



# Physiological state influences evaporative water loss and microclimate preference in the snake *Vipera aspis*



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## HIGHLIGHTS

- A potential physiological tradeoff exists between thermoregulation and water balance.
- We studied the effects of physiological state on water loss and metabolism in a snake.
- We determined if microclimate preferences were adjusted to reduce water loss.
- Physiological state influenced water loss, metabolism, and microclimate preference.
- Water constraints are crucial to understand animals' responses to climatic changes.

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## ABSTRACT

Animals typically respond to environmental variation by adjusting their physiology, behavior, or both. Ectothermic animals are particularly sensitive to microclimatic conditions and behaviorally thermoregulate to optimize physiological performance. Yet, thermoregulation can be costly and may obligate a physiological tradeoff with water loss. Presumably, this tradeoff intensifies when animals undergo necessary life-history events (e.g., pregnancy or digestion) that impose significant behavioral and physiological changes, including shifts in behavioral thermoregulation and increased metabolic rate. Thus, behavioral responses, such as modified microclimatic preferences, may help mitigate the physiological tradeoff between thermoregulation and water loss. Herein, we examined the influence of major physiological states (specifically, pregnancy, ecdysis, and digestion) on evaporative water loss and on behavioral adjustments in a viviparous snake, *Vipera aspis*. First, we used open-flow respirometry to measure the effects of physiological states and microclimatic conditions (temperature and humidity) on the rate of total evaporative water loss (TEWL) and metabolic rate (rate of O<sub>2</sub> consumption,  $\dot{V}O_2$ ). Then, we experimentally tested the influence of physiological state on microclimate selection. We found that energy-demanding physiological states were associated with i) an increased rate of TEWL and  $\dot{V}O_2$  compared to control states and ii) a slight preference (statistically marginal) for both warm and humid conditions compared to controls, suggesting a state-specificity in behavioral response. Overall our results underline the impact of physiological state on water loss and demonstrate the potential for behavior to mitigate the physiological tradeoff between thermoregulation and water balance.

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## 1. Introduction

Ambient temperature influences numerous physiological processes from enzymatic kinematics and metabolic rate to physiological performance (e.g., locomotion, digestion, immunity, and reproduction) [6–8, 22,41]. To perform critical activities (e.g., feeding, breeding, and migration), animals must adjust both their physiology and their behavior to

natural temperature fluctuations. Ectotherms are not able to produce sufficient metabolic heat to compensate for thermal variation; therefore, they mainly rely on behavioral thermoregulation [12–14,43]. Although it benefits physiological processes [7,39,42,44,83], thermoregulation is also known to present several costs (reviewed in [8]). Ecological costs associated with thermoregulation include increased predation risks [8,38, 92] or reduced time available for other activities [1,8,36].

Thermoregulation can also induce physiological constraints, such as a tradeoff with water balance [8,51,52,54]. The maintenance of high body temperature passively increases the rate of water loss due to

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increased ventilation (higher respiratory water loss) and increased transpiration (lower skin resistance) [37,56,86]. Among ectotherms, major physiological processes related to fitness, such as digestion, gestation or immune response, induce significant shifts in thermoregulation via increased basking behavior [7,8,12,55,59,67,75,79,81,89]. However, basking behavior often increases exposure to drier ambient conditions. Importantly, increases in temperature-dependent metabolic rate are also often associated with major physiological processes, such as digestion (see [78] for a review on specific dynamic action), gestation [10,32,88], and immune response [9,65]. Such metabolic shifts may, in turn, affect evaporative water loss through an increase in respiratory gas exchange [66]. However, the potential tradeoff between temperature-dependent physiological processes and water balance remains grossly overlooked [37].

A diversity of studies have focused on water balance and water-conserving mechanisms, including major changes in activity [18,27,29,40,58,73], shifts in thermoregulatory strategies [6,20,27,51,54,58], and/or metabolic depression [24,25,49,69,86]. These adjustments aim to lower the rate of total evaporative water loss (TEWL) [68] and eventually the rate of dehydration. TEWL results from two main physiological routes: respiratory EWL (REWL) and cutaneous EWL (CEWL) [66]. Although CEWL has been previously shown to account for the majority of TEWL [26,31,47,56], both REWL and CEWL increase with temperature [66]. Yet, as discussed above, energy-demanding physiological states often impose a preference for warmer temperatures, which likely result in an increased rate of TEWL. One solution to these dual constraints is the selection of a microhabitat that is both warm and humid [51,82]. Behavioral adjustments that mitigate physiological tradeoffs are therefore also crucial to investigate when determining animals' responses to environmental changes.

