Do female newts modify thermoregulatory behavior to manipulate egg size?

Eliška Toufarová\textsuperscript{a}, Lumír Gvoždík\textsuperscript{b,∗}

\textsuperscript{a}Institute of Vertebrate Biology AS CR, Květná 8, 603 65 Brno, Czech Republic
\textsuperscript{b}Department of Botany and Zoology, Masaryk University, Kotlářská 267/2, 611 37 Brno, Czech Republic

\textbf{A R T I C L E   I N F O}

Article history:
Received 2 December 2015
Received in revised form 15 February 2016
Accepted 19 February 2016
Available online 3 March 2016

Keywords:
Amphibians
Egg size
Gravidity
Jelly coat
Mother-offspring conflict
Oviparity
Oviposition rate
Parental effect
Preferred temperature

\textbf{A B S T R A C T}

Reproductive females manipulate offspring phenotypes by modifying conditions during embryogenesis. In ectotherms, the environmental control over embryogenesis is often realized by changes in maternal thermoregulation during gravidity. To determine if reproduction influences thermoregulatory behavior in species where females lay eggs shortly after fertilization (strict oviparity), we compared preferred body temperatures \(T_p\) between reproductive (egg-laying) and non-reproductive female newts, \textit{Ichthyosaura alpestris}. Next, we exposed reproductive females to temperatures mimicking \(T_p\) ranges of reproductive and non-reproductive individuals to find out whether the maternally modified thermal regime influences ovum and jelly coat volume, and early cleavage rates at the time of oviposition. In the thermal gradient, reproductive females maintained their body temperatures within a narrower range than non-reproductive individuals. The exposure of ovipositing females to temperatures preferred during their reproductive and non-reproductive period had a negligible influence on egg size and early cleavage rates. We conclude that the modification of maternal thermoregulatory behavior provides a limited opportunity to manipulate egg traits in newts.

© 2016 Elsevier Ltd. All rights reserved.

\section*{1. Introduction}

Mothers affect their offsprings’ phenotype not only via genes but also with environmental conditions during embryonic development (maternal effect). Countless studies have demonstrated that maternally-manipulated environments benefit developing embryos (Lorioux et al., 2013; Madsen and Shine, 1999; Rieger et al., 2004). On the other hand, females can also behave in the opposite way, i.e. by providing detrimental conditions for individual offspring (Gripenberg et al., 2007; Rosenheim et al., 2008; Scheirs et al., 2000). Current conceptual advances in maternal effect studies resolved this paradox by viewing the adaptive maternal effect from the female rather than offspring’s lifetime reproductive success (Marshall and Uller, 2007). From this view, maternally-provided conditions may be beneficial, neutral, or even detrimental to developing embryos. Despite the conceptual progress in this field, benefits of some maternal behaviors remain unclear.

In many ectotherm taxa, gravid females often modify the mean or variance in body temperatures that benefit her offspring (Shine and Downes, 1999; Mathies and Andrews, 1997; Rodriguez-Díaz and Braña, 2011). Originally, the thermoregulatory shift was explained as a solution to the conflict between disparate thermal optima for mother and offspring phenotypes (Beuchat and Ellner, 1987). In agreement with the current view (see above), it has been recently proposed that maternal thermoregulation may favor not only offspring survival, the maternal manipulation hypothesis (Shine, 1995), but also female performance (Schwarzkopf and Andrews, 2012). However, because females of most examined taxa, squamate reptiles, deposit their eggs in later developmental stages (stages 30–40 after Dufaure and Hubert, 1961), determining whether maternal thermoregulatory behavior primarily increases female performance or offspring survival seems a challenging task. One previously omitted solution to the ‘chicken and egg’ problem is the focus on maternal thermoregulation in non-squamate taxa with the most primitive reproductive mode, when a female oviposits shortly before or after fertilization (strict oviparity). If strictly oviparous females modify their thermoregulatory behavior during reproduction, it clearly shows that maternal thermoregulatory adjustments evolved primarily for other reasons than the manipulation of offspring phenotypes.

