

**Two lines of evidence for physiological control of insensible evaporative water loss by a tiny marsupial.**

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## Abstract

We present two independent lines of evidence that a tiny dasyurid marsupial, the ningau ( *Ningau* spp.), has acute physiological control of its insensible evaporative water loss below and within thermoneutrality. Perturbation of the driving force for evaporation by varying relative humidity, and therefore the water vapour pressure deficit between the animal and the ambient air, does not have the expected physical effect on evaporative water loss. Exposure to a helox atmosphere also does not have the expected physical effect of increasing on evaporative water loss for live ningaus (despite it having the expected effect of increasing heat loss for live ningaus), and increasing evaporative water loss for dead ningaus. We discuss the relative advantages and disadvantages of both experimental approaches for demonstrating physiological control of insensible evaporative water loss. An appreciation of physiological control is important because insensible evaporative water loss contributes to both water and heat balance, is clearly under environmental selection pressure, and potentially impacts the distribution of endotherms and their response to environmental change.

## Introduction

Evaporative water loss (EWL) is important for terrestrial animals. It is critical for their water budget and impacts heat balance (e.g. Schmidt-Nielsen and Schmidt-Nielsen, 1952; MacMillen, 1990; Withers et al., 2016). For endotherms, the thermoregulatory role and control of increased EWL at ambient temperatures ( $T_a$ ) above the thermoneutral zone (TNZ; the range of  $T_a$  at which the animal can maintain heat balance with basal or resting metabolic rate and minimal EWL; Scholander et al. 1950; IUPS Thermal Commission 2003; Riek and Geiser 2013) is well understood (Morrison and Nakamura, 2011; Gerson et al., 2014). However EWL in and below the TNZ (insensible EWL, IEWL; Monteith, 1973; IUPS Thermal Commission, 2003) has been considered a passive physical process, with IEWL proportional to the water vapour pressure differential ( $\Delta wvp$ ) between the animal and its environment (Cossins and Bowler 1987; Campbell and Norman, 1998; Withers et al., 2016) as a consequence of the inevitable permeability of the skin and respiratory surfaces to water vapour. Some studies of IEWL for birds and mammals, which modified the  $\Delta wvp$  by manipulating the ambient relative humidity (RH), concluded that IEWL conforms to this physical model (Chew Dammann, 1961; Edwards and Haines, 1978; Webster and King, 1987; Powers, 1992; Klüg-Baerwald and Brigham, 2017). Other studies, however, suggest that IEWL at and below the TNZ is under physiological control (Webster et al., 1985; Webster and Bernstein, 1987; Withers and Cooper, 2014; Cooper and Withers, 2017; Eto et al., 2017; Cooper et al. in press), identifying a previously unappreciated physiological process.

It is important to understand the dynamics of IEWL at and below the TNZ because it is the primary form of evaporation for many endotherms for the majority of the time (even small desert species are often at  $T_a \leq TNZ$ ; e.g. Schmidt-Nielsen, 1975; Cooper et al., 2019). Correlations between IEWL and ecological and environmental factors (Williams, 1996;

Withers et al., 2006; Van Sant et al., 2012; Song and Beissinger, 2020) indicate selective pressure on IEWL, similar to those observed for body temperature ( $T_b$ ) and metabolic rate (MR e.g. basal MR, field MR (Nagy, 1987; McNab, 2003; White and Seymour, 2004; Withers et al., 2006; Withers et al. 2016). Consequently, understanding regulation of IEWL is just as important for understanding the distribution of endotherms and their potential response to environmental change as regulation of metabolic heat production (MHP) for proportional thermoregulatory control, especially considering control of IEWL is likely to have a thermoregulatory role (Eto et al., 2017; Cooper and Withers, 2017; Cooper et al. in press). However, assessing if IEWL is under physiological control (i.e. deviates from the physical model) is not straightforward, as there is no expected slope for EWL vs RH (Eto et al., 2017). This can be resolved by determining the slope for  $EWL/\Delta wvp$  vs RH, since a slope of 0 indicates conformity to the physical model, and a slope  $>0$  indicates physiological control. However, calculation of  $\Delta wvp$  requires an estimation of the wvp saturation at the animal's mean evaporative surface temperature ( $T_{evap}$ ), which is between  $T_a$  and body temperature ( $T_b$ ); overestimating  $T_{evap}$  (by using  $T_b$ ) underestimates the slope, underestimating  $T_{evap}$  (by using  $T_a$ ) overestimates the slope (Cooper et al. in press).

