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RESEARCH ARTICLE

Thermoregulatory consequences of salt loading in the lizard *Pogona vitticeps*

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ABSTRACT

Previous research has demonstrated that dehydration increases the threshold temperature for panting and decreases the thermal preference of lizards. Conversely, it is unknown whether thermoregulatory responses such as shuttling and gaping are similarly influenced. Shuttling, as an active behavioural response, is considered one of the most effective thermoregulatory behaviours, whereas gaping has been proposed to be involved in preventing brain over-heating in lizards. In this study we examined the effect of salt loading, a proxy for increased plasma osmolality, on shuttling and gaping in *Pogona vitticeps*. Then, we determined the upper and lower escape ambient temperatures (UET_a and LET_a), the percentage of time spent gaping, the metabolic rate ($\dot{V}_{O_2}$), the evaporative water loss (EWL) during gaping and non-gaping intervals and the evaporative effectiveness (EWL/$\dot{V}_{O_2}$) of gaping. All experiments were performed under isotonic (154 mmol l^{-1}) and hypertonic saline injections (625, 1250 or 2500 mmol l^{-1}). Only the highest concentration of hypertonic saline altered the UET_a and LET_a, but this effect appeared to be the result of diminishing the animal's propensity to move, instead of any direct reduction in thermoregulatory set-points. Nevertheless, the percentage of time spent gaping was proportionally reduced according to the saline concentration; $\dot{V}_{O_2}$ was also decreased after salt loading. Thermographic images revealed lower head than body surface temperatures during gaping; however this difference was inhibited after salt loading. Our data suggest that EWL/$\dot{V}_{O_2}$ is raised during gaping, possibly contributing to an increase in heat transfer away from the lizard, and playing a role in head or brain cooling.

KEY WORDS: Gaping, Shuttling, Metabolic rate, Evaporative water loss, Brain cooling

INTRODUCTION

All physiological variables and processes are either directly or indirectly influenced by body temperature (T_b); as a result, T_b maintenance is vital to the physiological and biochemical functioning in the body. However, T_b not only influences but also can be influenced by physiological processes. In particular, because the most efficient thermolytic (i.e. heat-loss) mechanisms are water-related effectors, such as panting, gaping, sweating and salivation, it is not surprising that osmoregulation is one of the physiological functions that can affect T_b. If body water lost by evaporative cooling mechanisms is not replaced, dehydration ensues, the osmoregulatory system is stimulated and, consequently, effectors to restore the body fluid loss will be activated, probably causing inhibition of those water-dependent thermolytic effectors (McKinley et al., 2008). As a result of a decrease in evaporative water loss (EWL), a concomitant rise in T_b is expected. In fact, the interaction between osmoregulation and thermoregulation has been shown for almost all vertebrate groups (Schmidt-Nielsen et al., 1957; Parmenter and Heatwole, 1975; Kleinhaus et al., 1985; Preest and Pough, 1989) and the expected increase in T_b during dehydration has been found in many species of mammals, including humans, camels, African ungulates, goats, dogs and sheep (Schmidt-Nielsen et al., 1957; Taylor, 1970; Baker, 1989; Baker and Turlejska, 1989; Jessen et al., 1998; McKinley et al., 2008). Amphibians and reptiles also have thermal preferences influenced by hydration status (Malvin and Wood, 1991; O'Connor and Tracy, 1992; Ladyman and Bradshaw, 2003; Bradshaw et al., 2007), although in these cases, defended T_b is reduced through a behavioural selection of lower temperatures. Evidence about the central control of these interactions was found by Silva and Boulant (1984) when they described that temperature-sensitive neuronal activity in isolated hypothalamic slices of rats is altered by perfusion with hyperosmotic saline; some warm-sensitive neurons are excited whereas others are inhibited by hyperosmotic stimulus.

Other effects of osmoregulation on T_b regulation have also been found in reptiles, especially xeric-adapted desert-dwellers. Parmenter and Heatwole (1975) demonstrated that an agamid lizard, *Pogona barbatus* (formerly *Amphibolurus barbatus*), has its panting threshold raised when it is dehydrated; the same result was observed by Dupré and Crawford (1986) in dehydrated iguanas. In these cases, T_b would be expected to rise in the water-stressed state. In contrast, the snake *Notechis scutatus* (Ladyman and Bradshaw, 2003) and the lizard *Ctenophorus ornatus* (Bradshaw et al., 2007) select lower ambient temperatures (T_a) when body water availability is reduced. All of these effects indicate that body water is conserved at the expense of a finely tuned T_b maintenance when both homeostatic systems are competing.

Despite the fact that hydration state influences two important thermoeffectors in lizards (i.e. behaviours associated with homeostatic control, panting and choosing a preferred T_a), it is not clear whether hydration state modifies thermoregulatory set-points consistently for multiple thermoeffectors, such as shuttling and gaping behaviours. Shuttling in lizards is an active form of thermoregulation involving the movement between warm and cool areas that leads to an overall regulation of T_b; it is known to be a very important behavioural mechanism observed in reptiles. Optimal T_a is rarely found in the field, so it is common for reptiles to move back and forth between areas of high and low thermal intensity. This behaviour is also very well documented in the laboratory (Berk and Heath, 1975; Barber and Crawford, 1979; Cadena and Tattersall, 2009a) and is proving to be a tractable

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List of abbreviations and symbols

CC	cold compartment of shuttle box
EHT	evaporative heat transfer (J min^{-1})
EWL	evaporative water loss (mg H_2O min^{-1})
$EWL/\dot{V}_{O_2}$	evaporative effectiveness of gaping (mg ml^{-1})
FE_{CO_2}	excurrent CO_2 fractional
FE_{H_2O}	excurrent H_2O fractional
FE_{O_2}	excurrent O_2 fractional
FI_{CO_2}	incurrent CO_2 fractional (from baseline)
FI_{H_2O}	incurrent H_2O fractional (from baseline)
FI_{O_2}	incurrent O_2 fractional (from baseline)
FRI	incurrent flow rate (ml min^{-1})
HC	hot compartment of shuttle box
HP	heat production (J min^{-1})
i.p.	intra-peritoneal injection
LET_a	ambient temperature when the lizard moved from the cold to the hot compartment
SBC	selective brain cooling
STP	standard temperature and pressure
T_a	ambient temperature
T_b	body temperature
T_{body}	lizard's body skin temperature
T_{head}	lizard's head skin temperature
UET_a	ambient temperature when the lizard moved from the hot to the cold compartment
$\dot{V}_{O_2}$	metabolic rate assessed as the rate of oxygen consumed by the animal (ml O_2 min^{-1} STP)
WVDE	excurrent water vapour density (μg H_2O ml^{-1})
WVDI	incurrent water vapour density (μg H_2O ml^{-1})

approach for the study of temperature sensing and the cost of thermoregulation in reptiles (Cadena and Tattersall, 2009a).

