

Evaporative respiratory cooling augments pit organ thermal detection in rattlesnakes

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Abstract Rattlesnakes use their facial pit organs to sense external thermal fluctuations. A temperature decrease in the heat-sensing membrane of the pit organ has the potential to enhance heat flux between their endothermic prey and the thermal sensors, affect the optimal functioning of thermal sensors in the pit membrane and reduce the formation of thermal “afterimages”, improving thermal detection. We examined the potential for respiratory cooling to improve strike behaviour, capture, and consumption of endothermic prey in the South American rattlesnake, as behavioural indicators of thermal detection. Snakes with a higher degree of rostral cooling were more accurate during the strike, attacking warmer regions of their prey, and relocated and consumed their prey faster. These findings reveal that by cooling their pit organs, rattlesnakes increase their ability to detect endothermic prey; disabling the pit organs caused these differences to disappear. Rattlesnakes also modify the degree of rostral cooling by altering their breathing pattern in response to biologically relevant stimuli, such as a mouse odour. Our findings reveal that low humidity increases their ability to detect endothermic prey, suggesting that habitat and ambush site

selection in the wild may be influenced by external humidity levels as well as temperature.

Keywords Thermosensation · Pit organ · Thermal imaging · Respiratory cooling · Heat detection

Introduction

Pit vipers possess one heat-sensitive pit organ on each side of the head, located between the eye and the nostril. Neural information from the pit organs is integrated with their visual system (Chiszar et al. 1977; de Cock Buning 1983a), and although this sensory system can aid snakes in orienting toward thermally favourable environments (Krochmal and Bakken 2003; Krochmal et al. 2004), endothermic prey detection and capture remain the hallmark features of thermal sensing in many snakes, particularly pit vipers (de Cock Buning et al. 1981; de Cock Buning 1983a). Indeed, on moonless nights when rattlesnakes are most active (Clarke et al. 1996) or in darkened burrows, where visual cues are unavailable, thermal radiation is the main source of information about the location of live prey. Moreover, in the absence of visual cues, rattlesnakes and pythons are capable of striking prey based solely on thermal detection, with no effects on the accuracy or efficacy of the strike (Kardong and Mackessy 1991; Kardong 1992; Grace et al. 2001).

The pit organs of Crotalinae snakes provide the highest level of thermal sensitivity ever described for a biological system (Molenaar 1992; Ebert and Westhoff 2006; Ebert et al. 2007). In the case of pit vipers, the sensitive region of the pit organ is a membranous structure located within the pit. The heat sensors in the membrane appear to operate as thermal receptors, as opposed to photonic infrared (IR)

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receptors, such that temperature changes in the membrane of the pit organ evoke changes in the discharge frequency of the heat-sensitive units (Bullock and Diecke 1956; de Cock Buning et al. 1981; Pappas et al. 2004). IR radiation reaches the membrane and induces miniscule changes in membrane temperature, which are sensed and transduced by metabolically active (i.e., mitochondria-rich) neural mast cells (Bleichmar and de Robertis 1962; Ebert et al. 2006). Therefore, the pit organ detects changes in heat flux from the stimulus which in turn, depends on pit membrane temperature (de Cock Buning 1983b). Furthermore, pit membrane temperature will affect the opening probability of the TRPA1 ion channels located on the pit membrane, which have been implicated in the molecular sensation of heat flux (Gracheva et al. 2010). It has also been suggested that rapid heat removal from the membrane is important to reduce thermal “afterimages” by allowing the thermal receptors to return to their resting state after activation from a thermal stimulus, thereby increasing spatial or temporal resolution (Amemiya et al. 1999; Goris et al. 2003). Cooling of the pit membrane via evaporation may enhance thermal detection via any of these mechanisms.

We hypothesized that a decrease in rostral temperature (primarily around the nose or snout), and, therefore, in the pit organs, leads to enhanced temperature-based prey detection in rattlesnakes. Moreover, we also tested whether rattlesnakes are capable of modifying pit organ temperature via respiratory cooling, and whether they are capable of doing it rapidly enough to influence thermal detection and translate into a higher efficiency of predatory behaviours. To accomplish these objectives, we manipulated pit organ temperature by changing ambient humidity, thereby affecting the potential for respiratory evaporative cooling. In all cases, the effectiveness of this approach was validated by simultaneously tracking rostral and body surface temperatures with infrared thermography. Then, we tested predatory efficiency during staged predatory encounters with endothermic prey under conditions allowing for maximal (i.e., low relative humidity; RH) and minimal (i.e., high RH) respiratory cooling. Finally, we investigated whether South American rattlesnakes can rapidly lower pit organ temperature by changing their respiratory pattern as an alertness response to relevant stimuli, such as visual, mechanical, and chemosensory (prey odours) cues.

Materials and methods

Animals

South American Rattlesnakes ($N = 25$; mass: 741 ± 137 g, mean \pm SD; snout vent length: 98.6 ± 5.3 cm), *Crotalus durissus*, were collected from São Paulo state, Brazil, and

were housed for a period of 2 or 3 months at $\sim 28 \pm 2$ °C (daily range) ambient temperature (T_a) in individual wooden cages ($25 \times 26 \times 26$ cm; height \times width \times length) with a sliding glass door. Snakes were fed every other week with live mice and given free access to water. Two weeks prior to experimentation, all animals were fasted. Only non-ecdysial individuals were used in experimental procedures. Animals were obtained from snakes rescued from nearby sugar plantations, and research was performed at the Jacarezario Laboratory, which runs a conservation program for reptiles in captivity under the Brazilian Institute for Environment and Natural Renewable Resources (IBAMA; license number 1/35/94/1088-8). Approval to perform the animal studies involving feeding live prey to the rattlesnakes was issued by the Commission for Ethics in Animal Experimentation from University of São Paulo State (UNESP), SP, Brazil, Protocol Numbers: 03/08-CEEA and 021/2010.

Experimental procedures

All experiments were performed inside the snakes' home cages under normal housing conditions and as part of their normal care and feeding schedule. We used this setting rather than a larger experimental arena since more consistent behaviours could be elicited and preliminary experiments indicated that transferring the snakes to a novel test environment interfered with their normal predatory behaviour, precluding snakes from striking at prey for hours following the transfer (data not shown). The cages were devoid of foreign objects (e.g. water dish) starting 24 h before the beginning of each experiment. During all experiments cameras (thermal imaging camera and an infrared night vision camera; see details below) were located in front of the cage. Each measurement period started at least 5 min after the sliding glass door of the cage was gently removed. We also observed extreme care in minimizing disturbance from the presence of the experimenter; the observer was outside the snake's field of view, movements were slow and minimal and silence was kept all times to avoid disturbing the rodent prey.

