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 like shuttling and gaping are similarly influenced. Shuttling, as an active behavioural response, is considered one of the most effective thermoregulatory behaviours, while 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 mM) and hypertonic saline injections (625, 1250 or 2500 mM). 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.

Keywords: 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 does not only influence but can also 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 the 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 waterdependent 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. 1983; 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 Woods 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 while other 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), proving to be a tractable approach to studying temperature sensing and costs 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 that this behavior 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) and is a desert animal that likely faces seasonal plasma hypernatremia as has been described in other arid (Bentley, 1959) and agamid Australian reptiles (Bradshaw & 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 body water availability on shuttling and gaping, two extremely important thermoeffectors to $T_{\rm b}$ 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 warm 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 ones predicted by our mathematical calculations. The hypertonic saline doses, 625, 1250 and 2500mM, raised the plasma osmoconcentration by approximately 7, 12 and 22% compared to the control saline (154mM), 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 (χ^2 (3, *N*=114) =45.65, p< 0.001). For control animals (154 mM) only 5 out of 38 animals drank water (13%); for 625 mM injection, 6 out of 19 (31%); for 1250 mM, 14 out of 22 (63%) and for 2500 mM, 31 out of 35 (88%).

Effect of salt loading on shuttling behaviour:

The injection of 1250 mM hypertonic saline had no effect on UET_a (Upper Escape Ambient Temperature; See "Methods"; p= 0.882) nor on LET_a (Lower Escape Ambient Temperature; See "Methods"; p=0.357) compared to control animals (Fig. 2A); however the highest concentration (2500 mM) increased the UET_a and decreased the LET_a (interaction of direction x treatment: p< 0.001. $F_{2,30}$ = 18.116; Fig. 2A) and decreased the number of shuttles (treatment effect: p= 0.01. $F_{2,30}$ = 5.463; Fig. 2B) compared to isotonic saline injections.

In order to verify if 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 mM) had no effect on UET_a and LET_a (Fig. 2A). The salt loading significantly reduced the amount of exploratory shuttling (p= 0.004; Fig. 2C).

Effect of salt loading on gaping

Salt loading decreased the propensity for gaping in lizards in a dose and timedependent manner (interaction of time x treatment: p< 0.001. $F_{3,40} = 5.498$; Fig. 3A,B). The two highest concentrations of saline injections (1250 and 2500 mM) reduced the gaping at least 90% in the end of experiment compared to the pre injection values while the lowest concentration (625 mM) diminished gaping at 50% (Fig. 3A).

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. 4A,B,C) The T_{head} was significantly increased after saline injections with the highest effect of the 2500 mM treatment compared to control and 625 mM groups; the increase in T_{head} of 625 mM treated-animals was higher than that of control ones (interaction of time x treatment effect: p= 0.003; $F_{2,17} = 2.57$; Fig. 4A). Salt loading also caused a rise in the lizard's T_{body} compared to controls (interaction time x treatment effect: p= 0.002; $F_{2,17} = 2.78$; Fig. 4B), but no difference was observed between 625 and 2500 mM treatments.

Furthermore, control group and animals treated with the lowest hypertonic saline (625 mM) exhibited a significantly lower T_{head} than T_{body} compared to those animals that received the highest concentration of saline (2500 mM; 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. 1A,B,4C). For salt-loaded animals (2500 mM), T_{head} and T_{body} were virtually the same after injection (Fig. 1B and 4C).

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 "Methods" for details). Because gaping intervals shorter than 15 seconds were not considered for these calculations (see "Methods" for details), only control animals (154 mM) 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 gaping evaporative effectiveness ($EWL/\dot{V}O_2$; p= 0.01; Table 1). The T_b was 37.7±0.06°C throughout the experiment.

The hypertonic saline injection (625 mM) 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 to isotonic saline effect 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 rise in plasma osmolality along with a strong, proportional drive to drink. We expected that the high salt-loaded lizards 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 to 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 cooler T_a when water availability is reduced. However, in the present study, the highest concentration of saline injection (2500 mM) altered both LET_a and UET_a of *P. vitticeps*, but in opposite directions (Fig. 2A) and decreased the number of shuttles (Fig. 2B). A lower LET_a and a higher UET_a suggest that either the thermal preference of extreme voluntary temperatures was decreased and increased, at same time, or that the salt-loading was affecting the animal's propensity to move (Fig. 2B), and not targeting thermoregulatory set-points.