In this study, we addressed the influence of physiological state on total evaporative water loss (TEWL), metabolic rate (rate of  $O_2$  consumption,  $\dot{V}O_2$ ) and behavior (microclimate selection) in a viviparous snake species, the aspic viper (*Vipera aspis*). Ecdysis (skin shedding), digestion, and reproduction are physiological processes that increase thermal requirements and metabolism in squamate reptiles [23,35,60–62,70,83,90] as in many animals [5,46,50]. To the best of our knowledge, the effects of energy-demanding physiological states on evaporative water losses have been poorly investigated (but see [26]). To determine the influence of energy-demanding physiological states on water loss, metabolic rate, and behavior, we conducted two independent experiments. In Experiment 1, we determined the rate of TEWL and  $\dot{V}O_2$  of snakes in different energy-demanding physiological states (i.e., during pregnancy, ecdysis, or digestion) and compared them to snakes in a post-prandial (non-digesting), non-reproductive, and non-shedding state (i.e., control). We hypothesized that energy-demanding physiological states would impact rates of water loss and metabolism. Specifically, we expected pregnancy, ecdysis, and digestion to increase the rate of TEWL and  $\dot{V}O_2$  compared to control snakes. In Experiment 2, we determined whether physiological state influences microclimate selection. We hypothesized that physiological state would affect snakes' microclimate selection with the prediction that energy-demanding states would orient snake preferences to a more humid microclimate. Our study integrates the impacts of two microclimatic variables (i.e., temperature, humidity) and physiological state on physiological and behavioral responses thus, it should provide new insight into the complexity of animals' responses to environmental conditions [87].

## 2. Material & methods

### 2.1. Study species

We studied the aspic viper (*V. aspis*), a medium-sized viviparous snake distributed in western Europe [71]. The aspic viper is an ambush forager that feeds mainly on rodents [60,70,76]. In natural conditions,

foraging activity occurs between April and August [70,76] and the mean duration of digestion ranges from 5–6 days [70]. In adults, skin ecdysis occurs every 2–3 months, and the duration of epidermis renewal approximates 2 weeks [77]. This process is characterized by the succession of easily detectable sub-stages (e.g., dullness of skin or opaque-eyes) [63,85]. The species is a typical capital breeder [15], which means that females amass energetic reserves for 2–3 years before reproducing [15–17,60,61]. Vitellogenesis (i.e., synthesis of yolk) begins in spring (March–April) [17]. Then, ovulation occurs in mid-June immediately followed by pregnancy until parturition in late August–early September [60,61].

Importantly, digestion, ecdysis, and pregnancy are energy-demanding physiological states that induce increased metabolic rate and thermal preferences [3,11,32,35,59,84,85]. Previous studies on *V. aspis* provide background on these physiological states. Therefore, *V. aspis* is a good candidate to examine the possible physiological tradeoff between thermoregulation and water balance in the context of variable physiological states.

### 2.2. Capture and maintenance

We caught snakes in west-central France from neighboring populations (Vendée, Loire-Atlantique and Deux-Sèvres districts). From late April to mid-June 2012, we captured 77 individuals (31 males, 46 females) to measure TEWL and  $\dot{V}O_2$  (Experiment 1, see below). From early-May to late-June 2013, we captured 78 different individuals (32 males, 46 females) to test microclimate selection (Experiment 2, see below). We placed all snakes in a semi-natural outdoor enclosure (4 m × 4 m × 2.5 m; 10 individuals maximum per enclosure) at the Centre d'Etudes Biologiques de Chizé (CEBC). We fed snakes with mice once every two weeks, and we provided *ad libitum* access to water by misting enclosures 30 min per day. Two weeks before experimentation, we housed each viper individually in a plastic box (30 cm × 16 cm × 10 cm) in the laboratory, with a natural thermal gradient (20–33 °C) provided by a heat cable, water *ad libitum* in water bowls, and an opaque polyvinyl shelter (half cylinder PVC pipe, diameter = 15 cm, length = 25 cm). In both experiments, we followed all laws and rules relating to the conservation and welfare of the animals (Permit # 792, Direction Service Vétérinaire des Deux-Sèvres). After each experiment, we released the snakes (with their respective litter for pregnant females) at the exact site of capture.

### 2.3. Morphology

Before both experiments, we measured (snout-to-vent length, SVL ± 0.5 cm) and weighed (body mass, BM ± 1 g) all snakes. In Experiment 1, we also estimated the total skin surface area and the total body volume of each snake given the influence of these variables on TEWL. To do so, we used a non-invasive method based on morphological measures as detailed in the Methods section of Brischoux and Shine [21]. We divided the snakes into six equivalent sections, and we determined the length and width (diameter) of each section using a tapeline and a digital caliper, and the corresponding shape (i.e., truncated cone for body sections and cone for tail section). We summed the surface and volume of all sections to estimate total snake skin surface area and total snake volume. We did not measure head surface/volume for safety reasons and because cutaneous water loss is negligible compared to the rest of body [31].

### 2.4. Experimental design

#### 2.4.1. Experiment 1: gas exchange

We measured the rate of snake TEWL and  $\dot{V}O_2$  using an open-flow respirometry system. We distinguished thermal and humidity effects by measuring TEWL and  $\dot{V}O_2$  at two targeted ambient temperatures

**Table 1**  
Summary of observed conditions (ambient temperature:  $T_a$ , relative humidity: RH, water vapor pressure: WVP, and water density:  $\rho$ ) in each microclimatic category from the two experiments.

