

# Intraspecific differences in thermal tolerance of the diamondback watersnake (*Nerodia rhombifer*): effects of ontogeny, latitude, and sex

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## Abstract

Ontogenetic shifts in microhabitat use are widespread among taxa and can result in drastic shifts in thermal habitat among age classes. Likewise, geographic variation in climate along latitudinal gradients can cause differences in thermal environments among populations of a species. Using a common garden design, we examined four populations of a single species of semi-aquatic snake, *Nerodia rhombifer*, to determine whether ontogenetic shifts in habitat use (and/or body size) and latitudinal differences in ambient temperature have resulted in evolutionary changes in thermal tolerance. We found ontogenetic differences in thermal tolerance for all populations, with neonates tolerating temperatures 2 °C higher than adults, a pattern that is consistent with ontogenetic shifts in body size and microhabitat use in this species. There were differences in thermal tolerance among latitudes in neonates, suggesting genetic differences among populations, but adults showed no latitudinal differences. In combination, the increased thermal tolerance of neonates and the age-specific response to latitude suggest individuals may be most sensitive to selection on thermal tolerance as neonates. Although latitudinal differences exist in neonates, their tolerances were not ranked according to latitude, suggesting the effects of some other local factor (e.g., microclimate) may be important. Lastly, among neonates, females tolerate higher temperatures than males.

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**Keywords:** Age; Critical thermal maximum; CTMax; Genetic variation; Geographic variation; Heat tolerance; Latitude; Natricinae; Temperature; Thermal physiology

## 1. Introduction

Understanding the evolution of thermal tolerance in ectothermic organisms has become a central theme in evolutionary and ecological physiology (Huey and Kingsolver, 1993; Angilletta et al., 2002). For species that are not suitable for laboratory selection experiments (i.e., most vertebrate organisms), comparative methods generally must be used to infer evolutionary changes in thermal tolerance as a response to changes in thermal environments associated with habitat use or geographic variation in climate (Huey

and Kingsolver, 1993; Bennett and Lenski, 1999; Feder et al., 2000; Angilletta et al., 2002). In particular, comparative approaches that use a single species from different habitats and geographically separated populations offer a better alternative than interspecific comparisons, because among-species comparisons may be confounded by variables other than habitat differences (Pearson et al., 2002).

Ontogenetic shifts in microhabitat use are widespread among taxa and can sometimes result in drastic shifts in thermal habitat among size or life history stage classes (Middendorf and Simon, 1988; Paulissen, 1988a; Krebs et al., 1998). For example, most larval amphibians are constrained to their natal aquatic habitat, which can limit thermoregulatory options and at times be vastly hotter than other microhabitats available to the more terrestrial adults (Lillywhite, 1970; Tracy, 1976; reviewed in Ultsch et al., 1999). Similarly, larvae and pupae of *Drosophila* are constrained to thermally stressful necrotic fruit, while adult

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flies have the ability to minimize thermal stress through behaviors such as microhabitat selection (Krebs et al., 1998). In both of these cases, the larval stages, which are constrained to the more thermally stressful temperatures, have higher heat tolerance than the adult stages (Delson and Whitford, 1973; Berkhouse and Fries, 1995; Krebs et al., 1998; Ultsch et al., 1999). In aquatic snakes (e.g., *Nerodia*) and fish ontogenetic shifts in microhabitat use can result from ontogenetic changes in prey resource use or predator avoidance (McCauley and Huggins, 1979; Mushinsky et al., 1982; Plummer and Goy, 1984; Scott et al., 1989; Jobling, 1994; Lind and Welsh, 1994; Olson, 1996; Savitzky and Burghardt, 2000). In some freshwater fish there is a corresponding ontogenetic change in heat tolerance and selected body temperature, with smaller fish selecting and/or tolerating higher temperatures (McCauley and Read, 1973; Cox, 1974; Reynolds and Casterlin, 1978; McCauley and Huggins, 1979; Jobling, 1994). Aside from microhabitat differences in environmental temperature, species that exhibit ontogenetic changes in body size might be expected to exhibit ontogenetic changes in body temperatures because of their basic biophysical properties (Stevenson, 1985). Smaller ectotherms usually have faster heating and cooling rates than larger ectotherms (Hutchison, 1961; Ballinger et al., 1970; Stevenson, 1985; Carrascal et al., 1992; Tosini and Avery, 1993), which may make them more susceptible to thermal stress (Stevenson, 1985). As a result, increased heat tolerance might be adaptive in smaller/younger ectotherms.

