was negative and positive in 1993 and 2000, respectively, but this cannot be understood without considering the effects of mean temperature on the length of the incubation and subsequent effects on the stage of eye development at hatching. The direct effects of temperature are best understood in the context of our path model (Fig. 4, Table 2). Now, we also see that increasing egg size

TABLE 1. Means, standard deviations, and samples sizes for the phenotypic variables analyzed in this study. See the text for an explanation of the sample size.

Year	Time to hatch (days)	Eye development (Gosner stage)	Snout-vent length (mm)	Tail length (mm)	Tail height (mm)	Speed (cm/sec)	Probability of surviving
1993							
mean	4.19	20.3	5.15	3.56	2.01	6.02	0.67
SD	1.39	0.6	0.23	0.67	0.29	1.93	0.47
<i>n</i>	188	188	187	187	187	912	878
2000							
mean	3.95	20.4	5.26	3.31	2.09	5.08	0.65
SD	1.18	0.5	0.33	0.59	0.26	1.80	0.48
<i>n</i>	177	177	177	177	177	621	613

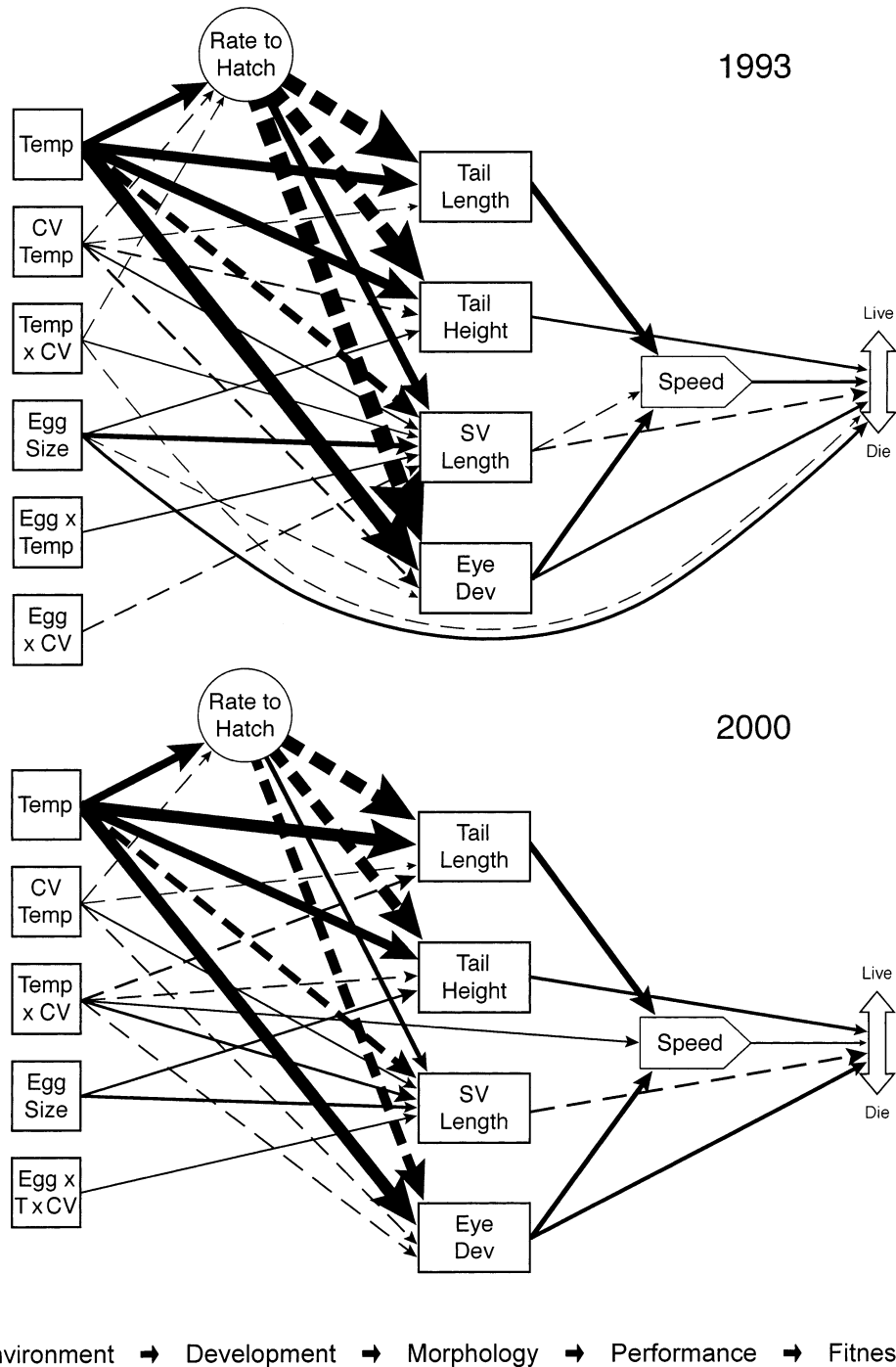


FIG. 4. Path analytic models for environmental effects on development, growth, sprint speed performance, and survival during each of the two years. Thickness of path shows magnitude of significant effects. Solid lines show positive effects, dashed lines show negative effects. Standardized regression coefficients for each path are presented in Table 2.

leads to a decrease in developmental stage in 1993 (Fig. 4, Table 2), with a similar but nonsignificant trend in 2000.

Linear measurements of size.—Temperature had a simple negative effect on SVL, tail length, and tail height after pooling both years (SVL, $F = 12.1$, $df = 1, 362$, $P < 0.001$; tail length, $F = 20.9$, $df = 1, 362$, $P < 0.001$; and tail height, $F = 24.1$, $df = 1, 362$, $P < 0.001$). The simplicity of these relationships, which is expected in poikilotherms, belies the