An alternative approach for perturbing the evaporative environment is to compare EWL in helox (21% oxygen in helium) and air (Cooper and Withers, 2014). Helox theoretically increases an endotherm's EWL for two reasons. Water diffuses 2-3x faster in helox due to its lower density (Kingdon, 1963; Paganelli and Kurata, 1977; Parkhurst and Mott, 1990), increasing cutaneous EWL (CEWL). Helox is also 4x more thermally-conductive, increasing heat loss (Leon and Cook, 1960; Rosenmann and Morrison, 1974), which requires increased metabolic heat production (Rosenmann and Morrison, 1974; Thomas et al., 1998) hence increased respiratory EWL (REWL) through accommodation of the higher  $O_2$  demand.

Therefore, we expect EWL to be higher in helox than air, due to enhancement of both CEWL and REWL. Helium mixtures have been used to modify the evaporative environment of plants (Egorov and Karpushkin, 1988; Parkhurst and Mott, 1990; Mott and Parkhurst, 1991), but only one study has examined this effect of helox for a mammal (Cooper and Withers, 2014).

We examine here the dynamics of IEWL at or below the TNZ ( $T_a \leq 30^\circ\text{C}$ ; Geiser and Baudinette 1988; Riek and Geiser 2013, Cooper and Withers unpublished data) for a tiny arid-habitat dasyurid marsupial, the ningai (*Ningai* spp.) using both altered RH and helox to perturb the evaporative environment. The basic thermoregulatory physiology of the ningai is typical of other small dasyurid marsupials (Geiser and Baudinette 1988), and as two other species of small dasyurid (little red kaluta, *Dasykaluta rosamondae*; Withers and Cooper 2014 and red-tailed phascogale, *Phascogale calura*; Cooper and Withers 2017) control their IEWL, the ningai is useful model for this study. We apply the two methodologies of both altered RH and helox together for the first time to seek strong evidence of IEWL control, compare the relative merits of each approach, and consider the importance of identifying EWL regulation.

## Methods

Ten ningais (*Ningai ridei* and/or *Ningai yvonnae*; these species cannot be reliably distinguished in the field at this site, where they are sympatric) were captured in the Goldfields (30°24'S, 119°38'E), Western Australia. They were maintained at Curtin University with *ad lib.* food (invertebrates, kangaroo meat, cat food) and water for a period of 3 months, during which time experiments were conducted. Ningais were fasted overnight, but had access to *ad lib.* water, before measurement for 6-8 hours the following day, during their inactive period to ensure resting values were achieved (e.g. Cooper and Withers 2009; Connelly and Cooper

2014). Each ningauai was measured at one experimental treatment per day, with at least 3 days between measurements.

We used open-flow respirometry (Withers 2001) to measure EWL, oxygen consumption ( $\text{VO}_2$ ) and carbon dioxide production ( $\text{VCO}_2$ ) of ningauis in air at varying RH, and in helox. Live ningauis were measured at  $T_a$  of 20, 25 and 30°C (below or within thermoneutrality; Geiser and Baudinette 1988; Riek and Geiser 2013, Cooper and Withers unpublished data), at five RHs of 7 to 81 % in air, and in helox, in random order. Not every ningauai was measured under each experimental condition for logistical reasons;  $N = 7-9$  for the various RH/ $T_a$  combinations and  $N = 6-7$  for the helox measurements. At the conclusion of the study, ningauis were killed with an intraperitoneal injection of sodium pentobarbitone, and EWL of seven individuals immediately measured in both dry air and helox at  $T_a = 33^\circ\text{C}$  (approximate  $T_b$  of live ningauis), to quantify the physical effects of helox on EWL for animals that were not physiologically regulating. Four of the ningauai carcasses were measured in air first and then switched to helox, and *vice versa* for the other three; the gas mix was switched once EWL had stabilised (~120 min).