On a larger scale, there can also be significant differences in thermal environments across a species' geographic range. By comparing populations that occur across a range of climatic regimes, such as along an altitudinal or latitudinal gradient, it is possible to determine whether a species has responded to historical differences in thermal habitat through local genetic adaptations in thermal tolerance. There are a limited number of studies that have demonstrated within species variation in heat tolerance among vertebrate populations (e.g., Hutchison, 1961; Brattstrom, 1968; 1970; Delson and Whitford, 1973; Miller and Packard, 1977; Hoppe, 1978; Hertz et al., 1979; Garland and Adolph, 1991; Meffe et al., 1995; Schwarzkopf, 1998). However, few studies have performed common garden experiments designed to distinguish between genetic and acclimation-induced differences in physiology (Garland and Adolph, 1991). Furthermore, most studies have examined organisms from populations in close proximity to each other (i.e., along an altitudinal gradient), which may have high rates of gene flow that could decrease the likelihood of interpopulational differences evolving (Lenormand, 2002).

We examined four populations of a single species of semi-aquatic snake, *Nerodia rhombifer*, to determine how it has evolved to deal with local variation in its thermal environment associated with both ontogenetic shifts in body size and habitat use and differences in environmental

temperature along a latitudinal gradient. We predicted that thermal tolerance would exhibit a latitudinal gradient in both neonates and adults, with snakes from lower latitudes having higher thermal tolerance than snakes from higher latitudes. We also predicted that neonates would have higher heat tolerance than adults as a mechanism to cope with ontogenetic differences in heating rates (i.e., body size) and thermal environments. *N. rhombifer* spend most of their time in or near the water or basking in branches above the water (Gibbons and Dorcas, 2004). They exhibit ontogenetic shifts in diet and microhabitat use, using shallow (warmer) habitats to forage for smaller fish and perhaps to avoid predators as neonates and juveniles, but shifting to deeper (cooler) habitats to forage on larger fish as adults (Mushinsky et al., 1982; Plummer and Goy, 1984; Savitzky and Burghardt, 2000). Because we found strong ontogenetic differences in thermal tolerance we also examined the effects of body size on thermal tolerance, to see if smaller snakes (within age class) had higher thermal tolerance. Lastly, we examined the effects of neonatal sex on heat tolerance.

## 2. Materials and methods

### 2.1. The species

Diamondback watersnakes (*N. rhombifer*) are well-suited for studies of ontogenetic and latitudinal variation in thermal tolerance. First, operative temperatures, as measured using snake models, frequently exceed 45 °C in microhabitats where *N. rhombifer* are found, suggesting that elevated thermal tolerances may be ecologically important in this species (Keck, unpublished data for a population in central Texas). Second, nearly all *Nerodia* exhibit strong ontogenetic variation in habitat and food resource use and thus offer an excellent opportunity to determine if ontogenetic variation in heat tolerance exists in reptiles (Mushinsky et al., 1982; Plummer and Goy, 1984; Scott et al., 1989; Savitzky and Burghardt, 2000). Third, *N. rhombifer* are geographically widespread, very abundant, and easily captured in many aquatic habitats (particularly lentic systems) throughout much of their range, which includes 14 states in the central United States and northern Mexico (Gibbons and Dorcas, 2004). In contrast, most terrestrial snakes are extremely secretive and difficult to systematically capture in large numbers during a short time period, as was required for this study. Lastly, *N. rhombifer* are easy to maintain in laboratory conditions, which is a necessary requirement for common garden experimental designs.

### 2.2. Collection and animal care

During May and early June of 2000, we collected adult male and adult pregnant female *N. rhombifer* from

four latitudes along a south–north transect that spanned 12°:26′03″ (South Texas, STX); 31°45′ (Richland Creek Wildlife Management Area in East Central Texas, CTX); 35°13′ (Central Oklahoma, OK); and 38°03′ (Cheyenne Bottoms Wildlife Area in Central Kansas, KS) (Fig. 1). At all latitudes, we chose specific collecting localities based on snake abundance and similarity of habitat characteristics. All localities were at low elevations (less than 560 m) in flat to gently rolling terrain, with few shade trees; no site had a well-developed tree canopy that would have provided deep shade along the shoreline. All sites frequently experience sunny and rainless summer days. Environmental temperatures of the localities followed a latitudinal cline, with higher mean ambient and mean maximum ambient temperatures at lower latitudes (National Climatic Data Center, unpublished data; Fig. 1).

Snakes from each locality were transported to the laboratory immediately after capture and kept under identical conditions (25 °C; 14L:10D) until the beginning of their experimental trial or until parturition. All snakes were housed singly in polypropylene containers (adult: 35×54×14.5 cm; neonate: 16.5×30.5×9.7 cm) fitted with mesh openings to reduce ambient humidity levels, and newspaper as a substrate. Adults were provided water bowls sufficient for submergence at least 2 days per week, and neonates had water available at all times. This slight difference in husbandry between adults and neonates likely had no effect on our results because water was always

provided 24 h prior to trials to ensure that all snakes had the opportunity to be fully hydrated during trials. Adults were fed 10% of their mass in sunfish (*Lepomis* sp.) weekly; however, food was withheld for 10 days prior to data collection to ensure that all adult measurements were made on postabsorptive individuals. Neonates were tested 9 to 15 days after birth and therefore were not fed prior to trials.

