# Herpetological Journal

https://doi.org/10.33256/hj29.3.138145

FULL PAPER



# The effect of thermal gradient design on the evaluation of thermoregulation in snakes

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Hertz et al. (1993) designed what is now the most widely used protocol to analyse the thermal strategies and efficiency of small squamates. Preferred temperature range ( $T_n$ ) is one of the most important variables required for determining the thermal efficiency index, and is calculated by monitoring the body temperature of the individuals in an enclosure containing a thermal gradient. Although thermoregulation studies of lizards have traditionally employed thermal gradients under laboratory conditions, this approach is not suitable for snakes given that such thermal gradients do not accurately represent their natural thermal environment and thus may result in snakes selecting suboptimal temperatures. Here, we compare the results of this thermal efficiency protocol using a laboratory thermal gradient (LG) and a semi-captivity thermal gradient (SCG) in the rattlesnake Crotalus polystictus. We found traces of seasonal variation in the SCG T<sub>a</sub>, but this could not be assessed in the LG. T<sub>a</sub> from the LG was much higher (29 – 34.3 °C) than from the SCG (22.5-30.9 °C). Values for the accuracy of thermoregulation  $(d_{\rm p})$  and thermal quality of the environment  $(d_{\rm e})$  indices from the LG were consistently higher than from the SCG. However, the efficiency of thermoregulation (E) was higher when calculated from the SCG. T\_ estimates were wider than most that have been obtained from other snake species, suggesting that C. polystictus is eurythermic. The Blowin Demers and Weatherhead index was nearly identical in both gradients. Results from the LG indicated that C. polystictus is an inaccurate and inefficient thermoregulator, due to the higher temperatures chosen in this environment. In contrast, results from the SCG suggested that it is a highly accurate and active thermoregulator. We suggest that the LG could represent a stressful environment for snakes, and, as a consequence, they might select higher temperatures to increase anti-predatory performance at the expense of less efficient thermoregulation. Generally, a thermal gradient that more accurately replicates the natural habitat of snake species should reduce stress and result in more robust estimates of thermoregulatory variables.

Keywords: behaviour, Crotalus polystictus, preferred temperatures, snakes, refuges, thermal efficiency

# INTRODUCTION

In non-avian reptiles, behaviour and physiological processes depend mainly on temperature (Webb & Whiting, 2005). Thus, it is critically important for reptiles to maintain their body temperature ( $T_b$ ) at or near their physiological optimum (Blouin-Demers & Weatherhead, 2001; Brown & Weatherhead, 2000; Harvey & Weatherhead, 2011). To achieve this, reptiles use a variety of thermal strategies like heliothermy, tigmothermy, and behavioural or passive thermoregulation (Fitzgerald et al., 2003; Garrick, 2008; Molina & Leynaud, 2017; Shine & Madsen, 1996). Behavioural thermoregulation, wherein individuals modify their position in the environment to maximise heat gain or loss, is the most frequent thermoregulatory strategy used by reptiles to regulate their  $T_b$  (Navarro-García et al., 2008; Vitt & Caldwell,

2014; Woolrich-Piña et al., 2006).

Hertz et al. (1993) designed what is now the most widely used protocol to analyse the thermal strategies of small and active ectotherms and how efficient they are in maintaining their temperature within their optimal range (Lara-Reséndiz et al., 2015). This protocol requires three basic sets of data: operative environmental temperatures ( $T_e$ ), the organisms' preferred temperature range ( $T_p$ ) and in situ active  $T_b$ . By comparing the  $T_p$  range with both  $T_b$  and  $T_e$ , researchers can calculate indices of accuracy of thermoregulation ( $d_b$ ), thermal quality of the environment ( $d_e$ ) and efficiency of thermoregulation (E) (Hertz et al., 1993).