Among the few studies on non-squamate systems examining the influence of reproduction on preferred body temperatures \(T_p\), Gvoždík, 2005; Webber et al., 2015), tailed amphibians, newts, seem particularly suitable for this task. Reproductive females of the Italian crested newt, \textit{Triturus carnifex}, modified both their \(T_p\)
mean and range relative to their non-reproductive counterparts (Gvoždík, 2005). Newts are strictly oviparous species and their eggs are laid before or shortly after the first cleavage (Griffiths and de Wijer, 1994). Accordingly, fertilized eggs stay in the oviduct for 4.5–12 h depending on temperature (Epperlein and Junginger, 1982; Bonacci et al., 2005). In contrast, newt embryonic development lasts about 15 days under thermally fluctuating conditions (Měrková and Gvoždík, 2009), and thus maternal thermoregulatory behavior provides a negligible opportunity for maternal thermal manipulation of offspring phenotypes during embryonic development. However, female body temperatures affect ovum size (Kaplan, 1987), and thereby offspring fitness-related traits, in amphibians (Bernardo, 1996; Kaplan, 1980; Semlitsch and Gibbons, 1990). Hence, newt maternal thermoregulation may affect offspring phenotypes in this previously unexplored way. In addition, because temperature influences most functions and processes within an organism (Bolins and Bowler, 1987), it may also modify the production of egg jelly coats. The jelly coating has many functions ranging from fertilization to protection (Shu et al., 2015), and thus their volume surrounding the oocyte is potentially adaptive. Whether maternal-modified temperatures affect newt ovum or jelly coat size is unknown.

In this study, we examined if reproductive alpine newt females, Ichthyosaura alpestris, modify egg size and early cleavage rates by modifying their thermoregulatory behavior. We expect that in strictly oviparous ectotherms, i.e. not carrying embryos in their bodies, the only possibility to effectively manipulate offspring phenotypes through maternal thermoregulatory behavior is via its effect on egg traits. Our aim was twofold. First, we tested the influence of reproductive state on $T_{sp}$ in female newts. Second, we exposed reproductive female newts to temperatures mimicking $T_{sp}$ ranges of reproductive and non-reproductive females to determine whether various thermal regimes affect the size and early cleavage rate of their eggs.

2. Materials and methods

2.1. Study species and maintenance

Ichthyosaura alpestris is a medium-sized (total length = 90–120 mm) newt that is widely distributed across most of continental Europe. The aquatic reproductive period lasts from April till June. Females oviposit 200–300 eggs during this period. Eggs are laid individually and wrapped in aquatic vegetation, providing eggs with protection against predators (Orizaola and Braña, 2003) and UV-radiation (Marco et al., 2001). This species shows distinct thermoregulatory behavior both in the laboratory and in semi-natural conditions (Balogová and Gvoždík, 2015; Marek and Gvoždík, 2012). Female oviposition preferences are also guided by water temperature (Dvořák and Gvoždík, 2009; Kurdlíková et al., 2011), which affects offspring performance and life-history traits (Kurdlíková et al., 2011; Měrková and Gvoždík, 2009).

We captured adult newts (snout-vent length [SVL] = 45–55 mm) by hand from a population near Jihlava, Czech Republic. In April 2009, we used 25 pairs (1:1 sex ratio) for testing the influence of reproduction on female preferred body temperatures. In April 2011, we took another 26 pairs for testing the effect of temperature on egg size. Newts (one male and one female) were placed in aquaria (50 × 30 × 18 cm high) filled with non-chlorinated water. Each aquarium was equipped with clumps of aquatic plants (Egeria densa) to provide egg laying substrate and shelter, and a piece of Styrofoam to allow newts to leave the water. Aquaria were placed in a room with light and thermally fluctuating (12–22 °C) regimes mimicking conditions in their natural habitat (Dvořák and Gvoždík, 2010). In November 2009, newts were reshuffled to keep both sexes separate and aquaria were transferred to a walk-in climatic chamber for wintering at 4 °C until the end of March 2010. Newts were fed with live food, Tubifex worms, earthworms (Eisenia sp.), and chironomid larvae, once or twice per week. Water was changed on a weekly basis.