### 2.3. Experimental design

We used both laboratory acclimated field-captured adults (males only; due to logistical constraints) and neonates born in a common garden environment. All adult males were acclimated to identical laboratory conditions (25 °C; 14L:10D) for a minimum of 2 weeks prior to heat tolerance trials. Ectotherms are known to show phenotypic plasticity in their physiological responses to temperature (Hutchison, 1976; Lillywhite, 1987; Garland and Adolph, 1991). Although it seems likely that our laboratory acclimation regime should have removed any effects of developmental temperature or acclimatization (Hutchison, 1976; Garland and Adolph, 1991; Lutterschmidt and Hutchison, 1997), relatively little is known concerning whether these effects can be persistent. Therefore, to determine if genetic differences in thermal tolerance exist among latitudes, we also used a common garden experimental design with neonatal snakes that had not experienced the normal developmental and environmental temperatures of their natural habitat (Garland and Adolph, 1991). To do this we captured pregnant females early during gestation (May–early June) and kept them under identical conditions (25 °C; 14L:10D) where they were not allowed to behaviorally thermoregulate until after parturition, which occurred 83 to 137 (mean±S.E.=120±3.2) days after first being housed in the laboratory. Although all females had enlarged follicles/ova when collected, only one CTX female gave birth suggesting that we collected females from that population prior to their mating. Additionally, at the time of collection we sacrificed and dissected one female from each locality and found medium to large ova, with no evidence of embryonic development. Neonates were subsequently kept under identical conditions until their trials.

Neonate thermal tolerance trials were conducted from early September to early November 2000 and adult male heat tolerance trials were conducted from November to mid-December 2000. The consistency of the laboratory photoperiod and temperature regime should have eliminated the possibility that differences between neonate and adult heat tolerance would result from testing adults slightly later in the season (Hutchison, 1976; Lutterschmidt and Hutchison, 1997). We conducted all trials between 12:00 and 16:00 to minimize possible circadian effects on thermal tolerance (Hutchison, 1976).

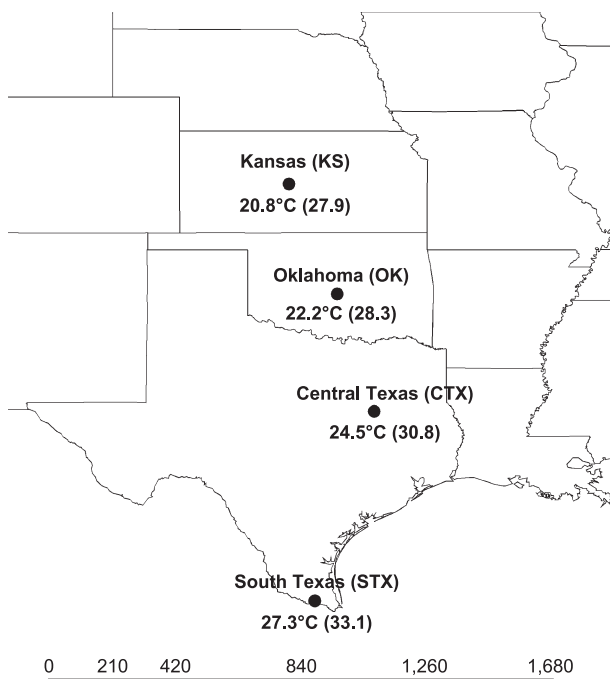


Fig. 1. Map showing the geographic origin of *N. rhombifer* used in the laboratory experiments. The mean ambient temperature (first temperature) and the mean daily maximum ambient temperature (in parentheses) during the activity season (April–October) are provided below each locality (National Climatic Data Center, unpublished data). Map scale is in km.

