Northern natterjack toads (*Bufo calamita*) select breeding habitats that promote rapid development

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Abstract

It is generally believed that harsh climate inhibits growth and development of ectotherms at high latitudes. However, this environmental effect may be counterbalanced by countergradient genetic variation and habitat selection. While there is laboratory evidence for genetically based increases in growth and development rates in amphibians living at high latitudes, it remains unclear how environmental and genetic effects combine in the field to produce gradients of phenotypic variation. We performed a field study on the growth and development of tadpoles and habitat selection of breeding adults of the natterjack toad (*Bufo calamita*) in Denmark and in Estonia where the average onset of breeding differs by 12 days. Although growth and development of Estonian tadpoles trailed Danish conspecifics during the first half of the larval period, Estonian tadpoles caught up in both body length and developmental stage to Danish tadpoles in the last third of the larval period. Breeding ponds in Denmark were significantly larger and deeper than in Estonia, however, the water temperatures in the ponds did not vary substantially between the countries. In Estonia the toads selected shallower breeding ponds with higher temperature and oxygen concentrations, suggesting, that at higher latitudes active selection of specific water bodies ensures faster growth and development of larvae. The results, together with previous laboratory evidence, indicate that amphibian distribution limits are shaped both by adaptive differences in developmental rates and behavioural plasticity. To cope with harsh climates, amphibians in the north apparently require shallow water bodies — a habitat particularly vulnerable to anthropogenic degradation.

1. Introduction

Direct physiological effects of ambient temperature on ectotherms have been relatively well studied but their ecological and evolutionary consequences are still debated (Angilletta et al., 2004). An intuitive expectation that, in colder regions, ectotherms grow slower and attain smaller body sizes is confronted by selective pressures associated with typically negative fitness consequences of small size and slow development (Altwegg & Reyer, 2003). For example, prolonged hibernation in the north causes severe weight loss in amphibians (Holenweg & Reyer, 2000) despite that a large body size could be particularly advantageous at higher latitudes (Merilä et al., 2004). Furthermore, the period available for growth is also limited at higher latitudes, creating selection for rapid growth and development (Berven & Gill, 1983).

Laboratory experiments indeed indicate that both phenotypic plasticity and genetic adaptations modify geographical variation in ectotherm growth. At suboptimally low temperatures ectotherms have slower, but prolonged growth periods (Atkinson, 1994) whereas numerous studies have also demonstrated heritable increased growth and development rates for higher-latitude populations (Conover et al., 2009). The latter findings reveal a pattern known as counter-gradient variation: the genetic variation for growth and development is opposed by environmental influences on those traits over the latitudinal gradient (Conover et al., 2009). In the wild, however, latitudinal increase in growth rate has seldom been reported (Riha & Berven, 1991) and field studies complementing the laboratory evidence are therefore required (Laugen et al., 2003). In particular, while focusing on the developmental mechanisms, the studies have not included a major maternal effect that may contribute to fast growth: active selection of breeding habitats by adults (Mousseau & Fox, 1998).

In this study, we provide evidence that amphibians can cope with adverse climatic conditions by adjusting breeding habitat selection, which promotes growth and development rates of tadpoles in the field at high latitudes. We (i) compare the growth and development of the natterjack toad (*Bufo calamita*) tadpoles in Estonia (at its northern range edge) and Denmark, and (ii) explore the contribution of breeding-habitat selection to geographical variation in development by comparing the toad's habitat preferences in the two countries and utilising a habitat restoration study in Estonia.

2. Material and methods

2.1. Study species

The natterjack toad is distributed from the Iberian Peninsula in the southwest to the Baltic coast in the east, reaching the northern range edge in Estonia (Sinsch, 1998; Figure 1). In the northern part of the toad's distribution range, the onset of reproduction varies from March on the west coast of England to May in Sweden and Estonia (Sinsch, 1998). Across these regions the growing season decreases from 252 to 180 days respectively (MetOffice UK; Estonian State Meteorological Institute), lasting on average 225 days in Denmark (Christensen, 2006). A toad population spawns over several months with 2–3 pulses that depend mainly on rainfall (Sinsch, 1998), though many populations make their main breeding effort in spring (April–May) and later pulses are indistinct or absent (Banks & Beebee, 1986; Sinsch & Seidel, 1995).

