Microgeographic variation in the effects of larval temperature environment on juvenile morphology and locomotion in the pool frog

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Keywords
complex life cycles; locomotor performance; metamorphosis; temperature; population differentiation.

Abstract
In animals with complex life cycles, the environment experienced early during the development may have strong effects on later performance and fitness. We investigated the intraspecific variation in the effects of larval temperature environment on the morphology and locomotory performance of juvenile pool frogs *Rana lessonae* originating from three closely located populations of the northern fringe metapopulation in central Sweden. Tadpoles were raised individually at two temperatures (20 and 25 °C) until metamorphosis. We measured the morphology of the metamorphs and tested the jumping performance of the froglets after complete tail absorption. We found that early temperature environment affected juvenile morphology, metamorphs from high-temperature environments having relatively longer hindlimbs (tibiofibulas) and longer tails when weight at metamorphosis was accounted for. In absolute terms, froglets from low temperature jumped significantly longer; however, after correcting for size differences the relationship was reversed, individuals raised at high temperature performing better. In both temperatures, relative jumping performance was positively associated with tibiofibula and body length. Populations differed both in metamorphic traits and in jumping capacity, especially at low temperature, suggesting microgeographical variation in temperature sensitivity within the metapopulation. Our results indicate that the temperature environment experienced during the early aquatic stages can influence the morphology and performance of juvenile frogs, and that these effects can be population specific.

Introduction
Complex life cycles consisting of discrete life stages are a common strategy in the animal kingdom, which allow the organisms to exploit different habitats during their ontogeny (Wilbur, 1980). The habitat shift during the ontogeny is often associated with metamorphosis, an abrupt change in the physiology, morphology and behaviour (Wilbur, 1980; Moran, 1994). While metamorphosis has traditionally been viewed as a way to decouple the phenotypes developed during different parts of the life cycle (Podolsky & Moran, 2006), evidence for significant carryover effects of the early environmental conditions to the later stages is now accumulating (Pechenik, 2006). Examples of coupling between life-history stages include the effect of larval growth conditions on juvenile growth rate in gastropods (Pechenik, Jarrett & Rooney, 2002), the impact of larval feeding on juvenile performance in crustaceans (Emlet & Sadro, 2006), the influence of larval growth conditions on the juvenile growth and survival in fish (McCormick & Hoye, 2004) and the effect of early predation environment on later growth and performance in amphibians (Relyea & Hoverman, 2003; Orizaola & Braña, 2005; Nicieza, Álvarez & Atienza, 2006).

In ectotherms, development and growth are highly dependent on the temperature, individuals growing slower and maturing later, but attaining a larger size, at lower temperatures (Atkinson, 1994, 1996; Angilletta, Steury & Sears, 2004). In species with complex life cycles, early temperature environment may influence the physiological and developmental mechanisms, and may have a strong effect on the later performance of the individual. For example, high temperature during the embryogenesis leads to a smaller size at hatching, lower larval growth rates and a smaller size at metamorphosis in crustaceans (reviewed in Giménez, 2006), whereas in insects high pupal temperature leads to an increased development rate and an altered adult phenotype (Chakir et al., 2002; Stevens, 2004). However, although there is ample evidence for intraspecific variation in temperature effects within life stages (see e.g. Conover & Schultz, 1995), few studies have addressed intraspecific variation in temperature response across different life stages.

The typical amphibian life cycle contains aquatic embryonic and larval stages and terrestrial juvenile and adult stages...
stages (Wilbur, 1980). Environmental conditions during the aquatic stage (e.g. temperature, competition and predation) can have a strong effect on the timing of and size at metamorphosis, and may influence juvenile growth and performance, as well as adult fecundity and maturation (Semlitsch, Scott & Pechmann, 1988; Altwegg & Reyer, 2003; Nicieza et al., 2006). As in other ectotherms, in amphibians, higher temperature leads to early metamorphosis at a smaller size, whereas lower temperature leads to later metamorphosis at a larger size (Atkinson, 1994, 1996). Consequently, environmental temperature experienced by larvae may influence the later stages of the amphibian life cycle, affecting, for example, juvenile locomotion and thus survival. However, studies on early temperature effects on later performance in amphibians have been few (Blouin & Brown, 2000; Alvarez & Nicieza, 2002; Walsh, Downie & Monaghan, 2008a) and have focused on single populations. Other studies have shown that temperature conditions can vary significantly among closely located ponds (Skelly, 2004; Orizaola & Laurila, 2009), and that embryonic and larval stages of amphibians can adapt to the local temperature conditions at a microgeographic scale (Freidenburg & Skelly, 2004; Skelly, 2004).