#### 2.4. Thermal tolerance trials

We used the critical thermal maximum (CTMax) or “dynamic” method as described by Lutterschmidt and Hutchison (1997) to measure heat tolerance of adult male and neonatal *N. rhombifer*. All snakes were individually heated in a cylindrical arena (adult arena: 51.5 cm tall, 41 cm diameter; neonate arena: 19 cm tall, 20 cm diameter) containing a sand substrate (approximately 2.5 cm deep for adults, 1 cm deep for neonates). A 30-gauge copper-constantan Teflon<sup>®</sup> coated thermocouple wire (model TT-T-30 special limits of error, Omega Engineering, Stamford, CT, USA) was inserted into the snake’s cloaca (6 cm for adults; 1.9 cm for neonates) and fixed into position with a small strip of adhesive tape. Body temperatures were monitored using a Campbell Scientific CR10 datalogger (Logan, UT, USA) with real-time digital display output occurring every 3 s. Because neonate body temperatures increased during handling associated with insertion of the thermocouple (through conduction of human body heat), we allowed each animal to cool to ambient temperature (25 °C) before initiating tolerance trials. Heat was supplied by one (neonates) or three (adults) 250-W infrared heat lamps shining directly on the snake from 37 cm (neonates) or 62 cm (adults) above the substrate. We heated all snakes at approximately 1 °C per minute (body temperature), using a rheostatic dimmer control connected to the heat lamps, to avoid thermal shock and acclimation during the trials (Lutterschmidt and Hutchison, 1997).

We used both the “loss of righting response” (LRR) and the “onset of muscular spasms” (OS) as our criteria of CTMax as recommended by Brattstrom (1968) and Lutterschmidt and Hutchison (1997). We defined LRR as the temperature at which snakes lost muscular coordination and were unable to right themselves in a coordinated manner when placed on their dorsum. We defined OS as the temperature at which muscular spasms began and characterized spasms as shallow muscular twitches that typically began on the posterior-lateral or posterior-ventral portion of the snakes’ bodies. One of us monitored and regulated each snake’s body temperature gain to 1 °C/min, while the other independently monitored the behavioral and physiological status of each snake until LRR or OS was reached (i.e., the person determining LRR and OS was unaware of the snake’s body temperature, latitude of origin, and sex). Each snake was heated until both LRR and OS occurred; in cases where OS was not observed, snakes were heated to approximately 3 °C above LRR before being removed from the trial (lack of OS response in some snakes resulted in unequal sample sizes between LRR and OS analyses). Immediately after OS, we removed snakes from the heat source and placed them in water at room temperature until recovery. Recovery was usually rapid and snakes appeared to function and behave normally after the trials. Only in a

few cases did trials result in death; in those cases the data were excluded from the analyses because LRR and OS had been exceeded. All snakes followed the sequence of behaviors typically associated with thermal tolerance trials (described in Lutterschmidt and Hutchison, 1997, p. 1566) and despite the species’ normal reliance on aggressive defense behavior when handled they were not aggressive towards the person manipulating the snake during the trials. We measured snout-vent length (SVL, distance between the tip of the snout and the posterior edge of the vent) and mass within 24 h of each trial.

#### 2.5. Statistics

We measured thermal tolerance on 56 adult male *N. rhombifer* from four latitudes (KS, OK, CTX, and STX) and 145 neonatal *N. rhombifer* from three latitudes (10, 6, and 7 litters from KS, OK, and STX, respectively). Only one female from CTX gave birth and therefore CTX was not represented in neonate and age class comparisons (their inclusion does not alter the results). We used litter means in all analyses of variance (ANOVAs) because siblings within a litter do not represent independent samples. We used two-way ANOVAs to determine the effect of locality and age class on LRR and OS (KS, OK, and STX localities). We subsequently performed separate one-way ANOVAs and analyses of covariance (ANCOVAs—with mass as the covariate) on LRR and OS for neonate litter means (KS, OK, STX) and adults (KS, OK, CTX, STX) to determine if effects of locality were attributable to neonates, adults, or both. Because the addition of mass (or SVL) as a covariate did not alter the results we only present the results of the ANOVAs. Assumptions of homogeneous variances were not satisfied in the two-way ANOVA of locality and age class for LRR; instead variance and sample size were positively correlated. According to Glass et al. (1972), a positive correlation between variance and sample size indicates that the actual alpha (Type I error rate) is less than the nominal alpha and therefore Type I errors due to assumption violations should not be a concern when significant differences are detected.

To determine if smaller snakes (within age class) have higher thermal tolerance than larger snakes we tested for relationships between body size (SVL and mass) and thermal tolerance (LRR and OS) using simple linear regression for each population. To make regression lines directly comparable among adult males and neonates, we used only litter means of male neonates for these regressions (results were not altered with the inclusion of females). We also performed two-way ANOVAs (sex and locality as grouping variables) using sex means for each litter to determine the effect of neonatal sex on LRR and OS, excluding litters that did not contain at least one data point for each sex. Statistical significance was recognized at  $P < 0.05$ . All statistical tests were performed