2.2. Preferred body temperatures

To test the influence of reproduction on $T_{sp}$, we paired randomly chosen females ($n = 11$) with males in the beginning of April 2010. After a few days, the females started egg-laying, and thus they were denoted as reproductive. The unmated females ($n = 14$) represented the non-reproductive group.

Preferred body temperatures were measured in a stainless steel tank (240 × 60 × 60 cm high). The tank was divided into three longitudinal lanes. Each lane was partially separated into 12 partitions. The tank was filled with water to 4 cm. A computer controlled series of Peltier modules and a heat recuperation system maintained a horizontal thermal gradient (5–32.5 °C) in steps of 2.5 °C per partition. Each partition contained a clump of Java moss, V. dubyana, (3 g of wet mass) to provide reproductive females with laying substrate equally across temperatures. Water was kept at oxygen-saturated levels using an aeration stone in each partition. The tank was located in a room with stable temperature conditions (18 ± 2 °C). Lighting was provided by fluorescent bulbs (300 lx on water surface) during the day (6:00–18:00) and two red bulbs (<5 lx) allowed the recording of newt behavior during the night.

Because feeding and digestion influence $T_{sp}$ in newts (Gvoždík, 2005), and newts generally have food in their stomachs (Griffiths and Mylotte, 1987), newts were fed 24 h before the beginning of $T_{sp}$ trials. Three randomly selected females were placed into the tank (17.5 °C) 12 h before the beginning of a trial (20:00) for habituation to tank conditions. Female behavior was continuously (12 fps) recorded using a digital surveillance system (V-Guard RT4, Chateau Tech. Corp., Taiwan) for the following 24 h (8:00–20:00). After a trial, egg numbers and oviposition temperatures were recorded for each female. Oviposition temperatures were characterized by their mean and range (max–min) for each female. Later, we analyzed video files to obtain information about newt body temperatures by interpolating horizontal positions, the partition number, with known water temperatures. Because body temperatures of small to medium sized aquatic ectotherms closely match surrounding water temperature (Lutterscheidt and Hutchison, 1997), we considered this indirect method as a reliable, non-invasive, and non-disturbing approach to obtain body temperatures in this system. We recorded newt positions at 10 min intervals, which yielded 144 temperatures from each individual. From an individual $T_{sp}$ distribution, we calculated mean, and boundaries of the $T_{sp}$ range as 10th and 90th percentiles. Previous studies (Hadamová and Gvoždík, 2011; Gvoždík, 2015) proved that this method is sufficient to eliminate possible outliers from individual $T_{sp}$ distribution in newts. Motor activity index was estimated as the number of crosses between partitions during a trial.