role of other environmental and developmental factors. In particular, development at higher temperatures increased both the length and height of the tail, but was associated with decreased SVL when all environmental factors were included in the model (Fig. 4, Table 2). The effects of temperature variability showed the opposite relationship (SVL positive and tail characteristics negative). The interaction of average incubation temperature with CV revealed similar effects, al-

TABLE 2. Standardized regression coefficients for the paths shown in Figure 3. Only coefficients that were significant at $P < 0.05$ are shown. Percentages are coefficients of determination for the sample size indicated (see text for further explanation).

Trait or effect	Rate to hatch		Eye maturity		Snout-vent length		Tail length		Tail height		Sprint speed		Survival	
	1993	2000	1993	2000	1993	2000	1993	2000	1993	2000	1993	2000	1993	2000
Egg size														
Temperature	0.95	0.984	-0.028	1.53	0.619	0.365	1.211	1.515	0.189	0.26			0.184	
CV temperature	-0.153	-0.16	-0.295	-0.14	0.969	-0.827	-0.127	-0.061	1.251	1.133				
Temp × CV temp	-0.14			-0.166	0.188	0.158		-0.279	-0.227		0.152			
Egg size × temp					0.155	0.28								
Egg size × CV temp					0.191									
Egg size × temp × CV temp					-0.186									
Rate to hatch	88.7%	83.8%	-2.21	-1.47	1.043	0.156	-1.87	-1.657	-1.821	-1.46				
Eye maturity			64.5%	42.6%	49.1%	52.7%	74.9%	54.6%	0.293	0.225	0.17	0.231	-0.123	-0.147
Snout-vent length											-0.075	0.386	0.149	0.186
Tail length											0.436		0.185	0.104
Tail height									69.6%	45.7%	48.7%	34.0%	22.4%	24.1%
Sprint speed													87.8	613
Survival	188	177	188	177	188	177	188	177	188	177	912	621	878	613
Sample size														

though the strength of this interaction varied between years. In general, higher daily variation had a more complex effect at warmer temperatures, as indicated by decreasing length and height of tail but increasing SVL.

