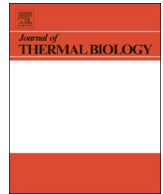




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Do ring-necked snakes choose retreat sites based upon thermal preferences?

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ABSTRACT

Biochemical reaction rates are highly sensitive to temperature, and the body temperatures of ectotherms covary with their immediate environment. Therefore, ectotherms should choose microhabitats that permit the maintenance of physiological function. While some previous studies have found that squamate reptiles choose retreat sites that allow them to maintain physiologically optimal body temperatures, this research has been limited in context and taxonomic scope. We sought to test these empirical patterns by studying the properties of retreat sites in the context of physiological preferences and tolerances in a population of semifossorial ring-necked snakes (*Diadophis punctatus*). We measured environmental temperature distributions of retreat sites, field body temperatures, thermal preferences, and both upper voluntary temperature and critical thermal minima of snakes. We found that ring-necked snakes are under larger and warmer rocks, but that body temperatures in the field do not match thermal preferences measured in the laboratory. Specifically, we found aggregated ring-necked snakes (those occurring with multiple conspecifics) select rocks providing environmental temperatures averaging 3 °C higher than their preferred temperature. By contrast, solitary snakes select rocks that allowed them to maintain their body temperatures very close to their preferred temperatures. These results imply that there is substantial within and among-species variation in the role of thermal considerations in retreat-site selection. Our work also highlights the complex tradeoffs between physiological and ecological requirements that organisms must navigate in heterogeneous habitats.

1. Introduction

The choices that animals make in selecting habitats can have substantial impact on their fitness. Animals are predicted to choose habitats that reduce competition, decrease predation risk, increase foraging efficiency, and optimize physiology (Giliam and Fraser, 1987; Huey, 1991; Morris, 2003). For ectotherms, the thermal environment can play a crucial role in habitat selection, because their internal body temperatures covary with their immediate surroundings and biochemical reaction rates are extremely sensitive to temperature (Gillooly et al., 2001; Hochachka and Somero, 1973; Huey and Slatkin, 1976; Pough, 1980). That is, animals should choose microhabitats that permit the maintenance of physiological function, and failure to do so may compromise survival and reproduction (Huey, 1991; Huey et al., 1989; Logan et al., 2014a, 2016).

Thermoregulatory behavior is typically considered in the context of animal activity, where an individual is either “active,” whereby they use behavior to maintain a narrow range of body temperatures, or they are “inactive,” whereby they retreat to hidden refugia and can no longer actively thermoregulate (Logan et al., 2014b; Sabo, 2003). Indeed, recent models of the impacts of climate change (Sinervo et al., 2010) assume that when ambient temperatures exceed some critical maximum, ectotherms move to cooler refugia where they can no longer acquire food resources or mate (activities critical for maintaining positive population growth). However, some retreat sites can also be very effective at conducting heat (Huey et al., 1989). For example, many squamate reptiles use retreat sites (rocks and rock crevices) which vary dramatically in temperature over space and time and can be used for thermoregulatory purposes (Huey et al., 1989; Webb and Shine, 1998).

Several studies have found that squamate reptiles choose retreat

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sites that allow them to maintain physiologically optimal body temperatures. In the first study of its kind, Huey et al. (1989) demonstrated that western garter snakes (*Thamnophis elegans*) hide under rocks of intermediate size which allow them to maintain body temperatures within the optimal range for digestion and metabolism. A handful of subsequent studies (Kearney, 2002; Shah et al., 2004; Webb and Shine, 1998) have shown that other species of semi-fossorial snakes and lizards tend to choose retreat sites that optimize thermal performance. Nevertheless, ectotherms can also choose retreat sites for reasons other than thermoregulation (e.g. social interaction, predator avoidance, moisture availability, etc.), and tradeoffs among these factors may reduce the thermoregulatory benefit of a particular site (Davis Rabosky et al., 2012; Riddell and Sears, 2015). Notably, prior studies have been limited in their taxonomic, ecological, and geographic scope, and additional studies of the supposed thermoregulatory benefits of retreat sites are crucial to understand the generality of physiological considerations in driving retreat site selection.

