Acta Herpetologica 17(2): 159-164, 2022
DOI: 10.36253/a_h-12660

Sunny-side up: ontogenetic variation in egg mass temperatures of the wood frog *Rana sylvatica*

Ryan Calsbeek*, Ava Calsbeek, Isabel Calsbeek

Department of Biological Sciences, Dartmouth College, Hanover, New Hampshire 03755 USA
**Corresponding author*. E-mail: ryan.calsbeek@dartmouth.edu

Submitted on: 2022, 24th January; revised on: 25th May 2022; accepted on: 26th May 2022
Editor: Raoni Rebouças

Abstract. The efficacy of most biological processes is temperature dependent and, within physiological limits, on average, warmer is better. This axiom of biology has led to a wide range of adaptations for dealing with temperatures that are outside of an organism's preferred temperature. Many pond-breeding amphibians lay their eggs during early spring, when water temperatures are near freezing. Communal nest-site selection has been proposed as a mechanism to increase developmental temperatures, and temperatures near the center of egg-mass aggregations are elevated relative to egg-masses on the aggregation's periphery. It is unclear whether this spatial variation in temperature is due to concentration of metabolic heat, absorption of solar radiation, or both. Here, we explore finer scale spatial variation within egg masses of the wood frog *Rana sylvatica*, one of the earliest amphibians to breed during the North American spring. We compared peripheral and core temperatures of egg masses that were exposed either to 1) ambient sunlight from above, or 2) sunlight reflected by a mirror from below. We found that differences between core and peripheral temperatures were higher in the control than in the mirror treatment, but core and peripheral temperatures were statistically indistinguishable in both cases. Moreover, the difference in peripheral and internal temperatures increased significantly over the course of development. However, these trends were only significant in ambient sunlight and actually decreased in the mirror group. Our results suggest that the benefits of communal nesting are also experienced by individual egg masses, albeit to a lesser extent. In addition, the lack of effect in shaded egg masses suggests that the thermal advantage is tied to sun exposure and not due to concentration of metabolic heat.

Keywords. Anurans, egg-mass aggregations, developmental temperatures.

INTRODUCTION

A central tenet of biology is that all physiological processes are temperature dependent (Huey, 1991). This is because enzymes that regulate physiology have specific temperature ranges over which they can operate (Knies et al., 2009). The vast majority of these thermal-performance ranges are left-skewed and show improving physiological performance with increasing temperature, until some threshold 'optimal temperature' (T_{opt}) is reached, at which point physiological performance drops off rapidly. The generality of this pattern underlies the adage that, within physiological limits, hotter is better (Frazier et al., 2006; Angilletta et al., 2010).

Organisms in environments characterized by extreme temperatures face unique challenges around life-history and reproduction, since sub-optimal environmental conditions should constrain physiological performance (Skelly, 2004; Tryjanowski et al., 2006; Benard 2015). These challenges are especially prevalent for ectotherms (Huey and Tewksbury, 2009), which depend on environmental sources of warmth to maintain metabolic activity. Temperatures that exceed a critical threshold (e.g., CTmax) may be lethal and during hot periods

ISSN 1827-9635 (print)
ISSN 1827-9643 (online)

www.fupress.com/ah

ectotherms may require shaded habitat or underground refugia to behaviorally thermoregulate (Díaz-Ricaurte et al., 2022; Sinervo et al., 2010). High temperatures may lead to dehydration which itself can change thermoregulatory dynamics (Guevara-Molina et al., 2020). Many ectotherms, including insects, reptiles and amphibians, buffer themselves from the negative consequences of sub-optimal temperatures by estivating or entering diapause (Blanckenhorn and Fairbairn, 1995; Ellner et al., 1998; Storey and Storey, 2012) during colder periods of the year. Nevertheless, these same organisms may often face extremely cold conditions that occur at the peak of their reproductive activity in early spring (Costanzo and Lee, 1993).