Increasing egg size primarily increased SVL and to a lesser extent tail height but had little effect on tail length in either year. There were significant interactions between egg size and both average incubation temperature and CV with the year effect, resulting in differences in both two-way and three-way interactions (Fig. 4, Table 2).

Locomotory Performance

Tail length had a strong positive influence on sprint speed performance (Table 2). Furthermore, the more developed a larva was at hatching (as measured by developmental stage), the faster it swam, whereas the longer its SVL, the slower it swam (significantly so only in 1993; Fig. 4, Table 2). In addition to these direct effects of morphology on sprint speed performance, we explored the impact of environmental factors on sprint speed separate from the developmental and morphological traits that we measured. We only found one marginal effect of the average temperature × CV interaction in 2000 (Fig. 4, Table 2). Thus, most of the effects of the environment on sprint speed performance (after subtracting the effects of immediate test temperature during sprint trials; see Materials and Methods) are mediated directly through development and morphology, as suggested in Figure 1.

Survival

We tuned the experimental treatment (five predatory tadpoles per 1 L) so that the approximate probability of survival would be 0.5 (the actual value is closer to 0.65; Table 1). Sprint speed performance had a strong positive effect on the probability of survival (Fig. 4, Table 2). In addition to its effects on sprint speed, developmental stage also had a direct positive influence on survivorship, with more-developed larvae surviving significantly better than less-developed larvae at common swimming speed and morphology. Similarly, tail height had a direct positive influence on the probability of surviving predation, while SVL had an additional negative effect that goes beyond its direct negative effect on speed (Fig. 4, Table 2). In 1993, there were additional environmental effects on survival that were not explained by morphology and sprint speed performance. Egg size had a positive effect in that year and an average incubation temperature × CV interaction had slight additional negative effects on survivorship.

Predicted Outcomes

The overall picture of the influence of environment, development, and morphology on performance and fitness was remarkably consistent across the two years (Fig. 4, Table 2). By and large, not only did the paths have the same sign but they were also usually very similar in relative magnitude. To interpret the complex interactions underlying variation in all of these variables, we constructed a single overall statistical model pooling over both years and then used this model to illustrate larval developmental rates, morphology, sprint