Our subsequent experiment testing behaviour in an isothermal shuttle box (maintained at 34°C) using the 2500 mM solution reduced the overall number of shuttling events (Fig. 2C), 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 similar to that 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 mM), which reduced 90% of the gaping response, did not alter LET_a and UET_a nor the number of shuttles, indicating a different threshold for salt loading influencing these thermoregulatory responses, and that some potential 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. 3A,B), which is interesting because the 1250 mM saline concentration did not affect behavioural thermoregulation in the shuttle box, while causing a large reduction in gaping. Even the lowest concentration (625 mM) 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, like behavioural shuttling. In this way, gaping reduction 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 reduced by 25% when the animal gaped. An even 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 the lizards. On the other hand, 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 nongaping 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, not reliable for instantaneous respirometry comparisons. Although hypertonic saline respirometry recordings were not ideal for gaping analyses, they were consistent for assessing the overall effect of salt on metabolism (Table 2). Salt-loading (625 mM) decreased the $\dot{V}O_2$ compared to the isotonic injection effect. Metabolic rate has also being found to be reduced in dehydrated camels, cats and goats (Schmidt-Nielsen et. al., 1967; Doris and Baker, 1981; Dmi'el R., 2002). EWL tended to be lower in salt-loaded animals (625 mM) which agrees with the reduction in time spent gaping after salt injection (Fig. 3A,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 mM), 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 has been shown being 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.

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 mM) was even higher compared to control animals (Fig. 1A,B,4A); the same pattern was also observed in the T_{body} (Fig. 1A,B,4B). 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 mM) than in the animals that received the highest hypertonic saline treatment (Fig. 1A,B,4C). Therefore, at levels of salt-loading that completely suppress gaping (2500 mM), T_{head} and T_{body} equalize, which may have significant

consequences for the defense 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, 2005). With a cooler brain, the EWL is not activated since warm-sensitive neurons are not triggered. In this way, it has been suggested that SBC is not only a neural protection mechanism but, 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. 1) which may be sufficient to cool the carotid blood on its way to brain. At low or preferred T_a the encephalic blood descends by the internal jugular vein and then, due to 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 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. 4C), but the cooling was not preserved when gaping was completely inhibited (Fig. 4C). 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 (goat and sheep), even when exposed to heat stress rarely present SBC, but dehydrated ones 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*, head cooling (and by inference, the brain) happened when water was available (i.e., when animals exhibited gaping

– 154 and 625 mM injections; Fig. 1A,4C) and, in an opposite direction, it was diminished when water was not available, i.e., when gaping was abolished (2500 mM injection; Fig. 1B,4C). 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. 3A). Therefore, it seems that it is not osmoregulation that drives brain cooling in lizards, but rather it is 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 similarly 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. 4).

In conclusion, we found that an intermediate concentration of hypertonic saline can affect EWL mechanisms, with no effect on non-EWL mechanisms. Since gaping are 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, while water is available, gaping may play a role as a local cooling mechanism, important to avoid encephalic superheating in reptiles that engage in thermoregulatory basking, such as *Pogona vitticeps*.

Material and Methods

Animals

A total of 19 animals (9 females and 10 males, *Pogona vitticeps*, Ahl, 1926, body mass: 200-500g) were randomly used in four different protocols. The lizards were housed in terraria with corn cob bedding, containing a 40W 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 12L:12D light:dark cycle (lights on at 8:00) 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 48h 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 same animals were used in almost all the protocols, but they had, at minimum, a 14 days interval between experiments. All experiments were run between 8:30 and 16:30 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 (1mL/100g) intra-peritoneal (i.p.) injection of either normal (154 mM NaCl – isotonic saline) 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 mM) 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 (Smits and Kozubowski, 1984).

Plasma concentration:

To assess the plasm concentration, blood samples were taken from the lizard's tail vein after one hour of either normal (154mM) or the hypertonic saline injections (625, 1250 and 2500mM). Blood was collected in micro-haematocrit glass tubes and centrifuged in a haematocrit centrifuge for 2 minutes to separate the plasma. The plasma was stored at 0°C and 10 μ L aliquots (triplicate) were analysed in a Vapro Vapor pressure osmometer (5520, Wescor, Inc, Logan, UT, USA) using a 291 mosmol/L standard.

Experimental setup and design

As mentioned before, after the experiments, all animals received a lukewarm bath and the number of animals that drank water within a 5 minute period of time was counted for comparison across all salt loading levels.

Shuttle Box experiments:

The shuttle box (see Cadena and Tattersall, 2009a,b for details) was a wooden chamber (119 x 61 x 45cm) with two compartments separated by a transparent partition. There was a hole (11.5 x 14cm) 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 (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 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). Ambient temperature was measured by a platinum resistor thermometer in each compartment and recorded by the same electronic system every 30 seconds and whenever the animal moved from one side to the other one along all 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 laboratory for bearded dragons; Cadena and Tattersall, 2009a) throughout the entire

experiment, and the total number of spontaneous shuttles produced with no thermoregulatory drive was recorded (see detailed description of *protocol #2 below*).