The respirometry system consisted of a ~400 ml glass metabolic chamber, inside a temperature-controlled room, through which flow of air (dried with drierite; W. A. Hammond Co., Xenia, OH, USA) was regulated at  $118 \text{ ml min}^{-1}$  with an Aalborg GFC17 (Orangeburg, NY, USA) mass flow controller. At each  $T_a$ , incurrent air RH was controlled by saturating the air with water using an aerator in a water-filled portable refrigerator (Engel, Varsity Lakes, QLD, Australia), that was regulated at a known temperature using a Ratek heater circulator, (Boronia, VIC, Australia), then warmed to the experimental  $T_a$  with RH calculated using the equations of Parish and Putnam (1977). Excurrent RH and  $T_a$  were measured with a Vaisala

HMP45A (Helsinki, Finland) probe, then a subsample was dried with drierite, passed through a carbon dioxide analyser (Sable Systems CA-2A; Las Vegas, NV, USA) and finally an paramagnetic oxygen analyser (Sable Systems PA-10). Analysers were interfaced to a PC via a Sable Systems UI2 A/D converter and excurrent O<sub>2</sub>, CO<sub>2</sub>, RH and T<sub>a</sub> were recorded every 20 seconds throughout the experiment, using custom-written Visual Basic (Microsoft VB v6; Redmond, WA) software. At the end of each experiment, when O<sub>2</sub>, CO<sub>2</sub> and RH data indicated that the ningau was resting and had attained minimal and steady-state MR and EWL, the animal was removed from the chamber and T<sub>b</sub> measured immediately. A lubricated plastic-tipped thermocouple, connected to a RadioSpares 611–234 thermocouple meter (Smithfield, NSW, Australia), was inserted into the cloaca while the animal was restrained by hand by the scruff, a process that was typically achieved within seconds of removing the animal from the chamber. For measurements in helox, incurrent air was replaced by a helox mix (21.1% oxygen in helium; BOC Gases, Perth, WA, Australia). Baselines of background O<sub>2</sub>, CO<sub>2</sub> and RH were established for at least 30 min before and after each experiment.

Mass flow meters were calibrated volumetrically using a Gilibrator 2 (Sensidyne, Clearwater, FL, USA) for both air and helox, corrected to standard temperature and pressure. RH probes were calibrated by comparing the measured and theoretical RH of the initial baselines at each RH. The Vaisala temperature sensor and thermocouple meter were calibrated against a mercury thermometer traceable to a national standard. Gas analysers were two-point calibrated with compressed nitrogen (BOC Gases; 0% O<sub>2</sub> and CO<sub>2</sub>) and dry ambient air (20.95% O<sub>2</sub>) and a certified gas mix (0.53% CO<sub>2</sub>; BOC Gases). We used a custom-written (P.C. Withers) VB v6 programme to calculate EWL, VO<sub>2</sub> and VCO<sub>2</sub> for a single ~20-min period towards the end of each experiment, when all variables were constant and minimal, after Withers (2001). Respiratory exchange ratio (RER) was calculated as VCO<sub>2</sub>/VO<sub>2</sub>, and this was used to convert

MR to metabolic heat production (MHP) after Withers et al. (2016). Wet ( $C_{\text{wet}}$ ) and dry ( $C_{\text{dry}}$ ) thermal conductance were calculated as  $\text{MHP}/(T_b - T_a)$  and  $(\text{MHP} - \text{EHL})/(T_b - T_a)$  respectively, with evaporative heat loss (EHL) calculated from EWL assuming  $2.4 \text{ J mg H}_2\text{O}^{-1}$  (Monteith, 1973). Hygrometeorological equations (Parish and Putnam, 1977) were used to calculate saturation wvp at  $T_b$ , and ambient wvp was calculated as saturation wvp  $T_a \times \text{RH}/100$ . The  $\Delta\text{wvp}$  was then calculated as (saturation wvp animal - ambient wvp).