Gaping involves a proportional increase in mouth opening with increasing T_a, accompanied by no apparent changes in ventilation (Spotila et al., 1977; Tattersall et al., 2006). Panting, which is an open-mouth form of rapid, shallow breathing usually initiates at extreme or near lethal T_a, acting as a last resort to survival; gaping typically starts at temperatures very close to preferred T_a (Heatwole et al., 1973), apparently contributing to the fine-tuning of T_b regulation (Tattersall and Gerlach, 2005). By opening the mouth, the EWL should increase and be important to prevent the brain from overheating (Spotila et al., 1977; Tattersall et al., 2006). One consequence that this additional cooling mechanism (gaping) can provide to lizards is that they would spend longer periods of time at elevated or optimal T_b before having to seek shade.

Gaping has been described previously in *Pogona vitticeps*, an agamid lizard, naturally found in central Australia. In the field, this lizard prefers T_b close to 33°C (Melville and Schulte, 2001). In the laboratory, it was found that as T_a increases, the time spent gaping is increased (Tattersall and Gerlach, 2005), suggesting a higher EWL as T_a is elevated. Additionally, the gaping threshold is decreased during hypoxia (a scenario known to reduce the T_b set point), reinforcing the idea that this behaviour acts as a heat-loss mechanism (Tattersall and Gerlach, 2005). Besides gaping, bearded dragons (*P. vitticeps*) also present a very well defined shuttling behaviour (Cadena and Tattersall, 2009a,b); they are desert animals that probably face seasonal plasma hypernatremia, as described in other arid (Bentley, 1959) and agamid Australian reptiles (Bradshaw and Shoemaker, 1967). All these characteristics make the bearded dragon an ideal species to study the factors that can influence gaping and shuttling. Therefore, we aimed to assess the effect of decreased availability of body water on shuttling and gaping, two extremely important thermoeffectors of T_b regulation in lizards, in order to ascertain whether hyperosmolarity impacts thermoregulatory control. We also investigated the effectiveness of gaping for EWL when the animals were exposed to high T_a. We used hypertonic saline injections to induce a decrease in the availability of body water (Rice, 1982; Baker and Dawson, 1985; Dupré and Crawford, 1986; Nagashima et al., 2001; Konishi et al., 2003; Bradshaw et al., 2007) and we predicted that: (1) animals treated with salt loading would shuttle from cool to warm areas and/or from warm to cool areas in the shuttle box at lower T_a thresholds, since hyperosmolarity has been shown to decrease thermal preference in ectotherms; (2) the percentage of time spent gaping would be lower after salt loading; and (3) that gaping would be a significant source of EWL.

RESULTS

Plasma concentration and water consumption

The increases in plasma osmolality were very similar to those predicted by our mathematical calculations. The hypertonic saline doses, 625, 1250 and 2500 mmol l^{-1}, raised the plasma osmoconcentration by approximately 7, 12 and 22% compared with control saline (154 mmol l^{-1}), resulting in a high linear relation between the measured plasma osmolality and the expected values (r^2=0.95; P<0.001; y=1.0886x−4.4073; data not shown). The proportion of animals that consumed water was strongly influenced by the salt loading level they were injected with (χ^2_3=45.65, N=114; P<0.001). For control animals (154 mmol l^{-1}) only 5 out of 38 animals drank water (13%); for 625 mmol l^{-1} injection, 6 out of 19 drank water (31%); for 1250 mmol l^{-1}, 14 out of 22 drank water (63%) and for 2500 mmol l^{-1}, 31 out of 35 drank water (88%).

Effect of salt loading on shuttling behaviour

The injection of 1250 mmol l^{-1} hypertonic saline had no effect on the upper escape ambient temperature (UET_a; see Materials and methods; P=0.882) nor on the lower escape ambient temperature (LET_a; P=0.357) compared with control animals (Fig. 1A); however the highest concentration (2500 mmol l^{-1}) increased the UET_a and decreased the LET_a (interaction of direction×treatment: P<0.001; $F_{2,30}$=18.116; Fig. 1A) and decreased the number of shuttles (treatment effect: P=0.01; $F_{2,30}$=5.463; Fig. 1B) compared with isotonic saline injections.

In order to verify whether the salt loading affected the propensity to move, a similar experiment was performed in the shuttle box held at a constant temperature of 34°C. In this series, only the highest and the isotonic saline concentrations were used because the other concentration (1250 mmol l^{-1}) had no effect on UET_a and LET_a (Fig. 1A). The salt loading significantly reduced the amount of exploratory shuttling (P=0.004; Fig. 1C).

Effect of salt loading on gaping

Salt loading decreased the propensity for gaping in lizards in a dose- and time-dependent manner (interaction of time×treatment: P<0.001; $F_{3,40}$=5.498; Fig. 2A,B). The two highest concentrations of saline injections (1250 and 2500 mmol l^{-1}) reduced the gaping at least 90% at the end of the experiment compared with the pre-injection values whereas the lowest concentration (625 mmol l^{-1}) diminished gaping at 50% (Fig. 2A).

Effect of salt loading on head and body skin temperatures

Lizard's body (T_{body}) and head (T_{head}) skin temperatures were recorded during the salt-loading effects on gaping experiments (Fig. 3A–C). The T_{head} was significantly increased after saline

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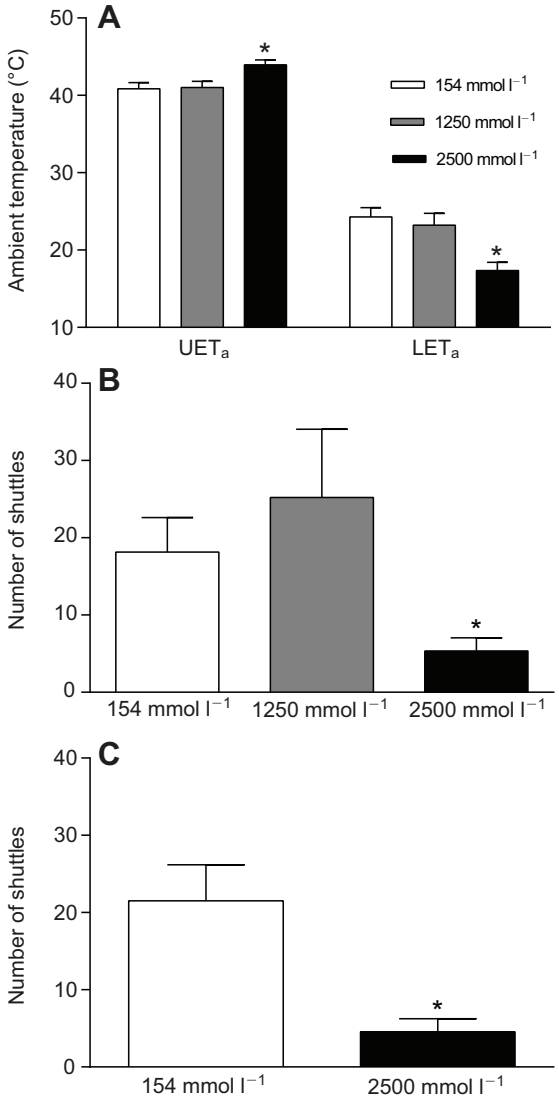