 $T_{p}$  is the range of body temperatures that ectotherms prefer for optimal behavioural and physiological performance, and it is calculated by monitoring the body temperature of individuals in an enclosure containing a thermal gradient. The assumption is that, without

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biological interactions and environmental constraints, individuals will select temperatures most suitable for them. It is important to note that the Hertz et al. (1993) protocol states that  $T_{p}$  is calculated under laboratory conditions, where no factor could limit thermoregulation. However, most habitats possess a variety of features that could limit the ability of individuals to thermoregulate effectively, including habitat structure (e.g. rocks, logs, and vegetation), the thermal quality of the environment (Weatherhead et al., 2012), and biological interactions (Vitt & Caldwell, 2014). As Hertz et al. (1993) mention, a laboratory setting for the thermal gradient will never perfectly replicate the natural environment of a species. Researchers should be cautious when interpreting the biological significance of  $T_n$  estimates obtained using a laboratory thermal gradient, as individuals might behave unnaturally in such artificial settings. This problem is even more salient in snakes, as they commonly experience prolonged bouts of stress in captivity (Sparkman et al., 2014) and, in their natural habitats, can often spend substantial lengths of time within refuges or retreat sites (Fitzgerald et al., 2003). When basking, some rattlesnakes use ground cover to avoid detection from predators or potential threats (Shoemaker & Gibbs, 2010). Additionally, reptiles often have variable basking patterns that are difficult to replicate under laboratory conditions. Reptiles can bask in open areas to increase exposure to UV radiation and stimulate vitamin D3 production (Seebacher & Franklin, 2005). However, they must be careful to avoid overexposure to UV radiation, which can cause eye and skin damage (Ferguson et al., 2010).

For these reasons, we propose that researchers should carefully consider the ecological and behavioural tendencies of snakes when designing thermal gradients to obtain more robust estimates of their  $T_n$ . Here, we describe and test a new approach to estimate the thermal efficiency of snakes using a semi-captivity thermal gradient (SCG) and compare its results with those obtained using a traditional laboratory thermal gradient (LG). We estimated thermal efficiency in the rattlesnake Crotalus polystictus, a species that is commonly associated with rocky areas and uses mammal burrows as shelters (Campbell & Lamar, 2004; Meik et al., 2012). As these microhabitat associations likely affect the species thermoregulatory behaviour, we expect estimates of thermal efficiency to differ depending on the type of thermal gradient used.

## **METHODS**

#### Species and study site

*Crotalus polystictus* is a medium sized viperid endemic to central Mexico (Mociño-Deloya et al., 2009; Setser et al., 2010). Individuals are commonly between 50 and 60 cm in total length, but some large individuals can reach 80 cm. Their dark spotted colour pattern over a pale brown or yellow base colour is unique among rattlesnakes (Campbell & Lamar, 2004). It inhabits dry and humid grasslands, pine-oak forests and scrublands of the Mexican Plateau between 1450 and 2739 m (Meik et al.,

2012). They can be active by day or at night (Campbell & Lamar, 2004), but individuals are primarily diurnal at the highest extent of their altitudinal distribution, such as at our study site. However, the activity patterns of this species have not yet been formally described. Setser et al. (2010) report that parturition in this species is synchronised with seasonal rains in a locality of central Mexico, near our study site.

The study was conducted in San Bartolo Morelos, located in the municipality of Morelos, north of Estado de Mexico (19.77 N, 99.65 W). This area is composed of croplands surrounded by natural and induced grasslands and oak forest patches at 2660 meters above sea level. The weather is temperate sub-humid with a rainy season that ranges from June to October (INEGI, 2009). Rocky areas between croplands provide suitable and numerous shelters for rattlesnakes.

#### Fieldwork

We sampled the locality monthly in March – October 2017 and March – July 2018. Each visit was performed by 3-4 people during three days. Active searches for rattlesnakes ran from 0900 to 1900. Within the first 30 seconds after capture, we measured  $T_{\rm b}$  with a digital thermometer (Fluke model 52-II, ± 0.1 °C) whose sensor was inserted 1 cm into the cloaca. We also measured substrate temperature ( $T_{\rm s}$ ) and air temperature ( $T_{\rm a}$ ) 10 cm above the substrate of the capture site. We recorded time of capture, mass (g), snout-vent length (SVL, mm), sex and geographic coordinates (Garmin Etrex GPS).