Many pond breeding amphibians emerge from winter hibernacula as soon as temperatures exceed freezing (Waldman, 1962; Herreid and Kinney, 1967). The first amphibians to emerge at the end of North American winters often breed while ponds are still ice-covered, and females lay eggs in water that is very close to its freezing point (Costanzo and Lee, 1993). Aggregating egg masses in communal nest sites is one potential adaptation to cold. Previous studies have shown that egg masses at the center of these communal aggregations are warmer compared to those on the periphery (Savage, 1961; Hassinger, 1970). Warmer temperatures should be advantageous to developing embryos, since warmer temperatures speed development and tadpoles that reach metamorphosis more quickly will have a higher probability of escaping vernal pools before they dry (Goldstein et al., 2017). Other work (Arrighi et al., 2013) has shown that the influence of diel fluctuations in temperature may be as (or more) important as that of average temperature on developmental rate. In addition, amphibian eggs often have higher concentrations of melanin in their dorsal hemisphere, which may serve as both protection from ultraviolet radiation and a means of absorbing and retaining heat (Licht, 2003).

Although a handful of studies have documented a thermal advantage to aggregated egg masses (Waldman, 1982; Skelly, 2004), the source of thermal variation (e.g., solar vs. metabolic) remains unclear as does the degree to which this thermal advantage occurs at smaller spatial scales (i.e., within individual egg masses). Moreover, all of these studies have been conducted as point estimates in time and so we currently have no information about how developmental progression impacts temperature of the egg mass itself. Developmental stage may be important for impacting both the generation of metabolic heat as well as greater thermal absorption by larger embryos. Finally, it is possible that egg mass aggregations are not at all adaptive but are the result of space constraints within a pond, aggregation due to wind currents, or other neutral exaplanations.

Here we build on previous work to address these shortcomings. The wood frog, *Rana sylvatica*, is among the earliest amphibians to breed in North America, emerging from winter hibernacula as soon as temperatures exceed freezing (Slough and Mennell, 2006). Wood frogs at our study populations near Norwich Vermont, USA, elevation ca. 300m; 43.7153°N, 72.3079°W (WGS 84 web Mercator), typically emerge during the end of March-early April (Brady et al., 2019; Goedert and Calsbeek, 2019). Males and females arrive at breeding ponds and breed explosively (Swierk et al., 2014), most oviposition occurring within a few days of arrival, and eggs are often laid while ponds are still partially covered in ice. These life history traits make wood frogs especially relevant for understanding the effects of temperature on embryo development. First, we test whether individual egg masses also exhibit warmer internal compared to peripheral temperatures. Next, we experimentally test the hypothesis that egg masses warm by absorbing radiant heat from above (i.e., via the pigmented dorsal surface) more efficiently than from below. Lastly, we provide temperature measurements over the time-frame from oviposition to hatching to assess the degree to which development itself may influence temperature variation (e.g., by metabolic warming) within egg masses

METHODS

We collected 10 wood-frog egg masses from a single pond within 36 hours of oviposition in April of 2021. Egg masses were collected by hand and carefully transferred to a plastic holding tank along with ~6L of unfiltered water from the same pond. We assessed Gosner's stage (Gosner, 1960), recording the average the developmental stage of five embryos scored under a microscope for each egg mass. All embryos were at Gosner's stage 10 at the start of the experiment. Developmental stage was thereafter scored by visual inspection to minimize disturbance over the course of the experiment. Egg masses were sectioned into two groups of ca. 75 embryos each (i.e., 150 eggs total from each egg mass) and these two sections of egg mass were then split randomly (by coin toss) into the two groups, such that ~75 individuals from of each of the 10 egg masses were represented in both groups. Each set of 75 embryos in the first group were placed in separate 500 ml cylindrical plastic containers (10 cm diameter, 10 cm deep), covered with an opaque lid and placed outdoors on wire shelving that was suspended approximately 30 cm above a mirror. The mirror group was designed to reverse the direction of sun exposure from the melanic dorsal to the white ventral side of the embryo. Each group of 75 embryos in the second group were likewise placed in individual 500 ml plastic containers but were covered with a clear plastic lid and placed on wire shelving over a dark green substrate that was covered with leaf litter from the adjacent forest. The two groups were placed in the same outdoor location with no canopy cover. Photoperiod during the experiment was approximately 14:10 (L:D). All sections of egg mass were suspended in approximately 450 ml of pond water, which was changed once, seven days into the experiment.