Protocol #1- The purpose of this protocol was to examine whether thermoregulatory control was altered in salt-loaded lizards. The first 3 hours after the animal was placed in the shuttle box were used to allow habituation to the novel environment, considered as exploratory shuttling and were not considered in the analyses (Cadena and Tattersall, 2009a). After this interval, animals received an i.p. injection of isotonic (154 mM) or hypertonic (1250 or 2500 mM) saline solutions and were placed again in the Shuttle Box (in the same compartment where they were before the injection) for four more hours. 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 mM) first thing in the morning and were 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 Tattersal, 2009a). The experiment lasted 4 hours and the number of shuttling events was analyzed in this protocol. Fourteen lizards were injected with each saline concentration.

Gaping experiments:

Lizards were placed in an acrylic box (24 x 24 x 40 cm) which had 3 of its 4 sides covered with paper to prevent animal's distraction and reduce reflections. On the top of the box, an infra-red thermal imaging camera (Mikron 7515; Oakland, NJ, USA) and accompanying MikroSpec RT® (Mikron Instruments, Oakland, NJ, USA) software were used to monitor the T_{head} and T_{body} . Two representative infra-red thermal images used to calculate T_{head} and T_{body} can be observed on figure 1A and 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 each 10 seconds (HandyAvi; Tempe, AZ, USA) and used to assess the percent time engaged in gaping.

These experiments were conducted to assess whether salt-loading influenced the lizard's gaping behaviour. The infra-red thermal camera allowed us to monitor the lizard's T_{body} and when it reached at least 37°C (usually 3-4 hours 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 mM) and were placed back in the observation chamber for 3.5 more hours. 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 analyzed from the webcam images monitoring the lizard (percent time was estimated over 30 minute intervals from images captured every 10 seconds) 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 by the animal 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 NafionTM 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. A subsample of this air was pushed through the H₂O, CO₂ and O₂ analyzers at 180 mL/min (FlowBar-8; Sable Systems, Las Vegas, NV, USA). The water vapor density (WVD; μ g/mL) was the first parameter analyzed (RH200; Sable Systems, Las Vegas, NV, USA) and this record was later used to calculate the EWL. Then, the CO₂ percentage (CA-2A; Sable Systems, Las Vegas, NV, USA) and, finally, the O₂ 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 analyzer (8-12 second delay determined by a bolus injection of nitrogen into the respirometer). A baseline, sample of dry incurrent air, was taken for 5 minutes every 25 minutes to ensure that the water, O₂ and CO₂ in the air offered to the animal remained constant over the experiment, and to provide the estimates of *F*_iO₂, *F*_iCO₂, and *F*_iH₂O. 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 analyzers. 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 by a data logger (iButtons DS1922L, Maxim Integrate, San Jose, CA, USA) attached to its ventral surface with a medical tape (TransporeTM, 3MTM).

The analyzers were calibrated weekly or whenever was necessary (O₂ was calibrated daily prior to experimentation). The O₂ analyzer was calibrated using dried air (20.95%); the CO₂ was calibrated using pure nitrogen as a zero value and a certified, pre-mixed gas source (1% CO₂) as a span value, and lastly, the H₂O analyzer 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, 2001). The $\dot{V}O_2$ (mL O₂ min⁻¹ STP) and EWL (mg H₂O min⁻¹) were calculated using the following equations (Lighton, 2008):

$$\dot{V}_{O_2} = FR_i \left[F_i O_2 - \left(F_e O_2 \frac{1 - F_i O_2 - F_i C O_2 - F_i H_2 O}{1 - F_e O_2 - F_e C O_2 - F_e H_2 O} \right) \right]$$
(1)

$$EWL = \frac{[FR_i (WVD_e - WVD_i)]}{1000}$$
(2)

Where:

 FR_i = incurrent flow rate;

 F_iO_2 = incurrent fractional concentration of oxygen (from baseline);

 F_eO_2 = excurrent fractional concentration of oxygen;

F_iCO₂ = incurrent fractional concentration of carbon dioxide (from baseline);

 F_eCO_2 = excurrent fractional concentration of carbon dioxide;

 F_iH_2O = incurrent fractional concentration of water vapor (from baseline);

 F_eH_2O = excurrent fractional concentration of water vapor;

 WVD_e = excurrent water vapor density;

 WVD_i = incurrent water vapor 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):

$$HP = \dot{V}O_2 \times 20 \tag{3}$$

$$EHT = EWL \times 2.5 \tag{4}$$

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 nongaping, before and after isotonic (154 mM) or hypertonic (625 mM) 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 infra-red 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. $\dot{V}O_2$ and EWL were recorded for 90 minutes, then animals received a saline injection (154 or 625 mM) and oxygen consumption and water loss were registered for another 90 minutes. The percentage of the time spent gaping was analyzed during the last 30 minutes 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 mean \pm s.e.m.