Within each sampling occasion, the snakes were kept in cloth bags and released at the end of the visit. We performed temperature preference trials in the SCG on the last day of the visits made in May (n = 12  $\bigcirc$ , 3  $\bigcirc$ ), June (n = 8  $\bigcirc$ , 5  $\bigcirc$ ), August (n = 5  $\bigcirc$ , 3  $\bigcirc$ ), and September 2017 (n = 11  $\bigcirc$ , 3  $\bigcirc$ ), and April 2018 (n = 13  $\bigcirc$ , 4  $\bigcirc$ ). Twelve snakes captured between April 20-22, 2018 (9  $\bigcirc$ , 3  $\bigcirc$ ) were transported to a laboratory in Mexico City. We kept the snakes on crystal terrariums where water was provided ad libitum. We conducted five daily temperature preference trials in the LG from May 1-5, 2018.

#### Operative environmental temperatures (T<sub>c</sub>)

We used biophysical models to determine the range of potential body temperatures available to snakes in the absence of thermoregulatory behaviour (Bakken, 1992; Hertz et al., 1993; Peterson et al., 1993). We made the models with green-coloured, polyvinylchloride (PVC) pipes, filled with tile adhesive and sealed with tape. The models were similar in shape and size (50 cm long x 2 cm wide) to C. polystictus, and were previously calibrated under field conditions during the activity period of the snake following the methods of Adolph (1990), Díaz de la Vega-Pérez et al. (2013), Lara-Reséndiz et al. (2014) and Sinervo et al. (2010). Our models accurately mimic the gain and loss of heat by C. polystictus at our study site ( $r^2 > 0.95$ , P < 0.01). We deployed two biophysical models at the study site, one under refuge cover and one in sunlit conditions. The models were connected to a data-logger with two sensors (HOBO® Pro V2-U23-003) which was set to record  $T_e$  every 15 minutes from June 2017 to April 2018. For the thermoregulation analyses we selected only the  $T_e$  data corresponding to the activity period of the snakes based on our in-situ observations (09:00 – 18:30).

#### Preferred temperature range (T<sub>n</sub>)

The LG was built in three adjacent  $120 \times 40 \times 40$  cm crystal enclosures, each containing a layer of approximately 2 cm of potting soil (Fig. 1A). Using cardboard barriers, we divided each tank into four racks to prevent individuals from interacting or competing for basking sites. Each rack was 10 cm wide, which allowed the snakes to rest in a coiled position. In each enclosure, we hanged two heat lamps, at 55 cm above the soil and placed one heat mat under the tank. Thus, the gradient consisted on a total of six lamps and three mats. At the other end of the tanks we placed 15 frozen refrigerant gel packs, five per enclosure, on the outside. These refrigerant packs were replaced every four hours during the experiments (Fig. 1A). This design generated a thermal gradient from ~ 15 °C to 45 °C.

The gradient was set up at 0730, and all organisms were maintained in it for 1.5 hours to adjust to gradient temperatures. We began the measurements at 0900 and, from then on, we recorded the preferred body temperatures every two hours until 1900. We used an infrared thermometer (Fluke 561, ± 0.1 °C) to take temperature recordings to minimise time, handling and stress of the snakes during the experiment. Unpublished data from a similar study with C. triseriatus showed no significant difference between temperatures measured with the Fluke 52-II and Fluke 561 thermometers. Every measurement was made in the middle of the body, under shade, and at a distance of 15 cm away from the snake. Each day of the LG test, we moved the snakes to a different rack and cleaned the tanks superficially to minimize the possible effect of olfactory signals.