This study examines the effects of environmental temperature during the larval stage on early juvenile morphology and locomotor performance in three closely located subpopulations of the Swedish pool frog *Rana lessonae* metapopulation in a laboratory common garden experiment.

**Materials and methods**

*Rana lessonae* lives in most of continental Europe from southern France and Volga river basin to the Baltic Sea. In addition, isolated populations exist in both southern Norway and central Sweden (Zeisset & Beebee, 2001; Snell, Tetteh & Evans, 2005). Our study populations belong to the central Swedish *R. lessonae* metapopulation situated at the northern fringe of the species’ distribution (Sjögren, Elmingberg & Berglind, 1988; Sjögren, 1991; Zeisset & Beebee, 2001). *Rana lessonae* is one of the most warmth-demanding amphibian species in Europe (Sinsch, 1984; Sjögren et al., 1988), and temperature effects on juvenile performance are especially relevant for this severely time-constrained high-latitude population (Sjögren, 1991). Start of reproduction requires high water temperatures (>16°C; Sjögren et al., 1988), and breeding in our populations does not usually commence until late May (Sjögren et al., 1988). As *R. lessonae* cannot overwinter at the larval stage (Sjögren et al., 1988; G. Orizaola & A. Laurila, pers. obs.), the larvae have to metamorphose in late summer before the water temperature falls below levels that prevent further development. Consequently, the Swedish metapopulation is exposed to strong developmental constraints, and the timing and conditions of the metamorphosis are expected to have a strong effect on the growth and survival of the terrestrial juveniles.

Freshly laid egg clumps were collected in the spring of 2005 in three localities in Central Sweden (Tierp municipality), situated within a range of about 5 km. We collected parts of these in three ponds: 15 clumps in Björkfjärden (60° 29’N, 18° 0’E, henceforth BJ), 10 in Klungsten (60° 32’N, 18° 1’E, henceforth KL) and 12 in Klungsten Hamn (60° 32’N, 18° 1’E, henceforth K LH; see Orizaola & Laurila, 2009 for more details). The localities were selected from a larger number of populations investigated in a previous study (G. Orizaola et al., unpubl. data) representing the range of thermal conditions experienced by *R. lessonae* in the study area. BJ has the warmest temperatures at the onset of the breeding season, but later in the season the temperature is similar to that in KL. KLH pond has consistently the lowest and the most variable temperature of the three populations (e.g. c. 2°C lower than BJ; Orizaola & Laurila, 2009). Eggs were brought to the laboratory, and 2 weeks after hatching, eight randomly chosen larvae from each family were placed individually in 1 L plastic containers in each of two experimental temperatures: 20°C (low temperature) and 25°C (high temperature). These temperatures are within the normal range in natural ponds (water temperature range in the localities in June–August 2007: 10.4–27.8°C; Orizaola & Laurila, 2009). To account for temperature variation within the treatments, the experiment was divided into eight and four blocks in low- and high-temperature treatments, respectively, and at least one individual from each family was present in each of the blocks. Every third day, water in the vials was changed and food (chopped boiled spinach) was added ad libitum. The photoperiod was set at 18 h light:6 h dark, corresponding to the conditions in the area at that time. For more details about the larval experiment, see Orizaola & Laurila (2009).

Containers were checked daily for the metamorphs. At metamorphosis, determined by the emergence of forelegs (stage 42, Gosner, 1960), individuals were weighed to the nearest 0.1 mg with a digital balance. A dorsal image was taken with a digital camera and later analysed with image analysis software to examine metamorph morphology. Four morphological dimensions were measured from the dorsal view: body length, tail length, head width and tibiofibula (hindlimb) length. The metamorphs were returned to the experiment until they had completed tail reabsorption (Gosner stage 46). Consequently, our estimates on the effects of the temperature environment during the larval period also include the period of tail reabsorption. This is in accordance with the natural situation as the juveniles do not leave the pond before completion of tail absorption (G. Orizaola & A. Laurila, pers. obs.). The vials were filled only to a depth of c. 0.5 cm to avoid drowning of the metamorphs, and a small stone was provided for resting.