2.3. Egg oviposition

Reproductive females captured in 2011 were individually placed in aquaria (41 × 23 × 17 cm high) filled with 3 L of water and equipped with a thermostat 50 W heater (Eheim Jäger, Wüstenrot, Germany) and a clamp of aquatic vegetation to provide substrate for oviposition. Females were randomly distributed between two groups that were exposed to temperatures matching the $T_{sp}$ range of reproductive and non-reproductive females (Fig. 1). Aquaria of each group were placed in a separate climatic room. The required thermal regime was achieved by heating water to...
From the egg photographs, we obtained four measures (resolution 0.001 mm) to calculate the volume of the fertilized egg cell (hereafter ovum) and surrounding jelly coats using DinoLite 2.0 (AnMo Electronics Corporation, Taiwan); ovum length ($l_{ovum}$; the longest diameter), ovum width ($W_{ovum}$; the shortest diameter, perpendicular to $l_{ovum}$), total length ($l_{tot}$; the longest egg diameter including jelly coat), total width ($W_{tot}$; the shortest egg diameter including jelly coat perpendicular to length). We calculated the ovum volume ($V_{ovum}$) as $V_{ovum} = \pi l_{ovum} W_{ovum}$ ($3c^2 + 14c + 35)/210$ (Maritz and Douglas, 1994), where $c=\lambda/(E-1)$, $\lambda$ and $E$ are parameters characterizing the ovoid shape. Both parameters were independently estimated from a template (Maritz and Douglas, 1994) by two persons with 100% concordance. Analogically, we calculated the total egg volume ($V_{tot}$) as $V_{tot} = l_{tot} W_{tot}$, where $l_{tot}$ and $W_{tot}$ respectively. We obtained the volume of jelly coats ($V_{coat}$) as $V_{coat} = V_{tot} - V_{ovum}$. The early cleavage rate was measured as the proportion of eggs laid before and after the first cleavage for each female.

2.4. Statistical analyses

Because our sample size prevented the determination of the distribution of examined variables, we applied a semi-parametric, randomization approach for statistical analyses. The influence of reproduction on $T_p$ characteristic was tested using a permutation general linear model (9999 permutations) with reproductive state as a fixed factor. Because the mean $T_p$ is associated with the $T_p$ range ($T_p$ boundaries) and vice versa (Hadamová and Gvoždík, 2011), we added the respective covariate and its interaction with reproductive state to the full model. If the interaction explained negligible variance of the examined variable, it was excluded from the final model. The full model for testing the influence of thermal regimes on $V_{ovum}$ and $V_{coat}$ contained the thermal regime as a fixed factor and female identity as a nested random factor. Because egg size increases with female body size in tailed amphibians (Kaplán and Salthe, 1979), female snout-vent length was added as a covariate. The exact $F$ distribution is unknown in a permutation model, and thus we refer to the calculated statistics as pseudo-$F$. Data are shown as means $\pm$ 95% CIs. Non-parametric confidence intervals were calculated using bias-corrected and accelerated methods (9999 bootstrap replications). Statistical modeling was realized using the PERMANOVA module in Primer package (version 6; Primer E, Lutton, UK). Confidence intervals were calculated using library ‘boot’ (Canty and Ripley, 2014) in R (R Development Core Team, 2014).

3. Results

We obtained preferred body temperatures from all reproductive and non-reproductive females ($n=25$). Mean, range, and boundaries of $T_p$ were intercorrelated (Fig. 1a), and so they were statistically analyzed using their respective covariate. Reproductive state affected the mean $T_p$ and the lower boundary of the $T_p$ range (mean: pseudo-$F_{1,22}=6.04$, $P=0.015$; lower boundary: pseudo-$F_{1,22}=12.62$, $P=0.003$; upper boundary: pseudo-$F_{1,22}=1.20$, $P=0.27$). Reproductive females maintained a lower mean $T_p$ and a narrower $T_p$ range than non-reproductive females (Fig. 1a). Females from both groups showed similar motor activity levels during $T_p$ trials (pseudo-$F_{1,22}=1.26$, $P=0.29$; Fig. 1b). Individual females deposited 1–23 eggs (mean $\pm$ SD = 17 $\pm$ 2) during a trial. Individual means of $T_p$ and oviposition temperatures (mean $\pm$ SD = 17.4 $\pm$ 2.3°C) were positively associated (Spearman $p=0.78$, $P=0.003$), whereas a statistically nonsignificant correlation was found between their ranges ($p=0.39$, $P=0.20$).
explained as a conflict between optimal temperatures for female and offspring traits (Beuchat and Ellner, 1987). Reproductive female newts maintained on average lower and less variable temperatures than their non-reproductive counterparts. However, thermal conditions simulating body temperatures of reproductive females had a non-detectable influence on early cleavage rates, and the volume of ovum and jelly coats. Hence, mothers modify their thermoregulatory behavior without affecting egg size and early cleavage rate in this strictly oviparous species.