Series I: Feeding trials: intact pit organs

The purpose of these experiments was to evaluate the effect of rostral cooling on the predatory efficiency of rattlesnakes. Each snake was tested on two separate days (at least 2 days apart) at low (LH; 40–45 % RH) and high humidity (HH; 90–95 % RH), presented in random order. Low humidity was obtained by fitting the room with dehumidifiers while high humidity was achieved with open source water heaters and water vapourisers (T_a was constant between treatments). RH and T_a were measured with a digital humidity/temperature meter (Model HHH81,

Omega Engineering Inc., Laval, Canada). To eliminate visual cues that could influence the snakes' predatory ability, the rattlesnakes were kept in complete darkness throughout the feeding. At the beginning of each experiment, a mouse (Strain CD-1; mass: 38 ± 6 g) was gently placed at the corner of the cage furthest from the snake. The snake was then allowed to strike, search, and attempt feeding on the mouse.

Predatory behaviour in venomous snakes is usually structured into the following identifiable stereotyped phases (Chiszar et al. 1977; de Cock Buning 1983a): (1) the strike: this is defined as a rapid projection of the snake's head followed by physical contact between the mouth of the snake with the mouse, resulting in its envenomation; (2) prey relocation: since rattlesnakes usually release their prey after envenomation, they subsequently must initiate a post-strike search (de Cock Buning 1983a); for our purposes, a snake was considered to have relocated the prey when its rostrum was within ~ 1 cm of the mouse; (3) attempt to eat after prey inspection: after relocation of the prey, snakes spend considerable time inspecting it before attempting to eat it. To determine predatory effectiveness relative to time 0 (time when the mouse was placed inside the cage), the latency (in seconds) to strike (S), relocate (R), and eat (E) the prey were scored from the video recordings. This was done by re-playing the videos frame by frame and using the time-stamp to determine the duration (to the nearest second) of each of the behaviours of interest.

We also determined the accuracy of the strike (i.e. how many snakes struck the warmer head vs. the cooler body of the mouse), and the snakes' propensity to strike (whether the snake struck within 15 min). We also calculated the distance from the head of the snake to the mouse immediately before the strike and the beginning of the relocation phase (obtained from videos) to test for possible differences in these distances between humidity treatments. Tongue flick frequency was also recorded; tongue flicks were visible from the video recordings (see description below) and were counted manually and binned across 10-s intervals. These were then averaged over the 10 s prior to a strike, the 60 s following a strike, and the 60 s prior to finding the prey.

Once the snake had made an attempt to eat, the mouse was removed from the cage. Snakes were not allowed to feed until the completion of the study, to maintain motivational state, and thus allow the same individuals to be used at both humidity levels. Since some snakes struck and held onto mice, data on search and eat times were not available for all animals (Table 1). Since we were interested in the latency between the strike and relocation and eating of the prey, we were unable to use these data. For this reason, and to maintain a repeated measures design, only snakes that struck and released prey in both humidity conditions were formally analysed for behavioural latencies.

Table 1 Number of snakes exhibiting different strike parameters during feeding trials at low (LH) and high humidity (HH)

	Intact pits		Blocked pits	
	HH	LH	HH	LH
Strike ^a	23	23	7	6
No strike ^a	2	2 ^b	7	4
Struck and held onto	7	9	2	1
Struck and released	16	14	5	5
Nosed live prey ^c	2	0	1	0
Attempted to swallow live prey	1	0	1	2

^a Propensity to strike (whether the snake struck or not within a period of 15 min) was not significantly affected by humidity

^b One of these two snakes struck the prey but was unsuccessful at envenomating it

^c Refers to events in which, after having struck, the snake immediately re-approached and started touching the mouse with its nose

Series II: Feeding trials: blocked pits

To account for potential effects of vomerolfactory senses being influenced by humidity, and thus, driving any differences in predatory performance between humidity levels, we repeated the experiments from Series I, but in animals devoid of pit organ thermosensation. To accomplish this we blocked the pit organs of snakes (Krochmal and Bakken 2003) using dental amalgam (3M ESPE, ImpregnumTM F) covered with a small piece of aluminium duct tape. Snakes were anaesthetized briefly with CO₂ (Andrade et al. 2004), while the amalgam mix was inserted into the pit organs. Mass and snout vent length of each snake were also recorded at this time. The entire procedure took <5 min and at least 17 h were allowed to pass before the beginning of the first experiment. Due to their reluctance to strike without thermal detection, snakes with blocked pits were allowed to strike in a lit environment. Lights were turned off immediately after the strike, and since the remaining portion of the experiments was conducted in complete darkness, the primary sense available to search and attempt to eat the prey was vomerolfaction. Animals that did not strike within 15 min of being presented with a mouse, or experiments in which the snake held onto the mouse after having struck, or in which the pit plugs fell out (three animals) were not included in the analysis. As a result, out of 20 snakes of which the pit organs were blocked, only 10 were used in the analysis ($N = 5$ per humidity level, not paired).

Series III: Disturbance trials

The objective of these experiments was to quantify transient changes in rostral and body surface temperature in response to disturbance. We tracked dynamic changes in

rostral (T_r) and body surface temperatures (T_s) of seven snakes, at each humidity level using infrared thermography (details below). Five minutes after removing the glass door of the cage the first disturbance was applied. This first disturbance consisted of simply turning the lights off in the room; after a 4- to 5-min period of darkness, a stream of air was forced over the snake for 10 s using a hand held electrical fan (diameter 10 cm), followed by another 4–5 min of no disturbance. Tongue flick frequency was recorded as in Series I.