Fig. 1. The effect of different concentrations of saline injection on UET_a and LET_a and on the number of shuttles in the lizard *Pagona vitticeps*. (A) UET_a, mean ambient temperature in which the animal escaped from the hot compartment. LET_a, mean ambient temperature at which the animal escaped from the cold compartment. (B) Number of shuttles when animals were placed in the shuttle box with thermoregulatory drive. Results of A and B are from protocol 1. In A and B, number of animals=11 for all groups. (C) Number of shuttles when both compartments of shuttle box were at 34°C (protocol 2); N=14 for all groups; *P<0.05, mean treatment effect.

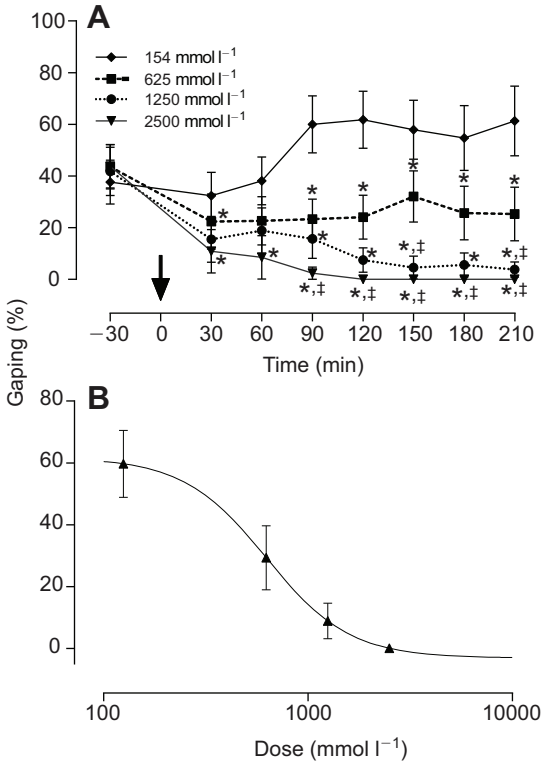

Fig. 2. Effect and dose response of different saline concentrations on time spent in gaping in *P. vitticeps* exposed to 37–39°C. Effect (A) and dose response (B) on percentage of time spent gaping. Arrow indicates time of injection. N=11 for all treatments; *P<0.05 compared with 154 mmol l^{-1} concentration. ‡P<0.05 compared with 625 mmol l^{-1} group.

injections with the highest effect of the 2500 mmol l^{-1} treatment compared with control and 625 mmol l^{-1} groups; the increase in T_{head} of 625 mmol l^{-1} treated-animals was higher than that of controls (interaction of time×treatment effect: P=0.003; $F_{2,17}$=2.57; Fig. 3A). Salt loading also caused a rise in the lizard's T_{body} compared with controls (interaction time×treatment effect: P=0.002; $F_{2,17}$=2.78; Fig. 3B), but no difference was observed between 625 and 2500 mmol l^{-1} treatments.

Furthermore, the control group and animals treated with the lowest hypertonic saline (625 mmol l^{-1}) exhibited a significantly lower T_{head} than T_{body} compared with those animals that received the highest concentration of saline (2500 mmol l^{-1}; treatment effect: P=0.005; $F_{2,17}$=7.362; time effect: P=0.192; $F_{2,17}$=1.449; no interaction effect: P=0.559; $F_{2,17}$=0.901; Fig. 3C and Fig. 4A,B).

For salt-loaded animals (2500 mmol l^{-1}), T_{head} and T_{body} were virtually the same after injection (Fig. 3C and Fig. 4B).

Effectiveness of gaping for EWL and the effect of salt loading on overall metabolism and on EWL

To verify how effective gaping was with respect to EWL, metabolic rate ($\dot{V}_{O_2}$) and EWL were measured during spontaneous periods of gaping and non-gaping [heat production (HP) and evaporative heat transfer (EHT), were calculated based on $\dot{V}_{O_2}$ and EWL values; see Materials and methods for details]. Because gaping intervals shorter than 15 s were not considered for these calculations (see Materials and methods for details), only control animals (154 mmol l^{-1}) were utilized for these analyses (comparisons between gaping and non-gaping); salt-loaded animals presented gaping episodes too brief after hypertonic injection to be confident that instantaneous correction could robustly capture the differences in water produced and $\dot{V}_{O_2}$.

Although EWL did not differ between gaping and non-gaping intervals (P=0.706), a significant reduction in $\dot{V}_{O_2}$ (P=0.023) contributed to an overall increase in the evaporative effectiveness of gaping (EWL/$\dot{V}_{O_2}$; P=0.01; Table 1). The T_b was 37.7±0.06°C throughout the experiment. The hypertonic saline injection (625 mmol l^{-1}) decreased $\dot{V}_{O_2}$ (P=0.025). However, because the EWL also tended to be lower after injection (P=0.12), the EWL/$\dot{V}_{O_2}$ was not affected by salt loading (P=0.195) when compared with the effect of isotonic saline on the same variables (Table 2).