Every one to two days we chose half of the ten containers in each group at random for temperature measurements. We recorded air temperatures using an outdoor Accu-Rite™ thermometer, and egg-mass/water temperature using a digital thermometer (Thermoworks model RT600C-N) on the periphery of the egg mass and inserted into the center of the egg mass at the same water depth (~2 cm). We recorded air temperatures outside of the containers, and water temperatures at the time of each measurement. We monitored rates of development by recording changes in Gosner stage (Gosner, 1960) during each temperature recording. Temperature measurements continued until hatching or 14 days, at which point the experiment was ended and the hatchlings and a few unfertilized eggs were transferred to larger volume holding tanks. In total we recorded 110 temperatures during 11 of the 14 days.

To minimize the problem of pseudo-replication, we calculated the mean temperature for each group on each day and used these mean values as our unit of observation in all analyses: 11 averages per group, 22 total observations. All data met the criteria for parametric statistics (normality, homoscedasticity, and independence; Sokal and Rohlf, 1995) based on testing in JMP Pro v.16. We tested for relationships between time elapsed since the start of the experiment (days) and air/water temperature using simple linear regression. We tested for a difference in peripheral temperature and core temperature between groups using ANOVA with 1 and 19 degrees of freedom. We tested for a difference in temperature from the center and periphery of the egg mass by subtracting the latter from the former and then using these difference values as the dependent variable using ANOVA with 1 and 19 degrees of freedom. We included Gosner's stage and tested for an interaction between Gosner's stage and experimental group to test whether there was a stage specific difference in temperature between groups in an ANOVA with 1 and 18 degrees of freedom. All tests were two-tailed and were conducted in JMP Pro v.16 (SAS Institute, Cary, North Carolina, USA 1989-2022).

RESULTS

Owing to the vicissitudes of spring temperatures during 2021 (typical of the NE United States), there was no significant correlation between measurement date and either air temperature ($r^2 = 0.03$, $t_{20} = 0.74$, $P = 0.47$) or water temperature ($r^2 = 0.07$, $t_{20} = 1.23$, $P = 0.23$). Mean temperature at the periphery of the egg mass section was not significantly different between the two groups (X ± SE: control = 13.13 ± 1.09 °C, mirror = 12.45 ± 1.05 °C; ANOVA: $F_{1,19} = 0.04$, $P = 0.80$, effect of Gosner's stage $P = 0.23$; Fig. 1A). Mean temperature at the center of the egg mass section was likewise not significantly different (control = 13.21 ± 2.47 °C, mirror = 12.32 ± 2.36 °C; ANOVA: $F_{1,19} = 0.87$, $P = 0.79$, effect of Gosner's stage $P = 0.21$; Fig. 1B). However, the differences between core and peripheral temperatures were significantly larger in the control than in the mirror group (0.39 ± 0.08 vs.

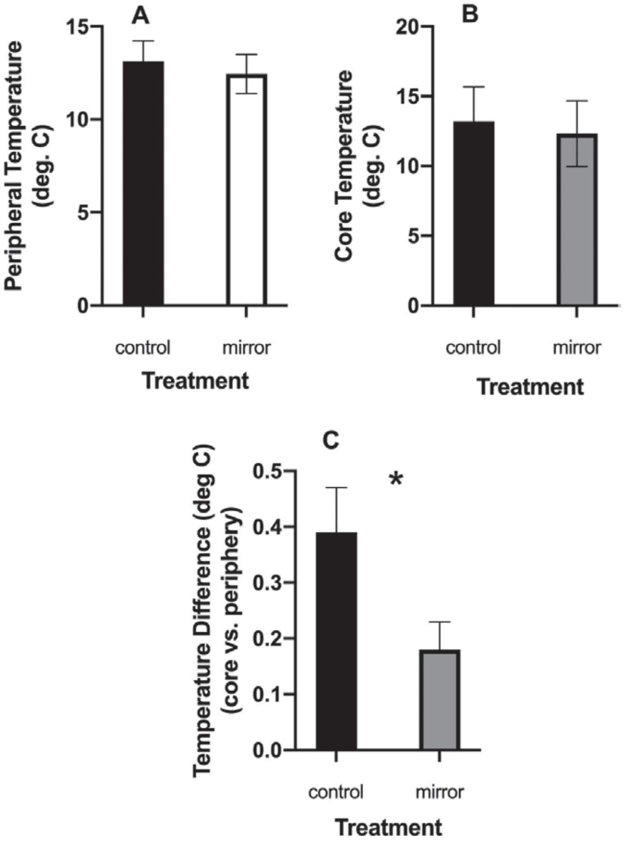

Fig. 1. Temperatures did not differ at the periphery of the wood frog egg mass (A), nor at the core of the egg mass (B) in either group. However, the difference in core and peripheral temperatures (that is, comparing means in panels A and B was significantly higher (* $t_{20} = 2.29$, $P = 0.03$) for the control group compared to the mirror group (C). Histograms bars show mean values ± one standard error.