2.2. The tadpole study

Tadpole growth and development were studied between latitudes 54–59°N in two countries (separated by ca. 1000 km): in seven of the 15 known populations of the natterjack toad in Estonia and in six populations in Denmark (Figure 1). Corresponding to the species' habitat use in these countries (Briggs, 2004; Rannap et al., 2007) two Estonian and four Danish study populations inhabited coastal meadows and the rest were in sand or gravel pits.
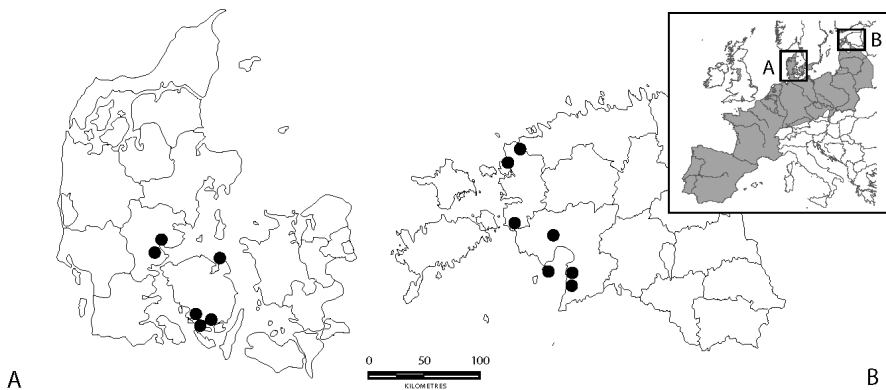

Figure 1. Distribution of the natterjack toad (shaded area; Gasc et al., 1997) and locations of the study populations/sites (filled circles) in Denmark (A) and in Estonia (B).

All the 13 populations were visited 2–4 times in April 2007 and 2010 to detect the onset of reproduction, and four times from May to June in 2007 to record growth and development of eggs and tadpoles. For each site (habitat of a population; may include several breeding ponds), the onset of breeding was identified according to the first amplexus or egg-laying attempt observed. Similar methods have been used by R.R. and L.B. to detect breeding onset in these sites since 2000.

In 2007 the mean monthly air temperatures in Denmark exceeded the values for Estonia by 4.4°C in April, 0.2°C in May and 0.6°C in June. In 2010, the differences were similar for April (2.7°C) and June (0.2°C), but May was extremely warm in Estonia: on average +18.1°C compared with +9.5°C in Denmark (Danish Meteorological Institute; Estonian State Meteorological Institute).

Ponds with spawn-strings were sampled (1–4 per study site; a total of 10 ponds from each country) and visited in both countries simultaneously four times in 2007 (1–2 May, 15–16 May, 1–2 June and 15–16 June). During every visit, 20 tadpoles from each pond were caught, photographed in a plastic box (equipped with dimensional grids and filled with 5 mm of pool water), and released to their natal pond. The developmental stage of the spawn and larvae was determined based on Gosner (1960), i.e., a value was assigned to each animal from egg-string (stage 10) to the completion of metamorphosis (stage 46). From the photographs, snout-vent body length of tadpoles was measured.

Geographic differences in tadpole length were analyzed using a mixed ANOVA (SAS procedure MIXED; Littell et al., 2002) with site as a random effect and country and, where appropriate, date of observation as categorical explanatory variables. Analogously, generalized linear models with random effects (SAS procedure GLIMMIX: ordered multinomial distribution, *cum-logit* link function) were used to analyze differences in the developmental stage. The quantitative analyses below were focused on the second and third sampling turns because there were no hatched tadpoles yet in early May in Estonian study sites, whereas most individuals in mid-June were already metamorphosing, which precluded using length as a developmental metric (see Große, 1994). Statistically significant country × date interactions were considered indicative of geographical difference in growth and development rates. The Kenward–Roger method for determining degrees of freedom (Littell et al., 2002) was applied to assure that site was considered an independent

observation and avoid overestimation of denominator degrees of freedom thereby.