	Microclimatic conditions	n	Observed conditions				TEWL (mg·h <sup>-1</sup> )	$\dot{V}O_2$ (ml·h <sup>-1</sup> )
			$T_a$ (°C)	RH (%)	WVP (kPa)	$\rho$ (g·m <sup>-3</sup> )		
Experiment 1	Cool × Humid	76	20.8 ± 0.04 <sup>a</sup>	53.8 ± 1.6 <sup>a</sup>	1.73 ± 0.01 <sup>a</sup>	13.8 ± 0.03 <sup>a</sup>	10.5 ± 0.8 <sup>a</sup>	5.9 ± 0.5 <sup>a</sup>
	Warm × Dry	73	30.5 ± 0.04 <sup>b</sup>	28.6 ± 1.5 <sup>b</sup>	1.75 ± 0.01 <sup>a</sup>	13.9 ± 0.03 <sup>a</sup>	41.8 ± 2.9 <sup>b</sup>	16.1 ± 1.4 <sup>b</sup>
	Warm × Humid	75	30.4 ± 0.04 <sup>b</sup>	51.4 ± 1.6 <sup>a</sup>	3.18 ± 0.01 <sup>b</sup>	25.4 ± 0.05 <sup>b</sup>	22.1 ± 1.1 <sup>c</sup>	16.0 ± 1.4 <sup>b</sup>
Experiment 2	Cool × Dry	78	25.1 ± 0.1 <sup>a</sup>	47.5 ± 1.1 <sup>a</sup>	1.52 ± 0.04 <sup>a</sup>	12.0 ± 0.3 <sup>a</sup>	–	–
	Cool × Humid	78	25.5 ± 0.1 <sup>a</sup>	82.0 ± 0.3 <sup>b</sup>	2.67 ± 0.02 <sup>b</sup>	21.2 ± 0.1 <sup>b</sup>	–	–
	Warm × Dry	78	30.8 ± 0.1 <sup>b</sup>	40.6 ± 1.0 <sup>c</sup>	1.80 ± 0.05 <sup>c</sup>	14.3 ± 0.4 <sup>c</sup>	–	–
	Warm × Humid	78	30.8 ± 0.1 <sup>b</sup>	65.8 ± 0.7 <sup>d</sup>	2.92 ± 0.03 <sup>d</sup>	23.2 ± 0.2 <sup>d</sup>	–	–

We included sample sizes (n) relative to analyses of physiological state effects in each microclimatic condition. For Experiment 1, the rate of total evaporative water loss (TEWL) and oxygen consumption ( $\dot{V}O_2$ ) are reported regardless of physiological state. Different superscripted letters are used to show significant differences in thermal or humidity conditions and in the rate of TEWL or  $\dot{V}O_2$  between microclimatic conditions. All values are displayed as mean ± SE. See text for details.

( $T_a$  = 20 or 30 °C) and humidities (absolute humidity,  $\rho$  = 14 or 25 g·m<sup>-3</sup>) (Table 1). These conditions are ecologically relevant since free ranging *V. aspis* selects basking sites with overlapping thermal and humidity ranges (18–39 °C & 5–25 g·m<sup>-3</sup>; see [37]; Lourdaïs et al. unpublished data). We compared TEWL and  $\dot{V}O_2$  at three microclimatic conditions, named thereafter Cool × Humid, Warm × Dry, and Warm × Humid (see details of thermal and humidity conditions in Table 1).

We measured each individual in only one physiological state but in all three of the microclimatic conditions. We randomized the order of microclimatic conditions, snake physiological state, and sex in each measurement session. We placed each snake in a test chamber (500 ml glass jar to minimize hygroscopicity), which was housed in a temperature controlled climatic chamber (± 1 °C; Cryosystem, air conditioned system Carel MasterCella). Snakes were introduced in the chamber 3 h prior to testing to reach thermal equilibrium and avoid exploratory activity. To obtain air with target humidity, we used the same method described in a previous study [37]. Briefly, we saturated the air with water vapor using a double bubbling system in a climatic chamber set at a targeted temperature (i.e., dew point temperature  $T_{DP}$ ). In this experiment, we used two  $T_{DP}$ s ( $T_{DP}$  = 15 and 25 °C) to obtain the two targeted humidity conditions ( $\rho$  = 14 and 25 g·m<sup>-3</sup>). We used an eight-channel flow controller and meter (± 1 ml·min<sup>-1</sup>) (FlowBar-8, Sable Systems, Las Vegas, USA) to provide air at a constant influx (82.7 ± 6.6 ml·min<sup>-1</sup>) to each test chamber. We previously used a soap bubble system to calibrate the flow meter [86]. Efflux of each test chamber was sequentially selected with a gas switcher (RM-8 Multiplexer, Sable Systems, Las Vegas, USA). The efflux was then passed through a hygrometer (RH 300, Sable Systems, Las Vegas, USA) and a column of desiccant (self-indicating drierite) before entering a CO<sub>2</sub> analyzer (CA10, Sable Systems, Las Vegas, USA), and an O<sub>2</sub> analyzer (FC10A, Sable Systems, Las Vegas, USA). We left the eighth test chamber empty to serve as reference for Baseline. We continuously recorded output signals from the different hardware with a specific interface (UI-2, Sable Systems, Las Vegas, USA). We analyzed each test chamber for 30 min before switching to another test chamber. We determined the Baseline influx for each test chamber from a 10 min sample of the empty test chamber before and after we measured efflux from the seven test chambers containing snakes. We repeated the sequence (10 min Baseline, 30 min each snake, 10 min Baseline) a second time to ensure that snake TEWL and  $\dot{V}O_2$  were stable during measurements. Finally, we used equations derived from Withers [93] and adapted it to our setup to calculate TEWL (mg·h<sup>-1</sup>) and  $\dot{V}O_2$  (ml·h<sup>-1</sup>).