DISCUSSION

In the present study, salt loading was used as a proxy for dehydration stress. Its success was verified by a highly predictable

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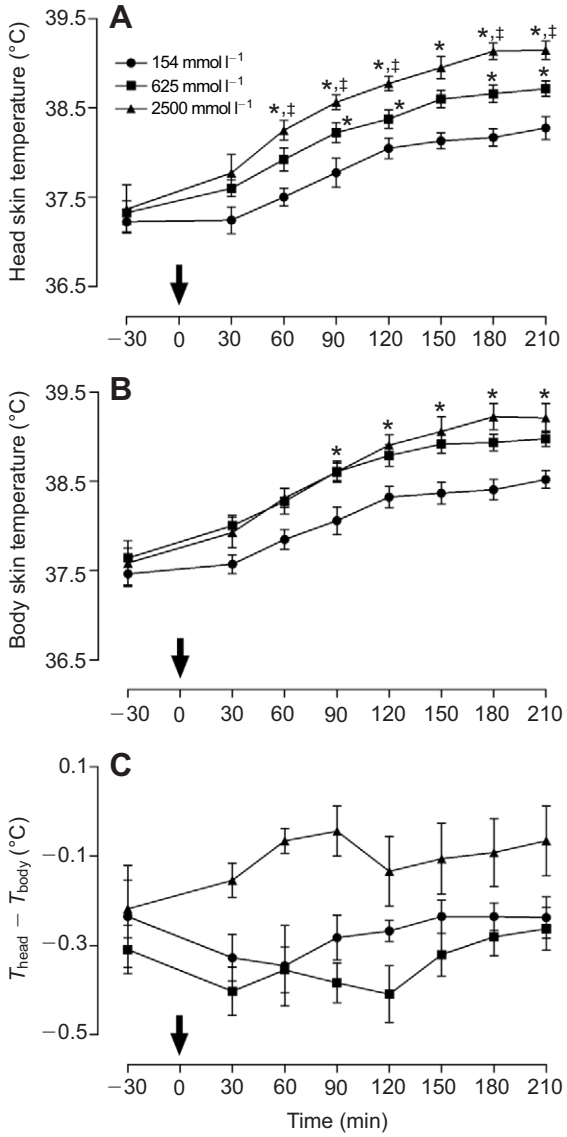

Fig. 3. Effect of different concentrations of saline injection on temperature over time. (A) Head skin temperature, (B) body skin temperature and (C) difference (T_{head}–T_{body}). N=11 (154 mmol l^{-1}), N=4 (625 mmol l^{-1}) and N=5 (2500 mmol l^{-1}). Differences in interaction effect for A and B: *P<0.05 compared with 154 mmol l^{-1} at the same time; ‡P<0.05 compared with 625 mmol l^{-1} at the same time. In C, 2500 mmol l^{-1} was different from the other groups (effect of treatment, P<0.05; no interaction between factors).

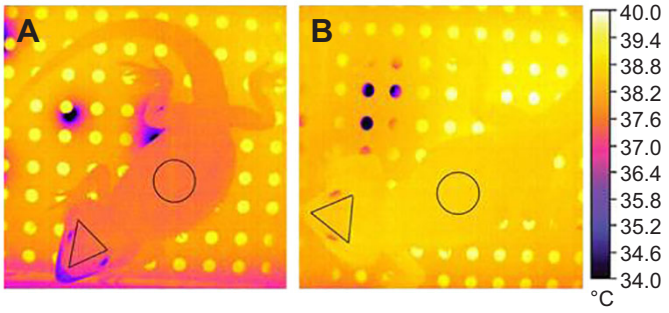

Fig. 4. Representative infrared thermal images recorded after saline injections in *P. vitticeps*. (A) Control animal (154 mmol l^{-1}). (B) Salt-loaded animal (2500 mmol l^{-1}). Both images were recorded 125 min after saline injection when control animals gaped close to 60% of the time and salt loaded ones had no gape. Note the cooler temperature surrounding the mouth in A. Head skin temperature was taken from the area indicated by the triangle and the body skin temperature was taken from the circled area indicated in the image.

rise in plasma osmolality along with a strong, proportional drive to drink. We expected that lizards loaded with high levels of salt would escape from the cold to the hot compartment and from the hot to the cold one at a lower T_a, i.e. the lizards would have a lower LET_a and UET_a compared with control animals, reflective of an overall reduction in their defended T_b. Previous research has shown that rats (Konishi et al., 2007), toads (Malvin and Wood, 1991), lizards (Crowley, 1987; Bradshaw et al., 2007) and snakes (Ladyman and Bradshaw, 2003) prefer a cooler T_a when water availability is reduced. However, in the present study, the highest concentration of saline injection (2500 mmol l^{-1}) altered both the LET_a and UET_a of *P. vitticeps*, but in opposite directions (Fig. 1A) and decreased the number of shuttles (Fig. 1B). A lower LET_a and a higher UET_a suggest that either the thermal preference of extreme voluntary temperatures was decreased and increased, at the same time, or that the salt loading was affecting the animal's propensity to move (Fig. 1B) and not targeting thermoregulatory set-points.

Our subsequent experiment testing behaviour in an isothermal shuttle box (maintained at 34°C) using the 2500 mmol l^{-1} solution reduced the overall number of shuttling events (Fig. 1C), indicating that salt loading affected the animal's predisposition to move, instead of the thermoregulation, per se. In the field, iguanid and agamid lizards abandon all activity and do not thermoregulate when they are starving or facing an extreme reduction in water availability (Bradshaw, 1997), a behaviour that is similar to the one found in the present study. It is still not certain whether the reduction in shuttling after salt loading is based on a diminished motivation to move, or a physiological inhibition of neuromuscular function. Based on our results, we suggest that the highest saline concentration used in the present study was high enough to inhibit any overt locomotory thermoregulatory responses. In contrast, the intermediate concentration (1250 mmol l^{-1}), which reduced 90% of the gaping response, did not alter the LET_a and UET_a nor the number of shuttles, indicating that a different threshold for salt loading influences these thermoregulatory responses and that water conservation could occur with mild dehydration stress without changes in shuttling thermoregulatory set-points.

The effect of salt loading on gaping confirmed our hypothesis, with gaping exhibiting a well-defined dose response (Fig. 2A,B), which is interesting because the 1250 mmol l^{-1} saline concentration did not affect behavioural thermoregulation in the shuttle box, but did cause a large reduction in gaping. Even the lowest concentration (625 mmol l^{-1}) was able to reduce gaping by approximately 50%. Although considered a different response from gaping, panting frequency and panting threshold temperatures were also altered by dehydration in endotherms such as fowls (Arad, 1983), emus (Maloney and Dawson, 1998) and sheep (McKinley et al., 2008). The effects of salt loading and dehydration suggest that the water-related thermoeffectors (gaping/panting) are more sensitive to salt levels in the circulation than the 'dry' thermoeffectors, such as behavioural shuttling. In this way, reduction of gaping induced by salt loading seems to be a strategy for saving water. Based on these results, the expectation is that gaping would be a significant source of water loss for the bearded dragon.