Series IV: Olfactory stimulation

To determine whether a mouse odour would induce respiratory cooling similar to those observed with light and airflow disturbances, the respiratory cooling responses to a mouse odour was tested on a subset of snakes ($N = 15$) under total darkness (at ~ 50 – 60 % RH). A plastic tube was connected to the rear of the snake's cage, which was fed back to an air pump in another room (5 m away with mechanical and auditory isolation) and a three-way valve, and subsequently returned to the front of the snake's cage, approximately 20 cm from the snake. Once the lights were turned off and the glass covering the cage was removed, the snakes were left undisturbed for a minimum of 5 min before applying the olfactory stimulus. Air was drawn from the snake's cage at approximately 200 mL/min and allowed to flow back toward the snake for approximately 5 min before measurements began. A subsequent control olfactory (snake smell) stimulation lasted 30 s, after which the valve was switched, allowing the air to pass through a chamber (~ 2 L) containing a live mouse before it was directed back to the snake's cage. To eliminate mechanical stimuli from air vibrations, the air flow was diffused by cotton gauze attached to the end of the outflow. The mouse odour was delivered continuously for a period of 3 min after which the valve was switched back allowing for the snake's own cage air smell to be re-circulated for a further 4 min. Because of the tubing dead space, there was a 30-s delay from the moment the valve was turned and the stimulus was delivered to the snake's cage. Throughout the procedure, changes in body and rostral surface temperature were monitored by infrared thermography as described below.

Thermal imaging of surface temperatures and video capture

Thermographic images were collected using an IR thermal imaging camera (Mikron Instruments, Model 7515, Vista, California for all experiments except Series IV in which a Model SC640, FLIR Systems, Wilsonville, Oregon was used) connected continuously to a video acquisition

program (MikrospecRT, Mikron Instruments or Thermacam Researcher Pro). Images were collected at 4 (Series I) and 1 frames s^{-1} (Series II–IV). The camera was calibrated prior to every experiment against an internal thermocouple (NIST standard), and in all cases emissivity was assumed to be 0.95 (Tattersall et al. 2004). Using specialized software (MikroSpec RT, Mikron Instruments or Thermacam Researcher Pro), regions of interest on the snake's body and the background were digitally 'drawn' on each frame to obtain the average surface body (T_s) and rostral (T_r) temperatures (corresponding to the surface along the body axis, and the region around the nares and surrounding the pit organs, respectively), as well as ambient temperature (T_a). Similarly, average mouse surface temperatures (T_m) were obtained for Series I. Maximum mouse head (T_{mh}) and mouse body (T_{mb}) for Series I were also compared before the strike. Simultaneously with the thermal imaging camera, snakes were recorded during experimental Series I–III using a digital IR (night vision) video camera (Model DCR-HC90 Handycam, Sony Corporation, Tokyo, Japan) at 30 frames s^{-1} .

Statistical analysis

Series I and II Latencies to strike, relocate, and eat were analysed for the influence of humidity using one-way analysis of variance (ANOVA) with humidity treated as a between-subjects factor when appropriate. The propensity to strike (i.e., whether the snake struck within a 15-min period) between blocked vs. intact pit organs was compared with a χ^2 test. The location accuracy of the strike in Series I and II was compared between humidities with χ^2 tests. T_a , T_s and T_r for Series I were compared between humidities using paired t -tests. T_s and T_r were assessed from the thermal video frames 5 s prior to strike (–S), 30 s after strike (+S), and 30 s prior to relocating the mouse (–R). The influence of humidity on $T_r - T_s$ was then analysed using two-way repeated measures ANOVA (RMANOVA; humidity and predatory event as factors). T_m from Series I at –S, +S, and R were analysed with two-way RMANOVA (humidity and predatory event as factors). Maximum mouse head vs. body surface temperatures were compared with a two-way RMANOVA with humidity and body region (i.e. head vs. body) as factors. The distance between the snake and the mouse immediately before the strike and before initiating the relocation phase was compared using two-way RMANOVA.

Series III T_s and T_r were assessed for the 60-s immediately before the beginning of darkness (no disturbance, ND), and for the 90 s after turning the lights off (LO) and the air flow (AF) disturbances. $T_r - T_s$ was analysed for the influence of humidity using a two-way RMANOVA (with humidity and disturbance as factors).

Series IV $T_r - T_s$ were calculated and averaged over 30 s immediately preceding the olfactory stimulus (NS), for 60 s during exposure to the mouse odour (OS), and for the 60 s following removal of the stimulus (PS). $T_r - T_s$ values before, during and after the stimulus were compared using RMANOVA.

In all cases, normality was evaluated by residual analysis using the D’Agostino-Pearson test. If log transformation could not improve the residual distribution, statistical tests were performed on ranked data. Post hoc multiple comparisons were made using the Holm-Sidak test. Statistical tests were considered significant at $\alpha = 0.05$ and Bonferroni corrections were applied where multiple variables were tested within the same experimental series. Data are presented as mean \pm SD.

Results

Series I and II: Feeding trials

Respiratory cooling during feeding: effects of humidity

Humidity proved to be an effective way of manipulating the degree of respiratory evaporation and, therefore, the temperature around the pit organ ($t_6 = 7.18, P = 0.0037$;

Fig. 1). However, T_a and T_s remained unchanged between humidity levels (T_a : $t_6 = -1.196, P = 0.277$; T_s : $t_6 = 0.47, P = 0.652$) and, therefore, the $T_r - T_s$ decreased (i.e. was more negative) at low humidity (Fig. 2). This was true whether snakes had intact ($F_{1,12} = 81.96, P = 0.0001$) or blocked pit organs ($F_{1,27} = 106.33, P < 0.001$). However, there was no change in $T_r - T_s$ between behavioural events (i.e., -S, +S and -R) in either the intact (Series I) or the blocked pit (Series II) treatment ($F_{2,12} = 0.65, P = 0.538$ and $F_{2,27} = 0.40, P = 0.676$, respectively; Fig. 2).