To make the SCG, we built a 3 m diameter circular enclosure bounded by polycarbonate plates (Fig. 1B). The enclosure was placed approximately 4 km away from the study site on a flat, uncovered area. The enclosure was prepared with multiple rocks of similar size and shape as those found in the natural habitat of C. polystictus. The rocks functioned as refuges in which the rattlesnakes could thermoregulate as normally as possible, without most of the stress associated with captivity in a LG. We placed six heat lamps at one end of the gradient, approximately 100 cm from the ground, which also was uncovered from sunlight (Fig. 1B). We covered the other end of the gradient with a canvas to provide shade during the whole experiment. We tied up the canvas to two poles at 2-3 m from the ground and diagonally to the gradient, so that sunlight was blocked but the snakes had no direct cover from the canvas. The conditions described above generated a relatively smooth thermal gradient from ~ 23 °C to ~ 45 °C. The temperature transition was smoother from the cold area to the middle (9 °C in 150 cm) than from the middle to the hot area (13 °C in 150 cm). The locality can experiment considerable drops of temperature during night, specially towards the end of



**Figure 1.** Diagram of the laboratory thermal gradient **(A)** and the semi-captivity thermal gradient **(B)** used to test the  $T_p$  range of *C. polystictus*. Heat lamps, heat mats and refrigerant gel packs in the laboratory gradient were used on all three enclosures.

the year. On a particular cold morning, we worked with a colder thermal gradient (from ~ 18 °C to ~ 42 °C) during the first measurements in the  $T_p$  trial of September 2017. The SCG stabilised around noon to the normal range. As in the LG, rattlesnakes were subjected to an acclimation period prior to experimental testing.  $T_p$  was recorded using the same procedure as described for the LG. If the snake was under a rock, we carefully moved the rock, took the measurement and repositioned the rock. We also repositioned the snake, if needed.

#### Thermoregulation indices and statistical analyses.

We calculated indices for thermal quality of the environment ( $d_e$ ) and accuracy of thermoregulation ( $d_b$ ) (Hertz et al., 1993).  $d_e$  and  $d_b$  are the mean of all individual deviations between  $T_e$  and  $T_b$  and the interquartile range of  $T_p$ , respectively. Deviations were calculated as follows: if  $T_e$  or  $T_b > T_p$  interquartile range, then  $d_b = T_b - T_p$  and  $d_e = T_e - T_p$ . If  $T_e$  or  $T_b < T_p$ , then  $d_b = T_p - T_b$  and  $d_e = T_p - T_e$ . If  $T_e$  or  $T_b$  and  $d_e$  values equal to or similar to zero represent high thermoregulatory accuracy and ideal thermal environments for the organisms. High values of the indices indicate low thermal quality.

We then calculated the Hertz et al. (1993) index for efficiency of thermoregulation:  $E = 1 - (\bar{d}_b/\bar{d}_e)$ . An *E* value close to one indicates that environmental temperatures available for the individuals do not match their physiological requirements, and therefore active thermoregulation is necessary to achieve optimal temperatures. In contrast, an *E* value close to zero indicates that the organisms find themselves in an ideal thermal environment and therefore must resort to thermoconformity. We also calculated the index developed by Blouin-Demers and Weatherhead (2001), which represents the effectiveness of thermoregulation and the deviation from thermoconformity. This index is expressed directly in °C and is calculated simply by subtracting  $d_b$  from  $d_a$ .

We used Spearman correlations to test whether  $T_s$  and  $T_a$  are correlated with  $T_b$ . We tested for a difference between male and female  $T_b$  using a student's *t*-test. We used a Mann-Whitney's test to analyse differences between male and female  $T_p$  within each gradient and differences in overall  $T_p$  between gradients. We used Welch's ANOVA and a Games-Howell post hoc test to analyse differences between  $T_p$  of the different trials from the SCG. We tested normality and homoscedasticity with Shapiro-Wilk and Bartlett tests, respectively. All analyses were done in R, version 3.3.2 (R Core Team, 2018).