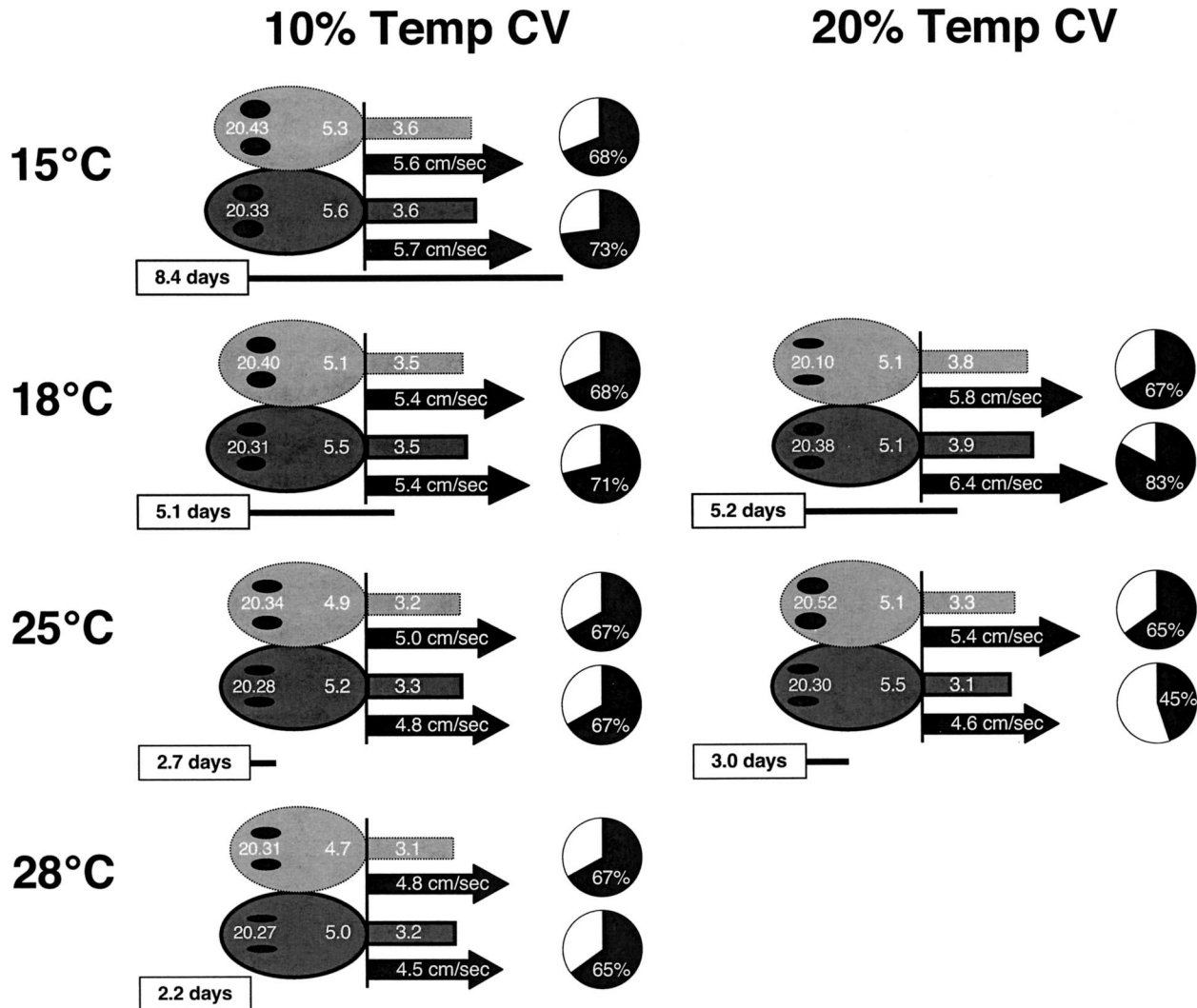


FIG. 5. Predicted developmental, morphological, sprint speed performance, and survival characteristics at six unique thermal combinations representing four constant temperatures and two levels of temperature variability, all to scale. Time to hatching is shown by the rectangular box and horizontal line (in units of days). The light shading shows individuals derived from small eggs, dark shading shows those derived from large eggs. Length of the oval shows average snout-vent length (mm), and length of the rectangles shows average tail length (mm). The eyes get proportionally narrower with decreasing Gosner stages (actual stage indicated numerically between the eyes). The arrow depicts sprint speed in centimeters per second, and the pie diagram shows the probability of surviving predation in black. (See Table 2 for approximate prediction coefficients and the text for further explanation.)

speed performance, and survivorship across several critical combinations of temperature, temperature variability, and egg size (Fig. 5). It is evident that survivorship did not vary much across temperatures when the overall level of variability in temperature was low ($CV = 10\%$). However, substantial differences in survivorship were generated when large and small egg individuals were raised in hot and cold ponds with high levels of temperature variability. In particular, larvae from large eggs perform and survive markedly better in cold, high-variability ponds than in any other combination of environmental variables. In stark contrast, these same individuals perform poorly and suffer from much higher mortality when raised in hot ponds with high thermal variability. These differences in sprint speed performance and survivorship now can be directly ascribed to the changes in morphology and developmental stage at hatching—in partic-

ular to the difference in the relationship between SVL and tail length in large and small egg individuals in these environments (Fig. 5).