The locomotor performance was examined at the same day the juveniles reached Gosner stage 46 by measuring the jumping capacity of each individual. This trait was selected as a measure of individual fitness because of its influence on juvenile survival via effects on food acquisition (Walton, 1988) and escape ability from predators (Wassersug &
Jumping was tested at 20 °C, a temperature within the range of optimal performance for frogs (Rome, Stevens & John-Alder, 1992), after a 6-h acclimatization period. The froglet was removed from its vial and placed individually in a small Petri dish with a neutral food dye positioned in a linear test track (120 × 60 cm) with plastic walls (height 7 cm) and the floor covered with white paper. After a few seconds, the froglet was gently prodded on the urostyle with a pen to induce jumping. The froglets were induced in the same way to jump until they reached the end of the test track. Using this protocol, the individuals left colour marks in the paper at the beginning and at the end of each jump, allowing the measurement of each individual jump. Jumping performance was examined by scoring the maximum jump distance recorded for each individual from two jump series recorded with a 1-hour interval. After the tests, juveniles were weighed again to the nearest 0.1 mg.

**Statistical analyses**

We used a nested block design, where the terms population and temperature treatment were considered as fixed effects and the effects of family (nested within the population) and experimental block (nested within the temperature treatment) as random effects. Interactions between fixed and random effects were considered as random effects. Population was considered as a fixed factor as they were selected on the basis of a previous study to represent the range of thermal conditions experience for the species in the area (G. Orizaola et al., in prep.). Linear mixed models with restricted maximum likelihood estimation procedures were used to examine the effect of the different factors on juvenile morphology and jumping performance using the SPSS 13.0 software package (SPSS, 2004). Morphological traits were analysed accounting for size, using metamorph (stage 42) weight as a covariate. Phenotypic correlations between the maximum jump distance and body and tibiofibula lengths were estimated as Pearson’s product–moment correlations, both with absolute values and when controlling for weight at stage 46. Deviation from normality was tested with Shapiro-Wilk tests. The significance level was set at α = 0.05 for all tests.

**Results**

Absolute body length was strongly affected by temperature treatment (P<0.001), high temperature leading to a shorter body length, but no differences were detected among populations (P = 0.881; Fig. 1a). The analysis of size-independent morphology showed that temperature treatment had no overall effect on relative body length at metamorphosis (Table 1). However, there was some indication of heterogeneity among the populations (Fig. 1b). BJ individuals had relatively longer bodies than the other two populations at low temperature (Tukey HSD test, P<0.029), but body length did not differ among populations at high temperature (P>0.211). Relative tail length was strongly influenced by rearing temperature, with metamorphs having 5–10% longer tails at high temperature (Table 1, Fig. 1d). There were also differences among populations; however, a significant population × temperature interaction indicated that variation was not uniform across the temperatures (Table 1, Fig. 1d). BJ had longer tails than the other populations at both temperatures; KLH individuals metamorphosed with longer tails than KL at low temperature whereas the populations did not differ at high temperature. Relative head width did not differ among populations, and was not influenced by temperature (Table 1, Fig. 1c). Temperature affected relative tibiofibula length, individuals having 2–7% longer tibiofibulas at high temperature (Table 1, Fig. 1e). However, a significant population × temperature interaction showed that whereas only BJ and KL differ in tibiofibula length at low temperature (P = 0.024; Table 1, Fig. 1e), at high temperature both KL and KLH populations had longer tibiofibulas than BJ (P>0.05; Fig. 1e). Significant family effects were found only for tail length, suggesting within-population genetic variation for this trait although differences in non-genetic maternal effects cannot be excluded (Table 1).

Absolute jumping performance was significantly lower for individuals reared at high temperature (Table 2, Fig. 2a). A significant population × temperature interaction indicates that BJ individuals tended to have a higher maximum jump length at low temperature than the other two populations (P<0.05); however, at high temperature there were no differences (P>0.211; Table 2, Fig. 2a). As locomotor performance may depend on body size, we repeated the analysis using weight at stage 46 as a covariate. We found a significant effect of rearing temperature on jump length (Table 2); however, the size-corrected maximum jump length was now higher for individuals reared at high temperature (Fig. 2b). Differences among populations as well as a significant population × temperature interaction were also found for the relative jump length (Table 2). BJ juveniles again had longer jumps at low temperature than the other populations (P<0.001), whereas there were no differences among the populations at high temperature (P>0.128). In KL and KLH populations, jump length was higher for larvae reared at high temperature than at low temperature (P<0.040), while no differences were observed for BJ (P = 0.225; Fig. 2b). No family differences or family × temperature interactions were detected (Table 2).