We used linear mixed effect models, with Helmert and reverse Helmert *a priori* contrasts (Withers and Cooper, 2011), with RH as a fixed factor and individual as a random factor, to determine if IEWL and other physiological variables were effected by RH, and if  $\text{IEWL}/\Delta\text{wvp}$  deviated from the physical model i.e. if the slope of  $\text{IEWL}/\Delta\text{wvp}$  vs RH  $>0$ . We analysed these data separately at each  $T_a$  because of non-equivalence of RH, and of wvp relative to saturation, at different  $T_a$ . We used lme4 (Bates et al., 2014) and lmerTest (Kuznetsova et al., 2014) in R Studio (R Studio Team, 2015). Values in helox were compared to those in dry air (7% chamber RH), to achieve similar chamber RH for the two treatments, using a linear mixed effect model, with air/helox and  $T_a$  as fixed factors and individual as a random factor. Due to significant interactions between  $T_a$  and air/helox, differences in physiological variables in air and helox were explored separately at each  $T_a$  for live animals, and at  $T_a = 33^\circ\text{C}$  for dead ningauis, using a paired t-test, accomplished with statistiXL (www.statistiXL.com, Perth, WA, Australia). Expected rates of EWL in helox were calculated assuming 50:50 REWL/CEWL partitioning (Chew, 1955; Tracy and Walsberg, 2000) and the measured diffusive (1.3x) and respiratory (1.5x) effects of helox for ningauis. Values are presented as mean  $\pm$  SE, with N = number of individuals and n = number of measurements.



## Results

The mass of ningauis ( $N = 10$ ,  $n = 145$ ) was  $7.8 \pm 0.09\text{g}$ . Relative humidity had no effect on  $T_b$ , MR or  $C_{\text{wet}}$  at any  $T_a$  ( $F_{1,24-37} \leq 3.47$ ,  $P \geq 0.072$ ). There was a significant negative linear effect of RH for EWL at all  $T_a$  ( $F_{1,29-34} \geq 11.37$ ,  $P \leq 0.002$ ), although this was driven by lower IEWL at higher RH (reverse Helmert contrasts;  $F_{1,27-32} \geq 5.72$ ,  $P \leq 0.023$ ; Fig 1A). Relative humidity had a positive linear effect on IEWL/ $\Delta w_{\text{vp}}$  at all  $T_a$  ( $F_{1,28-30} \geq 5.22$ ,  $P \leq 0.030$ ), although the effect was attenuated at the two highest RH (reverse Helmert contrasts;  $F_{1,27-30} \leq 1.93$ ,  $P \geq 0.062$ ; Fig 1B)

For live ningauis,  $T_b$  was maintained constant in air and helox ( $F_{1,36} = 1.97$ ,  $P = 0.169$ ), but there was an overall  $T_a$  effect ( $F_{1,36} = 6.97$ ,  $P = 0.012$ ; Fig 2A). Wet conductance was higher ( $\sim 1.68\text{x}$ ) in helox compared to air (Fig. 2B) below ( $t_{5-6} \geq 3.96$ ,  $p \leq 0.007$ ), but not in the TNZ ( $t_6 = 0.05$ ,  $p = 0.958$ ). Consequently, MR was influenced by both  $T_a$  ( $F_{1,30} = 190$ ,  $P < 0.001$ ) and helox ( $F_{1,30} = 17.8$ ,  $P < 0.001$ ; Fig 2C), increasing both at low  $T_a$ , and in helox compared to air, but with a significant interaction ( $F_{1,30} = 11.2$ ,  $P < 0.001$ ) as the helox/air differential ( $\sim 1.5\text{x}$ ) was only apparent below the TNZ ( $< 30^\circ\text{C}$ ). Insensible evaporative water loss was not influenced by helox ( $F_{1,30} = 0.561$ ,  $P = 0.460$ ), although there was a  $T_a$  effect ( $F_{1,30} = 21.8$ ,  $P < 0.001$ ; Fig 2D). Predicted rates of IEWL in helox were 1.15-1.4x those measured in air. The IEWL of dead ningauis was higher in helox ( $3.04 \pm 0.366\text{ mg g}^{-1}\text{ h}^{-1}$ ) than in air ( $2.30 \pm 0.308\text{ mg g}^{-1}\text{ h}^{-1}$ ;  $t_6 = 9.82$ ,  $p < 0.001$ ; Fig 2D).