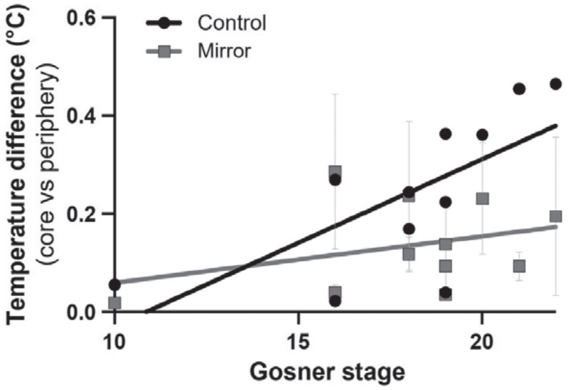

Fig. 2. *Top*. The difference in temperature between core and periphery increased throughout embryonic development of wood frogs (*Rana sylvatica*) for the control group but not in the mirror group. Data points show mean values ± one standard error. *Bottom*. Picture of the study species.

0.18 ± 0.05 °C respectively, ANOVA: $F_{1,19} = 6.44$, $P = 0.02$, effect of Gosner's stage $P = 0.03$; Fig. 1C). Moreover, this difference increased over the course of development resulting in a significant group × Gosner's stage interaction (ANOVA: $F_{1,18} = 6.42$, $P = 0.04$; Fig. 2). Hatching success at the conclusion of the experiment did not differ between groups; nearly all containers showed 100% hatching success. Whereas developmental stage and hatching in the mirror group lagged the control group by one day, developmental stage did not vary with position in egg mass section in either group. Nor did hatching dates vary within groups. Given this lack of variation, these results were not analyzed statistically.

DISCUSSION

The challenges associated with life in cold climates select for a variety of adaptations that facilitate heat retention and speed development (Skelly 2004, Sparks et al. 2006, Benard 2015). Ectotherms, which rely on external sources of heat to sustain metabolic processes, exhibit patterns in nature that may represent a limited set of strategies to maximize heat retention. Concentrating melanin and other dark pigments in the dorsal hemisphere of amphibian eggs may enhance thermal absorption within individual egg masses (Licht 2003). Likewise, communal nest sites, in which aggregations of egg masses are formed, may concentrate temperatures on their interior (Hassinger 1970).

We have shown that both of these patterns occur not just in aggregate, but also at the smaller spatial scales of individual portions of egg masses. Eggs in our control group experienced significantly warmer temperatures at the core of the egg mass compared to their peripheral counterparts but the same was not true in the mirror group. This suggests that the darker dorsal side of the developing embryo may briefly enhance thermoregulation during development compared to the white ventral side of the embryo. This effect is likely to be short-lived in nature since the dark pigment quickly subsumes the entire embryo. Wood frog embryos appear to maintain this thermal advantage even following disturbance sufficient to re-orient the embryos in their horizontal plane. We included the mirror group in our study to account for the rotational behavior of flipped embryos. It is worth noting that embryo rotation is likely a result of differences in density and not a response to light, since all embryos retained their normal orientation in both the mirror and control group.

The difference between internal and peripheral temperatures increased significantly over the course of development in our control group but not in the mirror group. This further supports a role for the pigmentation of embryos in enhancing thermoregulation (Clusella-Trullas et. al., 2007, 2009; Stuart-Fox et. al., 2019). The initial difference between dorsal and ventral pigment persists up to about the 13th Gosner's stage in wood frogs (Gosner, 1960), which occurs about one week after oviposition. After stage 13, the embryo enters gastrulation and the distribution of pigment is more evenly distributed throughout the entire embryo (Altig, 1972). As differentiation proceeds, the surface area to volume ratio of the embryo increases dramatically and the uniformly dark body acts as a heat sink. The fact that this ontogenetic shift was absent in the mirror group suggests that despite our attempts to maintain similar degrees of light exposure in the two groups, a mirror may have been insufficient to match the thermal energy absorbed in the control group. An alternative hypothesis is that the difference in peripheral and core temperatures arises due either all, or in part, to metabolic heat production. There are at least three reasons to think that this may not be true: first, ectotherms produce negligible amounts of metabolic heat (Andrade et al., 2015). Second, we see no reason why differences in metabolism should have arisen between groups. Third, egg masses held in a dark, temperature-controlled room as part of a separate experiment (Calsbeek, unpublished) showed no variation in temperature between the center and periphery of the egg mass.