DISCUSSION

Complex interactions between variable environments and development are to be expected but have only recently become the focus of studies meant to elucidate factors that promote or hinder the evolution of phenotypic plasticity (e.g., Halkett et al. 2004; Stillwell and Fox 2005). With an amphibian model, we focused on differences in the variability of both the thermal environment during early development and its interaction with variability in ovum size, a maternally transmitted environmental effect (Dziminski and Alford 2005; Dziminski and Roberts 2005). We used both forms of

naturally occurring variation to investigate the influence of environment on phenotypic variation and subsequent larval performance and natural selection. In the case of thermal variation, we used naturally occurring diel variability and a response surface experimental design. We combined this in the case of ovum size with a naturally occurring but statistically engineered ANOVA approach (Benedetti-Cecchi 2005; Inouye 2005), thereby increasing the power to detect environmental interactions during early development (e.g., Sinervo and Huey 1990; Wade and Kalisz 1990) and our understanding of the developmental/ecological context in which selection is occurring.

Survivorship in a Variable Environment

Temperature has ramifications for all biological systems, especially during early ontogeny (Hochachka and Somero 2002). The role of average temperature over extended periods of early development has been more extensively investigated than that of fluctuations in temperature. But, increasing attention is currently being given to daily thermal variability in the field, and it is not surprising to find important effects of variability at every level of biological organization from gene expression (e.g., Podrabsky and Somero 2004) to life-history traits (e.g., Shine and Elphick 2001; Ashmore and Janzen 2003). The potentially complex interactions between temperature variation and egg size investigated here have ramifications that can cascade across the entire developmental system on into performance and fitness. If we collapse causation across all levels and simply look at the relationship between the environmental variables and fitness, a striking pattern emerges (Fig. 6). Larvae that hatched from small eggs survived predation best if they developed in environments that tended to be warm (26–28°C) and of relatively high thermal variability (CV = 14–16%; Fig. 6B). In contrast, larvae that hatched from large eggs had higher survival rates in relatively moderate environments, but showed a marked decline in survivorship under increased temperature and temperature variability (Fig. 6A; see also Kaplan 1992). We can readily visualize this most striking interaction involving average temperature, temperature variation, egg size, and survival as the crossing of the two fitness surfaces in Figure 6C.

Variation in diel temperature was largely driven by diurnal variation, but also by weather patterns, with sunny weather increasing the CV in temperature. A likely interpretation of the above results is that increased thermal variation at low average temperatures (about 17°C) tends to increase the time spent in warmer favorable growth environments, while increased variation at high average temperatures (about 26°C) tends to increase sojourns into critically high temperature ranges at which developmental processes become disrupted, especially for individuals that developed from large eggs. Large eggs, with their accompanying larger yolk reserve (a major part of the SVL) at this stage of development, seem to possess a liability in such environments. Diminished oxygen delivery to the dorsal side of the gut and overlying tissues running along the midline of the peritoneal cavity could be one possible result of this inert gut yolk. Another possibility could be thermal inertia resulting in a locally increased heat exposure as a result of the insulating properties

of yolk fat. These possible fine-scale and asymmetrical effects of thermal variation can produce deviations in variable environments beyond, or even separate from, those predicted by the response to average temperatures (e.g., Ruel and Ayres 1999). Further study will be required to sort out these alternatives (see also Mitchell and Seymour 2000, 2003).

Effects on later fitness.—We only expect these fitness surfaces (Fig. 6) to apply during the first 24 h after hatching, when overall predator susceptibility is high and overall levels of locomotion are low. After 24 h, continued post-hatching development temporally moves the larvae into a stage where they are no longer susceptible to predation by other tadpoles (Parichy and Kaplan 1995), and the fitness landscape should change dramatically as this important selective factor changes. In this system, the next predatory hurdle is posed by salamander larvae (Hynobiidae), which sit and wait for attractive prey.

Building the Paths from the Ontogenetic Environment to Fitness

The complex relationship between environmental variation and fitness illustrated in Figure 6 can be more fully understood by decomposing the intermediate steps through which: variation in survivorship stems from sprint speed performance, variation in sprint speed performance stems from morphology, and variation in morphology (and phenotype) is driven by the environment as mediated by the developmental system (Fig. 1).