There are two potential problems with such straightforward analyses; thus, alternative analyses were carried out to check for robustness of the results. First, the country × date interaction for body length unambiguously confirms differential growth rates only when the growth trajectories are linear. Based on Große (1994) the linearity assumption is satisfactorily met for the natterjack toad tadpole length < 2.3 cm but growth slows down considerably in larger tadpoles (slope 3.7-times lower; note that these are whole lengths, not snout-vent lengths). Therefore, additional analyses considering non-linearity were carried out; however, these did not alter the results qualitatively. Secondly, because of the pulsed breeding activity, some spawn-strings detected could have been laid after the main spawning period. If that was more frequent in Denmark than in Estonia, a detected 'slower growth' of Danish tadpoles might represent an artefact related to the presence of younger larvae. To check for this effect, alternative analyses excluded, for each site × date subsample separately, all tadpoles with lengths either below the lower quartile or median of that subsample. Such breaking points were justified by the observations that the second spawning wave (late May–June), if it occurs, typically yields <30% of the offspring (Sinsch & Seidel, 1995), i.e., we aimed at exclusion of all larvae that might represent the second spawning.

2.3. The breeding-habitat selection study

Breeding site selection of the natterjack toad was explored using three approaches.

(1) In the sites of the tadpole study we measured the maximum depth and surface area of every pond (37 in Estonia, 16 in Denmark) in April 2007. Although the depth and the area of the ponds were correlated (Spearman correlation, $r_s = 0.32$; $p < 0.0001$), both these variables were considered in the analyses (the area being log-transformed for distribution normality) due to their potentially different effects on the toads. General linear models were used to explore the variation in pond characteristics according to the country and occupancy by toads.

(2) To confirm that the occupancy patterns detected are indeed produced by active habitat selection, we analyzed the incidence of colonization by the toads of 20 constructed ponds in Estonia in relation to their depth and

size (logistic regression; Type III approach). Those ponds were created in 2000–2006 in two coastal meadows and in five sandpits to support the existing natterjack toad populations there. The median depth of these ponds was 35 cm and the median area was 300 m^2 in 2007; they were located within 5–30 m of existing breeding ponds. Evidence for pond colonization was recorded when spawn-strings and/or tadpoles of the species were found during 2001–2009.

(3) We performed two sets of measurements of environmental conditions in the ponds to explain the habitat-selection patterns observed. (a) In spring 2010 thermo-loggers (2010 TGX-3020) were used to record water temperature in eight Estonian and six Danish ponds of the tadpole study. Water depth maxima were 55–80 cm in four Estonian and five Danish ponds, and 30–40 cm in the rest (four ponds in Estonia, one in Denmark). In Estonia, natterjack toads bred only in those shallower ponds. The sensors of the loggers were settled on shallow sun-exposed margins of the ponds into 10–12 cm depth of the water (considering the depths used by tadpoles and the expectable water loss during the larval period). The temperatures were recorded every half an hour between 16 April and 21 June (i.e., until the completion of metamorphosis). General linear models were used to analyze daily minimum and maximum water-temperatures in relation to country and pond depth, using the mean values for each month as a repeated measure. (b) In spring 2011 five pond characteristics (water temperature, conductivity, oxygen concentration, pH and maximum depth) were measured in each pond in all Estonian study sites. Every site was visited at least twice and all five parameters were measured during each visit. In daytime each available pond was measured within 30 min for uniformity of conditions, later at night the number of calling males and spawn-strings was recorded in every pond. The measurements were taken 8–9 cm below water surface during the first peak of breeding (24 April–13 May) using conductivity-meter (CD-4302), oxygen-meter (DO-5510) and pH-meter (PH-212). Because water oxygen concentration, temperature, pH and conductivity depend on natural conditions at the measuring time, relative values were calculated for each pond as differences from the average values in that site. We used general linear modelling (with site included as a random effect) in SAS procedure MIXED to test the difference between ponds with and without breeding attempt.