We sought to address this issue by studying 1) the physical dimensions and thermal properties of retreat sites, and 2) physiological preferences and tolerances in a population of semifossorial snakes. Our focal species, the ring-necked snake (*Diadophis punctatus*), is a diminutive snake that uses cover objects (including rocks) as retreat sites throughout the year, but is concentrated under these sites following emergence from hibernacula. We combined field estimates of body temperature and habitat use with laboratory assays of thermal physiology to test 1) whether snakes select rocks of certain physical dimensions, 2) whether thermal preferences or tolerances are related to retreat site selection, and 3) whether snake thermal physiology or retreat site selection is driven by organismal traits (sex, shedding cycle, or evidence of recent predator attacks). Our results lend insight into how thermal ecology and other factors shape retreat site and habitat selection among ectotherms.

2. Materials and methods

2.1. Field collection

Our study site was an isolated rocky hillside surrounded by mature deciduous forest at Mountain Lake Biological Station near Pembroke, VA, USA. We sampled this site a total of four times from May 31st to June 2nd of 2017 as part of a Field Herpetology class. We sampled during daylight hours between 1000 and 1730 h, during the diurnal activity period of these snakes (Ernst and Ernst, 2003). We exhaustively searched all of the rocks for the bottom 3 m of the hill, flipping and collecting data for every rock that was visible. We also haphazardly searched a few rocks further up the hillside. We painted a unique numerical ID on each rock, regardless of whether the rock contained a snake. We measured the temperature of the top surface of the rock from a distance of ca. 0.5 m using an infrared thermometer (General Tools and Instruments, IRT207, resolution of 0.1 °C, accuracy of 1 °C of temperature, 8:1 distance to spot ratio) prior to flipping it over. After flipping the rock, we measured the temperature of the bottom surface, and lastly we measured the temperature of the substrate underneath the rock. We also measured the length (length of the longest axis of the rock), width (length of the shortest axis) and height at the thickest part of the rock. We then multiplied these dimensions together to calculate rock volume (approximated as a rectangular prism; Eq. (1)),

$$V = L \times W \times H \quad (1)$$

where L is length, W is width, and H is the height or thickness of the rock.

Any snakes found under a rock were immediately captured and temperature was measured by inserting a thermometer (Taylor Compact Folding Thermometer, Taylor US, Oakbrook, Illinois, USA, accuracy and resolution of 0.1 °C) into the cloaca (T_b). We then recorded sex, snout-vent length (SVL), tail length (TL), ecdysis state,

gravity, presence of body scars, whether or not they were missing their tail, and whether the snake was solitary or with other snakes at the time of capture. Sex was noted by length of the tail and by gently evert the hemipenes in males. We note that the majority of snakes in this study were female (24 out of 30). SVL and TL were measured using standard methods, where snakes were extended alongside a fixed measuring tape and SVL was recorded as the distance between the tip of the snout and the cloaca (vent), and TL was recorded as the distance between the tip of the snout and the tip of the tail. Ecdysis was noted when snakes were actively shedding or if their eyes became translucent. Gravity was noted by gently palpating the snake's abdomen for oviductal eggs. This same procedure was used to check snakes for the presence of stomach contents, and no snakes had any palpable stomach contents. While the reproductive biology of this population is not known, ringnecked snakes can breed from May-September and generally lay eggs in June and July (Ernst and Ernst, 2003). Snakes were then brought to the laboratory and maintained for up to 72 h for physiological assays. Snakes were maintained simply in one liter plastic containers, with a two cm layer of dampened substrate from near the site of capture, maintained at 25 C, with a 12 h light:dark cycle. Snakes were given a 12–24 h acclimation period prior to the onset of physiology trials. After physiological assays were complete, all individuals were marked with a unique color code using paint pens and released under the same rocks where they were captured to avoid recapture and pseudoreplication. This study was performed under University of Virginia IACUC Protocol 3927-04-15 to Edmund D. Brodie III (Director of Mountain Lake Biological Station).