Fitness and performance (sprint speed).—Locomotor capacity has long intrigued students of adaptation, especially in light of the near codification of a theoretical framework known as the performance paradigm (reviewed by Irschick and Garland 2001). Although a recent call for undertaking studies within the ecological context of the organism is well founded (e.g., Irschick 2003), we also feel it fundamental to consider the ontogenetic environmental history that precedes the actual moment that fitness is measured (Podolsky 2003). Toward this goal, the first variable moving back from fitness in our integrative model (Fig. 1) is a traditional performance measure, sprint speed, as influenced by rearing temperature. In Figure 5 we can see the predicted decrease in speed at increasing average rearing temperatures when the CV in rearing temperature is 10%. We can also see the change in sprint speed interacting with average temperature and egg size when the CV is 20%. This interaction pattern parallels that of survival discussed above and can be seen by comparing the simple relationship between the survival pie diagrams and the arrows depicting sprint speed in Figure 5. In particular, increased maternal investment through egg size can decrease sprint speed performance in some environments (Parichy and Kaplan 1995), yielding reduced survivorship.

Other prey characteristics, such as neuromuscular physiologically related response and recovery times and maneuverability, should also play a role in both swimming performance and survival. In fact, our model (Fig. 4) illustrates that other direct effects of measured variables, such as the stage of eye development (which we expect to be related to overall neuromuscular physiological capacity) as well as tail height and SVL (which we expect to play a role in maneu-