We also observed a larger thermal gradient between the rodent prey and the area surrounding the pit organs at low than at high humidity ($F_{1,12} = 24.01, P = 0.003$; Table 2). This was caused by the decrease in rostral temperature of the snake since T_m was unaffected by humidity ($F_{1,12} = 1.77, P = 0.239$; Table 2). However, T_m fell significantly from the time immediately before the strike to the time when snakes attempted feeding ($F_{2,12} = 7.036, P = 0.00951$; Table 2), but was not affected by humidity. Maximum mice head surface temperature was $\sim 2^\circ\text{C}$ higher than maximum body surface temperature before the strike ($T_{mh} = 35.3; T_{mb} = 33.3; F_{1,7}, P < 0.001$; Table 2). There were no significant differences in either maximum head or body temperatures between humidity treatments ($P = 0.387$). The distance between the snake and the

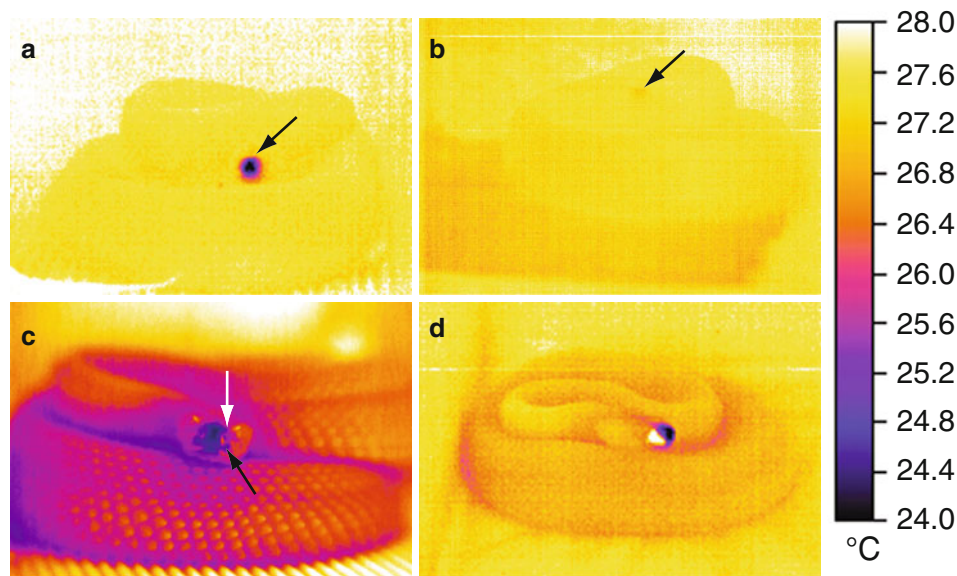


Fig. 1 Sample thermal images of South American rattlesnakes held at different humidity levels, exhibiting different degrees of respiratory cooling (black arrows in a and b). **a** Snake at low humidity (40–45 % RH) with extensive respiratory cooling (dark area shows the entire rostrum including the pit organs of the snake as cooler than the body surface); **b** a snake at high humidity (90–95 % RH with minimal respiratory cooling and nearly uniform surface temperature throughout the body); **c** a snake at low humidity, where it has been exposed to

a brief draft of warm air which heats those parts of the skin with low thermal inertia and more distinctly highlights the specific regions of intense respiratory cooling around the nares (white arrow), upper airways, and pit organs (black arrow); **d** a snake at low humidity with blocked pit organs. In **d**, the pit organs are blocked with a triangular piece of aluminium foil adhered with dental amalgam to the outside of the pit organ, thereby partially reflecting mouse surface temperature (white triangular warm area; mouse not shown)

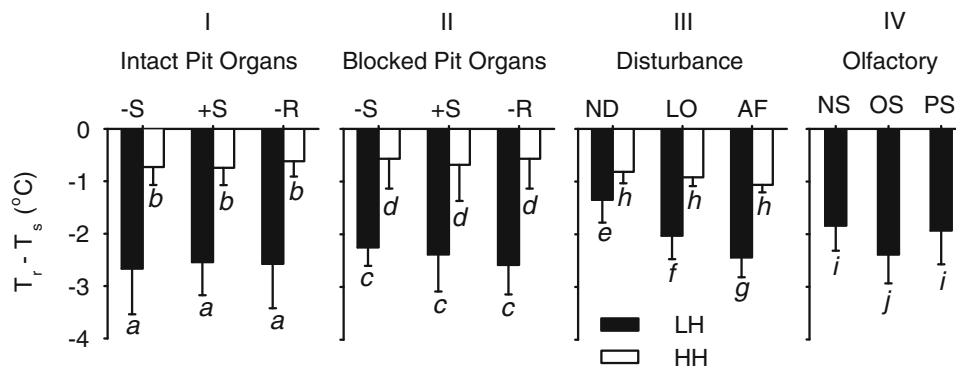


Fig. 2 Respiratory cooling ($T_r - T_s$; mean \pm SD) from South American rattlesnakes during Series I–IV experiments at low (LH; 40–45 % RH) and high (HH; 90–95 % RH) humidity. Series I values ($N = 9$) were taken during feeding trials from snakes with intact pit organs before striking prey (–S), after striking prey (+S), and immediately before relocating prey (–R). Series II values ($N = 6$) were taken during feeding from snakes with blocked pit organs. Values for Series III ($N = 17$) were derived from disturbance trials before exposure to a disturbance in lit conditions (no disturbance,

ND), after turning room lights off (LO) and after being presented with a 10 s air flow disturbance (AF). Series IV experiments ($N = 15$) were only conducted at one humidity level (~ 60 % RH). Data for Series IV were derived from the olfactory stimulus trials during no olfactory stimulus (NS), 60 s following introduction of the mouse stimulus (OS), and 60 s following cessation of the olfactory stimulus (PS). *Small letters* denote statistically similar values (post hoc tests performed within each experimental series)

Table 2 Effect of humidity on surface temperatures during the predatory behaviour trials

Behaviour	Low humidity			$T_m - T_r$	High humidity			$T_m - T_r$
	T_m	T_{hmax}	T_{bmax}		T_m	T_{hmax}	T_{bmax}	
–S	32.1 \pm 0.2 ^a	35.3 \pm 0.6 [‡]	33.2 \pm 0.4	7.5 \pm 1.1	31.9 \pm 0.5 ^a	35.3 \pm 0.6	33.5 \pm 0.5 [‡]	4.6 \pm 0.4 [†]
+S	31.7 \pm 0.6 ^a			6.4 \pm 1.3	32.2 \pm 0.7 ^a			4.5 \pm 0.4 [†]
–R	31.3 \pm 0.3 ^b			6.4 \pm 1.1	31.9 \pm 0.6 ^b			4.9 \pm 0.6 [†]

Average mouse surface temperatures (T_m), maximum mouse surface head (T_{hmax}) and body (T_{bmax}) and the resultant thermal gradient between mouse and the area around the pit organs of South American Rattlesnakes ($T_m - T_r$) at different humidity levels, prior to a strike (–S), 30 s following a strike (+S), and immediately before prey relocation (–R). All values in $^{\circ}\text{C} \pm$ SD. *Small letters* indicate significant differences within T_m ($P = 0.0163$)

[†] Significant differences in $T_m - T_r$ between humidities ($P = 0.00067$)

[‡] Indicate significant differences between T_{hmax} and T_{bmax} within a humidity treatment ($P < 0.001$)

mouse immediately before the strike and before initiating the relocation phase were not significantly different between humidity treatments either before the strike (HH: 13.7 \pm 6.3 cm; LH: 9.7 \pm 4.9 cm) or at the beginning of the relocation phase (HH: 10.1 \pm 3.5 cm; LH: 15.2 \pm 6.9 cm; interaction between humidity \times treatment $F_{1,7} = 4.98$, $P = 0.061$).