## Discussion

It is important to understand the control of IEWL because it is a contributor to the overall heat and water budgets of endotherms, which in turn relate to their distribution and response to environmental change. We demonstrated, for the first time using two independent methodologies (RH and helox), that IEWL is under physiological control. If IEWL was not controlled, we would expect it to follow physical predictions i.e. be higher in helox than in air, and decrease in proportion to a decrease in the WVP deficit with increasing RH. As neither of these physical effects were observed, we conclude that IEWL must be under physiological control.

Although EWL decreased overall with increasing RH, this was driven by decreases in EWL at higher RH. Clearly a small  $\Delta wvp$  near saturation makes the maintenance of constant EWL challenging; this has been observed for other marsupials (Cooper and Withers, 2008; Withers and Cooper, 2014) and a bird (Eto et al., 2017). At lower RH (higher  $\Delta wvp$ ), EWL remained constant. Consequently there was an overall linear relationship for  $EWL/\Delta wvp$  vs RH, indicating that  $\Delta wvp$  alone was not determining IEWL, which deviated significantly from the linear relationship with  $\Delta wvp$  predicted by the physical model. It was only at high RH that the  $\Delta wvp$  was sufficiently small to inhibit EWL. This is particularly compelling evidence for IEWL control as we used a conservative approach to calculate the  $\Delta wvp$  gradient by using  $T_b$  for the “animal end”, which underestimates the  $EWL/\Delta wvp$  vs RH slope (Cooper and Withers, 2017; Eto et al., 2017; Cooper et al. in press). Estimating  $T_{evap}$ , which provides a more accurate calculation of  $\Delta wvp$  and a steeper, more significant slope, requires partitioning of IEWL into REWL and CEWL, and estimation of the animal’s surface ( $T_{surf}$ ) and expired air temperatures, all of which are difficult to measure non-invasively (e.g. Muñoz-García et al., 2012; Minnaar et al., 2014), especially for small mammals. Physiological regulation of EWL was sufficient to

maintain MR,  $T_b$  and  $C_{wet}$  independent of RH, by minimising the impact on evaporative heat loss.

The thermal and metabolic responses of ningauis to a helox atmosphere were typically endothermic. Values for MR and  $C_{wet}$  were equivalent in both air and helox for ningauis at  $T_a = 30^\circ\text{C}$ , consistent with the results of previous studies (e.g. Leon and Cook, 1960; Rhoades et al., 1967; Holloway and Geiser, 2001; Cooper and Withers, 2014) that there is no helox effect on  $C_{wet}$  or MR in thermoneutrality. Below the TNZ, normothermic responses to  $T_a$  were typical for this and other very small dasyurids (Dawson and Wolfers, 1978; Geiser and Baudinette, 1988; Warneke et al., 2010). Helox increased  $C_{wet}$ , but MR increased sufficiently (1.5-1.7x) to maintain  $T_b$ . For small endotherms this helox/air MR ratio does not exceed 2.6, and a low ratio is expected for a tiny, poorly-insulated mammal (Rosenmann and Morrison, 1974; Cooper and Withers, 2014). The significant increase in EWL of dead ningauis in helox was also expected since there was no possibility for physiological control, and although the increase was lower (1.3x) than predicted for a purely diffusional effect (2.3x; Parkhurst and Mott, 1990) it was similar to the increase in EWL for a water-filled plastic vial in helox compared to air (1.7x; Cooper and Withers, 2014). This suggests that there were convective as well as diffusional effects on EWL.

Despite the expected thermal and metabolic effects of helox for live ningauis, and physical effects on evaporation for carcasses, we did not observe the increase in EWL predicted by a physical model for live ningauis; EWL in air was indistinguishable from that in helox. Increased respiratory ventilation that must accompany a higher MR in helox (Cooper and Withers, 2014) should increase REWL, and the increased diffusion observed for dead ningauis should increase CEWL. Thus, exposure to helox also provides additional strong evidence that

insensible EWL is controlled, and is not simply a passive consequence of the evaporative environment.