#### 2.4.2. Experiment 2: behavioral paradigm

We designed a maze that consisted of four inter-connected compartments with different thermal and humidity conditions (Cool × Dry,

Cool × Humid, Warm × Dry, Warm × Humid; see details of thermal and humidity conditions in Table 1). Contrary to Experiment 1, we included a cool and dry condition. Although this microclimate is not ecologically relevant, we used this condition as a negative control in individual microclimate selection. We created thermal conditions that vipers regularly encounter in the field and that approximate the thermal preference of control (~25 °C) vs. energy-demanding states (>30 °C) [53,59]. Unlike in Experiment 1, fine control of humidity was not feasible with the design of Experiment 2 given the connectedness of compartments (described below) obligated by the choice paradigm. Although humidity control was coarse, we ensured that absolute humidity conditions were significantly different among the four compartments (Table 1).

Each compartment consisted of two plastic boxes (length × width × height: 30 cm × 20 cm × 10 cm), one stacked inside the other. This disposition allows to create an empty space that facilitates the control and stability of microclimatic conditions. To create the two thermal conditions (~25 °C and 30 °C), we equipped the bottom box of each compartment with a 25 W thermal cable covered with adhesive aluminum to homogenize temperature that was controlled by a thermostat (Thermo Control PRO II, Lucky Reptile, Germany). To create humid conditions, we placed a set of five sponges (2 cm × 2 cm × 0.4 cm) in each bottom box—'Humid' compartments contained water-saturated sponges and 'Dry' compartments contained dry sponges. The floor of the upper box was comprised of a metal grid and no substrate to allow heat and water vapor transfer from the bottom box (Table 1). We used two thermocouples (Type T standard) per compartment connected to a data logger (21X, Campbell Scientific Instruments, Logan, UT, USA) to monitor temperature variation before each trial. We programmed three miniaturized temperature-humidity data loggers (Hygrochron DS1923, Maxim Integrated Products, Sunnyvale, CA, USA) per compartment to record temperature (intra-box coefficient of variation = 5.2%) and humidity (intra-box coefficient of variation = 3.2%) conditions every 10 min. We used opaque plastic tubes (diameter = 5 cm, length = 15 cm) to connect the four compartments to one another (each compartment was connected to two others).

We measured each individual in one physiological state to avoid any learning process. For each trial, we randomly assigned the four combinations of microclimatic conditions to the four compartments. In the morning (9:00 am), we started the system and let temperature and humidity conditions stabilize for 90 min. Then, we placed each snake in one randomly chosen compartment to avoid possible bias on final preference. After 6 h of acclimation, we noted each animal's position at the end of its trial (*sensu* [82]). This duration is ecologically relevant since snakes typically adjust their thermoregulation behavior over a day. A previous experimental study demonstrated rapid thermoregulatory responses in this species [59]. Between each trial, we washed the maze with warm water and a mild detergent to avoid chemical communication.

## 2.5. Physiological states

We first identified pregnant females (Experiment 1: 19 females, SVL =  $46.0 \pm 0.7$  cm, BM =  $94.4 \pm 5.0$  g; Experiment 2: 11 females, SVL =  $50.2 \pm 1.2$  cm, BM =  $127.8 \pm 11.7$  g) in the field with manual palpation of the abdomen and then checked in the laboratory with high resolution ultrasonography (Sonosite microMaxx, Inc., Bothell, WA, USA). We experimented on females in the final trimester of pregnancy during the period of rapid embryonic somatic growth (Experiment 1:  $19.4 \pm 3.2$  days before parturition; Experiment 2:  $22.5 \pm 2.8$  days before parturition).

We selected pre-shedding snakes (Experiment 1: 10 males, 6 females, SVL =  $47.0 \pm 0.9$  cm, BM =  $76.5 \pm 5.2$  g; Experiment 2: 11 males, 12 females, SVL =  $51.2 \pm 1.0$  cm, BM =  $116.3 \pm 6.6$  g) with dull skin and during the most detectable sub-stage of ecdysis cycle in *V. aspis* (i.e., blue-belly and opaque eyes). We regularly checked snakes to find pre-shedding individuals.

We measured digestive snakes (Experiment 1: 9 males, 12 females, SVL =  $45.2 \pm 0.9$  cm, BM =  $80.8 \pm 4.1$  g; Experiment 2: 11 males, 11 females, SVL =  $49.4 \pm 1.6$  cm, BM =  $103.2 \pm 9.8$  g) 24 h after the ingestion of a mouse (Experiment 1: mice mass =  $23.9 \pm 1.3$  g,  $29.4 \pm 0.3\%$  of snake BM; Experiment 2: mice mass =  $26.8 \pm 2.0$  g,  $27.3 \pm 0.9\%$  of snake BM) when specific dynamic action (SDA: post-prandial hypermetabolism) is maximal [3].

We selected control snakes (Experiment 1: 12 males, 9 females, SVL =  $45.8 \pm 1.1$  cm, BM =  $77.1 \pm 5.8$  g; Experiment 2: 10 males, 12 females, SVL =  $50.3 \pm 1.3$  cm, BM =  $105.5 \pm 8.1$  g) in a non-reproductive, non-shedding, and post-absorptive state (>7 days after meal).

In both experiments, snakes were not different in SVL (all  $P > 0.518$ ) but BM was significantly higher in reproductive snakes from Experiment 1 compared to that in control and pre-shedding snakes (all  $P < 0.023$ ) and the difference was marginal when compared to digestive snakes ( $P = 0.058$ ).