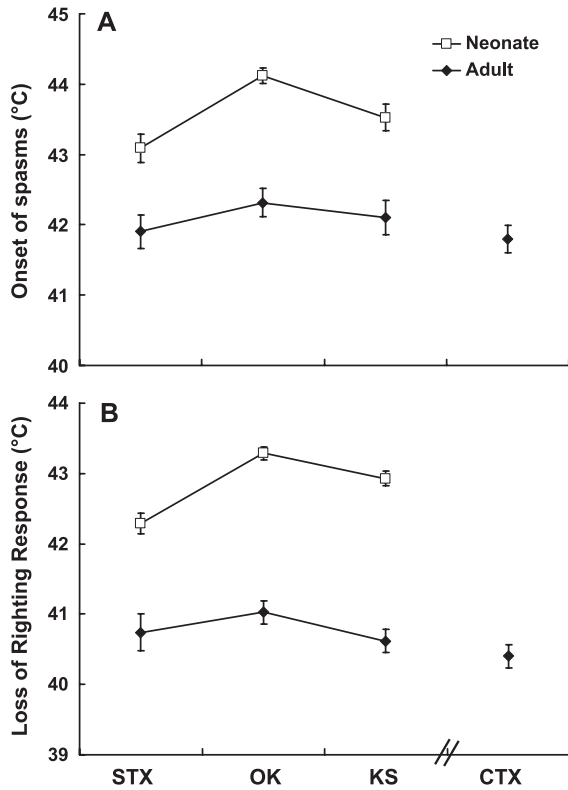


Fig. 2. Effects of locality and age class on the thermal tolerance (mean  $\pm$  1 S.E.) of *N. rhombifer*. Onset of spasms (A) and loss of righting response (B). Central Texas is shown disconnected because it was not included in the two-factor ANOVA. Note difference in scale.

using the STATISTICA for Windows software package (StatSoft, Tulsa, OK, USA, 1998).

### 3. Results

#### 3.1. Effects of locality

Overall, locality had a significant effect on LRR ( $F_{2,57}=5.19$ ;  $P=0.009$ ) and OS ( $F_{2,43}=5.2$ ;  $P=0.009$ ). There was no statistically significant locality  $\times$  age-class interaction in either two-way ANOVA (LRR:  $F_{2,57}=2.38$ ,  $P=0.10$ ; OS:  $F_{2,43}=0.97$ ,  $P=0.39$ ). However, a possible interaction is suggested by visual inspection (Fig. 2) and by the results of separate ANOVAs for each age class. There was a highly significant influence of locality on neonate thermal tolerance (one-way ANOVAs: LRR:  $F_{2,20}=15.61$ ,  $P<0.001$ ; OS:

$F_{2,19}=7.17$ ;  $P=0.005$ ), whereas there was no significant effect of locality on adult thermal tolerance (one-way ANOVAs: LRR:  $F_{3,52}=2.36$ ,  $P=0.083$ ; OS:  $F_{3,36}=1.62$ ,  $P=0.202$ ). Oklahoma neonates had higher thermal tolerances than KS (one-sided planned comparison: LRR:  $P=0.024$ ; OS:  $P=0.017$ ) and STX neonates (two-sided unplanned comparison: LRR:  $P<0.001$ ; OS:  $P=0.001$ ). Kansas neonates had higher LRR (two-sided:  $P<0.001$ ), but not OS (two-sided:  $P=0.092$ ), than STX neonates. Therefore, the neonatal differences were not consistent with a latitudinal gradient. Rather, the “middle” latitude (OK) exhibited the highest thermal tolerance, followed by the highest latitude (KS), with the lowest latitude (STX) having the lowest thermal tolerance (Fig. 2).

#### 3.2. Effects of ontogeny

Age class had a highly significant effect on both LRR ( $F_{1,57}=168.6$ ,  $P<0.001$ ) and OS ( $F_{1,43}=68.9$ ,  $P<0.001$ ). The mean LRR of adults from all localities combined was 40.8 °C, whereas the mean LRR of the neonates from all localities combined was a full 2 °C higher, at 42.8 °C (Fig. 2). The mean OS of adults and neonates from all localities was 42.1 and 43.6 °C, respectively (Fig. 2). The ontogenetic trends in thermal tolerance were consistent among all three localities. The effects of age class on thermal tolerance remained highly significant when female neonates were removed from the analyses (LRR:  $F_{1,57}=131.2$ ,  $P<0.001$ ; OS:  $F_{1,43}=42$ ,  $P<0.001$ ). Differences in body size between neonates and adults used in thermal tolerance trials are summarized in Table 1.