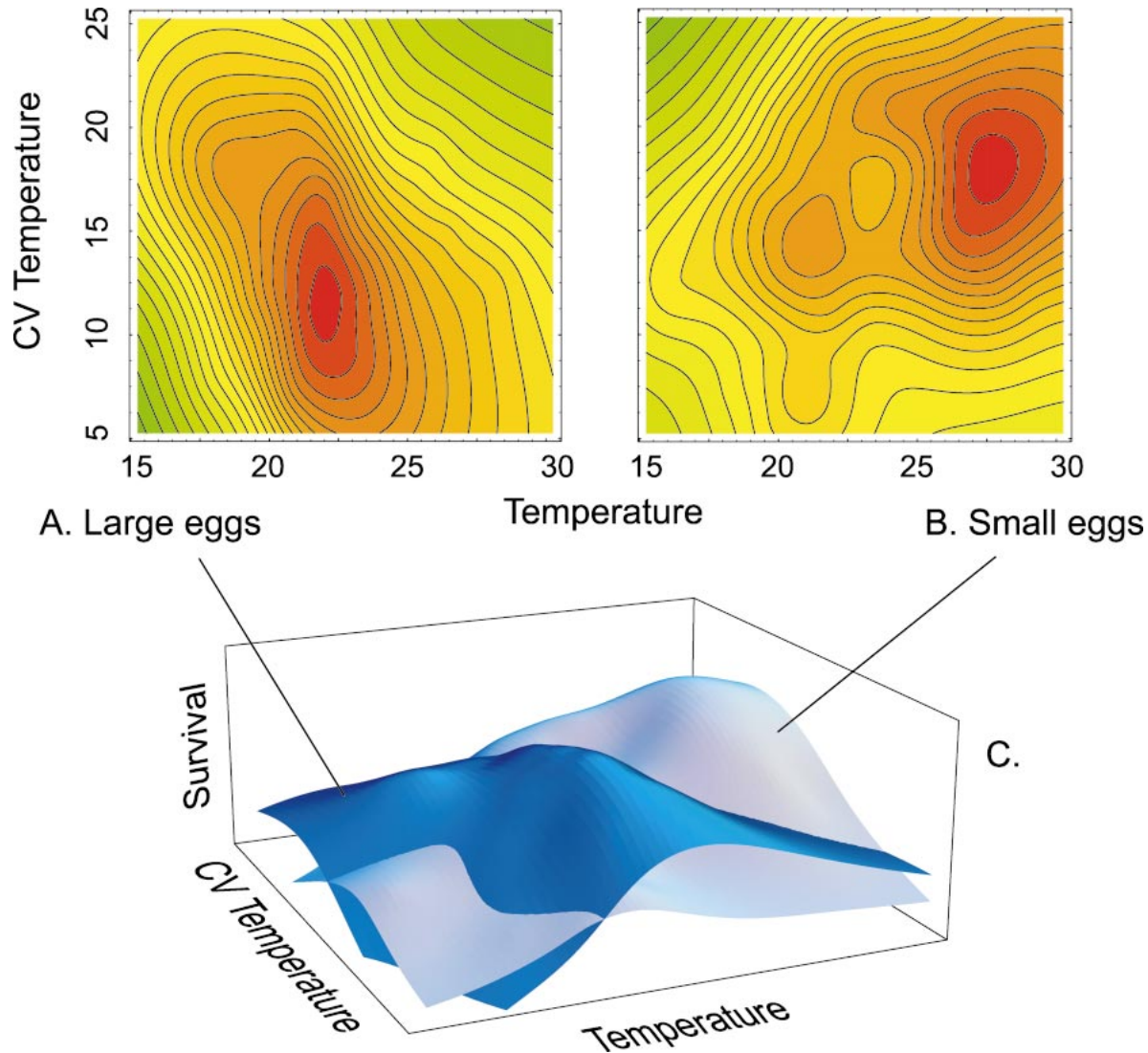


FIG. 6. The relationship between survival and temperature (x-axis) and coefficient of variation of temperature (y-axis) for (A) large eggs and (B) small egg size treatments illustrated as separate contour plots. Each plot shows the results of a cubic spline fit of survivorship data across all ponds, with each contour line representing an approximately 3.5% change in the probability of survival (red highest and green lowest). (C) Three dimensional representation of the fitness surfaces for both egg size classes that allows for a direct comparison of the influence of egg size on the response to variation in the thermal environment. Note that small eggs do best in hotter, more variable environments, whereas large eggs do best in somewhat cooler, less variable environments. The functional bases of these differences are illustrated in Figure 5.

verability), have direct effects on survival independent of their influence on sprint speed performance (also see Fitzpatrick et al. 2003). In addition, unmeasured variables that are influenced by the environment can also contribute to survival, as evidenced by the several paths leading from environment directly to survival in Figure 4 (e.g., 1993).

Sprint speed and morphology.—In keeping with the general morphology/performance paradigm (Fig. 1; Arnold 1983), much of the variation in sprint speed can be explained directly by morphological variation. Indeed tail length variation is the main explanatory factor (see also Wilson and Franklin 2000). Here, however, variation in morphology is generated in nonobvious ways via interactions among environmental features. For example, focusing on tail length in Figure 5, predicted tail length decreases with an increase in average

temperature at low levels of thermal variation ($CV = 10\%$), whereas at high levels of thermal variation ($CV = 20\%$) there is an additional nonadditive effect of average temperature and egg size. This result is made obvious by observing the predicted shorter tail in individuals that developed from large eggs at 25°C relative to the longer tail in individuals that developed from large eggs at both 25°C and 28°C at the lower level of thermal variation ($CV = 10\%$). There are similarly complex relationships involving SVL, which is strongly influenced by egg size (Table 2) but also has negative effects on sprint speed and survival (Fig. 4). Longer or shorter tails, relative to overall body size, lead to decreased sprint speed, which in turn is correlated with survival in the predatory trials.