3. Results

3.1. Tadpole development

The breeding season of the natterjack toad started earlier in Denmark than in Estonia by 10 days in 2007 (consistently with the 12-day average difference in 2000–2007; Figure 2) and by 12 days in 2010. Within country, the onset of breeding varied 6–17 days in 2007 and 8–16 days in 2010.

On 1–2 May 2007, only spawn-strings or newly hatched tadpoles (stages 10–19) were found in Estonia, while in Denmark tadpoles were swimming freely (stages 21–25) in all but one coastal-meadow pond (only egg-strings of stage 15–16), which was also the deepest pond studied (maximum depth 70 cm). On 14–15 May, Estonian tadpoles were mainly in developmental stages 21–25 (75%), but in two sites only spawn-strings were found (stage 11–17). In contrast, 71% of the Danish tadpoles were in the stages 27–33. Difference in the average developmental stage between countries was highly significant ($F_{1,12} = 22.1$; $p = 0.0005$) and, within country, it was clearly more advanced in shallower ponds (Spearman correlations with maximum depths of ponds that had tadpoles: $r_s = -0.76$; $N = 8$; $p < 0.028$ in Estonia; $r_s = -0.75$; $N = 7$; $p < 0.055$). The average length of tadpoles in Denmark was 0.67 cm, compared to 0.36 cm in Estonia ($F_{1,11.7} = 10.3$; $p = 0.0077$; Figure 3). However, by 1–2 June, Estonian tadpoles had caught
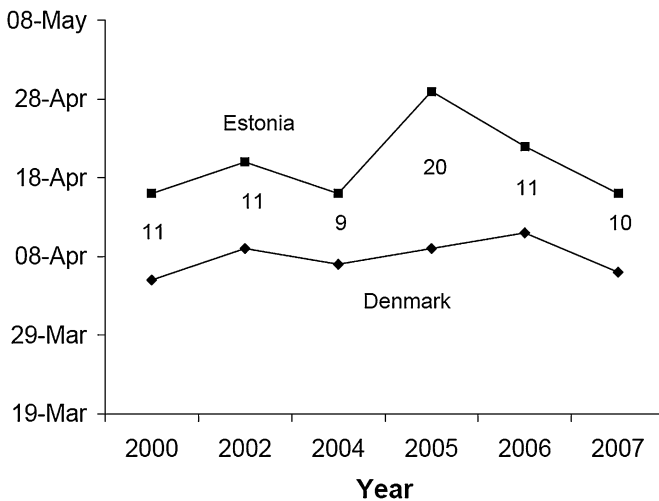

Figure 2. Onset of breeding at Estonian and Danish study sites by natterjack toad, 2000–2007. The numbers between the lines indicate differences between countries (number of days).
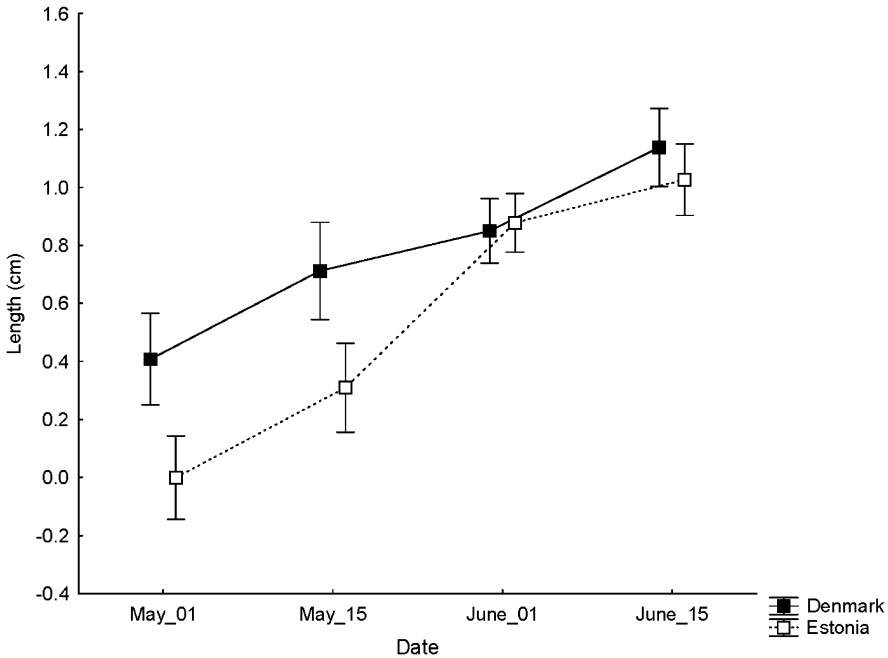
Figure 3. The mean length of the natterjack toad tadpoles/metamorphosed toadlets measured in 10 Estonian and 10 Danish study ponds in 2007. The vertical lines are 95% confidence intervals.