Plasma concentration and Water consumption: the predicted plasma concentration and the values found were analyzed by linear regression and the proportion of lizards that drank water within a 5 minute period after salt loading was analyzed by a chi-square test.

Shuttle box: Protocol #1: two- way RM ANOVA (factors: direction – UET_a or LET_a – and saline treatment) was used to compare the effect of treatment on the UET_a and LET_a and one way RM ANOVA was run to compare the number of shuttles between saline treatments (factor: treatment). Protocol #2: a paired T-test was used to compare the number of shuttles between saline treatments.

Gaping: two-way RM ANOVA was performed to analyze the effect of salt loading on percentage of time spent in gaping, on T_{head} , on T_{body} and on delta (T_{head} minus T_{body}) (factors for all comparisons: time and saline treatment) over time. **Respirometry:** a paired T-test was used to compare EWL and $EWL/\dot{V}O_2$ between gaping and non-gaping intervals and a Wilcoxon Signed Rank Test was run to analyze $\dot{V}O_2$ data. Only gaping periods longer than 15 seconds in duration were utilized for the determination of $\dot{V}O_2$ and EWL values. A t-test was performed to analyze the effect of salt injection on %*EWL* and % $\dot{V}O_2$ (post injection values compared to pre injection values) and a Mann-Whitney Rank Sum Test was performed to compare % *EWL/\dot{V}O_2* data.

Whenever RM ANOVA resulted in significant main or interaction effects, a Holm-Sidak *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 analyzed. Differences were considered significant when $p \le 0.05$. All the analyses were performed using SigmaPlot 11.

List of abbreviations:

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);
F_eCO_2	excurrent CO ₂ fractional;
F _e H ₂ O	excurrent H ₂ O fractional;
F_eO_2	excurrent O ₂ fractional;
F_iCO_2	incurrent CO ₂ fractional (from baseline);
F_iH_2O	incurrent H ₂ O fractional (from baseline);
F_iO_2	incurrent O ₂ fractional (from baseline);
FR _i	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;

Ta	ambient temperature;
T _b	body temperature;
T_{body}	lizard's body skin temperatures;

T_{head} lizard's head skin temperatures;

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 O₂ min⁻¹ STP);

WVD_e excurrent water vapor density ($\mu g H_2 O m L^{-1}$);

WVD_{*i*} incurrent water vapor density (μ g H₂O mL⁻¹).

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Author contributions: GJT, KCB and CSS: designed research, performed data analysis and prepared the manuscript prior to submission. CSS and GJT: performed experiments.

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Figures

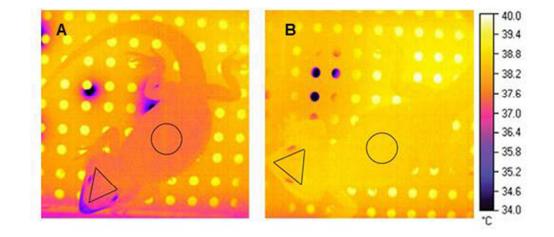
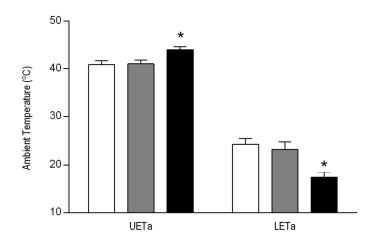
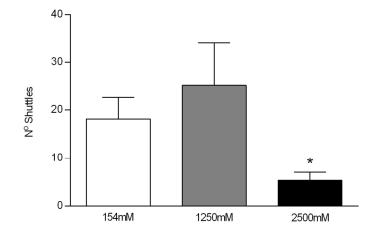


Fig. 1. Representative infra-red thermal images recorded after saline injections in *P. vitticeps.* A) Control animal (154 mM). B) Salt loaded animal (2500 mM). Both images were recorded 125 minutes after saline injection when control animals gaped close to 60% of the time and salt loaded ones had no gape. Note the cooler temperature surrounding the mouth in A. Head skin temperature was taken from the triangle and the body skin temperature was taken from the circle as indicated in the image.