The influence of reproduction on $T_p$ corroborates previous findings in other newt species, *T. carnifex* (Gvoždík, 2005). Although the mean $T_p$ showed the opposite shift, reproductive females reduced their $T_p$ range to a surprisingly similar extent in both newt taxa (*I. alpestris*: 16.2–21.4 °C; *T. carnifex*: 15.6–22.4 °C). The similar $T_p$ ranges suggest that the trait was inherited from their common ancestor 42 million years ago (Wiens et al., 2011) or both species became adapted to the same thermal conditions. Variation in mean annual air temperatures between examined populations (6.9 °C for *I. alpestris* and 9.8 °C for *T. carnifex* (Hijmans et al., 2005) and the slow evolution of $T_p$ in newts (Gvoždík, 2015) support the former option.

Given the prolonged egg-laying period, it may be argued that the modified maternal thermoregulatory behavior in fact follows temperature oviposition preferences. Because temperature-driven oviposition and its adaptive potential has been documented in this species (Dvůřák and Gvoždík, 2009, 2010; but see Kurdičková et al., 2011), the shifted thermoregulatory response appears to be a passive consequence of this beneficial maternal manipulation. The association between mean $T_p$ and mean oviposition temperature provides further support for this proposition. However, the maternal shift in mean $T_p$ was smaller than the shift in $T_p$ boundaries, which were independent on limits of temperature oviposition preferences. In addition, mean egg deposition time, i.e. time to lay a single egg, lasts about five minutes (Díaz-Paniagua, 1989), and thus females spent ovipositing 0.3–8% of the total trial time in the thermal gradient. Hence, similar to previous results (Gvoždík, 2005), time spent ovipositing was too short to explain the consistent shift in female thermoregulatory behavior.

Thermal regimes of reproductive and non-reproductive females, contrary to their individual identity, had a non-detectable influence on ovum and jelly coat size at the time of oviposition. This result seems surprising, because temperature is an important factor affecting egg size and developmental rates in amphibians (Kaplan, 1987; Bradford, 1990) including newts (Griffiths and de Wijer, 1994; Kurdičková et al., 2011; Smith et al., 2015). However, the thermoregulatory shift involved only the lower boundary of the $T_p$ range. thermal rates for various traits are usually less steep on the cold than the hot side of thermal performance curves (Martin and Huey, 2008), and thus 4 °C differences between the lower $T_p$ boundaries of reproductive and non-reproductive females were likely too small to affect egg traits. Because females have a more limited opportunity to maintain their target body temperatures in the field (Hadamová and Gvoždík, 2011), we predict that under natural conditions maternal thermoregulation would have an even smaller effect on egg traits than in our experimental settings.

Irrespective of thermal regime, female newts deposited 88–94% of eggs before the first cleavage. Because egg control intervals were relatively long (12 h), some eggs might start the first cleavage outside a female body. Hence, it is likely that the proportion of deposited eggs before the first cleavage was even higher than our results show. Given the extremely short time that fertilized ova spent in a female body, i.e., less than 3% of embryonic development period (Bonacci et al., 2005; Kurdičková et al., 2011), and relatively low $T_p$ variation between reproductive and non-reproductive females, it appears that the shift in female

4. Discussion

Changed thermoregulatory behavior during pregnancy, seen in many squamate taxa (Schwarzkopf and Andrews, 2012), has been
thermoregulatory behavior during reproduction has no opportunity to effectively manipulate offspring phenotypes in this system.