The two approaches for assessing regulation of IEWL, compared here for the first time, were consistent in indicating that IEWL is under physiological control. Of the two approaches, modification of wvp by controlling the inlet RH is a less expensive and more straightforward technique. However, there are logistical issues; it is difficult to generate and maintain low RHs at low  $T_{as}$ , and condensation at high RH and high  $T_{as}$  can be problematic. The major challenge with the RH experimental approach is interpreting the results. With no theoretical slope for EWL against RH, it is necessary to calculate the slope of  $EWL/\Delta wvp$  vs RH (or wvp). Calculation of  $\Delta wvp$  is not straightforward due to challenges associated with estimating  $T_{evap}$ ; direct efforts to physically partition EWL into REWL and CEWL and instrument animals to measure expired air and surface temperatures can overestimate IEWL (Munoz-Garcia et al., 2001; Minnaar et al., 2014; Cooper et al., 2018). The approach of using  $T_b$  for calculating  $\Delta wvp$  is conservative; it will avoid falsely claiming IEWL control (type I error) but it may fail to identify IEWL control (type II error; Cooper et al. in press). However, an advantage of using the slope for  $EWL/\Delta wvp$  vs RH to assess IEWL control is that the relative abilities of different species for IEWL regulation can be compared (Cooper et al. in press).

Using helox has the advantage of requiring fewer experiments to assess IEWL control over the same  $T_a$  range (most individuals were measured 6x for the helox/air experiment and 15x for the RH experiment), with ethical, logistical and financial benefits. It is also straightforward to interpret results; estimation of  $T_{evap}$  is not necessary. However, helox is relatively expensive and it is a finite resource (Nuttall et al., 2012) so its use is problematic for large species. It is difficult to detect helox effects on EWL within the TNZ, where there are no  $C_{wet}$  or MR impacts

on REWL. However, a sensitivity analysis (Cooper and Withers, 2014) demonstrated that elevated EWL should still be detected in helox if mammals conform to physical predictions.

The role of the acute control of IEWL that we have shown for this small arid-habitat marsupial could be related to either water conservation or heat balance. Although the purpose of this control is not yet understood, current data suggests that a thermoregulatory rather than water balance role is more likely for both birds and mammals (Eto et al., 2017; Cooper and Withers, 2017; Cooper et al. in press). If IEWL control has a thermoregulatory function, then the well-developed thermoregulatory feedback system of endotherms provides a possible sensory system for IEWL control, although we do not understand the specific effector mechanism(s) that achieve control. Control could result from changes to REWL and/or CEWL.

Respiratory EWL is determined by the temperature and RH of the expired air and by respiratory minute volume ( $V_I$ ). For ash-grey mice (Cooper and Withers 2014), changes in  $V_I$  in helox compared to air accommodated the increased  $VO_2$ , and there were no changes in oxygen extraction ( $EO_2$ ), suggesting that  $EO_2$  is already optimised in air and that there is little scope for modification of  $V_I$  to control IEWL. Cooper et al. (in press) also concluded that  $V_I$  was an unlikely mechanism of IEWL control for parrots at different RH. Changes in the temperature and RH of expired air is a more likely avenue of REWL control. REWL can be cooled below  $T_b$  by nasal counter-current water and heat exchange (e.g. Schmidt-Nielsen et al., 1970), and although the RH of expired air is assumed to be 100%, some mammals such as camels (*Camelus dromedarius*, Schmidt-Nielsen et al., 1981) and sheep (*Ovis aries*, Johnson et al., 1988) can expire unsaturated air. Cutaneous blood flow, posture, fur positioning and skin lipids may all affect CEWL via their impact on  $T_{surf}$  and the resistance of the skin, pelt and boundary layer. Modifying peripheral blood flow can change  $T_{surf}$ , and therefore CEWL, along with

changes in posture and piloerection, and these adjustments can be near-instantaneous and could account for the acute control of IEWL we observed here. Adjustments to skin lipid composition impact CEWL of bats (Muñoz-Garcia et al., 2012), and for birds these micro-structural changes in the skin occur within timeframes of hours to weeks (Menon et al., 1996; Muñoz-Garcia and Williams, 2008; Muñoz-Garcia et al., 2008), so possibly contribute to the control we observed here.