Survival during metamorphosis was almost twice as high at high than at low temperature (F1,492 = 74.59, P<0.001; Fig. 1f). There were significant differences among the populations in survival (F2,59.59 = 4.07, P = 0.022); however, the significant population × temperature interaction indicated that this was due to higher survival in BJ at low
temperature, whereas there were no differences at high temperature ($F_{2,492} = 5.19, P = 0.006$; Fig. 1f).

Phenotypic correlations between maximum jump distance and body and tibiofibula lengths showed overall a positive and significant relationship between the traits, both in low- and in high-temperature environments when body weight was not taken into account ($P < 0.006$). After controlling for weight at stage 46, correlations remained positive overall, but when considering data within populations a significant correlation was found only for BJ individuals reared at low temperature (body length: $r_p = 0.41$, tibiofibula length: $r_p = 0.38$, $P < 0.001$; $n = 75$). The correlations were also positive in the other two populations also, but remained insignificant possibly due to the lower statistical power in these populations (e.g. at low temperature, post hoc power test for $r_p = 0.41$ was 0.29 for KLH and 0.35 for KL).

**Discussion**

In organisms with complex life cycles, conditions experienced in early life-history stages can have strong effects on individual fitness later during the ontogeny. In our study,
Table 1: Linear mixed model tables showing the effects of population, family, temperature treatment and block on several morphological traits (body, tail and tibiofibula length and body width) of *Rana lessonae* metamorphs

<table>
<thead>
<tr>
<th>Trait</th>
<th>Fixed effects</th>
<th>Random effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body length</td>
<td>Population 2</td>
<td>Var ± SE</td>
</tr>
<tr>
<td></td>
<td>Temperature 1</td>
<td>Z</td>
</tr>
<tr>
<td></td>
<td>Weight 1</td>
<td></td>
</tr>
<tr>
<td>Tail length</td>
<td>Population 2</td>
<td>Var ± SE</td>
</tr>
<tr>
<td></td>
<td>Temperature 1</td>
<td>Z</td>
</tr>
<tr>
<td></td>
<td>Family 0.001</td>
<td></td>
</tr>
<tr>
<td>Head width</td>
<td>Population 2</td>
<td>Var ± SE</td>
</tr>
<tr>
<td></td>
<td>Temperature 1</td>
<td>Z</td>
</tr>
<tr>
<td></td>
<td>Family 0.001</td>
<td></td>
</tr>
<tr>
<td>Tibiofibula length</td>
<td>Population 2</td>
<td>Var ± SE</td>
</tr>
<tr>
<td></td>
<td>Temperature 1</td>
<td>Z</td>
</tr>
<tr>
<td></td>
<td>Family 0.001</td>
<td></td>
</tr>
</tbody>
</table>

Weight at metamorphosis was used as a covariate in the analyses.
Var, variance component estimate; d.f.N, numerator degrees of freedom; d.f.D, denominator degrees of freedom.
embryonic and larval amphibians suggest that populations can adapt to the local temperature conditions at a microgeographic scale (Freidenburg & Skelly, 2004; Skelly, 2004), but we are not aware of such studies conducted at the juvenile stage. Nevertheless, the differences found among the populations in locomotor performance indicate that the effect of thermal conditions experienced during the larval period can be population dependent in amphibians, and future studies should attempt to quantify the mechanisms, as well as the possible adaptive value of such differences.

The tail reabsorption stage was associated with significantly higher juvenile mortality at low than at high temperature. Higher mortality at a low environmental temperature could be due to developmental or physiological constraints and individuals exposed to low temperature may experience higher developmental stress (Smith-Gill, 1983). The higher survival at 25°C is likely to reflect the importance of relatively high temperatures in *R. lessonae* (Sinsch, 1984).