Given the rapid loss of polarity in the pigmentation of embryos, any thermal advantage to the dorsal orientation of the pigmented embryo should be short-lived (e.g., a few days). Combined with the lack of an effect in the mirror group, we suggest that our results are consistent with a brief thermal advantage to an egg that rests sunny-side up (i.e., darker side dorsal), followed by a rapidly increasing thermal advantage associated with shifts in the surface area to volume ratio of the developing anuran embryo.

Understanding the importance of incubation temperature for amphibians with different oviposition behaviors (e.g., communal versus solitary nesting) could prove important for understanding the potential impacts of climate warming. Future work should include tests for the joint roles of nesting behavior and incubation temperatures in tropical species, since temperatures in the tropics are both warmer and less variable than in the temperate zone (Sinervo et. al., 2010). These differences in thermal regime suggest that tropical ectotherms may be especially vulnerable to climate warming, since even subtle changes in temperature could surpass thermal maxima (Huey and Tewksbury, 2009). As such, small differences in temperature within egg masses could have important implications for rates of development and/or survival for tropical amphibians.

ACKNOWLEDGMENTS

We thank Madilyn Gamble, Ridhi Chandarana and two anonymous referees for helpful comments that improved this experiment. RC was supported by an award from the National Science Foundation NSF DEB-1655092. All research was conducted with permission from the Vermont department of fish and wildlife and IACUC protocol 00002097.

REFERENCES

Adrade, D.V., Gavira, R.S.B., Tattersall, G.J. (2015): Thermogenesis in ecothermic vertebrates. Temperature **4**: 454.

Altig, R. (1972): Notes on the Larvae and Premetamorphic Tadpoles of Four *Hyla* and Three *Rana* with notes on tadpole color patterns. J. Elisha Mitchell Sci. Soc. **88**: 113-119.

Angilletta, M. J., Huey, R.B., Frazier, M.R. (2010): Thermodynamic effects on organismal performance: Is hotter better? Physiol. Biochem. Zool. **83**: 197-206.

Arrighi, J.M., Lencer, E.S., Jukar, A., Park, D., Phillips, P.C., Kaplan, R.H. (2013): Daily temperature fluctuations unpredictably influence developmental rate and morphology at a critical early larvval stage in a frog. BMC Ecology **13:** 13-18.

Benard, M.F. (2015): Warmer winters reduce frog fecundity and shift breeding phenology, which consequently alters larval development and metamorphic timing. Glob. Change Biol. **21**: 1058-1065.

Blanckenhorn, W.U., Fairbairn, D.J. (1995): Life-History Adaptation Along a Latitudinal Cline in the Water Strider *Aquarius-Remigis Heteroptera*, Gerridae. J. Evol. Biol. **8**: 21-41.

Brady, S. P., Zamora-Camacho, F. J., Eriksson, F.A.A., Goedert, D., Comas, M., Calsbeek, R. (2019): Fitter frogs from polluted ponds: The complex impacts of human-altered environments. Evol. Appl. **12**: 1360-1370.

Clusella-Trullas, S., Wyk, J.H., Spotila, J.R. (2009): Thermal benefits of melanism in *cordylid* lizards: a theoretical and field test. Ecology **90**: 2297-2312.

Clusella-Trullas S., van Wyk, J.H., Spotila, J.R. (2007): Thermal melanism in ectotherms. J. Thermal Biol. **32**, 235-245.

Costanzo, J. P., Lee, R. E. (1993): Cryoprotectant Production Capacity of the Freeze-Tolerant Wood Frog, *Rana-Sylvatica*. Can. J. Zool. Rev. **71**: 71-75.