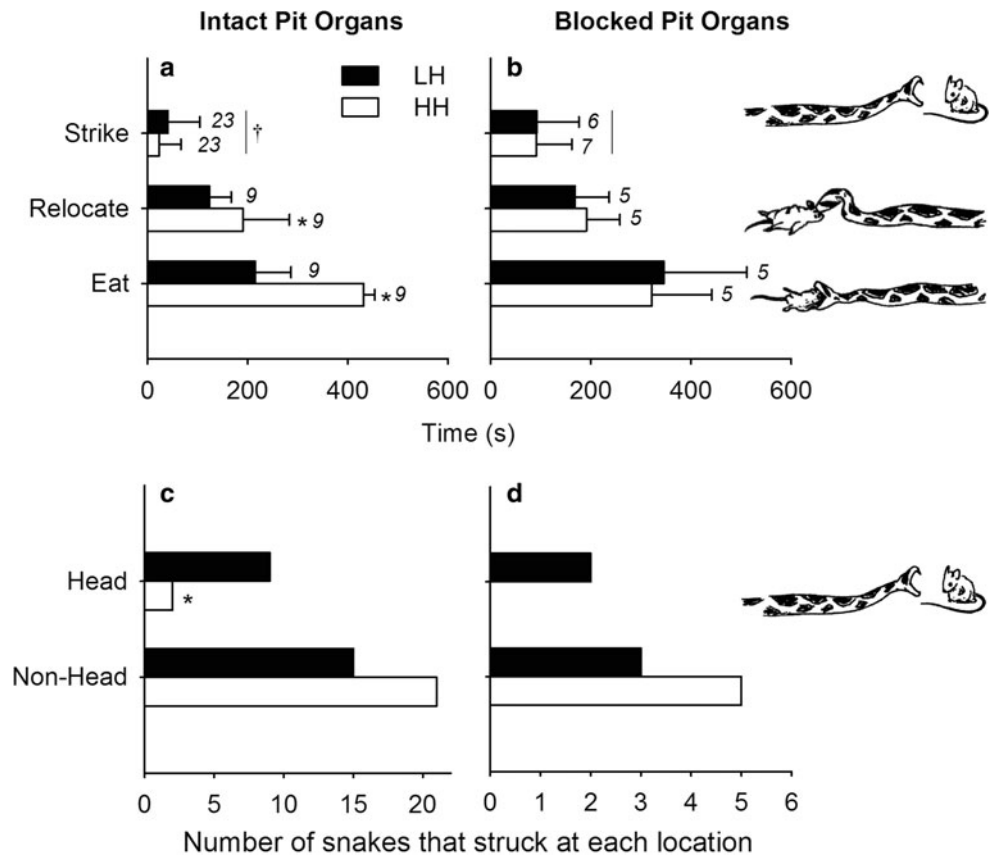
Effect of decreased pit organ temperature on predatory behaviour

Whether snakes had intact (Series I) or blocked (Series II) pit organs, the propensity to strike ($P = 0.7$, and $P = 0.46$, for intact and blocked pit organs, respectively; Table 1) and latency to strike (intact pit organs: $F_{1,6} = 2.97$, $P = 0.135$; blocked pit organs: $F_{1,8} = 1.71$, $P = 0.228$; Fig. 3) remained unchanged between humidity treatments. However, snakes with intact pit organs struck more

accurately at their prey (i.e., struck at the warmer head more often) at LH (Fig. 3; $P = 0.019$) than at HH; this difference was not observed in snakes with blocked pit organs (Fig. 3; $P = 0.114$). Furthermore, the times to relocate and attempt eating prey following a successful strike were significantly shorter in snakes at low humidity (i.e., with cooler rostra) with functional pit organs (latency to relocate prey: $F_{1,6} = 9.67$, $P = 0.021$; latency to attempt to eat prey: $F_{1,6} = 11.69$, $P = 0.0142$), but unaffected by humidity in snakes with blocked pit organs (relocation: $F_{1,8} = 0.29$, $P = 0.608$; eat: $F_{1,8} = 0.070$, $P = 0.798$; Fig. 3).

When we blocked the snakes' pit organs (i.e., disabled thermal detection), the snakes exhibited a dramatically diminished predatory drive and ability. The latency to strike was significantly longer in blocked-pit than in intact snakes ($F_{1,17} = 51.5$, $P < 0.001$; Fig. 3) and the propensity to strike was remarkably lower; only 50 % of snakes with

Fig. 3 Behavioural latencies (mean ± SD) and location of the strike on the body of the mouse (head vs. non-head) from South American rattlesnakes during feeding trials (Series I and II). Animals were exposed to low (LH) and high (HH) humidity with intact (a and c) and blocked pit organs (b and d). Asterisk denotes a significant effect of humidity within each experimental group. Cross denotes a significant increase in the latency to strike in snakes with blocked pit organs (a) with respect to snakes with intact pit organs (b). Drawings extracted from de Cock Buning (1981, 1983a). Numbers indicate sample sizes



blocked pit organs struck their prey. In contrast, only 8 % of the snakes with intact pit organs did not strike (Table 1).