Recently, non-adaptive and adaptive alternatives to the maternal manipulation hypothesis (Shine, 1995) have been explained to depict the thermoregulatory shift during reproduction (Schwarzkopf and Andrews, 2012; Shine, 2012). According to the adaptive scenario, females change thermoregulatory behavior to maximize lifetime reproductive success, not through providing a direct benefit to their offspring but by maximizing their own performance. The mean $T_p$ of reproductive females matched the mean preferred oviposition temperatures (Dvořák and Gvozdík, 2009), which maximize oviposition rates (Kurdišová et al., 2011). Because each egg is carefully wrapped in leaves of aquatic vegetation (Díaz-Paniagua, 1989), laying the whole clutch takes considerable time at the expense of other activities. In addition, the oviposition period is limited by the permanence of the water body and the duration of the larval period. This suggests that reproductive females shifted their body temperatures to maximize oviposition rates.

A non-mutually exclusive alternative proposes that females burdened with an egg load change body temperature to increase their escape performance (Shine, 2012). Although alpine newts have thermal optima for swimming velocity well above the $T_p$ range (Gvozdík, 2015), increasing the lower $T_p$ boundary may improve their locomotor performance. However, using a simple interpolation from published thermal performance curves for the study population (Šamajová and Gvozdík, 2009), the upward shift in the lower $T_p$ boundary only causes 8% increase in burst swimming velocity. In addition, egg load had a minor influence on swimming performance in other salamanders (Finkler et al., 2003). Hence, the egg-burden hypothesis seems an unlikely explanation for the maternal thermoregulation in our system.

According to the non-adaptive explanation, the $T_p$ shift results simply from changes in activity or thermal inertia of reproductive females. In newts, reproductive and non-reproductive females maintain similar motor activity levels in a thermal gradient (Fig. 1b). Moreover, given the high heat conductivity and capacity of water, body temperature closely follows water temperature in small aquatic organisms (Lutterschmidt and Hutchison, 1997). It appears that thermal inertia has a negligible influence on body temperature variation in alpine newts.

In conclusion, we showed that maternal thermoregulatory behavior has a negligible influence on egg size and early cleavage rate in newts. Accordingly, the opportunity for a female to manipulate offspring traits through her maternal thermoregulatory behavior is greatly limited in these strictly oviparous ectotherms. Our finding has at least three important implications. First, though the shifted $T_p$’s have been frequently cited as an example of behavioral maternal effects for decades, our results support our earlier claim (Gvozdík 2005) that the modified maternal thermoregulation in newts evolved independently of advanced reproductive modes, such as viviparity or oviparity with prolonged egg retention. An unsolved question remains whether newt maternal thermoregulation is an ancestral trait or if it evolved together with their unique parental care, egg-wrapping behavior. A further comparative study of salamandrid taxa with primitive and advanced reproductive modes would be highly informative. Next, the thermoregulatory shift in reproductive females may arise from disparate thermal optima not only for maternal and offspring traits but also for performance traits used by a female during reproductive and non-reproductive periods. The general model explaining thermoregulation in reproductive females needs further development to be applied also to strictly oviparous taxa. Finally, though maternal thermoregulation has been extensively studied in various squamates (reviewed by Schwarzkopf and Andrews, 2012), none of them provided a suitable ‘model’ to examine maternal thermoregulation in species with the ancestral reproductive mode, strict oviparity. Newts proved to be an excellent system to obtain a more general view on this phenomenon.

Acknowledgments

We thank J. Dvořák and R. Smolinský for their help with obtaining newts; anonymous reviewers for their comments on the previous versions of this paper. This study was funded by a grant from the Czech Science Foundation (P506/10/2170 and 15-07140S) and institutional support (RVO: 68081766) to LG. All experimental procedures were conducted under permission of the Expert Committee for Animal Conservation of the Institute of Vertebrate Biology AS CR (research protocol no. 113/2009). The Agency for Nature Conservation and Landscape Protection of the Czech Republic issued permission to capture the newts (1154/ZV/2008).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jtherbio.2016.02.001.

References