Timing of developmental events.—Part of the variation in

morphology and performance observed here is generated by developmental timing—not as a pure rate phenomenon, but as a discrete developmental transition. In our case, there can be variation in the actual hatching event after the attainment of hatching competence (Bradford 1990; Martin 1999; Laurila et al. 2002a). In amphibians, varied factors can be clumped into a broad category of stress-induced hatching, which can generate potentially large differences in hatching times partially decoupled from other developmental processes. Inducers include oxygen concentration (e.g., Petranka et al. 1982; Bradford and Seymour 1988; Mills and Barnhart 1999; Warkentin 2002), direct contact with predators (Warkentin 1995), indirect contact with predators (Chivers et al. 2001), and fungal attacks (Warkentin et al. 2001). Thermal stress that results from diel variation would fall into this category. At the average pond temperature of approximately 20°C (characteristic of the field site) changing the level of variation has no effect on either development time to or SVL at the hatching stage. At cooler average temperatures, increasing exposures to high temperature accelerates development and is associated with a decreased SVL. This would be characteristic of a heat-stress-induced hatching event. Above approximately 20°C, increasing exposure to high temperatures can result in a deceleration of development and hatching with a larger SVL at an earlier stage. This latter result could be the outcome of temperature exceeding the thermal tolerance of the embryo and would be expected to occur more frequently in embryos that developed from larger eggs and in the warmer year of 2000. Further study will be necessary to combine an understanding of specific developmental processes, such as the response to thermal stress, with the ecological setting in which those processes are expressed.

Incorporating the Environment into Selection Studies

The approach used here differs somewhat from previous methods devised to assess the strength of selection on patterns of phenotypic plasticity (Weis and Gorman 1990; Scheiner and Callahan 1999). These methods are based on measuring the strength of selection operating directly on the norm of reaction. As such, they require experimental designs in which families, clones, or inbred lines can be split and monitored across environments. The advantage with the Scheiner and Callahan (1999) approach is that the norm of reaction is explicitly incorporated into the overall path model, including the ability to study the consequences of varying the probability of encountering different environments. However, this relies on a coarse granularity of the environment such that different paths are fitted within specific environments. Thus, in addition to a simplified experimental design, an advantage of the method described here is the ease with which different environmental factors can be included in the model and, especially, the ability to view downstream phenotypic and fitness responses as direct functional consequences of environmental effects (Fig. 1). Both approaches are ultimately complementary to one another. In addition, although several authors have advocated measuring selection directly on genotypes or breeding values to eliminate the influence of unmeasured environmental factors (Rausher 1992; Stinchcombe et al. 2002), these approaches actually estimate the evolutionary response to selection rather than the natural selection itself, which is dependent on the entire range of phenotypic variation, not just that underlain by genetic variation (Lande and Arnold 1983). When possible, it is much more powerful to include environmental factors that might promote phenotypic covariances among traits and fitness, as was done here, although unmeasured environmental factors still have the potential to obscure the results. Ultimately, incorporating genetic, environmental, developmental, and phenotypic data into the overall analysis will provide the clearest insights into the evolution of environmentally labile complex traits.

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Conclusion

Although there have been renewed calls for integrating ecological information into developmental systems (e.g., Gilbert 2001), evolutionary ecologists have long studied the role of environmental variation in generating phenotypic plasticity throughout development. What is different now is the hope that more specific developmental mechanisms can be incorporated into the more general picture. Similarly, functional morphologists and physiological ecologists have increasingly sought to couch their analyses in terms of the fitness consequences of morphological and physiological variation. A fully integrated approach includes connections among all of these potential levels of causation (Fig. 1). In this study, the significant fitness consequences of the interaction between thermal variation and maternal investment can best be understood functionally in terms of the environment's influence on morphology and larval swimming performance (Figs. 5, 6). Similarly, the central role of these environmental factors illustrates how difficult it would be to understand individual variation in morphology and performance outside of the proper ecological context. Yet to be determined is how genetic variation and covariation among these characters influences the evolutionary response to this selection. The complex interplay between temperature variation, egg size, morphological development, swimming performance, and survivorship demonstrated here illustrates how each level in this temporal hierarchy can critically inform our understanding of the role of factors at other levels.

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