Series III and IV: Effects of disturbance and olfactory stimulation

Effects of humidity and disturbance on rostral temperature

We found a significant two-way interaction between type of disturbance and humidity ($F_{2,12} = 17.78, P = 0.00026$) on the $T_r - T_s$ differential. Overall, disturbances under LH conditions induced larger temperature differentials ($F_{1,12} = 36.94, P = 0.00901$) than did HH (Fig. 2). Small disturbances such as sudden changes in light levels or air flow blown toward the head of the snakes induced pronounced (0.7–1.4 °C) changes in rostral temperature at low humidity, which translated into changes in $T_r - T_s$ ($F_{2,12} = 55.64, P < 0.001$; Fig. 2). These changes occurred rapidly (<30 s) following disturbances and were clearly the result of altered ventilatory patterns (Fig. 4a).

Exposure to a mouse odour (Series IV) revealed a $T_r - T_s$ pattern similar to those observed during the disturbance trials. T_r decreased within 1 min of being exposed to mouse odours (Fig. 4) and $T_r - T_s$ was significantly more pronounced during the olfactory stimulation than during both the period before and after the stimulus (Fig. 2; $F_{2,27} = 5.96; P = 0.0072$).

Tongue flicking rates in feeding and disturbance trials

Series I and II: Humidity did not alter tongue flicking rates (Intact snakes: $F_{1,17} = 0.005, P = 0.941$; snakes with blocked pits: $F_{1,16} = 0.38, P = 0.553$). However, we did observe an elevated rate of tongue flicking immediately prior to striking, a low rate following the strike, and a ramping up of tongue flicking rate prior to relocating the prey (intact snakes: $F_{2,17} = 41.997, P < 0.001$; snakes with blocked pit organs: $F_{2,16} = 7.93, P = 0.00405$; Fig. 5).

Series III: A brief air flow stimulus and light disturbance both induced an increase in tongue flicking rates ($F_{2,10} = 12.39, P = 0.00196$). There was a significant interaction between disturbance and humidity ($F_{2,10} = 3.83, P = 0.0583$; Fig. 5). The influence of humidity during baseline and lights off stimulus was not significant but tongue flicking rates were significantly higher during the air flow disturbance at LH ($P = 0.028$, Holm-Sidak).

Discussion

In the present study, we demonstrate a number of distinctive features of thermal detection in rattlesnakes. Evaporative cooling of the area around the heat-sensing facial pits

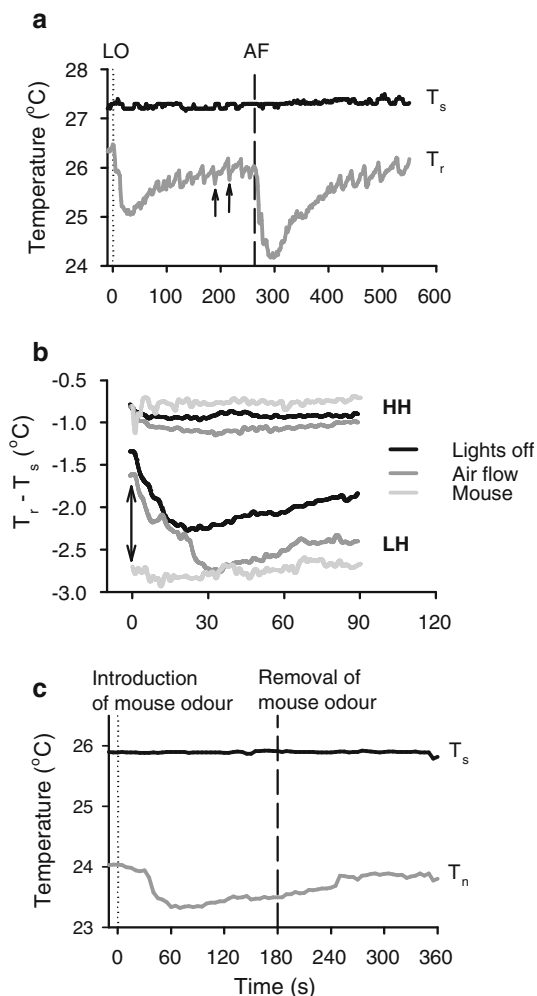


Fig. 4 Time course changes in rostral temperature of South American rattlesnakes held at low (LH) and high (HH) humidity during disturbance and olfactory stimulation trials. **a** Sample trace from one snake at low humidity during Series III (disturbance trials) showing body surface (T_s ; black line) and rostral surface temperature (T_r ; dark grey line) fluctuating in synchrony with inspiration (arrows), and decreasing moderately when room light was turned off (LO; dotted vertical line at 0 s) and quite severely when a brief (10 s) air flow stimulus was presented (AF; dashed vertical line at 260 s). **b** Average ($N = 17$) respiratory cooling responses ($T_r - T_s$) for snakes when the room light was turned off (black line), when an air flow stimulus was presented (dark grey line), and during the feeding trials when a mouse was presented (light grey line). **c** Average ($N = 15$) time course changes in nose temperature (T_n ; grey line) and body surface temperature (T_s ; black line) of South American rattlesnakes during Series III experiments before, during (0 s) and after removal of a mouse olfactory stimulus (180 s)

enhances some aspects of endothermic prey detection, and by inference, thermal detection. This was evident from the more accurate strikes and from the shorter latency of the post-strike behaviours in snakes with increased rostral cooling (i.e., in the area around the pit organs). It follows, then, that thermal sensation, a sense that is integrated with the visual system, may contribute to the post-strike

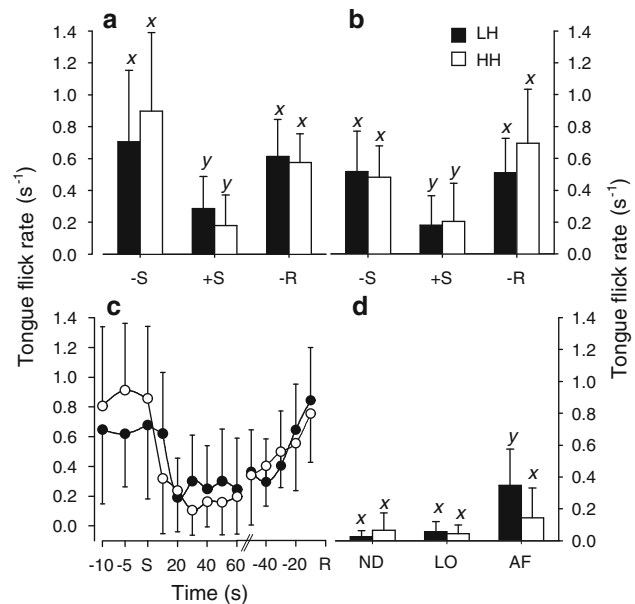


Fig. 5 Tongue flicking rates in South American rattlesnakes exposed to low (LH) and high (HH) humidity. Snakes with intact pit organs (Series I; $N = 17$) are shown in **a** and **c** (expanded time course; S denotes the time of strike and R denotes time of prey relocation), before striking ($-S$), after striking ($+S$), and before finding prey ($-R$). Snakes with blocked pit organs (Series II; $N = 5$) are shown in **b**. **d** Shows tongue flicking rates for snakes during the disturbance trials (Series III) under control lighted conditions (LO), following lights off (LO), and following an air flow stimulus (AF). Small letters denote statistically similar effects of humidity within an experimental series