## 2.6. Statistical analyses

All analyses were performed with R software [74]. TEWL and  $\dot{V}O_2$  data were  $\log_{10}$  transformed to achieve normality condition.

### 2.6.1. Experiment 1

We used linear mixed models (lme, package nlme) to assess the effects of physiological state and microclimatic conditions on the rate of TEWL and  $\dot{V}O_2$ , with physiological state, microclimatic condition, and their interaction as explanatory factors. We included morphological parameters as covariates because they are known to influence the rate

**Table 2**

Model selection comparing the influence of different covariates in analyses on the effect of microclimatic conditions and physiological states on total evaporative water loss (TEWL) and oxygen consumption ( $\dot{V}O_2$ ).

Variable	Covariate	df	AIC
TEWL ( $\text{mg} \cdot \text{h}^{-1}$ )	BM (g)	15	−215.7
	SVL (cm)	15	−221.2
	Surface ( $\text{cm}^2$ )	15	−213.1
	Volume ( $\text{cm}^3$ )	15	−212.3
	Residuals S ~ V	15	<b>−235.4</b>
$\dot{V}O_2$ ( $\text{ml} \cdot \text{h}^{-1}$ )	BM (g)	15	<b>−216.6</b>
	SVL (cm)	15	−170.7
	Surface ( $\text{cm}^2$ )	15	−182.0
	Volume ( $\text{cm}^3$ )	15	−180.7
	Residuals S ~ V	15	−162.8

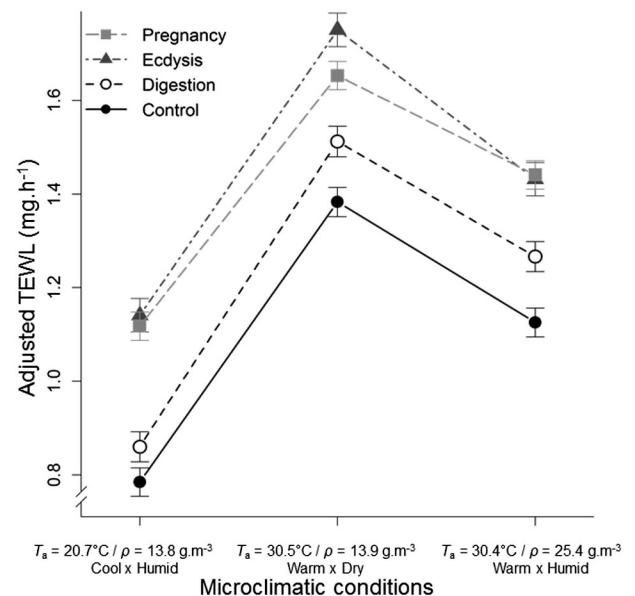
Boldfaced numbers represent the smallest values of AIC and the selected covariate.

We tested the influence of 5 morphological covariates to adjust TEWL and  $\dot{V}O_2$  values, with body mass (BM), snout-vent length (SVL), skin surface, snake volume, or the residuals derived from the relation between surface and volume.

of TEWL and  $\dot{V}O_2$ . We confronted 5 morphological parameters as covariate with BM, SVL, total skin surface, snake volume or the residuals of surface-to-volume relationship ( $F_{1,75} = 4137.7$ ,  $P < 0.001$ ,  $r^2 = 0.98$ ) (see Table 2). We used Akaike's information criterion (AIC) to select the appropriate covariate (Table 2). The best models were obtained when including the residuals of the surface-to-volume relationship (TEWL analyses) or BM ( $\dot{V}O_2$  analyses) as covariates (Table 2). Although each snake was measured in one physiological state, we considered the identity of each snake as a random factor because each snake was measured in all three microclimatic conditions. We conducted pairwise *post-hoc* comparisons after accounting for  $\alpha$ -inflation with Tukey's tests to determine the effects of physiological states (package lsmeans) on TEWL and  $\dot{V}O_2$ . In the figures, we used the least square means method to adjust values of TEWL by residuals of the surface-to-volume relationship, and  $\dot{V}O_2$  by BM. We found no impact of sex on TEWL ( $F_{1,73} = 0.2$ ,  $P = 0.622$ ) or on  $\dot{V}O_2$  ( $F_{1,73} = 0.0$ ,  $P = 0.921$ ), so we discarded this factor from the analyses. We also removed from the analyses individuals that were active during measurements in Experiment 1 (Cool × Humid:  $n = 1$ ; Warm × Dry:  $n = 4$ ; Warm × Humid:  $n = 2$ ; Table 1). We used linear regression model (lm, package stats) to test relationships between absolute values of TEWL and  $\dot{V}O_2$  for each physiological state. We analyzed these relationships in each microclimatic condition, but we only presented results obtained in the Warm × Humid condition because the statistical effects were similar in the other two conditions.