#### 3.3. Lack of body size effect

We found no consistent effect of body size on thermal tolerance. Only 2 of 16 separate adult regressions were significant (STX regressions of LRR on mass [ $b=-0.009$ ,  $r^2=0.714$ ,  $P=0.002$ ] and LRR on SVL [ $b=-0.097$ ,  $r^2=0.634$ ,  $P=0.006$ ]), and one other marginally significant (KS regression of OS on SVL [ $b=-0.070$ ,  $r^2=0.456$ ,  $P=0.046$ ]). Similarly, only 2 of 12 neonate regressions were significant, but significance occurred only in either LRR or OS, not both, indicating that it was not a consistent effect on all measures of thermal tolerance and/or size (STX: LRR on mass:  $b=1.416$ ,  $r^2=0.915$ ,  $P=0.043$ ; KS: OS on SVL:  $b=-0.582$ ,  $r^2=0.554$ ,  $P=0.033$ ). All other regressions were non-significant ( $0.953>P>0.074$ ), and none of the regres-

Table 1  
Body size of neonatal and adult male *N. rhombifer* (mean  $\pm$  1 S.E.) used in heat tolerance trials.

	South Texas		Central Texas		Oklahoma		Kansas	
	Neonate	Adult	Neonate	Adult	Neonate	Adult	Neonate	Adult
Mass (g)	9.4 $\pm$ 0.4	230.8 $\pm$ 23.1	–	269.5 $\pm$ 17.2	9.3 $\pm$ 0.5	174.7 $\pm$ 10.8	9.5 $\pm$ 0.4	223.4 $\pm$ 16.3
SVL (cm)	21.9 $\pm$ 0.3	68.2 $\pm$ 2.1	–	68.2 $\pm$ 1.6	21.8 $\pm$ 0.4	59.0 $\pm$ 1.2	21.3 $\pm$ 0.3	63.5 $\pm$ 2.0

There was no significant effect of neonatal sex on body size so neonate body size data were pooled for presentation.

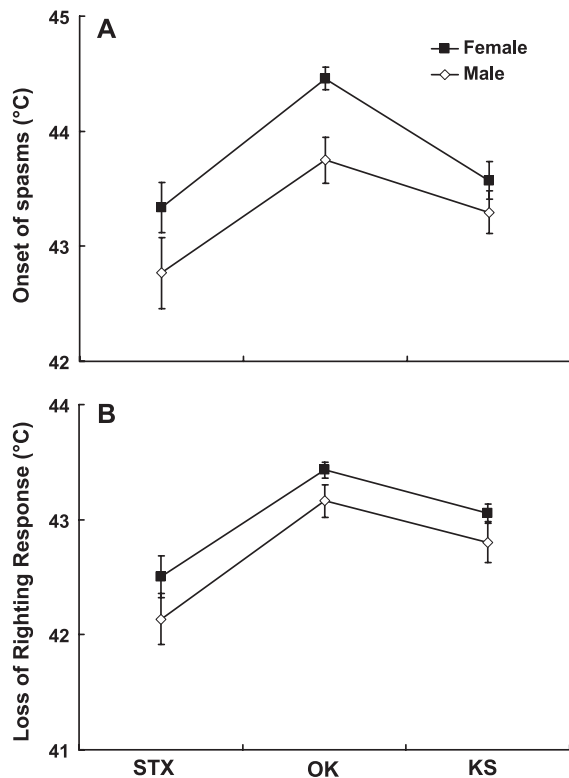


Fig. 3. Effect of neonatal sex on thermal tolerance (mean  $\pm$  1 S.E.) in *N. rhombifer*. Onset of spasms (A) and loss of righting response (B). Note difference in scale.

sions were significant after correcting the family-wise error rate using the sequential Bonferroni technique.

### 3.4. Effects of neonatal sex

Neonatal sex had a significant effect on both LRR ( $F_{1,38}=5.2$ ,  $P=0.029$ ) and OS ( $F_{1,33}=9.8$ ,  $P=0.004$ ) and, as in the above analyses, there were significant effects of locality on thermal tolerance (LRR:  $F_{2,38}=16.8$ ,  $P<0.001$ ; OS:  $F_{2,33}=12.9$ ,  $P<0.001$ ). The mean thermal tolerance was higher in females (LRR=43.0 °C; OS=43.8 °C) than males (LRR=42.7 °C; OS=43.3 °C), and this result was consistent among localities as the sex  $\times$  locality interaction term was not significant (LRR:  $F_{2,38}=0.1$ ,  $P=0.929$ ; OS:  $F_{2,33}=0.6$ ,  $P=0.535$ ; Fig. 3).