#### RESULTS

During the sampling period, we recorded 93 female and 36 male  $T_{\rm b}$  recordings. Most of the individuals were average-sized adults. Despite the high number of females captured, we only detected seven pregnant individuals, which were captured unevenly between March and July 2018. Due to logistical reasons, in that period we only made gradient experiments in April, so in the end only one pregnant snake was used in each gradient. Therefore, the effect of reproductive status on thermoregulation could not be assessed. The activity period of *C. polystictus* was between 0900 and 1830; the highest peak of activity was between 1000 and 1200. We detected a second lower activity peak from 1600 to 1800.

Mean  $T_b$ ,  $T_a$  and  $T_s$  are presented in Table 1. We found no significant differences between male and female  $T_b$ 's (t= -1.446, P = 0.152).  $T_b$  was significantly related with  $T_a(\rho$ = 0.218, P < 0.05) and  $T_s$  ( $\rho$  = 0.584, P < 0.05).  $T_p$  did not differ significantly between sexes in the LG (W = 11266, P= 0.192) nor in the SCG (W = 9848, P = 0.265).  $T_p$  was not equal through the five SCG trials (F = 4.798, P < 0.01).  $T_p$ from May ( $\mathbf{x}$  = 27.9 °C), June ( $\mathbf{x}$  = 26.1 °C) and August ( $\mathbf{x}$  = 27.2 °C) were higher than  $T_p$  measured in April ( $\mathbf{x}$  = 24.1 °C) and September ( $\mathbf{x}$  = 24.1 °C). However, differences were only significant between May and September (t = 3.02, P< 0.05) and May and April (t = 3.02, P < 0.05). Overall  $T_p$ differed significantly between gradients (W = 77830, P <0.001). Below we present the results from each thermal gradient type separately.

#### T<sub>p</sub> and thermoregulatory indices derived from the semicaptivity gradient

Mean  $T_p$  and the interquartile range of  $T_p$  are presented in Table 1. A total of 52 % of field body temperatures fell within this range, 43 % below it and only 5 % above it (Fig. 2A). Only 17 % of  $T_p$  fell within the  $T_p$  interquartile range, while 19 % fell above it and 64 % below it. (Fig.



**Figure 2.** Distribution of field body temperatures **(A)** and operative temperatures **(B)** of *C. polystictus* at San Bartolo Morelos, Estado de México. The arrows point to each temperature distribution's mean. The interquartile of the preferred temperature range obtained from the semicaptivity gradient (22.5 - 30.9 °C) is represented by diagonal lines, whereas the one obtained from the laboratory gradient (29 - 34.3 °C) is represented by a dotted area.

**Table 1.** Descriptive thermoregulatory data and thermoregulation indices for *C. polystictus* in San Bartolo Morelos, Estado de México. Means and SD are shown. \*shows the parameters that are significantly different between the gradients.

Variables	Temperature	
Field body temperature $(T_{\rm b})$	23.2 ± 4.5	
Air temperature $(T_a)$	$20.4 \pm 4.1$	
Substrate temperature ( $T_s$ )	20.2 ± 3.6	
Operative environmental temperatures ( $T_e$ )	22.5 ± 10.3	
	Thermal gradients	
	Laboratory gradients (LG)	Semi-captivity gradient (SCG)
Mean preferred temperature range $(T_p)$ *	30.9 ± 4.9	26.5 ± 6.1
Tp interquartile range $(T_p 25 - T_p 75) *$	29 - 34.3	22.5 - 30.9
Accuracy of thermoregulation $(d_{\rm b})^*$	5.84 ± 4.3	1.63 ± 2.3
Thermal quality of the environment $(d_e)$ *	$10.08 \pm 5.3$	5.88 ± 4.9
Thermoregulatory efficiency $(d_e - d_b)$	4.24	4.25
Thermoregulatory efficiency (E)	0.42	0.72

2B). The  $d_{b}$  indicates relatively accurate thermoregulation, while the de suggests that the habitat is of low thermal quality, but considerably more benevolent in comparison with the estimation from the LG (Table 1). *E* indicated a preference for active thermoregulation, which is consistent with the value of the Blowin-Demers and Weatherhead index.