2.2. Predicted body temperature

In order to generate an environmental temperature distribution (a distribution of temperatures available to snakes in the environment), we constructed a model that predicted the body temperature that a snake would achieve under every rock we sampled (even those that did not contain snakes). Using the rocks that did have snakes under them, we tested linear models using all combinations of rock thickness, rock volume, top of rock temperature, bottom of rock temperature, and substrate temperature to predict actual snake body temperature, and selected those with the highest R^2 as the best predictive models. Note that we included rocks with multiple thermal measurements (both for rocks with multiple snakes and for rocks that had thermal values measured during different days) as useful variation for our predictive model. We found that the model with the highest R^2 ($R^2=0.533$) incorporated both substrate temperature and log-transformed rock volume. We then predicted the body temperature of hypothetical snakes for all rocks in our dataset (Eq. (2)),

$$\text{Predicted } T_b = T_{\text{substrate}} \times 0.2886183 + \ln(\text{rock volume}) \times 1.1530628 + 2.3426157 \quad (2)$$

where T_b is body temperature and $T_{\text{substrate}}$ is the substrate temperature.

We note that an alternative approach would be to measure operative temperature distributions using operative temperature models (Bakken, 1976; Dzialowski, 2005), which is a more accurate method to assess the temperatures that an organism would reach in the environment. While this approach was beyond the scope and resources for this particular study, future research should incorporate operative temperature models to test the accuracy of our predicted body temperatures.

2.3. Thermal preference, upper voluntary temperature, and critical thermal minimum

To measure thermal preference (T_{pref}), we used an experimental setup that created a thermal gradient under cover objects, mimicking a situation that this species would experience naturally in the field. We used a large plastic arena (82 × 45 cm), filled to a depth of ca. 4 cm

with soil collected from the site of capture. Two floor tiles (45 by 45 cm) were placed above the substrate and a heat lamp (250 W) was placed over one end of the arena. This heat lamp created a thermal gradient of 48–55 °C on the hot end of the gradient substrate (measured using an infrared thermometer both under and on top of the tiles), and 16–20 °C on the cool end of the gradient substrate. Snakes were then introduced into the arena and allowed to crawl under the tiles. After a 30 min trial period, snake body temperature was measured by inserting a thermometer into the cloaca. Thermal preference was measured in two separate trials (separated by 2–4 h) for each snake, and we considered the mean of these two trials to represent the preferred temperature of an individual snake.

To assess critical thermal minimum (CT_{min}), we cooled snakes by placing them (in separate plastic containers) in an ice-filled insulated box. Snakes were then cooled until they could not right themselves when placed on their dorsal surfaces. As soon as snakes regained the ability to right themselves, their body temperature was measured by inserting a thermometer into the cloaca.

We measured upper voluntary temperature (UVT) by placing snakes under a heat lamp in an open enclosure. We then determined UVT as the temperature when the snakes attempted to escape the enclosure (Cowles and Bogert, 1944). Body temperature was measured by inserting a thermometer into the cloaca. Between individuals, the enclosure was returned to a standard substrate temperature (28 °C).

2.4. Statistical methods

We used a combination of parametric linear models (linear regression and ANOVA), nonparametric methods (Wilcoxon test, Welch's test), and contingency tests. We compared the physical attributes (dependent variables of rock length, width, depth, and volume) of occupied and unoccupied rocks (independent variable) using ANOVA on each individual variable. We also used ANOVA to compare occupied and unoccupied rocks using the most explanatory axes from a principal components analysis of the physical characteristics of rocks. We compared predicted T_b (dependent variable) for rocks with and without snakes (independent variable) using ANOVA and nonparametric Wilcoxon, and Welch's test. We used repeated-measures ANOVA to compare variables collected for the same individual (dependent variables of T_b , T_{pref} , UVT and CT_{min}). We tested whether the independent variables of aggregation, sex, ecdysis, or evidence of predation (presence of scars or missing tail tips) impacted T_b , T_{pref} , UVT, or CT_{min} (dependent variables) using ANOVA. Finally, we tested for a relationship between SVL or body mass (independent variables) and T_b , T_{pref} , UVT, or CT_{min} (dependent variables) using linear regression. Note that for comparing the physical characteristics of retreat sites, we used an average value per rock for cases where we had multiple measurements. In all cases, we confirmed that our data conformed to the assumptions of the statistical tests by examining frequency distributions of the raw data and residual plots. Rock volume and body mass were log-transformed for all analyses. Data are presented as means \pm SEM. All analyses were completed in JMP v 12.0.