## 4. Discussion

### 4.1. Latitudinal effects depend on age class

Historical weather data indicate that average yearly temperatures and average daily maximal temperatures follow a latitudinal gradient, and are approximately 10 and 9 °C, respectively, higher in STX than in KS (National Climatic Data Center, unpublished data). Considering only the months that *N. rhombifer* are typically active (April–October), the mean and mean maximum temperatures are

still 6.5 and 5 °C higher in STX than in KS (Fig. 1). Ambient and operative temperatures are highly correlated (Keck, unpublished data); however, the substantial among-site differences in ambient temperature may underestimate the differences in operative temperature because solar intensity, which also is highly correlated with operative temperature, should be greater at lower latitudes. Given these large differences in environmental temperatures across the geographic range of *N. rhombifer* we predicted there would be a clear latitudinal trend in thermal tolerance among populations of both adults and neonates. In contrast to our prediction, we found that our conclusions are dependent upon which age class is examined. Within *N. rhombifer*, neonates exhibited significant differences in thermal tolerance among latitudes; these results were obtained despite the neonates being born in a common garden environment, which suggests that the latitudinal differences may be genetic in origin (Garland and Adolph, 1991). In adults, however, we found no significant differences among latitudes, suggesting that individuals may be most sensitive to selection on thermal tolerance as neonates.

Life history stage-dependent responses to environmental gradients are not unprecedented. Evidence from *Drosophila pseudoobscura* also suggests that the extent of geographic variation in thermal tolerance can depend on the life history stages that are examined (Coyne et al., 1983). Larvae and pupae of *D. pseudoobscura* are more prone to thermal stress than adults, and they show adaptation to local environments, whereas the adults do not (Coyne et al., 1983). A similar phenomenon is known to occur in the tiger salamander, *Ambystoma tigrinum*. Delson and Whitford (1973) demonstrated that mature larval-form *A. tigrinum* (i.e., paedomorphic adults: sexually mature salamanders that retain aquatic characteristics of larvae) from a desert population had higher thermal tolerances than those from a montane population. There were no differences, however, between the thermal tolerances of these two populations in mature terrestrial-form (i.e., transformed) salamanders (Delson and Whitford, 1973).

Among both adults and neonates, we found no clear evidence of latitudinally driven genetic changes in thermal tolerance. First, we found no evidence of latitudinal variation among adult male *N. rhombifer*. Second, although we observed significant differences in thermal tolerance among latitudes in the neonates, they were not ranked in latitudinal order. Instead, the “middle” latitude (OK) exhibited the highest thermal tolerance, followed by the highest latitude (KS), with the lowest latitude (STX) having the lowest thermal tolerance. There are at least two interpretations of this observation: (1) the populations may have evolved in response to unexamined local factors (e.g., microclimates) that differ in rank order from the macroclimate data we used to generate our prediction, or (2) the population differences may be the result of genetic drift, founder effects, or some evolutionary force other than natural selection for a high CTMax.

Intraspecific geographic variation in thermal physiology does not always follow latitudinal or altitudinal gradients in other organisms. Laugen et al. (2003) found significant genetic variation in the thermal dependence of embryonic development and growth rate among four latitudinally separate populations of the frog *Rana temporaria*, but, except for hatchling size, the traits did not follow a latitudinal cline. Laugen et al. (2003) suggested that the observed non-latitudinally ordered differentiation may be due to unmeasured local environmental factors that were more important than latitudinal variation in temperature. Coyne et al. (1983) observed genetic differences among populations of adult flies that did not follow a latitudinal gradient. Also, Hertz and Huey (1981) found significant interpopulational differences in the thermal tolerance of field-acclimatized *Anolis cybotes* along an altitudinal transect; however, the population means were not ordered altitudinally. Nonetheless, there is evidence that some species do follow geographic clines in thermal tolerance (e.g., Miller and Packard, 1977; Hertz et al., 1979; Hoffman et al., 2002), while others do not exhibit variation in thermal tolerance among thermally diverse environments (e.g., Huey and Webster, 1976; Hertz, 1979; Gvozdik and Castilla, 2001).

#### 4.2. Stronger patterns of ontogenetic variation

Neonates born in a common garden environment exhibited significantly higher thermal tolerances than adults acclimated to the same laboratory temperature. The observed mean difference in thermal tolerance between neonates and adults (2 °C for LRR and 1.5 °C for OS) is approximately twice the greatest observed mean difference between geographic localities (1 °C for LRR and OS), suggesting that age influences thermal tolerance more than locality in *N. rhombifer*. These results appear to be robust measures of ontogenetic shifts in thermal tolerance, rather than methodology-induced ontogenetic differences. While LRR could be criticized by some as being a “behavioral” measure of physiological tolerance (which could be argued to be related to endurance), OS appears to be a purely physiological response to thermal stress (Lutterschmidt and Hutchison, 1997) and, like LRR, clearly differed between age classes of *N. rhombifer*. Further, endurance is approximately eight times lower in neonates than adults of a closely related species, *Nerodia sipedon* (Pough, 1978). Consequently, if the results of our thermal tolerance trials were due to differences in endurance rather than thermal tolerance, adults should have had higher thermal tolerance values than the neonates.