Although no significant difference between gaping and non-gaping intervals was detected in the EWL by itself, the $\dot{V}_{O_2}$ was

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Table 1. Effect of gaping in the lizard *Pogona vitticeps*

Gape state	EWL (mg min^{-1})	EHT (J min^{-1})	$\dot{V}_{O_2}$ (ml min^{-1} STP)	HP (J min^{-1})	EWL/$\dot{V}_{O_2}$ (mg ml^{-1} STP)	EHT/HP
Non-gaping interval	5.87±0.65	14.69±1.63	0.97±0.14	19.46±2.8	6.90±1.13	0.86±0.14
Gaping interval	6.07±0.43	15.18±1.06	0.73±0.09*	14.52±1.83	9.10±1.03*	1.14±0.13

These results (means±s.e.m.) are from lizards (N=8) that received isotonic injections. *P<0.05, difference between non-gaping and gaping intervals.

reduced by 25% when the animal gaped. A further decrease in $\dot{V}_{O_2}$ and a 10-fold increase in breathing frequency (=panting) has been reported in sheep when they were exposed to mild heat stress (40°C for 2–3 h). Such a decline in $\dot{V}_{O_2}$ was associated with a decrease in blood flow to the skeletal muscle and internal organs accompanied by an increase in blood flow to peripheral areas (Hales, 1973; Hales and Brown, 1974). Indeed, gaping has always been observed in our laboratory in very calm animals, showing no active movement concomitant with gaping. This reduction in movement, simultaneously with a possible increase of blood flow to the mouth region, could have contributed to the overall decrease in $\dot{V}_{O_2}$ in lizards. However, because the total EWL did not change while the $\dot{V}_{O_2}$ was reduced, the ratio EWL/$\dot{V}_{O_2}$ was augmented (Table 1) indicating that gaping does contribute to water loss when accounting for the contribution of metabolism itself (evaporative heat loss corresponds to 86% of the total HP during non-gaping intervals, rising to 114% when gaping; Table 1). Bradshaw (1997) previously reported that agamid lizards (*Ctenophorus ornatus*) lose normal thermoregulatory behaviours in the field when they face hypernatraemia associated with chronic dehydration. The present study corroborates Bradshaw's finding (1997) and provides further evidence that when faced with a dehydration stress, the osmoregulatory system looks to be preserved (gaping, a EWL source, was inhibited) at the expense of the thermoregulatory system. As mentioned, only animals injected with isotonic saline were considered for gaping evaporative potential (Table 1) because after hypertonic saline treatment, lizards presented very short periods of gaping, which were not reliable for instantaneous respirometry comparisons. Although such recordings were not ideal for gaping analyses, they were consistent for assessing the overall effect of salt on metabolism (Table 2). Salt loading (625 mmol l^{-1}) decreased the $\dot{V}_{O_2}$ compared with the isotonic injection effect. Metabolic rate is also reduced in dehydrated camels, cats and goats (Schmidt-Nielsen et al., 1967; Doris and Baker, 1981; Dmi'el, 1986). EWL tended to be lower in salt-loaded animals (625 mmol l^{-1}), which agrees with the reduction in time spent gaping after salt injection (Fig. 2A,B), once again suggesting that gaping normally contributes to water loss and is reduced when plasma osmolality rises. Because $\dot{V}_{O_2}$ was reduced and EWL tended to be smaller after hypertonic injection (625 mmol l^{-1}), the EWL/$\dot{V}_{O_2}$ ratio was not significantly altered (Table 2).

The neurological origins for the interactions between the osmoregulatory and thermoregulatory system are often found within the hypothalamus. The thermosensitivity of hypothalamic neurons is reduced during dehydration in cats, decreasing the evaporative heat loss (Doris and Baker, 1981). Furthermore, increased cerebrospinal fluid sodium concentration reduces the sweat rate in monkeys (*Erythrocebus patas*; Owen et al., 1989) and breathing frequency (=panting) in rabbits (Turlejska and Baker, 1986). In sheep, the inhibitory effect of hypertonic saline into the carotid artery on panting was abolished by lesion of the lamina terminalis, a region that includes the osmoreceptors and the median preoptic nucleus (McKinley et al., 2008), which, in turn, is involved in the regulation of thermoeffectors (Nakamura and Morrison, 2010) in mammals. Therefore, in many vertebrates, thermoregulation is linked to osmoregulatory status directly through central neurons. Similar pathways might have been activated in our lizards; in other words, salt loading might have increased the encephalic sodium concentration, changed the neuron's thermosensitivity and contributed to the reduction in the percentage of time spent in gaping.

Table 2. Percentage change in evaporative water loss (EWL, mg min^{-1}), oxygen consumption ($\dot{V}_{O_2}$, ml min^{-1} STP) and EWL/$\dot{V}_{O_2}$ (mg ml^{-1} STP) ratio after isotonic and hypertonic saline injections in the lizard *P. vitticeps*

Saline treatment	%EWL	%$\dot{V}_{O_2}$	%EWL/$\dot{V}_{O_2}$
154 mmol l^{-1}	−5.11±8.3	−12.96±8.1	16.1±21.3
625 mmol l^{-1}	−22.7±6.9	−40.9±7.5*	48.1±25.4

Percentage values were calculated based on the change post-injection compared to pre-injection; N=7 (154 mmol l^{-1}) and N=8 (625 mmol l^{-1}); * P<0.05, difference between treatments.

All of the lizards in the gaping protocol demonstrated an increase in T_{head} over the time they were inside the environmental chamber while they were still equilibrating to chamber temperature; however, the T_{head} of those animals that received the most concentrated saline injection (2500 mmol l^{-1}) was even higher compared with control animals (Fig. 3A and Fig. 4); the same pattern was also observed in the T_{body} (Fig. 3B and Fig. 4). It is interesting to note that the difference between T_{head} and T_{body} was higher in those animals that were injected with isotonic saline or lowest hypertonic saline (625 mmol l^{-1}) than in the animals that received the highest hypertonic saline treatment (Fig. 3C and Fig. 4). Therefore, at levels of salt loading that completely suppress gaping (2500 mmol l^{-1}), T_{head} and T_{body} equalize, which may have significant consequences for the defence of cranial temperatures and brain function, indicating that gaping might play a role as a local brain-cooling mechanism (Tattersall et al., 2006), in spite of the relatively minor changes in whole body EWL that accompany gaping (Table 1).

A mechanism called 'selective brain cooling' (SBC) has been described to be activated in mammals (Caputa et al., 1976; McConaghy et al., 1995; Jessen, 2001; Mitchell et al., 2002) when they face water stress. SBC is defined as brain temperature lower than arterial blood temperature (IUPS Thermal Commission, 2001), which is achieved by carotid blood cooling on its ascent to the brain (Willmer et al., 2005). With a cooler brain, the EWL is not activated because warm-sensitive neurons are not triggered. In this way, it has been suggested that SBC is not only a neural protection mechanism, but is also a water conservation strategy.