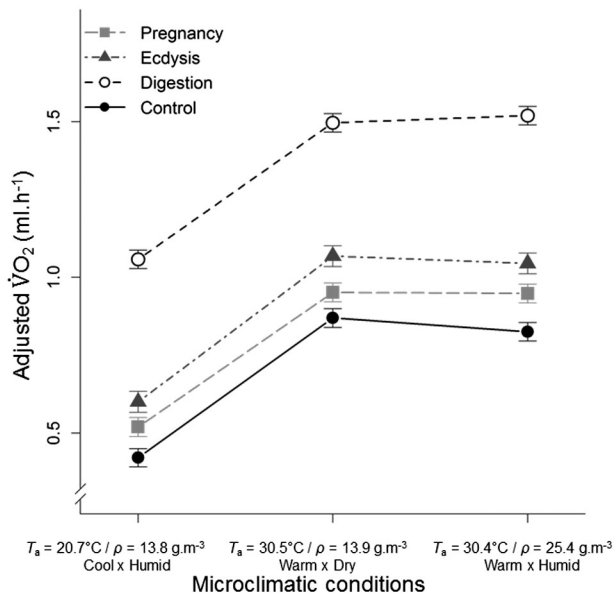
### 2.6.2. Experiment 2

To determine if physiological state impacted snake microclimate preferences, we used a global analysis on final microclimate selection—specifically,  $\chi^2$  tests to determine if snakes in energy-demanding states selected different microclimatic conditions than control snakes. Due to the low sample size of the pregnant snakes ( $n = 11$ ; thus, expected frequencies <5 in each of the four microclimates), we compared preferences (pregnant vs control snakes) for the Warm × Humid microclimate (the most commonly chosen microclimate, see Fig. 3) using Fisher's exact test.



**Fig. 1.** Effect of physiological state (Pregnancy:  $n = 19$ ; Ecdysis:  $n = 16$ ; Digestion:  $n = 20$ ; Control:  $n = 22$ ) and microclimatic conditions (combination of ambient temperature  $T_a$  and absolute humidity  $\rho$ ) on snake total evaporative water loss (TEWL). Points represent mean values ( $\pm$  SE) of  $\log_{10}$  TEWL adjusted by residuals from the surface-to-volume relationship. See text for details.





**Fig. 2.** Effect of physiological state (Pregnancy:  $n = 19$ ; Ecdysis:  $n = 16$ ; Digestion:  $n = 20$ ; Control:  $n = 22$ ) and microclimatic conditions (combination of ambient temperature  $T_a$  and absolute humidity  $\rho$ ) on snake metabolic rate ( $\dot{V}O_2$ ). Points represent mean values ( $\pm$  SE) of  $\log_{10} \dot{V}O_2$  adjusted by snake BM.

### 3. Results

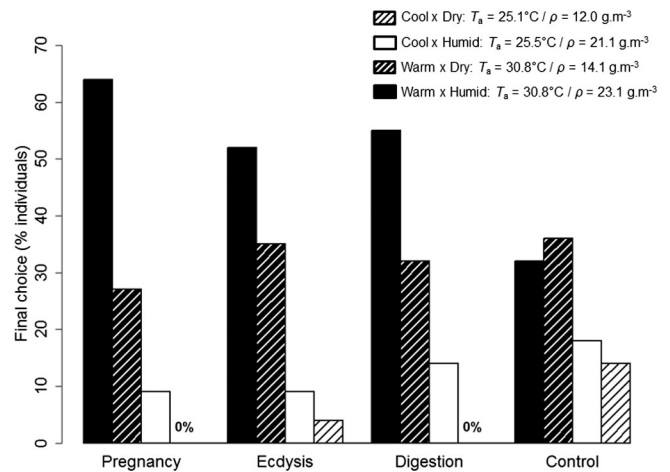
#### 3.1. Experiment 1

##### 3.1.1. Influence of physiological state

TEWL was significantly influenced by the residuals of surface-to-volume relationship ( $F_{1,134} = 44.1$ ,  $P < 0.001$ ).  $\dot{V}O_2$  was significantly influenced by BM ( $F_{1,134} = 113.8$ ,  $P < 0.001$ ). Snakes with high surface-to-volume ratios exhibited greater TEWL, and bigger snakes exhibited great  $\dot{V}O_2$ . We found a significant effect of the physiological state on the rate of TEWL ( $F_{3,134} = 39.2$ ,  $P < 0.001$ ; Fig. 1) and on  $\dot{V}O_2$  ( $F_{3,134} = 147.1$ ,  $P < 0.001$ ; Fig. 2). Compared to control snakes, snakes in demanding physiological states showed significantly higher TEWL (*post-hoc*, all  $P < 0.027$ ; Fig. 1) and  $\dot{V}O_2$  (*post-hoc*, all  $P < 0.001$ ; Fig. 2). Pre-shedding and pregnant snakes presented similar TEWL (*post-hoc*,  $z = -0.94$ ,  $P = 0.781$ ; Fig. 1) and different  $\dot{V}O_2$  (*post-hoc*,  $z = -2.80$ ,  $P = 0.026$ ; Fig. 2). In digesting snakes, TEWL was higher than that of control snakes (*post-hoc*,  $z = 2.80$ ,  $P = 0.027$ ; Fig. 1) and lower than that of both pre-shedding and pregnant snakes (*post-hoc*, all  $P < 0.001$ ; Fig. 1). The  $\dot{V}O_2$  of digesting snakes was higher than snakes in all other states (*post-hoc*, all  $P < 0.001$ ; Fig. 2). We found a significant relationship between  $\dot{V}O_2$  and the rate of TEWL for control ( $F_{1,18} = 44.8$ ,  $P < 0.001$ ,  $r^2 = 0.71$ ) and digesting snakes ( $F_{1,18} = 48.5$ ,  $P < 0.001$ ,  $r^2 = 0.73$ ), but this relationship was not significant in pregnant ( $F_{1,17} = 0.1$ ,  $P = 0.814$ ,  $r^2 = 0.00$ ), or in pre-shedding snakes ( $F_{1,14} = 0.4$ ,  $P = 0.536$ ,  $r^2 = 0.06$ ).