up in size and developmental stage with Danish tadpoles; 72% of the tadpoles in Estonia and 73% in Denmark had reached stages 30–40, and no between-country differences existed in developmental stage ($F_{1,13.2} = 1.6$; $p = 0.22$) or length (0.85 versus 0.87 cm, $F_{1,14.8} = 0.4$; $p = 0.53$). Accordingly, when data from these two sampling periods were combined, a highly significant country $\times$ date interaction both for the developmental stage ($F_{1,607} = 942$; $p < 0.0001$) and length ($F_{1,617} = 132$; $p < 0.0001$) reflected greater between-country differences on the earlier date. Importantly, on 1–2 June large (>1 cm) and small (<0.7 cm) tadpoles were similarly represented in both countries (11% of Estonian and 13% of Danish tadpoles were large, and 4% and 2% were small; Figure 4). This indicates that both ends of size distributions had shifted to produce overlapping distributions by that time; thus, it is unlikely that tadpole 'populations' in Estonia caught up the size of the Danish conspecifics due to selective mortality rather than a difference in growth parameters.
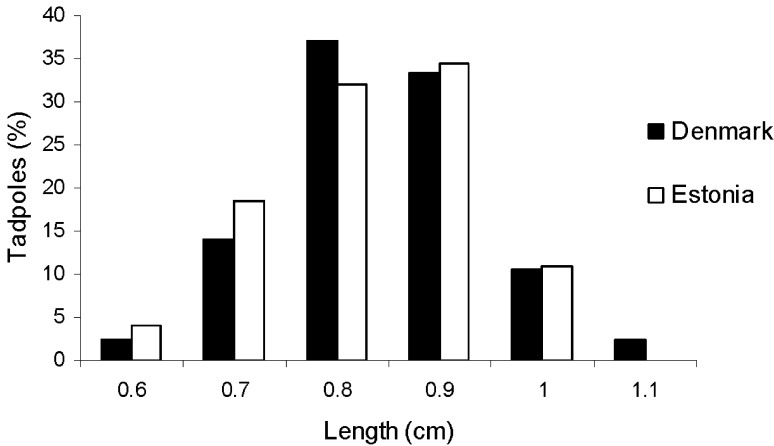
Figure 4. Frequency distributions of tadpole length on 1–2 June 2007 in Estonia and Denmark.

   All these analyses yielded qualitatively identical results when performed on the alternative data sets (see Methods). Hence, the conclusion that growth and developmental rates of the tadpoles were higher in Estonia appears robust in the light of potential pitfalls. It was further supported by the observation that, by 15–16 June 2007, most tadpoles had completed metamorphosis and left the ponds in both countries. Similarly, despite the 12-day difference in the onset of breeding in 2010, metamorphoses took place during the same week and were completed by 22 June in these countries. However, 2 out of 10 study ponds dried up before the metamorphosis of tadpoles in 2007 in Estonia, and one (the shallowest one) in Denmark.