Ontogenetic differences in thermal tolerance fit our predictions based on ontogenetic differences in body size and habitat use. The timing of birth for most *N. rhombifer* (late July through September; Gibbons and Dorcas, 2004) may be thermally stressful due to high environmental temperatures, low water levels, and small size at birth. Having higher thermal tolerance than adults is likely

important to neonates because the combination of their small body size (and thus fast heating rates) and reliance on warm shallow water for foraging likely makes them more vulnerable than adults to thermal stress. Further, because of their smaller body size and therefore slower locomotor speeds (Finkler and Claussen, 1999) neonates may not be able to escape dangerous temperatures during overland movements as readily as larger adults. Indeed, the ontogenetic patterns observed in *N. rhombifer* thermal tolerance follow patterns observed in other taxa including flies (Coyne et al., 1983; Krebs and Feder, 1997; Krebs et al., 1998; Gilchrist and Huey, 1999; Sorensen and Loeschke, 2002), anurans (reviewed in Ultsch et al., 1999), and salamanders (Delson and Whitford, 1973; Berkhouse and Fries, 1995). In nearly every case (see Berkhouse and Fries, 1995 for an exception), the ontogenetic stage that is most susceptible to heat stress is the stage with the highest thermal tolerance. Future studies should focus on possible physiological mechanisms and potential costs that could lead to decreased thermal tolerance among adults (e.g., Krebs and Feder, 1997; Krebs et al., 1998; Sorensen and Loeschke, 2002).

#### 4.3. Effect of age class not body size

After determining that *N. rhombifer* exhibit strong ontogenetic variation in thermal tolerance, we tested the hypothesis that smaller snakes (within age class) have higher thermal tolerances than larger snakes. We found no consistent significant linear relationship between body size and thermal tolerance within age classes of *N. rhombifer*. Our results are consistent with three amphibian studies that have documented ontogenetic variation in thermal tolerance with no effects of body size (within life history stage) on thermal tolerance (Delson and Whitford, 1973; Cupp, 1980; Berkhouse and Fries, 1995). Additionally, no effect of body size on thermal tolerance was found in adult *Pseudacris triseriata* (Miller and Packard, 1977), *Cnemidophorus sexlineatus* (Paulissen, 1988b), *Chelydra serpentina* (Williamson et al., 1989), and several species of salamanders (Hutchison, 1961; Hutchison et al., 1973).

#### 4.4. Neonatal sex affects thermal tolerance

We found a highly significant effect of neonatal sex on thermal tolerance, with females tolerating temperatures approximately 0.3 °C (LRR) and 0.5 °C (OS) higher on average than males. While it is unclear if this small difference in thermal tolerance is ecologically important, the strength and consistency of the trend suggests that thermal tolerance is truly different between the sexes at birth.

Thermal tolerance and heat shock protein expression are known to be higher in adult female than adult male flies (Coyne et al., 1983; Krebs et al., 1998), which might be related to differences in gonadal tissue heat shock protein

expression (Palter et al., 1986). In two species closely related to *N. rhombifer* there is evidence that females may be more resistant than males to non-optimal developmental temperatures. In *N. sipedon* there is a significant male-bias among stillborn young, likely due to a greater sensitivity to high temperatures among male embryos (Weatherhead et al., 1998). Also, in *Thamnophis elegans*, males may be more susceptible than females to temperature-induced abnormalities during development (Arnold and Peterson, 2002). Future studies should examine the possible links between sexual differences in neonatal thermal tolerance, sexual differences in temperature-induced developmental abnormalities, and sex ratios at birth.

#### 4.5. Conclusions

We have demonstrated that within *N. rhombifer* thermal tolerance can vary with locality, ontogenetic stage, and sex. From an ecological and evolutionary standpoint, ontogenetic shifts in habitat use and/or body size apparently have resulted in stronger selection on thermal tolerance within this species than has occurred from geographic separation of populations. The most parsimonious interpretation of the consistency of ontogenetic shifts in thermal tolerance among latitudes is that this trait evolved early within *N. rhombifer* or one of its ancestors. Our data also serve as an ideal illustration of why it is important to study more than two populations, and to focus on the life history stages that are most sensitive to selection in their thermal environment, when testing hypotheses of evolution along latitudinal gradients (Coyne et al., 1983; Garland and Adolph, 1994; Van der Have, 2002). If we had only used the OK and KS populations we might have incorrectly concluded that thermal tolerance in *N. rhombifer* does follow a latitudinal gradient. Similarly, if we had only examined latitudinal variation among adults we would have incorrectly concluded that there was no divergence among populations in thermal tolerance. Future studies should focus on the potential factors responsible for locally driven (non-latitudinal) genetic variation in thermal tolerance among neonates and the possible physiological mechanisms and potential costs that lead to decreased thermal tolerance in adults.

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