A combination of respiratory cooling and a by-passing vascular countercurrent system for heat exchange in the brain may work together and lead to encephalic cooling in lizards when these animals are exposed to high T_a (Tattersall et al., 2006). Gaping clearly contributes to water evaporation in the upper airways and buccal cavity (Fig. 4), which may be sufficient to cool the carotid blood on its way to the brain. At low or preferred T_a the encephalic blood descends by the internal jugular vein and then, because of its close proximity to the internal carotid artery, heat (from thermoregulatory basking) is transferred to this artery, thus warming the brain (Oelrich, 1956; Heath, 1964, 1966). At higher T_a, internal

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jugular vein is suggested to be constricted, thereby by-passing the blood through the external jugular, which is not very close to the carotid artery. In this case, the heat is not transferred to the carotid blood, but instead, is carried away from the brain (Heath, 1964; Tattersall et al., 2006). It is possible that these two mechanisms together (respiratory cooling and by-passing of the vascular heat exchanger) keep the brain cooler when bearded dragons gape at least 50% of time (Fig. 3C), but the cooling was not preserved when gaping was completely inhibited (Fig. 3C). The same evaporative/non-evaporative mechanisms might contribute to head cooling in a small lacertid lizard (*Podarcis muralis*) when they are heated (Sannolo et al., 2014), although this hypothesis remains to be experimentally tested.

There remain key differences between SBC in mammals and reptiles. In mammals, SBC can be observed when water is not available. Euhydrated mammals (goats and sheep), even when exposed to heat stress rarely present SBC, but dehydrated animals show more frequent and a larger degree of SBC, supporting the idea that such a mechanism has an osmoregulatory drive and might not play a thermoregulatory function in these animals (Jessen et al., 1998; Fuller et al., 2007). In contrast, in *Pogona*, cooling of the head (and by inference, the brain) happened when water was available (i.e. when animals exhibited gaping: 154 and 625 mmol l^{-1} injections; Fig. 4A and Fig. 3C) and, in an opposite direction, it was diminished when water was not available, i.e. when gaping was abolished (2500 mmol l^{-1} injection; Fig. 4B and Fig. 3C). Furthermore, the degree of gaping in bearded dragons is augmented as T_a increases in euhydrated animals and the difference between T_{head} and T_{body} increases as well (Tattersall and Gerlach, 2005), but this difference was observed to decrease when animals were salt loaded at the same T_a (Fig. 2A). Therefore, it seems that it is not osmoregulation that drives brain cooling in lizards, but rather thermoregulation; consequently, SBC may operate as a protective encephalic mechanism. Despite having a thermoregulatory drive, when lizards are faced with low water availability, body water is still conserved at the expense of thermoregulation, similar to what happens to mammals. Although a previous study has reported that gaping is an effective cooling thermoeffector for *Alligator mississipiensis* (Spotila et al., 1977), the present study is the first to show that gaping may work as a local (brain) cooling mechanism for desert lizards, based on the fact that salt loading reduced gaping and increased T_{head}, eliminating the difference between T_{head} and T_{body} (Fig. 3).

In conclusion, we found that an intermediate concentration of hypertonic saline can affect EWL mechanisms, with no effect on non-EWL mechanisms. Since gaping is more responsive to salt loading than shuttling, this may be an important response for saving water over a natural dehydration period, while still allowing for optimal thermoregulatory behaviours. However, when water is available, gaping may play a role as a local cooling mechanism that is important to avoid encephalic superheating in reptiles such as *Pogona vitticeps* that engage in thermoregulatory basking.

MATERIALS AND METHODS

Animals

A total of 19 animals (9 females and 10 males, *Pogona vitticeps* Ahl 1926; body mass: 200–500 g) were randomly used in four different protocols. The lizards were housed in terraria with corn cob bedding, containing a 40 W light bulb for thermoregulation and an additional UV light source for vitamin D synthesis, and enriched with a small and opaque tube and a cardboard material that provided shelter and extra climbing surfaces. The animals were kept on a 12 h:12 h light:dark cycle (lights on at 08:00 h) and fed three times a week with a combination of chopped vegetables and fruits and twice a week with insects (cockroaches), but they were fasted for at least 48 h before the experiments. The dragons also received a lukewarm bath before and after all the experiments and an extra bathing and drinking opportunity once a week to ensure they were hydrated. The number of animals that drank water within a 5 min period of time was counted for comparison across all salt loading levels. The same animals were used in almost all the protocols, but they had, at a minimum, a 14 day interval between experiments. All experiments were run between 08:30 h and 16:30 h and all procedures used in this study were approved by the Brock University Animal Care and Use Committee (Protocol #12-11-01).

Injections

Lizards received an isovolemic (1 ml/100 g) intra-peritoneal (i.p.) injection of either normal isotonic saline (154 mmol l^{-1} NaCl) or hypertonic saline, in order to induce an estimated 5, 10 and 20% increase on plasma osmolality. Three hypertonic concentrations were used (625, 1250 and 2500 mmol l^{-1}) based on previous studies (Konishi et al., 2003; Ford and Bradshaw, 2006) and on mathematical assumptions (75% body water, 50% extracellular fluid and rapid mixing of salt within the plasma) assuming that the baseline osmolality for bearded dragons was 308 mosmol l^{-1} (Smits and Kozubowski, 1984).

Plasma concentration

To assess the plasma concentration, blood samples were taken from the lizard's tail vein after 1 h of either normal (154 mmol l^{-1}) or hypertonic saline injections (625, 1250 and 2500 mmol l^{-1}). Blood was collected in micro-haematocrit glass tubes and centrifuged in a haematocrit centrifuge for 2 min to separate the plasma. The plasma was stored at 0°C and 10 µl aliquots (triplicate) were analysed in a Vapro vapor pressure osmometer (5520, Wescor, Inc, Logan, UT, USA) using a 291 mosmol $litre^{-1}$ standard.

Shuttle box experiments

The shuttle box (see Cadena and Tattersall, 2009a,b for details) was a wooden chamber (119×61×45 cm) with two compartments separated by a transparent partition. There was a hole (11.5×14 cm) at the bottom of the partition which connected both compartments and allowed the animal to move from one side to the other one (shuttling behaviour). The walls were oriented to naturally funnel the lizard toward the hole in the partition, which facilitated the shuttling movement by serving as a guide toward the transition point. A transparent lid was placed on the top of the box to avoid any disturbance and to help to maintain the internal temperature. Cameras were mounted over the lid for continuous monitoring, but no data were collected from them.