searching behaviour of stationary prey objects, a behaviour that has traditionally been thought to be mediated primarily by vomerolfaction (Chiszar et al. 1977; Chiszar et al. 1992; Haverly and Kardong 1996). We have provided evidence that *C. durissus* alters the degree of respiratory cooling by changing the depth or duration of breathing, as part of a behavioural ‘alertness’ response; this response provides for greater cooling of the region around the pit organs and may augment or at least influence the animal’s thermal detection. In this manner, South American rattlesnakes are able to capitalize upon a phenomenon (i.e., respiratory cooling) that is common among vertebrates (Robertshaw 2006; Tattersall et al. 2006) to increase their prey capture ability.

Effect of rostral cooling on thermal sensation

Our results demonstrate that the area around the upper airways and the pit organs can be cooled up to 2.6 °C below the surface temperature of the rest of the body. This was clearly the result of respiratory evaporation (Borrell et al. 2005; Tattersall et al. 2006) because an increase in environmental humidity diminished the magnitude of rostral cooling (Figs. 1, 2, 3, 4). Furthermore, evaporation

originates from the upper airways (Fig. 1c) and phasic changes in rostral temperature (Fig. 4a) occur at the same rate as normal breathing frequency (4 breaths/min) in this species (Andrade et al. 2004; Tattersall et al. 2006).

In addition to an overall thermal effect, respiratory cooling of the area around the pit organs may enhance heat removal from the pit membrane by facilitating conductive heat transfer to the surrounding skin. Due to the extreme thinness of the sensory membrane of the pit organ (8–6 μm ; Bullock and Fox 1957; Bleichmar and De Robertis 1962), temperature changes (both those that activate the thermosensors and those that remove the “thermal image”) are expected to occur almost instantaneously, increasing the effectiveness of a sudden decrease in pit organ temperature due to respiratory cooling. Indeed, the heat-sensitive neurons in the membrane are stimulated by phasic changes in temperature, exhibiting extremely high discharge rates with sudden changes in temperature (Bullock and Diecke 1956; de Cock Buning et al. 1981; Pappas et al. 2004). Processes that enhance the temporal resolution of thermal signatures should enhance thermal detection of warm prey.

We propose here three possible mechanisms by which heat removal of the pit membrane may increase thermal detection: (1) Enhanced heat flux between the prey and the thermal sensors: because the pit organ of rattlesnakes detects changes in heat flux which is dependent on the thermal gradient between the pit membrane and the prey (de Cock Buning 1983b), a decrease in the temperature of the heat sensitive membrane should increase thermal detection. (2) Changes in pit organ temperature will affect the function of TRP channel thermal sensors in the pit membrane: heat sensing TRPA1 channels abundantly present in the pit organ membrane of rattlesnakes have been proposed to be the sensors involved in rattlesnake thermal detection (Gracheva et al. 2010), although direct involvement of TRPA1 channels in prey capture behaviours or thermal detection has not been shown. In rattlesnakes, TRPA1 channels have an *in vitro* threshold temperature of $\sim 27.6^\circ\text{C}$ and do not respond to lower temperatures (Gracheva et al. 2010). Therefore, a decrease in the temperature of TRPA1 channels via respiratory cooling would “prepare” the channels for an upcoming stimulus, especially if cooling results in silencing all ion channel activity prior to a thermal stimulus. (3) Reduction of thermal “afterimages”: the presence of a dense capillary network associated with the pit membrane (Goris et al. 2003) highlights the importance of being able to modify heat flux to the pit organ, to maintain functional thermal detection (Amemiya et al. 1999). The dense blood supply to the pit organ membrane acts as a heat exchange mechanism that effectively removes heat from the heat receptors. As a new stimulus is conveyed to the membrane, this

heat exchange process allows the receptor to return to its resting state after activation from a thermal stimulus, preventing the formation of “afterimages” (Amemiya et al. 1999; Goris et al. 2003). The well-accepted “afterimage-suppressant” role of the microvasculature of the pit membrane supports the notion that a cool pit organ could also be important to maintain the high sensitivity of the heat receptors and our results are in agreement with this hypothesis.

Consequences for prey detection

Pit vipers preferentially target warmer temperatures (van Dyke and Grace 2010); in the case of a mouse, this would be the head (Table 2). Indeed, Kardong (1992) observed almost exclusive targeting of the head by rattlesnakes (*Crotalus viridis oreganus*), suggesting that snakes actually aim for the head or neck region of small rodents. Our results indicating that snakes in low humidity (with intact pit organs) are able to strike more accurately (i.e. strike at the head of the mouse) than at high humidity strongly support the notion that respiratory cooling enhances thermal sensation. Given the importance of thermal detection during the strike (Chiszar et al. 1977, 1992; Haverly and Kardong 1996), we were surprised to observe no effect of humidity on strike latency. However, snakes took significantly longer to relocate their struck prey and initiate feeding when exposed to high humidity. The fact that respiratory cooling did not allow snakes to strike faster but did decrease the latency for prey relocation suggests that high humidity does not impair thermal sensation per se, but rather, that thermal detection is dampened as a result of the higher pit organ temperature or decrease in prey-pit organ temperature differential (Table 2). Considering the distance threshold of thermal detection of rattlesnakes ($<100\text{ cm}$; Westhoff et al. 2006), the size of our experimental arena ($25 \times 26 \times 26\text{ cm}$), and the persistence of a still considerable thermal gradient between the pit organ and the mouse even at high humidity, any decrease in the effectiveness of thermal detection may have failed to cause any appreciable difference in striking behaviour. Moreover, thermal sensation in snakes is particularly suited for detecting mobile prey (Bullock and Diecke 1956) and the movement provided by the mouse during the strike phase would make it readily detectable. Rattlesnakes are able to aim their strikes at the anterior portion of rodents based on the direction of prey motion (Hayes and Hayes 1993), but experimental evidence shows that rattlesnakes prioritize infrared cues from their prey rather than using the direction of prey movement for aiming their strike (Schmidt et al. 1993), suggesting that warm regions are indeed preferential targets.