##### 3.1.2. Influence of microclimatic conditions

We found a significant effect of microclimatic conditions on TEWL ( $F_{2,134} = 828.4$ ,  $P < 0.001$ ; Table 1, Fig. 1) and on  $\dot{V}O_2$  ( $F_{2,134} = 517.3$ ,  $P < 0.001$ ; Table 1, Fig. 2). Within each physiological state, TEWL was significantly different among the three microclimatic conditions (*post-hoc*, all  $P < 0.001$ ; Table 1, Fig. 1). At 30 °C, the rate of TEWL in the humid condition (Table 1). In equivalent humidity conditions ( $\rho = 14 \text{ g} \cdot \text{m}^{-3}$ ), TEWL was higher in the warm than that in the cool condition (fold-increase due to a 10 °C



**Fig. 3.** Proportion of snakes in different physiological states (Pregnancy:  $n = 11$ ; Ecdysis:  $n = 23$ ; Digestion:  $n = 22$ ; Control:  $n = 22$ ) selecting varied microclimatic conditions (combination of ambient temperature  $T_a$  and absolute humidity  $\rho$ ).

increase in temperature:  $Q_{10} = 3.98$ , Table 1). The  $\dot{V}O_2$  was significantly influenced by temperature (*post-hoc*,  $z = -27.84$ ,  $P < 0.001$ ; Fig. 2) but not by humidity (*post-hoc*,  $z = -0.74$ ,  $P = 0.737$ ; Fig. 2). At 30 °C,  $\dot{V}O_2$  was higher compared to snakes at 20 °C ( $Q_{10} = 2.72$ , Table 1).

##### 3.1.3. Interaction between physiological state and microclimatic conditions

Physiological state and microclimatic conditions interactively affected TEWL ( $F_{6,134} = 2.49$ ;  $P = 0.026$ ; Fig. 1). For instance, microclimatic conditions affected TEWL by 22–43% during pre-shedding and pregnancy and by 10–13% during digestion (Fig. 1). We did not find a significant effect of the interaction between physiological state and microclimatic condition on  $\dot{V}O_2$  ( $F_{6,134} = 0.57$ ,  $P = 0.756$ ; Fig. 2).

#### 3.2. Experiment 2

Snakes from all physiological states chose microclimatic conditions non-randomly ( $\chi^2 = 37$ ,  $df = 3$ ,  $P < 0.001$ ; Fig. 3) but differences were not significant among physiological states (control vs. pre-shedding and digesting:  $\chi^2 = 7.3$ ,  $df = 2$ ,  $P = 0.29$ ; preference of control vs. pregnant for the Warm  $\times$  Humid microclimate:  $\chi^2 = 3.0$ ,  $df = 1$ ,  $P = 0.14$ ; Fig. 3). When pooling the snakes in the three energy-demanding physiological states and comparing them to control snakes, snakes in the energy-demanding states marginally preferred Warm  $\times$  Humid conditions ( $\chi^2 = 3.5$ ,  $df = 1$ ,  $P = 0.061$ ). Although we also found that control snakes were more likely to choose Cool  $\times$  Dry conditions ( $\chi^2 = 4.6$ ,  $df = 1$ ,  $P = 0.032$ ), our sample sizes for this test were relatively low—thus, this result is less interpretable (Fig. 3). There was no significant preference between control and energy-demanding physiological states for Cool  $\times$  Humid ( $\chi^2 = 0.79$ ,  $df = 1$ ,  $P = 0.38$ ) and Warm  $\times$  Dry ( $\chi^2 = 0.13$ ,  $df = 1$ ,  $P = 0.72$ ) (Fig. 3).

### 4. Discussion

Water is a vital resource, often overlooked in ecological studies [18, 29,33]. Yet, habitat humidity and water availability in the environment are critical to animal survival and reproduction [58,64], and behavioral adjustments (e.g., microhabitat or microclimate selection) are therefore central to optimizing physiological tradeoffs [37,51,82]. In this study, we conducted two independent experiments to assess the effects of physiological state and microclimatic conditions on i) total evaporative water loss (TEWL) and metabolic rate ( $\dot{V}O_2$ ), and ii) on microclimate preferences in a viviparous snake. We first hypothesized that physiological state would impact water losses and metabolic rates. Supporting our

associated prediction, we found direct effects of energy-demanding states (i.e., pregnancy, ecdysis, and digestion) that resulted in increased rate of TEWL and  $\dot{V}O_2$ . We then hypothesized that physiological state would drive snake microclimate selection, and we predicted that energy-demanding state will orient snake preferences to a more humid microclimate. Behavioral investigations revealed only marginal preference for warm and humid conditions in energy-demanding states compared to controls.