In the first protocol performed in the shuttle box, one compartment was always 10°C warmer than the other, creating a so-called 'hot compartment' (HC) and a 'cold compartment' (CC). The temperature inside the entire box was controlled by a treadle switch located on the floor, right below the transition point between the compartments. By stepping on this treadle, the lizards regulated the T_a and, consequently, their own temperature. Lizards were always placed in the HC in the beginning of an experiment to increase motivation and to prevent long lethargy from exposition to cold, as the animals had just emerged from the rest phase (dark phase) and the lights inside their terraria had only just been turned on, so the temperature within their housing environments was relatively cold.

Once the animal was placed in the HC, the temperature in both compartments automatically rose at 0.7°C min^{-1} (Cadena and Tattersall, 2009a), while maintaining a 10°C difference between them. The temperature rose until the animal moved to the CC, stepping on the treadle and activating the cooling system. At that moment, both compartments cooled down at 0.7°C min^{-1} until the animal moved back to HC. The maximum temperature allowed in the HC was 43°C and the minimum temperature allowed in the CC was 10°C, as a safety precaution for the animals and because these values fall well outside previously known limits for thermoregulation in this species (Cadena and Tattersall, 2009a).

Cooling and heating systems were controlled by an automated electronic system (Brock University, Electronics Shop, St Catharines, ON, CA).

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Ambient temperature was measured by a platinum resistor thermometer in each compartment and recorded by the same electronic system every 30 s and whenever the animal moved from one side to the other one during the experiment. The location of the lizard (HC or CC) was recorded at the same times as the T_a. The T_a inside the HC when the lizard moved from the HC to the CC was called UET_a and the T_a inside the CC when the lizard moved from the CC to the HC was named LET_a. Throughout the course of an experiment, a lizard exhibited numerous UET_a and LET_a values, based on how often it shuttled. Ambient escape temperatures were used to describe thermoregulatory behaviours based on previous work (Cadena and Tattersall, 2009a) showing that these escape T_a measures accurately reflect changes in T_b in this species.

The second protocol performed in the shuttle box had no difference in the temperature between the compartments. Both sides had the T_a fixed at 34°C (normal preferred T_b in the laboratory for bearded dragons; Cadena and Tattersall, 2009a) throughout the experiment and the total number of spontaneous shuttles produced with no thermoregulatory drive was recorded (see detailed description below).

Protocol 1

The purpose of this protocol was to examine whether thermoregulatory control was altered in salt-loaded lizards. The first 3 h after the animal was placed in the shuttle box were used to allow habituation to the novel environment and were considered to be exploratory shuttling and therefore were not used in the analyses (Cadena and Tattersall, 2009a). After this interval, animals received an i.p. injection of isotonic (154 mmol l^{-1}) or hypertonic (1250 or 2500 mmol l^{-1}) saline solutions and were placed again in the shuttle box (in the same compartment where they were before the injection) for a further 4 h. This last interval was taken into account for the analysis of UET_a and LET_a and the number of shuttles. For this experiment, 11 animals were injected with all the three doses of saline.

Protocol 2

The aim of this protocol was to examine whether salt loading prevented or impinged on the lizard's natural propensity to move and behave spontaneously. In this protocol, the animal received the i.p. injection of saline solutions (154 or 2500 mmol l^{-1}) first thing in the morning and was immediately placed in the shuttle box. The reason for this was to capitalize on the natural tendency for lizards to initially explore the shuttle box when they are first placed into it. Previous research has shown that following this exploration interval, lizards will generally cease all shuttling entirely when there is no thermoregulatory drive (Cadena and Tattersall, 2009a). The experiment lasted 4 h and the number of shuttling events was analysed in this protocol. Fourteen lizards were injected with each saline concentration.

Gaping experiments

Lizards were placed in an acrylic box (24×24×40 cm) which had three of its four sides covered with paper to prevent distraction and reduce reflection. On the top of the box, an infrared thermal imaging camera (Mikron 7515; Oakland, NJ, USA) and accompanying MikroSpec RT® (Mikron) software were used to monitor the T_{head} and T_{body}. Two representative infrared thermal images used to calculate T_{head} and T_{body} can be observed in Fig. 4A,B. The acrylic box was positioned in an environmental chamber (Thermo Forma, Marietta, OH, USA) which was used to maintain a narrow range of T_a [T_a inside the acrylic box was about 37–39°C: a level sufficiently high to induce gaping in bearded dragons (Tattersall and Gerlach, 2005)].

A webcam was attached to the internal wall of the environmental chamber, facing the only side of the acrylic box that was not covered by the paper. Images of the lizard were collected by the webcam every 10 s (HandyAvi; Tempe, AZ, USA) and used to assess the percentage time engaged in gaping.

These experiments were conducted to assess whether salt loading influenced the lizard's gaping behaviour. The infrared thermal camera allowed us to monitor the lizard's T_{body} and when it reached at least 37°C (usually 3–4 h after they were placed in the acrylic box inside the environmental chamber) the animals received an i.p. saline injection (154, 625, 1250 or 2500 mmol l^{-1}) and were placed back in the observation chamber for a further 3.5 h. Because the environmental chamber was slowly warmed, the i.p. injection happened before the chamber reached its complete thermal equilibrium. The percentage of the time spent in gaping was analysed from the webcam images monitoring the lizard (percent time was estimated over 30 min intervals from images captured every 10 s) prior to and following the injection. Eleven animals were injected in this protocol for each dose of saline.