Several studies have examined the effects of larval environment (i.e. predation, competition and desiccation) on the morphology of juvenile frogs (e.g. Blouin & Brown, 2000; Relyea, 2001; Van Buskirk & Saxer, 2001; Relyea & Hoverman, 2003; Nicieza et al., 2006; Richter-Boix, Llorente & Montori, 2006). However, the effect of environmental temperature during the larval stage on juvenile morphology has remained unexplored. Absolute body length was affected by temperature in all the studied populations, larvae being larger at low temperatures, which is a common rule for ectotherms (Atkinson, 1994, 1996). While we did not find significant temperature effects on the relative body length or head width, we detected relatively longer tails and tibiofibulas in metamorphs from high-temperature treatments. In addition, individuals from BJ had longer tails at both temperatures, and especially KL and KLH individuals had longer legs at high than at low temperature. These two traits may directly influence the duration of tail absorption and juvenile locomotion. A longer tail is correlated with a longer time to complete metamorphosis and to adopt a full juvenile morphology (Downie, Bryce & Smith, 2004), although it may also be associated with a smaller amount of weight loss during metamorphosis (Walsh, Downie & Monaghan, 2008); G. Orizaola and A. Laurila, in prep.). Several studies have reported a negative relationship between larval period and relative hindlimb length (Emerson, 1986; Blouin & Brown, 2000; Relyea & Hoverman, 2003; Nicieza et al., 2006; M. Tejedo, pers. comm.; but see Relyea, 2001; Ficetola & De Bernardi, 2006). Our results are in accordance with those studies as we found longer tibiofibulas at high temperature, conditions under which the duration of the larval period was shorter (Orizaola & Laurila, 2007).

**Table 2** Linear mixed model table showing the effect of population, family, temperature treatment and block on jumping performance for *Rana lessonae* juvenile frogs, as absolute values and when accounting for individual weight (weight at stage 46 used as a covariate in the analysis).

<table>
<thead>
<tr>
<th>Fixed effects</th>
<th>Maximum jump</th>
<th>Relative maximum jump</th>
</tr>
</thead>
<tbody>
<tr>
<td>Population</td>
<td>2</td>
<td>44.00</td>
</tr>
<tr>
<td>Temperature</td>
<td>1</td>
<td>20.57</td>
</tr>
<tr>
<td>Population × temperature</td>
<td>2</td>
<td>48.11</td>
</tr>
<tr>
<td>Weight</td>
<td>1</td>
<td>336.03</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Random effects</th>
<th>Var ± SE</th>
<th>Z</th>
<th>P</th>
<th>Var ± SE</th>
<th>Z</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Block (temperature)</td>
<td>57.52 ± 36.51</td>
<td>1.58</td>
<td>0.1152</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Family (population)</td>
<td>33.78 ± 42.34</td>
<td>0.80</td>
<td>0.4250</td>
<td>13.56 ± 28.62</td>
<td>0.47</td>
<td>0.6357</td>
</tr>
<tr>
<td>Fam. (pop.) × temp.</td>
<td>67.08 ± 59.81</td>
<td>1.12</td>
<td>0.2621</td>
<td>33.41 ± 40.01</td>
<td>0.83</td>
<td>0.4038</td>
</tr>
</tbody>
</table>

**Figure 2** Effects of rearing temperature on (a) absolute jumping performance and (b) relative jumping performance, expressed as least square (LS) means ± 1 SE of maximum jump length. BJ, Björkfjärden; KL, Klungsten; KLH, Klungsten Hamn.

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2009). As thyroid hormones regulate both metamorphosis and leg development, individuals with high growth and early metamorphosis could be exposed to higher concentrations of thyroid hormones during a shorter time period, consequently inducing the development of relatively longer limbs (Emerson, 1986).

In general, differences among populations detected in morphology and juvenile performance were not as large as those found in larval life-history traits in the same populations (Orizaola & Laurila, 2009), suggesting that compensatory mechanisms occurring after metamorphosis may reduce the differences among local populations. Knowledge on the role of compensatory mechanisms after metamorphosis is crucial for understanding larval life-history and morphological variation in amphibians. Field studies on *R. lessonae* have not found evidence for compensation for small metamorphic size by enhanced juvenile growth, and hence even small size differences at metamorphosis can have significant effects on survival (Altwegg & Reyer, 2003). Our results show that the conditions experienced early on in development may influence individual performance during the later stages and emphasize the need for more integrative studies across life stages.

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