3. Results

3.1. Physical attributes of retreat sites

We found evidence that ring-necked snakes are found under larger and warmer rocks. Rocks that contained snakes ($n=22$) were significantly greater in length (ANOVA: $F_{1,102}=5.56$, $P=0.0203$), width (ANOVA: $F_{1,102}=4.33$, $P=0.0400$), depth (ANOVA: $F_{1,101}=6.45$, $P=0.0126$), and total volume (ANOVA: log-transformed, $F_{1,102}=21.47$, $P=0.0017$) than rocks without snakes ($n=82$). Thermal characteristics of rocks with snakes did not differ significantly from rocks without snakes (ANOVA: all P s > 0.05).

Using principal components analysis, we found that the first two

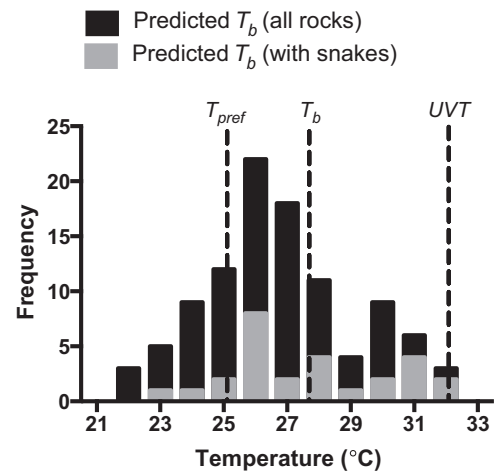


Fig. 1. Distribution of predicted temperatures for all rocks ($n=104$, black) and rocks with ring-necked snakes (*Diadophis punctatus*, $n=22$, grey). Mean actual daytime body temperature ($n=24$, T_b), thermal preference ($n=29$, T_{pref}), and upper voluntary temperature ($n=29$, UVT) are indicated with dashed lines. Mean critical thermal minimum ($n=29$, CT_{min}) is not indicated because it falls outside the range of the graph (11.48 °C).

orthogonal axes explained 77.9% of the variation in thermal and physical characteristics of rocks. The first axis explained 46.8% of the variance and was loaded with all temperature variables (coefficients from 0.80 to 0.89), while the second axis explained 31.1% of the variance and was loaded with physical characteristics of rocks (coefficients ranging from 0.62 to 0.71). Rocks with snakes differed significantly from rocks without snakes for both the first (ANOVA: $F_{1,102}=8.4550$, $P=0.0045$) and second principal components (ANOVA: $F_{1,102}=12.0180$, $P=0.0008$), suggesting that rocks without snakes differed from rocks with snakes in both thermal and physical characteristics.