3.2. Breeding-habitat selection

In 2007 the mean water-depth of the ponds at the study sites did not differ between the countries (ANOVA: $F_{1,49} = 0.1$; $p = 0.70$) but there were striking country-specific preferences of toads for breeding sites (country $\times$ occupancy interaction: $F_{1,49} = 56.5$; $p < 0.001$): the Estonian toads clearly preferred shallower ponds, while the Danish ones preferred deeper ponds. As a result, the country main effect on occupancy was not significant ($F_{1,49} = 0.1$; $p = 0.73$). The depth distributions of the occupied ponds in Estonia (median depth 28 cm; range 20–60 cm; median surface area 317 m^2) and in Denmark (median depth 50 cm; range 40–70 cm; median surface area 860 m^2; Figure 5a) were almost non-overlapping. Similar, but much weaker, effects
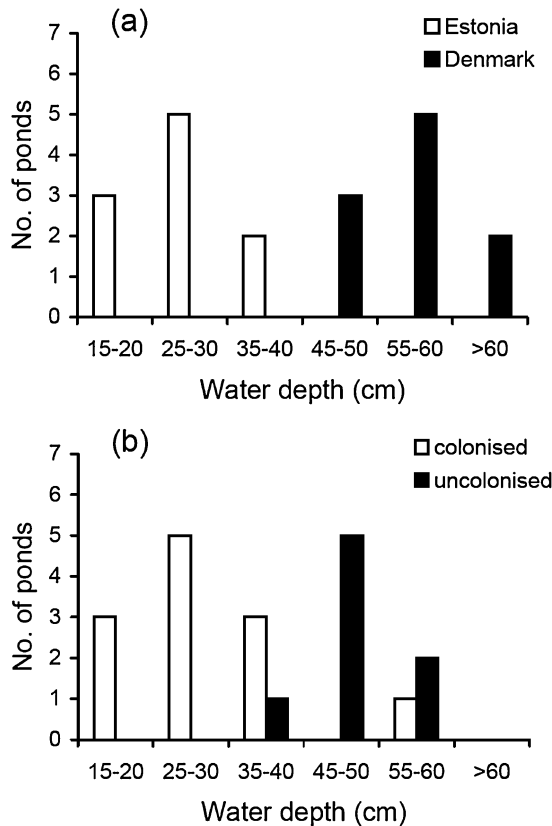
Figure 5. Maximum water depth in natterjack toad breeding ponds in April 2007 in Estonia and Denmark (a), and in the constructed ponds colonized and not colonized by toads in Estonia (b).

were obtained for pond area (country: $F_{1,49} = 1.4$; $p = 0.24$; occupancy: $F_{1,49} = 0.4$; $p = 0.55$; country × occupancy: $F_{1,49} = 9.4$; $p = 0.004$). However, this trend disappeared altogether when water-depth was included as a covariate into the area model. In 2010, when the pond sample only partly overlapped with that of 2007, all these key results on pond occupancy in relation to depth and area remained similar.

The pond colonization study in Estonia, where 12 of the 20 constructed ponds were colonized by the species, also revealed a preference for shallow ponds (logistic regression: coefficient = −0.47; SE = 0.29; $LL = −13.4$; $\chi^2 = 19.0$; $p < 0.0001$) with large surface area (coefficient = 139.1; SE = 94.7; $LL = −7.3$; $\chi^2 = 6.9$; $p = 0.009$). All the colonized water bodies but

Table 1.

Results of general linear models explaining daily minimum and maximum water-temperatures in 8 Estonian and 6 Danish ponds in April–June 2010.

Variable	Daily minimum temperature		Daily maximum temperature	
	F	*p*	*F*	*p*
Water depth	<0.1	0.959	<0.1	0.909
Country	<0.1	0.867	2.8	0.122
Month	9.3	0.001	6.6	0.006
Water depth × month	0.1	0.881	1.5	0.243
Country × month	6.8	0.005	9.5	0.001

Degrees of freedom: 1, 11 for the main-effects of Country and Water depth; 2, 22 for Month (repeated measure) and the interactions

one had a maximum water depth < 40 cm (Figure 5b). The exceptional pond (60 cm deep) had extensive shallow margins where spawn-strings were laid.

Consistently for daily minimum and maximum values in 2010, water temperatures did not differ between countries in general, but the Estonian ponds were warmer than Danish ponds in May (country × month interaction; Bonferroni-corrected post-hoc comparisons: $p < 0.05$) (Table 1). The comparison of water parameters in the Estonian study ponds in 2011 demonstrated that breeding ponds were shallower, having a combination of relatively warm water and high oxygen concentrations than non-breeding ponds (Table 2; Figure 6).