When compared to control snakes, we found that the snakes in states of pregnancy, ecdysis, and digestion exhibited increased rates of water loss (average, pregnancy: +31%, ecdysis: +33%, digestion: +11%) and oxygen consumption (average, pregnancy: +27%, ecdysis: +32%, digestion: +117%). It has been previously demonstrated that metabolic rate increases during ecdysis [11,84], pregnancy [10,32,88], and digestion (SDA reviewed in [78]) in snakes, but we are the first to report the shifts in metabolic rate and water loss due to all three of these physiological states in a single study. Importantly, higher oxygen consumption should result in higher ventilation and thereby increase respiratory water flux (i.e., REWL). However, digesting *V. aspis* presented the highest increase in metabolic rate due to SDA (Fig. 2), and they showed a lower TEWL rate than other energy-demanding physiological states (Fig. 1). Moreover, we detected a significant correlation between  $\dot{V}O_2$  and TEWL in digesting and control snakes but not in pregnant and pre-shedding individuals. Together, these results suggest that respiratory water loss is not the main component of the observed increase in TEWL. Instead, as previously suggested, skin surface may represent a more important avenue for water losses (i.e., CEWL) [26,31,47,56]. In support of this, pregnant and pre-shedding snakes undergo important skin modifications, which likely results in increased rate of CEWL. During pregnancy, embryonic growth causes significant abdominal burden and the distension of maternal skin [80]. Such mechanic distension increases the skin surface exposure and likely decreases skin's resistance (by reducing its thickness), which increases the rate of CEWL (e.g., [2]). During the epidermis renewal phase of the ecdysis cycle, the space between the old and next skin is filled with a fluid [63], which may increase the rate of CEWL. During the first days of digestion, snakes also face skin distension due to prey absorption. The unexpected low rate of TEWL associated with this physiological state might be caused by a much localized skin distension only in the stomach region. Yet, the impact of these physiological states on water balance remains understudied, and further studies should continue to investigate the functional properties of physiological states that induce water loss.

Our study allowed us to quantify the effects of microclimatic conditions on the rate of TEWL and  $\dot{V}O_2$ . We found a strong effect of temperature on TEWL and  $\dot{V}O_2$  with a higher thermal dependence of water loss ( $Q_{10} = 3.98$ ) than metabolic rate ( $Q_{10} = 2.72$ ), which is consistent with previous assumptions that respiratory water loss is not the main component of TEWL (see above). Water loss increases with temperature because of increased metabolism (see above) but mostly because of the decrease in skin resistance [56]. When thermal conditions were constant ( $T_a = 30^\circ\text{C}$ ), the rate of TEWL was 86% higher in dry ( $\rho = 14\text{ g}\cdot\text{m}^{-3}$ ) than in humid conditions ( $\rho = 25\text{ g}\cdot\text{m}^{-3}$ ) while  $\dot{V}O_2$  was not affected by humidity.

Considering the effects of microclimatic conditions on water loss, we expected snakes to optimize their microclimatic choices (i.e., choose a warm and humid microclimate) to enhance temperature-dependent physiological processes while limiting evaporative water loss [51,82]. Yet, we found a complex interaction between physiological state and microclimatic selection. Although marginal in our study, energy-demanding states induced a higher selection for both warm and humid conditions compared to control snakes. Marginal differences likely result from a reduced sample size that impacted the power of this test ( $1 - \beta = 0.78$ ). Ideally, we would have to test microclimate selection in at least 20 snakes for each physiological state, but we

were dependent on snake capture. However, pregnant snakes tended to select higher humidity than control snakes, which might highlight state-specific physiological constraints. For instance, pregnancy has been demonstrated to be a particularly energy-demanding state [32, 53,60,61] with a shift in maternal behavior and thermal preference [59,91]. Further, pregnant squamates are likely exposed to substantial water constraints because they exhibit higher TEWL than controls (this study) and because they provide water to their developing embryos over a prolonged period [28]. In comparison, ecdysis and digestion states may impose milder constraints on water balance because individuals are only exposed to a transient increase in water loss. Digestion is likely even less constraining for water balance because water loss is low (Fig. 1) and because snakes might rely on water content of the prey to maintain water balance (but see [94]).

Microclimate selection buffers physiological constraints and has been reported in a number of different taxa [19,51,57,72,82]. Our results suggest that different physiological processes impose different constraints on water balance. Importantly, snakes were provided with water *ad libitum* and therefore were not in a negative water balance. Organisms are regularly exposed to significant water restriction periods (i.e., summer droughts or heat waves). Under water deprivation, we expect that constraining physiological state will compromise water balance and likely induce dehydration. In response to negative water balance, we expect more marked microclimate selection than the response we observed here and possibly a decrease in thermal preference [52]. Future work should investigate the interaction between microclimate selection and physiological state following water deprivation.

Our results underline the impact of physiological state on water loss and demonstrate a potential tradeoff between thermoregulation and water balance. We considered the interaction of two major variables (temperature and humidity) on physiological and behavioral responses. Studies integrating the impacts of shifts in multiple environmental factors offer ecologically relevant insight because animals typically respond to multiple stressors in the field [87]. We demonstrate that energy-demanding physiological states negatively impact water balance. We found that these physiological states may drive microclimatic preferences, particularly during pregnancy. Such behavioral adjustments in response to state-specific physiological constraints are important to investigate given the increasing frequency or duration of extreme climatic events [34,45]. Determining and predicting animal responses to global changes is a major research focus [4,30,48,87]. The duration and frequency of water-constraining events are expected to increase in the near future because of global climate change [45]. Therefore, integrating the impact of physiological states on water balance, energy use (e.g., metabolic rate or  $\dot{V}O_2$ ), and behavioral responses provides key insight into the complexity of animals' responses to environmental shifts [87].

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