3.2. Actual and predicted T_b and T_{pref}

We found that neither predicted (for rocks with snakes) nor actual T_b s in the field matched thermal preferences measured in the laboratory (Fig. 1). Predicted T_b s of snakes for rocks that did not contain snakes ($n=108$, 26.43 ± 0.26 °C) were significantly lower than either predicted ($n=30$, 27.66 ± 0.48 °C, ANOVA: $F_{1,128}=11.0189$, $P=0.0011$; Wilcoxon: $P=0.0037$; Welch's test: $P=0.0025$) or actual T_b s ($n=24$, 27.77 ± 0.54 °C, ANOVA: $F_{1,125}=11.2319$, $P=0.0011$; Wilcoxon: $P=0.0015$; Welch's test: $P=0.0111$) for rocks that did contain snakes. However, actual T_b (27.77 ± 0.54 °C) was significantly higher than T_{pref} ($n=29$, 25.12 ± 0.28 °C, repeated-measures ANOVA, $F_{1,23}=8.1353$, $P=0.0037$), closer to the predicted T_b s of snakes (26.43 ± 0.26 °C) under rocks that did not contain snakes. We note that our statistical model predicted body temperatures (27.66 ± 0.48 °C) that were within 0.11 °C of actual body temperatures (27.77 ± 0.54 °C), with the difference between predicted and actual T_b s ranging from 0.21 to 3.2 °C.

3.3. UVT and CT_{min}

We found that both UVT and CT_{min} (both $n=29$) were largely outside of the range of predicted or actual daytime body temperatures. Mean upper voluntary temperature (32.09 ± 0.25) was 4.32 °C above mean T_b and 5.77 °C above T_{pref} , with 8% of T_b s and 2% of the predicted body temperatures (for all rocks) exceeding UVT. In contrast, mean CT_{min} (11.48 ± 0.41) was 16.29 °C below T_b and 14.84 °C below T_{pref} , with none of the T_b s or the predicted daytime body temperatures (for all rocks) within 9 °C of CT_{min} . Both thermal preference and actual daytime body temperatures were significantly different from both critical thermal minimum temperature (repeated-measures ANOVA,

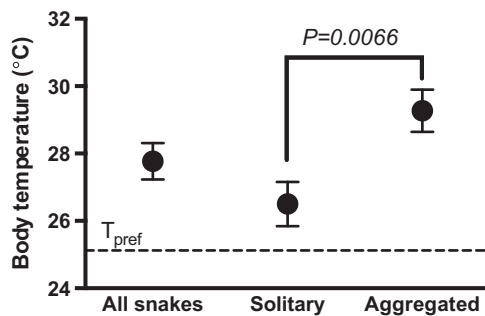


Fig. 2. Mean body temperature for all snakes ($n=30$), solitary snakes ($n=16$), and aggregated ring-necked snakes (*Diadophis punctatus*, $n=14$, under rocks with conspecifics). Mean thermal preference ($n=29$, T_{pref}) is indicated with a dashed line. Note that while body temperature of solitary snakes closely matches thermal preferences, mean body temperature of aggregated snakes is substantially higher than their mean thermal preference.

$F_{1,28}=26.93$, $P < 0.0001$ and $F_{1,23}=608.4492$, $P < 0.0001$, respectively) and upper voluntary temperature (repeated-measures ANOVA, $F_{1,28}=11.46$, $P < 0.0001$ and $F_{1,23}=50.2970$, $P < 0.0001$, respectively).

3.4. Snake aggregations, morphology and thermal ecology

Our research also uncovered evidence that the mismatch between T_b and T_{pref} was driven by differences in T_b between aggregating and solitary snakes (Fig. 2). Nearly half of the snakes were found under a rock with another snake (14 of 30 snakes), for a total of six aggregations. All except one aggregation had two snakes (one contained four snakes). Two aggregations had a single male, while the others were composed entirely of females (with all but one female gravid). We found that snakes that were aggregated (i.e., under rocks that contained other snakes) had higher $T_{b,s}$ (29.27 ± 0.63) than snakes that were solitary (26.50 ± 0.66 , ANOVA: $F_{1,24}=8.9946$, $P=0.0066$). Notably, $T_{b,s}$ of aggregated snakes were significantly higher than T_{pref} (ANOVA: $F_{1,10}=4.12$, $P < 0.0001$), while $T_{b,s}$ of solitary snakes were slightly lower than T_{pref} (ANOVA: $F_{1,10}=0.46$, $P=0.0364$), suggesting that solitary snakes, but not snakes in aggregations, are selecting rocks closer to their thermal preference. By contrast, T_{pref} , UVT, or CT_{min} did not vary between aggregated and solitary snakes (ANOVA: all $P_s > 0.05$).