Most individuals chose the refuges on the warm part of the gradient. However, they did not directly move to the warmest spot; most snakes actively changed refuges during the experiments. Generally, snakes selected the lowest temperatures at the beginning of the experimental period, higher temperatures in the middle and lower temperatures at the end of the trials. The highest temperatures were selected between 1200 and 1400.

# $\textit{T}_{\rm p}$ and thermoregulatory indices derived from the laboratory gradient

Mean  $T_p$  and the interquartile range of  $T_p$  are presented in Table 1. Only 13 % of field  $T_b$  fell within this range, while 87 % were below it and none above it (Fig. 2A). The overlap of  $T_e$  with the  $T_p$  interquartile range was minimal (5 %), while 79 % of  $T_e$  fell below it and 16 % above it (Fig. 2B). Both  $d_b$  and  $d_e$  were high values, indicating a low accuracy of thermoregulation and a habitat of thermal quality (Table 1). The efficiency index from Blouin-Demers and Weatherhead indicates that individuals are active thermoregulators. The index *E*, on the other hand, suggests that individuals have a tendency towards thermoconformism.

Snakes in the LG consistently chose higher temperatures. Despite this, snakes were more static, often choosing a spot near the lamps early in the day and staying there during the whole experiment. One snake, on a couple of occasions, climbed the cardboard barrier and accessed the next rack. We considered this to be an escape attempt and, therefore, did not use that period's temperature reading in the analyses.

### DISCUSSION

Due to the manifold biological implications of  $T_{\rm b}$  for reptiles, robust estimates of this physiological trait are critically important for ecological and evolutionary studies. Although  $T_{\rm a}$  and  $T_{\rm s}$  have been widely proposed to provide possible predictors of  $T_{\rm b}$ , some researchers have found them of little use (Seigel & Collins, 1993). We found a positive relationship between  $T_{\rm b}$  and both  $T_{\rm a}$  and  $T_{\rm s}$ , but the relationship was stronger with  $T_{\rm s}$ , suggesting that temperature regulation is tigmothermal.

We found traces of seasonal variation in the preferred temperatures. Within the SCG, snakes chose the highest  $T_p$  in May. This preference could be related to female reproduction. It is well known that reproductively active snakes prefer higher and less variable temperatures during pregnancy (Charland & Gregory, 1990; Setser et al., 2010; Shine, 2003). Parturition in this species begins in June (Setser et al., 2010), so it makes sense that snakes chose higher temperatures in May prior to giving birth. We did not detect pregnant females during this period, though;

but females might generally follow this pattern to some degree. Alternatively, we might have failed to diagnose some individuals as pregnant during fieldwork. We were not able to analyse  $T_{p}$  seasonality from the LG since we only obtained data from April 2018. It is possible that the seasonal pattern found in the SCG is also present in the LG. However, we suspect that seasonality could not have a large impact on the differences between the  $T_{r}$  from both gradients. Since the conditions of the SCG are closer to the natural conditions, we would expect this seasonal pattern to be the regular one for the population, and therefore a potential pattern in the LG could be similar. Among the SCG trials, some of the lowest temperatures were recorded in April. Nevertheless, the LG T in April was much higher than any of the  $T_n$  from the SCG. If the same seasonal pattern from the SCG happens in the LG, we would expect even higher LG  $T_{p}$  in subsequent months, so it is possible that the overall LG  $T_p$  would still be much higher than the SCG  $T_{p}$  even with seasonal variation.

Overall, *C. polystictus* chose a lower  $T_p$  interquartile range in the SCG than in the LG. Indeed, the upper limit of the SCG's range (30.9 °C) barely exceeded the lower limit of LG's range (29 °C). The range obtained from the SCG was 8.4 °C, the widest interquartile range yet to be reported among thermal studies of snakes (Blouin-Demers & Weatherhead, 2001; Brown & Weatherhead, 2000; Fitzgerald et al., 2003; Harvey & Weatherhead, 2011). The  $T_p$  range calculated from the LG was considerably lower (5.6 °C) but was still wider than most  $T_p$ 's estimated from other snake species. These findings indicate that *C. polystictus* is eurythermic at our study site. Eurythermy is commonly favoured when the energetic costs of thermoregulation are high and individuals live in thermally variable climates (Powell & Russell, 1985).