Motivational differences among snakes likely contributed to the variability in strike times as well. The possibility that vomerolfactory sensation was affected by humidity (e.g., that olfactory chemicals emanating from the mouse were more volatile at low humidity levels, or the vomerolfactory mucosa were influenced by humidity) and, therefore, driving these responses was ruled out because when we blocked the facial pits, the differences in prey relocation and eating between humidity levels disappeared. These differences cannot be attributed to differences in the distance between the snakes and the mice before the beginning of the relocation either, because there were no significant differences in these distances between treatments. The use of heat detection during the relocation phase, however, does not diminish the importance of chemical sensing via the vomero-nasal organ, which is likely used in conjunction with other sensory modalities (Lee et al. 1992). Indeed, in agreement with the strike-induced-chemosensory search response largely documented for venomous snakes (Chiszar et al. 1979; O'Connell et al. 1983), we found that tongue-flicking rate varied significantly between the different predatory phases, reinforcing the importance of chemosensory detection.

Although thermal sensation has been believed to play a minor role in prey relocation and handling (de Cock Buning 1983b; Haverly and Kardong 1996; Kardong and Berkhoudt 1999) our results do not support this contention. Indeed, prior evidence actually supports our viewpoint; de Cock Buning and colleagues (1981) noticed that snakes with blocked pits exhibited dramatic alterations in their feeding behaviour, touching live prey with their noses after having struck them and sometimes even attempting to eat live mice. We observed this phenomenon in 19 % of intact-pit snakes during the high-humidity trials and in 40 % of the blocked-pit trials (whether at high or low humidity) but never during the low-humidity trials in intact snakes. These observations further support the hypothesis that evaporative cooling of the face enhances thermal detection and corroborate the importance of thermal sensation during the post-strike feeding phases. Since mice quickly stop moving during the relocation phase, how is the thermal search image detected by the pit organs? While the receptors in the pit membrane respond strongly to phasic changes in thermal radiation (Bullock and Cowles 1952; Bullock and Diecke 1956), which would make the pit organ more suitable for detection of moving targets, movement of the receptors themselves would induce similar phasic signals by creating a dynamic contrast between the object and background, and thus increasing the conspicuousness of stationary objects. Indeed, snakes (Chiszar et al. 1977; de Cock Buning 1984; Lee et al. 1988) exhibit sweeping movements of the head during post-strike trailing behaviours, suggesting they can induce dynamic thermal signals

by moving their pit organs within the field of potential thermal stimuli. By inference, it is also possible that respiratory movements by the mouse are detected by the infrared sensors during the relocation phase.

In complex natural environments, post-strike chemosensory behaviours will clearly be superior to thermosensory ones, since a “line of sight” is required for thermal detection to be effective. However, behavioural studies have demonstrated that rattlesnakes can respond to thermal stimuli up to 100 cm away (Krochmal and Bakken 2003; Krochmal et al. 2004). Furthermore, these behavioural responses are likely to underestimate the actual heat detection range of the pit organ (Ebert and Westhoff 2006; Westhoff et al. 2006). In natural conditions, however, envenomated mice can travel a distance from 10 cm to several meters before becoming immobilized following a strike (Brock 1980; Estep et al. 1981; Chiszar et al. 1992). Neurophysiological studies have also shown that some heat sensitive units in the rattlesnakes' pit membrane are able to detect signals from as far as 500 cm or more (Goris and Terashim 1973), placing an immobilised, envenomated mouse within the heat detection range of the snake. The use of evaporative cooling to augment the function of the pit organ during prey re-location could be a strategy available to rattlesnakes in the wild in conjunction with other sensory modalities (i.e., chemosensory and visual), provided the prey does not move out of the snake's thermal detection range. Because the ultimate success of a predatory event is determined by the capacity of a snake to relocate its struck prey, rattlesnakes that select low-humidity sites to ambush their prey could be more successful in their feeding attempts. Den site selection and thermoregulation are also likely to be affected by humidity since heat sensing is known to play an important role in these activities (Krochmal and Bakken 2003; Krochmal et al. 2004); our results suggest that the detection of stationary objects is more strongly affected by changes in respiratory cooling. Whether this is the case in natural scenarios remains to be tested.

Control over respiratory cooling

Small disturbances induced pronounced respiratory cooling through alterations in respiratory patterns, which were also accompanied by an increased tongue flicking rates. Although transient changes in breathing pattern and thus, of pit organ temperature, undoubtedly bear the potential to improve thermal detection, it remains obscure whether such improvement in thermal sensitivity is the causal factor driving ventilatory changes. For the time being, we favour a more conservative interpretation in which respiratory cooling is one component of an orchestrated alertness response generally exhibited by snakes in response to disturbances or alterations in the environment.

Conclusions and perspectives

The original thermal imaging cameras used liquid nitrogen cooling to lower the temperature of the sensor and surrounding environment, to reduce stray IR radiation that would swamp the detector (Vollmer and Möllmann 2010). An analogous mechanism appears to occur in rattlesnakes, whereby respiratory cooling appears capable of improving thermal sensation. The nearby location of the pit organs to the nares allows for rapid cooling of the pit organ itself. By cooling the surrounding tissues, ‘contaminating’ local radiation would also be reduced, potentially increasing the detection capability of the pit organ membrane to resolve small temperature differences and reduce afterimages. Furthermore, respiratory cooling appears to form part of a behavioural response to disturbances or alterations in the environment. Visual, chemosensory (de Cock Buning 1983a) and thermal stimuli are recruited during this enhanced arousal state, which, among its responses, induces an increase in ventilation; the subsequent evaporative cooling appears to be most important during the re-location and eating phases of the predatory behavioural sequence. Also, respiratory evaporative cooling may play an important role in den site selection and thermoregulation in the wild and, therefore, in terms of temperature-related sensation, snakes may be influenced by the humidity of the selected environments.

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