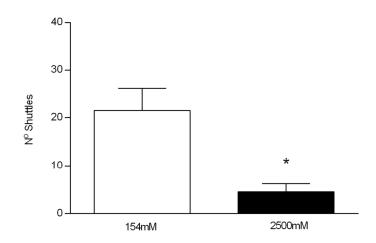


Fig. 2. The effect of different concentrations of saline injection on UET_a and LET_a and on number of shuttles in *P. vitticeps*. A) UET_a: mean ambient temperature in which the animal escaped from the hot compartment. LET_a: mean ambient temperature in which the animal escaped from the cold compartment. B) Number of shuttles when animals were placed in the Shuttle Box with thermoregulatory drive. Results of A and B are from protocol #1. 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; * means treatment effect (p<0.05).

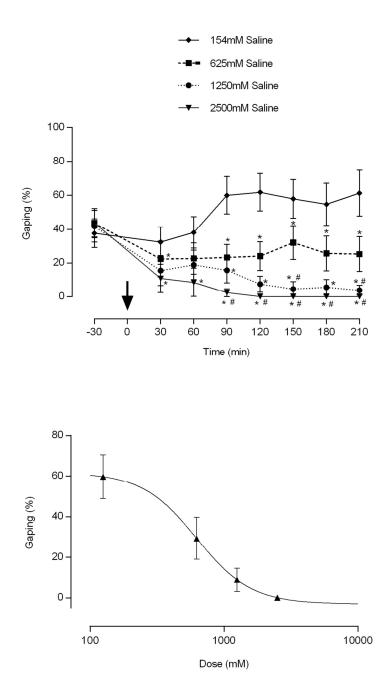
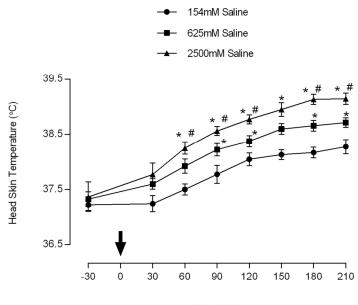
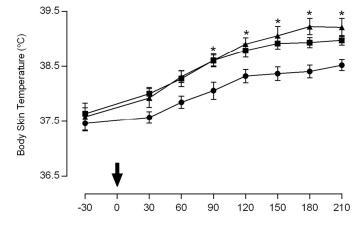


Fig. 3. Effect (A) and dose response (B) of different concentrations of saline injection on time spent (%) in gaping in *P. vitticeps* exposed to 37-39°C. Arrow indicates the time of injection. Number of animals = 11 for all treatments; * = p < 0.05 compared to 154 mM concentration. # = p < 0.05 compared to 625 mM group.



Time (min)





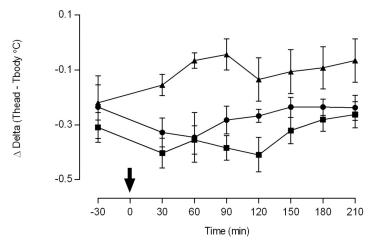


Fig. 4. Effect of different concentration of saline injection on head skin temperature (A), body skin temperature (B) and on delta (T_{head} minus T_{body} ; C) over time. Number of animals = 11 (154 mM), 4 (625 mM) and 5 (2500 mM); * and # mean differences on interaction effect for A and B. * = p< 0.05 compared to 154 mM at the same time. # = p< 0.05 compared to 625 mM at the same time. In C, 2500 mM was different from the other groups (effect of treatment, p< 0.05. No interaction between factors).

TABLES

Table 1. Effect of gaping on evaporative water loss (EWL; mg min⁻¹), evaporative heat transfer (EHT; J min⁻¹), oxygen consumption ($\dot{V}O_2$; mL min⁻¹ STP), heat production (J min⁻¹), *EWL*/ $\dot{V}O_2$ ratio (mg mL⁻¹ STP) and the *EHT*/*HP* in the lizard *P. vitticeps*

Gape State	EWL	EHT	ΫO ₂	HP	EWL/VO ₂	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

Number of animals = 8. These results are from lizards that received isotonic injections. * = difference between non-gaping and gaping intervals (p<0.05).

Table 2. Percent 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 (154mM) and hypertonic (625mM) saline injections in the lizard *P. vitticeps*

Treatment	%EWL	% <i></i> [†] 0 ₂	%EWL/VO ₂
154 mM	-5.11 ± 8.3	-12.96 ± 8.1	16.1 ± 21.3
625 mM	-22.7 ± 6.9	$-40.9 \pm 7.5^{*}$	48.1 ± 25.4

154mM and 625mM= saline concentrations; % was calculated based on the change post-injection compared to pre-injection; number of animals = 7 (154 mM) and 8 (625 mM); * = difference between treatments.