The operative environmental temperatures obtained from the biophysical models are indeed highly variable compared to  $T_{\rm h}$  at our study site. Our estimates of the thermal quality of the environment are low, especially when estimated from the LG (Table 1). A more in-depth study in which more models are deployed in different microhabitats should be done in the future, but the evidence found suggests that San Bartolo Morelos does not provide a thermally ideal habitat for C. polystictus. Nevertheless, according to the data obtained from the SCG, C. polystictus is considered a highly accurate and active thermoregulator. This finding is consistent with studies by Blouin-Demers & Weatherhead (2001) and Row & Blouin-Demers (2006), which have found that ectotherms thermoregulate more precisely within environments with low thermal quality.

Interestingly, estimates of the Blouin-Demers and Weatherhead index are nearly identical based on data obtained from both gradients, where the snakes have to compensate 4.2 °C to reach  $T_p$ . The scale of the  $d_b$  and the  $d_e$  differs between gradients, despite the actual difference between both indexes within each gradient being minimal. This finding indicates that there is a preference for active thermoregulation over thermoconformity by individuals in this population.

The important differences found in *E* are directly caused by the higher temperatures the snakes chose in

the LG. However, stress might explain why individuals selected high temperatures. Generally, reptiles often exhibit stress responses to handling and captivity (Franklin et al., 2003; Moore et al., 1991; Schuett et al., 2004). Many studies have confirmed that blood levels of corticosterone increase after capture and confinement in several snake species (Bailey et al., 2009; Dayger et al., 2013; Herr et al., 2017; Mathies et al., 2001; Schuett et al., 2004; Sykes & Klukowski, 2009).

Behavioural and anti-predator responses in reptiles depend on temperature (Mori & Burghardt, 2001). Snakes generally prefer escaping and showing more defensive behaviours such as striking when the temperatures are higher and motor abilities are optimal (Citadini & Navas, 2013; Llewelyn et al., 2010; Mori & Burghardt, 2001; Passek & Gillingham, 1997; Prior & Weatherhead, 1994; Schieffelin & De Queiroz, 1991). When cold, snakes rely more on crypsis given that motor capability in ectotherms decreases with lower temperatures (Brodie III & Russell, 1999; Keogh & DeSerto, 1994; Prior & Weatherhead, 1994). It is likely that, due to increased stress, snakes chose higher temperatures in the LG to enhance their ability to perform anti-predatory displays (Currin & Alexander, 1999), and also the addition of refuges to the SCG might have helped to reduce such stress. Further studies that evaluate the level of corticosterone in snakes during  $T_{r}$ experiments should be made.

Aside from the effect that refuges have in avoiding detection by predators, the use of refuges also has thermoregulatory considerations (Todd et al., 2016). Some have suggested that many squamates choose specific retreat sites that provide thermal variation and could be used for thermoregulatory purposes (Amo et al., 2004; Cox et al., 2018). For example, Cox et al. (2018) found that solitary Diadophis punctatus individuals select rocks that allow them to maintain a stable range of  $T_{\rm h}$ within their preferred range. Webb and Shine (1998) found that Hoplocephalus bungaroides selects thin rocks during the spring, but changes to thicker rocks during the summer as the temperatures under the refuges increase. Similarly, Huey et al., (1989) showed that Thamnophis elegans chooses rocks of intermediate thickness that improve energy gain and temperature regulation.