Respirometry experiments

$\dot{V}_{O_2}$ and EWL values were obtained by flow-through respirometry. The animal was placed in a cylindrical respirometer (total volume: 2.8 l) inside a temperature controlled environmental chamber (Sable Systems, Las Vegas, NV, USA). The T_a inside the respirometer was about 37.5°C. The incurrent air was dried through Nafion tubing using a counter-current water vapour extraction system (pure nitrogen was used to extract water vapour) and pushed into the respirometer at a rate of 1000 ml min^{-1}. A subsample of this air was pushed through the H_2O, CO_2 and O_2 analysers at 180 ml min^{-1} (FlowBar-8; Sable Systems, Las Vegas, NV, USA). The water vapour density (WVD; μg ml^{-1}) was the first parameter analysed (RH200; Sable Systems, Las Vegas, NV, USA) and this record was later used to calculate the EWL. Then, the CO_2 percentage (CA-2A; Sable Systems, Las Vegas, NV, USA) and, finally, the O_2 percentage were recorded (FC-1B; Sable Systems, Las Vegas, NV, USA). Following acquisition, the three channels of gases were time shifted to account for the delay in sample transfer from each analyser (8–12 s delay determined by a bolus injection of nitrogen into the respirometer). A baseline sample of dry incurrent air was taken for 5 min every 25 min to ensure that the water, O_2 and CO_2 in the air offered to the animal remained constant over the experiment, and to provide the estimates of $F_{I_{O_2}}$, $F_{I_{CO_2}}$ and $F_{I_{H_2O}}$. One gas flow distributor (RM8 Intelligent Multiplexer; Sable Systems, Las Vegas, NV, USA) was used to control which gas sample (from respirometer or baseline) entered the analysers. All data were collected by using a data-acquisition system (AcqKnowledge v.3.8.1, BIOPAC Systems, Goleta, CA, USA) collecting values every second. Prior to analysis, all time-aligned gas channel data were *Z*-transformed to obtain instantaneous values (Lighton, 2008). The temperature inside the environmental chamber was monitored by a thermocouple meter (TC-2000; Sable Systems, Las Vegas, NV, USA) and the lizard's surface temperature was measured using a data logger (iButtons DS1922L, Maxim Integrate, San Jose, CA, USA) attached to its ventral surface with medical tape (Transpore, 3M).

The analysers were calibrated weekly or whenever was necessary (O_2 was calibrated daily prior to experimentation). The O_2 analyser was calibrated using dried air (20.95%); the CO_2 was calibrated using pure nitrogen as a zero value and a certified, pre-mixed gas source (1% CO_2) as a span value, and lastly, the H_2O analyser was also calibrated using pure nitrogen as a zero value and air bubbled through water of a known temperature as the span value using WVD estimates (Dossat and Horan, 2001). The $\dot{V}_{O_2}$ (ml O_2 min^{-1} STP) and EWL (mg H_2O min^{-1}) were calculated using the following equations (Lighton, 2008):

$$\dot{V}_{O_2} = FR_I\left[F_{I_{O_2}} - \left(F_{E_{O_2}}\frac{1 - F_{I_{O_2}} - F_{I_{CO_2}} - F_{I_{H_2O}}}{1 - F_{E_{O_2}} - F_{E_{CO_2}} - F_{I_{H_2O}}}\right)\right], \quad (1)$$

$$EWL = \frac{[FR_I(WVD_E - WVD_I)]}{1000}, \quad (2)$$

where FR_I is incurrent flow rate, $F_{I_{O_2}}$ is incurrent fractional concentration of oxygen (from baseline), $F_{E_{O_2}}$ is excurrent fractional concentration of oxygen, $F_{I_{CO_2}}$ is incurrent fractional concentration of carbon dioxide (from baseline), $F_{E_{CO_2}}$ is excurrent fractional concentration of carbon dioxide, $F_{I_{H_2O}}$ is incurrent fractional concentration of water vapour (from baseline), $F_{E_{H_2O}}$ is excurrent fractional concentration of water vapour, WVD_E is excurrent water vapour density and WVD_I is incurrent water vapour density.

Assuming that 1.0 ml of consumed oxygen produces 20 J of heat and for each milligram of water evaporated, 2.5 J is dissipated, then heat production (HP; J min^{-1}) and evaporative heat transfer (EHT; J min^{-1}) were also

calculated as follows (Randall et al., 1997):

$$\mathrm{HP} = \dot{V}_{\mathrm{O_2}} \times 20, \quad (3)$$

$$\mathrm{EHT} = \mathrm{EWL} \times 2.5. \quad (4)$$

The objective of these experiments was to examine the overall changes in oxygen consumption and whole body water loss during spontaneous periods of gaping and non-gaping, before and after isotonic (154 mmol l^{-1}) or hypertonic (625 mmol l^{-1}) saline injection. In this protocol, the animals were also placed in the acrylic box inside the environmental chamber until the T_{body} reached 37°C (monitored with the infrared thermal camera), following which the animals were transferred to the glass respirometer and placed inside an environmental chamber (also at 37°C) connected to the respirometry system. $\dot{V}_{\mathrm{O_2}}$ and EWL were recorded for 90 min, then animals received a saline injection (154 or 625 mmol l^{-1}) and oxygen consumption and water loss were registered for another 90 min. The percentage of the time spent gaping was analysed during the last 30 min before the transfer to the respirometer and during all the time that the animals were inside the respirometer (i.e. 180 min; data not shown). The ventral surface temperature was recorded throughout the experiment using a data logger attached to the skin. Eight animals were used for each saline concentration.

Statistical analysis

All the results are presented as means±s.e.m. The predicted plasma concentration and the values found were analysed by linear regression and the proportion of lizards that drank water within a 5 min period after salt loading was analysed using a chi-square test. Protocol 1 results were analysed using a two-way RM ANOVA (factors: direction – $\mathrm{UET_a}$ or $\mathrm{LET_a}$ – and saline treatment) was used to compare the effect of treatment on the $\mathrm{UET_a}$ and $\mathrm{LET_a}$ and one-way RM ANOVA was run to compare the number of shuttles between saline treatments (factor: treatment). Protocol 2 results were analysed using a paired *t*-test to compare the number of shuttles between saline treatments. Two-way RM ANOVA was performed to analyse the effect of salt loading on percentage of time spent in gaping, on T_{head}, on T_{body} and on the difference in these values ($T_{\mathrm{head}}-T_{\mathrm{body}}$) (factors for all comparisons: time and saline treatment) over time. A paired *t*-test was used to compare EWL and EWL/$\dot{V}_{\mathrm{O_2}}$ between gaping and non-gaping intervals and a Wilcoxon signed-rank test was run to analyse $\dot{V}_{\mathrm{O_2}}$ data. Only gaping periods longer than 15 s in duration were utilized for the determination of $\dot{V}_{\mathrm{O_2}}$ and EWL values. A *t*-test was performed to analyse the effect of salt injection on %EWL and %$\dot{V}_{\mathrm{O_2}}$ (post injection values compared to pre injection values) and a Mann–Whitney rank-sum test was performed to compare % EWL/$\dot{V}_{\mathrm{O_2}}$ data.

Whenever RM ANOVA resulted in significant main or interaction effects, a Holm–Šidák *post hoc* test was performed to verify where the differences existed. Residuals were tested for unequal variance and normality. In cases where log transformation was insufficient in terms of model assumptions, ranked data were analysed. Differences were considered significant when $P \leq 0.05$. All analyses were performed using SigmaPlot 11.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

G.J.T., K.C.B. and C.d.S.S. designed research, performed data analysis and prepared the manuscript prior to submission. C.d.S.S. and G.J.T. performed experiments.

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