Table 2.

Mean (± standard deviation) characteristics of breeding ponds and non-breeding ponds in Estonia, their estimated mean difference (± standard error) and its significance (general linear models with random site effect).

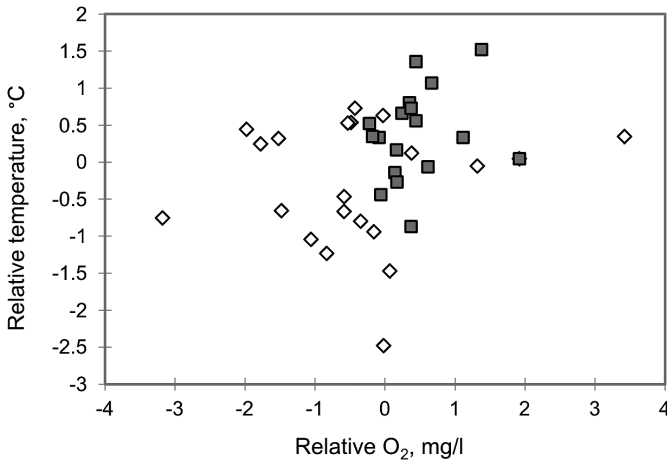|	O_2 (mg/l)	Water temperature (°C)	pH	Conductivity (S/m)	Max depth (cm)
Breeding	11.3 ± 0.6	14.1 ± 0.6	7.9 ± 0.3	0.28 ± 0.09	39.9 ± 13.0
Non-breeding	10.5 ± 1.4	13.5 ± 0.8	7.7 ± 0.6	0.33 ± 0.09	52.3 ± 13.6
Difference	0.87 ± 0.40	0.76 ± 0.27	0.28 ± 0.18	−0.058 ± 0.032	−12.1 ± 4.7
p-value	0.037	0.009	0.134	0.082	0.016

Figure 6. Relative oxygen concentration and water temperature in relation to the incidence of natterjack toad breeding (filled symbols, breeding; hollow symbols, no breeding, based on calling males or spawn-strings) in Estonian ponds in spring 2011. The relative values were calculated for each pond as differences from the average values in that site (cluster of ponds).

4. Discussion

We demonstrated that natterjack toad's tadpoles grew and developed faster and metamorphosed in a shorter time in the field in Estonia than in Denmark, and despite a 12-day average difference in the onset of breeding, the tadpoles completed metamorphosis nearly at the same time in both countries. The data also indicated that geographically varying breeding-habitat selection (basically a type of maternal effect; Mousseau & Fox, 1998) contributed to the faster growth and development rates of Estonian tadpoles — toads actively selected shallower ponds with higher temperatures and oxygen concentrations for breeding. Hence, at least in amphibians, in addition to genetic divergences (e.g., Laugen et al., 2003; Rogell et al., 2009), maternal effects via habitat choice can contribute to latitudinal gradients of phenotypic variation. Previously, synchronized metamorphoses despite asynchronous spawning have been recorded in British natterjack toad populations (Beebee, 1985) but the mechanism remains unexplored.

Water depth and area create a major gradient of pond characteristics, along which amphibians vary in their development times (Wellborn et al., 1996). The natterjack toad usually breeds in temporary, unshaded pools with warm shallow margins (Banks & Beebee, 1987). Our study indicated that, within species, habitat requirements also vary geographically and are subject

to active habitat selection: despite similar characteristics of available ponds in the breeding sites, shallower ponds were selected in Estonia, but not in Denmark, and also of the experimental ponds in Estonia (with one exception) only the shallow were colonised. Geographic variation in habitat selection has previously been demonstrated in various taxa, but these studies only occasionally report that a habitat preferred in one location may be avoided in another (Parody & Parker, 2002; Väli et al., 2004) and lack insights into causal mechanisms.

Conventionally, shallow water has been regarded to support tadpole growth because it warms up rapidly and is better oxygenated (Noland & Ultsch, 1981). Hence, use of such habitats might explain the paradoxically higher temperature optima reported for northern amphibians (Ståhlberg et al., 2001). In current study the natterjack toads actively selected shallower ponds for breeding in Estonia, where such ponds were also warmer and more oxygenated; while in Denmark deeper ponds were preferred. However, those deeper ponds in Denmark warmed up to the same extent than the shallow ponds in Estonia, so that the temperatures did not vary significantly within the 30–70 cm depth range used by the toads in Estonia or Denmark.