There was no relationship between body mass or SVL and either T_b , T_{pref} , or UVT (linear regression: all $P_s > 0.05$). However, there was a significant negative relationship (Fig. 3) between CT_{min} and both log-transformed body mass (linear regression: $F_{1,27}=4.9496$, $P=0.0346$) and SVL ($F_{1,27}=5.0176$, $P=0.0335$). Neither sex, ecdysis, nor evidence of predation (presence of scars or missing tail tips) impacted T_b , T_{pref} , UVT, or CT_{min} (ANOVA: all $P_s > 0.05$). Finally, snakes in aggregations did not differ in body size, were not significantly male or female biased, more likely to be in ecdysis, or scarred with evidence of predation relative to solitary snakes (ANOVA or contingency analysis: all $P_s > 0.41$).

4. Discussion

Contrary to previous research on retreat site selection in snakes and other squamates (Huey et al., 1989; Kearney, 2002; Shah et al., 2004; Webb and Shine, 1998), we found that not all snakes primarily choose retreat sites based on thermal considerations. Instead, aggregated ring-necked snakes (those occurring with multiple conspecifics) select rocks providing environmental temperatures averaging 3 °C higher than their preferred temperature. By contrast, solitary snakes selected rocks that allowed them to maintain their body temperatures very close to their preferred temperatures. These results highlight the complex tradeoffs between physiological and ecological requirements that organisms

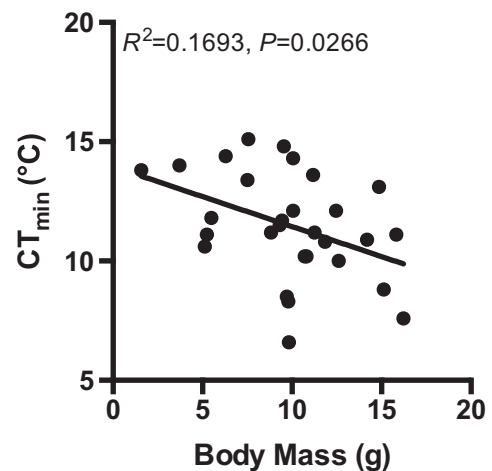
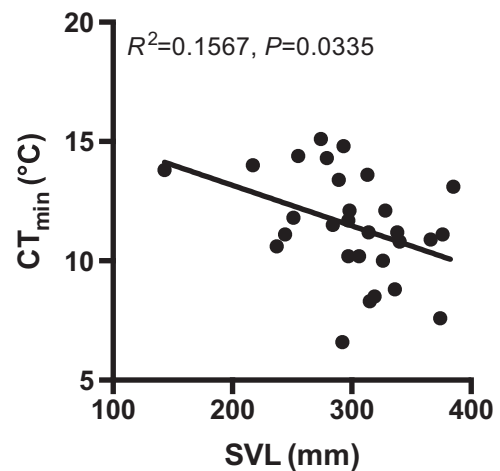


Fig. 3. The relationship between snout-vent length (top) or body mass (bottom) and critical thermal minimum ($n=29$, CT_{min}) of ring-necked snakes (*Diadophis punctatus*). CT_{min} is significantly negatively correlated with both length and mass.

must navigate in heterogeneous habitats.