For our study population of Crotalus polystictus, refuges appear to provide an important microhabitat for thermoregulation. Most of the individuals that we captured were found under rocks instead of basking out in the open, regardless of the time of day. Traditionally, snakes located under shelters would be considered inactive (Whitaker & Shine, 2002). However, this characterisation fails to consider that some snakes could prioritise the use of refuges for thermoregulatory reasons. Crotalus polystictus, in particular, is known to prefer burrowing rodents as prey (Meik et al., 2012), which might explain its high preference for dwelling under rocks as sit-and-wait predators. However, a trade-off between selecting refuges for foraging and thermoregulatory purposes can exist in some species (Huey et al., 1989). Thus, additional study of the effects that refuges have on the thermoregulation of C. polystictus is needed.

adapting an outdoors thermal gradient with considerably low temperatures at the cold end is difficult, since materials like refrigerant gel packs or ice are not suitable for this method. Making the thermal preference trials including colder parts of the day or on particularly cold days, like in our September 2017 trial, can help to mitigate this limitation. However, a more consistent method would be desired, since the lower limit of the gradient should be more stable. If a thermal gradient is not wide enough, results drawn from it could be misleading.

As we stated, a SCG design can help to reduce stress in the  $T_{p}$  trials. However, some interaction between researchers and snakes still had to occur during the measurements. The two-hour periods between measurements can mitigate this issue, giving the snakes plenty of time to adapt and resume their thermoregulatory behaviour if they felt too much disturbed. Other type of devices, like ultra-thin thermocouples or ibuttons, could help even further to reduce stress in the SCG gradient. Other issues like background noise and inadequate weather conditions can also affect the snakes' behaviour or even impede the SCG trials. All these considerations need to be addressed when designing this type of gradients. We consider that, with meticulous experimental design and careful planification, the possible limitations of these type of gradients can be overcome and adapted to many reptile species.

Overall, we suggest that a LG like the one used in most thermoregulatory studies of reptiles is not suitable for snakes. A thermal gradient in semi-captivity that generally resembles the original habitat can reduce the stress associated with captivity while also providing more thermal variability than a laboratory gradient. The addition of refuges also allows snakes to use the same substrates that they would naturally thermoregulate on. Most thermal gradients in snake studies have been made with wood and covered with various substrates (Aubret & Shine, 2010; Blouin-Demers & Weatherhead, 2001; Fitzgerald et al., 2003; Harvey & Weatherhead, 2011; Whitaker & Shine, 2002). However, different materials conduct heat at different and rates; thus, using substrates from the natural habitat of a species is ideal for estimating their  $T_{n}$ . Providing a more naturalistic thermal gradient allows snakes, and potentially vertebrate ectotherms in general, to thermoregulate as accurately as they would in their natural habitat, which, in turn, enhances the accuracy of preferred temperature range estimates.

We can conclude, therefore, that *C. polystictus* is an accurate thermoregulator despite living in a low thermal quality habitat. Although it is an active thermoregulator, it has a wider set of preferred temperatures than most other snakes that have been studied to date (Blouin-Demers & Weatherhead, 2001; Brown & Weatherhead, 2000; Fitzgerald et al., 2003; Harvey & Weatherhead, 2011). Our results also emphasise the importance of tailoring the design of thermal enclosures to each study species. In sum, our study shows that the Hertz et al. (1993) protocol can be robustly adapted to more species, thereby increasing the quality of data on thermoregulation obtained from a variety of non-avian reptiles.

# ACKNOWLEDGEMENTS

We would like to thank L. Jaramillo-Alba for methodological advice regarding the design of the thermal gradients and field support. We also thank M. García-Guerrero, L. E. Bucio-Jiménez, S. J. González-Ramos, J. I. Chiu-Valderrama, R. Sánchez-González and J. P. Bárcena-Maldonado for assistance on the field and during the gradient experiments. Special thanks to Moisés Castillo and his family for providing lodging and helping with logistics during the study. We thank Christopher Akcali for his helpful comments on this manuscript. Funding was provided by UNAM through the PAPIIT project no. IA 204416 and by CONACyT through the scholarship no. 450717 and the Catedras CONACyT program no. 883. This paper is a requisite to obtain the MSc degree on the Posgrado en Ciencias Biológicas, UNAM.

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Accepted: 21 March 2019