The geographically different growth and development of natterjack toad's tadpoles was not likely a response to the following factors of potential importance: (i) tadpole density, which in our study ponds was clearly below the high levels that are shown to delay tadpole metamorphosis (Reques & Tejedo, 1997); (ii) reduced water volume, given that natterjack toad's tadpoles normally do not respond to pond desiccation (Brady & Griffiths, 2000), except under conditions of high salinity (Rogell et al., 2009), which was not the case in our study sites; (iii) food scarcity in shallow ponds (Boorse & Denver, 2003) because rapid development of tadpoles was accompanied by their accelerated growth; (iv) geographically contrasting predator abundance (Laurila & Kujasalo, 1999), in which case one might expect differences in size and stage distributions (caused by selective mortality): such differences were not observed (Figure 4); (v) geographically different body size of females (Laugen et al., 2002) — anurans (particularly *Bufonidae*) do not show the general relationship between adult size and regional temperature (Ashton, 2002), and natterjack toads may even be smaller in the north than in the south (Leskovar et al., 2006); (vi) geographical differences in egg size: we believe this is unlikely as egg size does not influence larval growth rate,

metamorphic size or survival in this species (Tejedo & Reques, 1992). How-ever, whether this applies to our populations remain unknown.

An obvious reason for geographical variation in habitat selection is that similar habitats may pose risks that are outweighed only regionally. Shallow ponds, which were avoided by the toads in Denmark despite a similarly positive effect on larval development as observed in Estonia, are presumably more prone to desiccation there than in northern areas. Indeed, the shallowest pond used for reproduction in Denmark dried up before the metamorphoses of tadpoles. The risk of desiccation may be further magnified by pronounced intraspecific competition among tadpoles in ephemeral ponds (Rogers & Chalcraft, 2008). Such pressures may also explain why the Estonian toads in their cool and maritime climate still selected ponds that were not only shallow, but also extensive. These pond characteristics are related to the length of the hydroperiod (Brooks & Hayashi, 2002), a preference by this species which has similarly been recorded in northern England (Banks & Beebee, 1987). Also, in shallow water tadpoles may be more exposed to avian and mammalian predators (Day & Linn, 1972; Sherman & Morton, 1993) and damaging UV-B radiation (Blaustein & Bancroft, 2007). Those risks might be mitigated by the shorter time to metamorphosis and fewer predators at high latitudes (Laurila et al., 2008).

In conclusion, the effects of pond depth on the breeding of the natterjack toad reveal a link between habitat selection and geographic range. While habitat availability is known to limit species' ranges (Holt et al., 2005) our study illustrates how physiological constraints (i.e., that cool temperature reduces growth) create 'habitat' variation across the range, a pattern in-creasingly noticed but still poorly explained (Oliver et al., 2009). Clearly, delineating potential ranges according to the availability of 'typical' habi-tats is risky unless the determinants of habitat quality are explicitly known. Moreover, the correlative and mechanistic (physiologically based) models used for range explanation and prediction today do not incorporate flexible habitat selection (see Peck et al., 2009), which appeared a major factor for successful breeding of natterjack toads.

From a conservational point of view our study suggests that a common set of management recommendations should not be applied uniformly to protect all populations of widely distributed species (Parody & Parker, 2002; Whit-tingham et al., 2007). For amphibians, the importance of ephemeral water-bodies and impacts of their loss may be particularly pronounced at range

edges. In line with that, the natterjack toad in Estonia has now gone extinct in most coastal meadows, where drainage, fertilizer use and the cessation of traditional management have degraded particularly the lowest and wettest parts, the sites where the temporary breeding ponds were formed (Rannap et al., 2007). To summarize, our study supports the calls for geographically explicit habitat conservation strategies, which are based on analyses of local limiting factors (Väli et al., 2004).

Acknowledgements

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