Díaz-Ricaurte, J.C., Serrano, F.C., Martins, M. (2022): VTMaxHerp: A data set of voluntary thermal maximum temperatures of amphibians and reptiles from two Brazilian hotspots. Ecology **103**: e3602.

Ellner, S. P., Hairston, N.G., Babai, D. (1998):Long-term diapause and spreading of risk across the life cycle. Ergebnisse der Limnologie **0**: 297-312.

Frazier, M. R., Huey, R.B., Berrigan, D. (2006): Hotter is better: Thermodynamics constrains the evolution of insect population growth rates. Integ. Comp. Biol. **46**: E45-E45.

Goedert, D., Calsbeek, R. (2019): Experimental evidence that metamorphosis alleviates genomic conflict. Am. Nat. **194**: 356-366.

Goldstein, J.A., Hoff, K.V., Hillyard, S.D. (2017): The effect of temperature on development and behaviour of relict leopard frog tadpoles. Cons. Phys. **5**: Cow075.

Gosner, K.L. (1960). A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica **16**: 183-190.

Guevara-Molina, C., Gomes, F.R., Camacho, A. (2020): Effects of dehydration on thermoregulatory behavior and thermal tolerance limits of *Rana catesbeiana* (Shaw, 1802). J. Thermal Biol. **93:** 102721.

Hassinger, D. (1970): Notes on the thermal properties of frog eggs. Herpetologica **26**: 49-51.

Herreid, C.F., Kinney, S. (1967): Temperature and development of the Wood Frog, *Rana Sylvatica,* in Alaska. Ecology **48**: 579-590.

Huey, R.B. (1991): Physiological consequences of habitat selection. Am. Nat. **137**: S91-S115.

Huey, R.B., Tewksbury, J.J. (2009): Can behavior douse the fire of climate warming? Proc. Natl. Acad. Sci. U.S.A. **106**: 3647-3648.

Knies, J.L., Kingsolver, J.G., Burch, C.L. (2009): Hotter is better and broader: thermal sensitivity of fitness in a population of bacteriophages. Am. Nat. **173**: 419-430.

Licht, L.E. (2003): Shedding light on ultraviolet radiation and amphibian embryos. Bioscience **53**: 551-561.

Savage, R. (1961): The ecology and life history of the common frog, *Rana temporaria temporaria*. Hafner Publishing Co., New York.

Sinervo, B., Méndez-de-la-Cruz, F., Miles, D.B., Heulin, B., Bastiaans, E., Villagrán-Santa Cruz, M., Lara-Resendiz, R., Martínez-Méndez, N., Calderón-Espinosa, M.L., Meza-Lázaro, R.N., Gadsden, H., Avila, L.J., Morando, M., De la Riva, I.J., Victoriano Sepulveda, P., Rocha, C.F., Ibargüengoytía, N., Aguilar Puntriano, C., Massot, M., Lepetz, V., Oksanen, T.A., Chapple, D.G., Bauer, A.M., Branch, W.R., Clobert, J., Sites, J.W. Jr. (2010): Erosion of lizard diversity by climate change and altered thermal niches. Science **5980**: 894-899.

Skelly, D.K. (2004): Microgeographic countergradient variation in the wood frog, *Rana sylvatica*. Evolution **58**: 160-165.

Slough, B.G. Mennell, R.L. (2006): Diversity and range of amphibians of the Yukon territory. Can. Field. Nat. **120**: 87-92.

Sokal, R.R., Rohlf, F.J. (1995): Biometry: The principles and practice of statistics in Biological Research. 3rd Edition, W.H. Freeman and Co., New York.

Storey, K.B., Storey, J.M. (2012): Aestivation: signaling and hypometabolism. J. Exp. Biol. **215**: 1425-1433.

Swierk, L., Graham, S.P., Langkilde, T. (2014): The stress of scramble: sex differences in behavior and physiological stress response in a time-constrained mating system. Behav. Ecol. Sociobiol. **68**: 1761-1768.

Tryjanowski, P., Sparks, T., Rybacki, M, Berger, L. (2006): Is body size of the water frog *Rana esculenta* complex responding to climate change? Naturwissenschaften **93**: 110-113.

Waldman, B. (1982): Adaptive significance of communal oviposition in wood frogs (*Rana sylvatica*). Behav. Ecol. Sociobiol. **10**: 169-174.