While our results cannot determine the specific ecological drivers of aggregation in ring-necked snakes, squamate reptiles may aggregate for multiple reasons. First and perhaps most obviously, squamate reptiles can aggregate to facilitate breeding (Gardner et al., 2001; Gregory and Stewart, 1975). However, this explanation is unlikely to explain aggregation in ring-necked snakes, as most spring breeding would have occurred prior to our study (Ernst and Ernst, 2003), and most aggregations did not include males. Second, lizards and snakes can also form aggregations to minimize damage or lethality from cold temperatures (Davis Rabosky et al., 2012; Shah et al., 2003), which would not necessarily apply during the early summer when we conducted our study. Third, many snakes aggregate at both hibernacula and maternal denning sites (Graves and Duvall, 1995; Noble and Clausen, 1936), and it is possible that the rocks containing aggregated snakes contained ideal oviposition sites for ring-necked snakes. Fourth, snakes could aggregate in retreat sites to avoid predation or to heal wounds and recover from illness (Cox et al., 2009; Huey et al., 1989), but aggregated snakes did not differ from solitary snakes in the prevalence of attack scars. Fifth, snakes may have been aggregating for thermoregulation to facilitate digestion (e.g., Sievert and Andreadis, 1999; Tsai and Tu, 2005), but we found no stomach contents and snakes were acclimated to ensure that they were not digesting. Finally, snakes may have aggregated under rocks that provided some common resource, such as food or moisture (Riddell and Sears, 2015).

By contrast, solitary snakes achieved body temperatures that closely corresponded to their preferred temperature as measured in a

laboratory thermal gradient. Prior work has showed that squamate reptiles often choose retreat sites that enable optimal physiological performance (Huey et al., 1989; Kearney, 2002). During the time period of our study, none of the possible retreat sites were at temperatures below the CT_{min} of *D. punctatus*. Additionally, only a very small number of rocks achieved temperatures above UVT (Fig. 1), thus, individuals of this population were unlikely to encounter extreme thermal stress during the period of this study. Nevertheless, snakes clearly chose rocks that differ from non-occupied rocks to use as retreat sites (Fig. 1), and solitary individuals did seem to be choosing rocks that match thermal preference (Fig. 2).

We found that body size and CT_{min} were negatively correlated, which could either suggest an adaptation to low temperatures in larger snakes, or that the thermal inertia of larger snakes (due to decreased surface area to volume ratios) cause slower cooling and rewarming and decrease CT_{min} . Interestingly, evidence that CT_{min} scales with body size in other taxa is mixed (positive relationship, Doughty, 1994; negative relationship, Sanders and Jacob, 1981), with some previous research failing to detect any relationship between CT_{min} and body size in squamate reptiles (McConnachie et al., 2007; Weeks and Espinoza, 2013; Yang et al., 2008). Interestingly, our estimates of CT_{min} (11.48 °C) tend to be higher than that of previous studies in other snake species, which ranged from 2.5 –9.6 °C (e.g., *Regina septemvittata*, Butler, 1978; *Thamnophis* sp., Doughty, 1994; *Nerodia rhombifer*, *Thamnophis proximus*, *Salvadora hexalepis*, Jacobson and Whitford, 1970; Jacobson and Whitford, 1971; *Agkistrodon contortrix*, Sanders and Jacob, 1981). These studies focused on snake species that are larger than ring-necked snakes, suggesting that CT_{min} could scale with body size for both inter- and intraspecific comparisons. While the mechanisms underlying the pattern we document requires further research, our results do suggest that larger ring-necked snakes can maintain function at substantially lower temperatures than smaller snakes, which has implications for understanding seasonal and daily variation in individual activity.

Finally, our work underscores the importance of the comparative approach in thermal biology. Pioneering work had suggested that ectotherms choose retreat sites for thermal physiological considerations, while acknowledging there are other drivers of retreat-site selection (Huey et al., 1989; Kearney, 2002; Shah et al., 2004; Webb and Shine, 1998). By testing this well-established pattern in a different species of squamate reptile, we found that only solitary snakes, but not aggregated snakes, select rocks based upon thermal preferences. It is worth noting that because our study was limited in both geographic and temporal scope, we do not assert that all ring-necked snakes across their range have similar aggregations, or that aggregating behavior and thermal biology do not vary throughout the year. However, our results do demonstrate an interaction between thermal biology and aggregations, which is a pattern that was previously unknown in this species. Indeed, it is only by continuing to test both theory and well-established empirical patterns in different species that we can gain a holistic understanding of how organisms use the biotic and abiotic components of the environment to maximize fitness.

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