It is inconceivable that we would try to understand the distribution of endotherms without appreciating the acute thermoregulatory control afforded by proportional metabolic heat production; we propose that the consequences of IEWL control that we demonstrate here are also important. Evidence for acute physiological control of IEWL is growing (Webster et al., 1985; Webster and Bernstein, 1987; Cooper and Withers 2008, 2014, 2017; Withers and Cooper, 2014; Eto et al., 2017; Cooper et al. in press), and here we use two different techniques to unequivocally demonstrate this for a tiny arid-habitat dasyurid marsupial. Further studies are required to elucidate the extent, purpose, and importantly the mechanism, of this control system, by examining this phenomenon in a broad range of phylogenetic, ecological and allometric contexts.

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## **Competing interests**

The authors declare no competing or financial interests.

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## **Data availability**

Original data are available from the corresponding author on request.

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## Figures

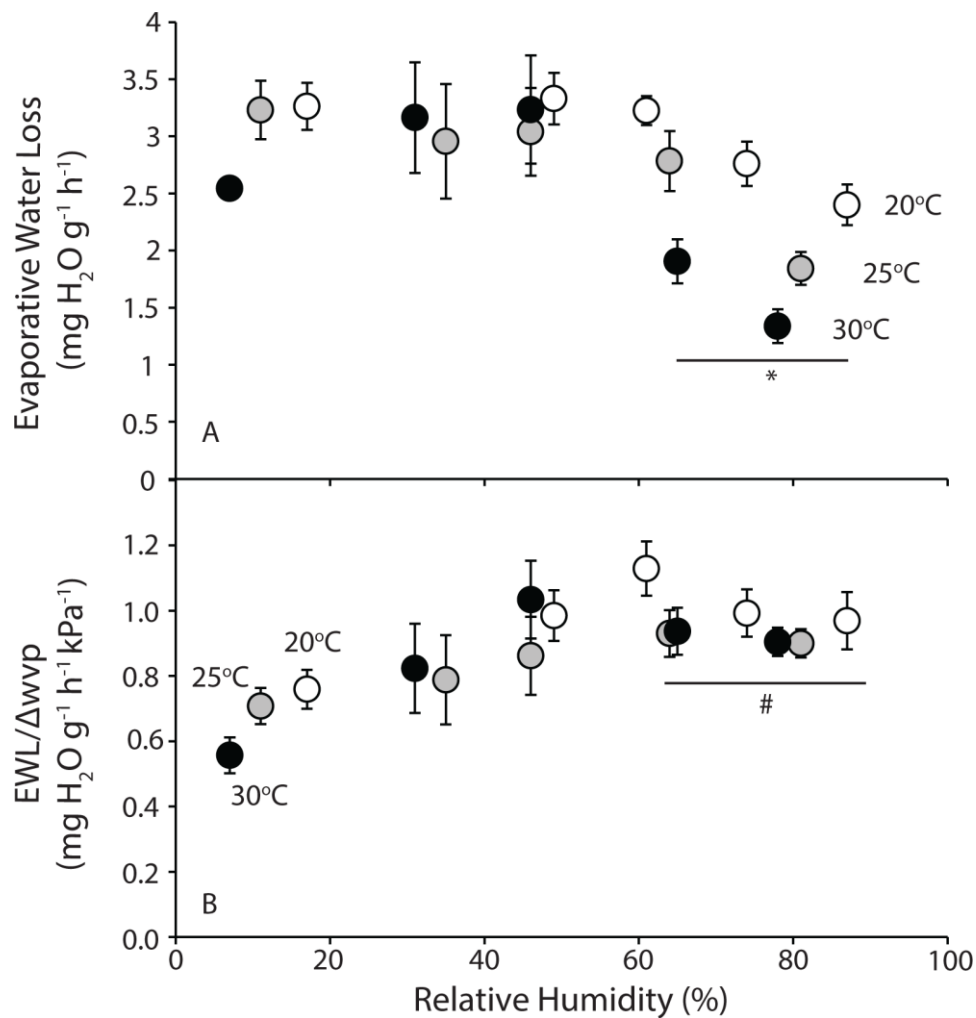


Figure 1. Effect of (A) relative humidity on evaporative water loss and (B) evaporative water loss per water vapour pressure deficit (EWL/Δwvp), for ningaus at ambient temperatures of 20, 25 and 30 °C. \* indicates values significantly lower than the mean of the previous levels, and # indicates values which do not differ from the previous levels (reverse Helmert contrasts). Values are mean ± SE (N = 10, n = 7-9).

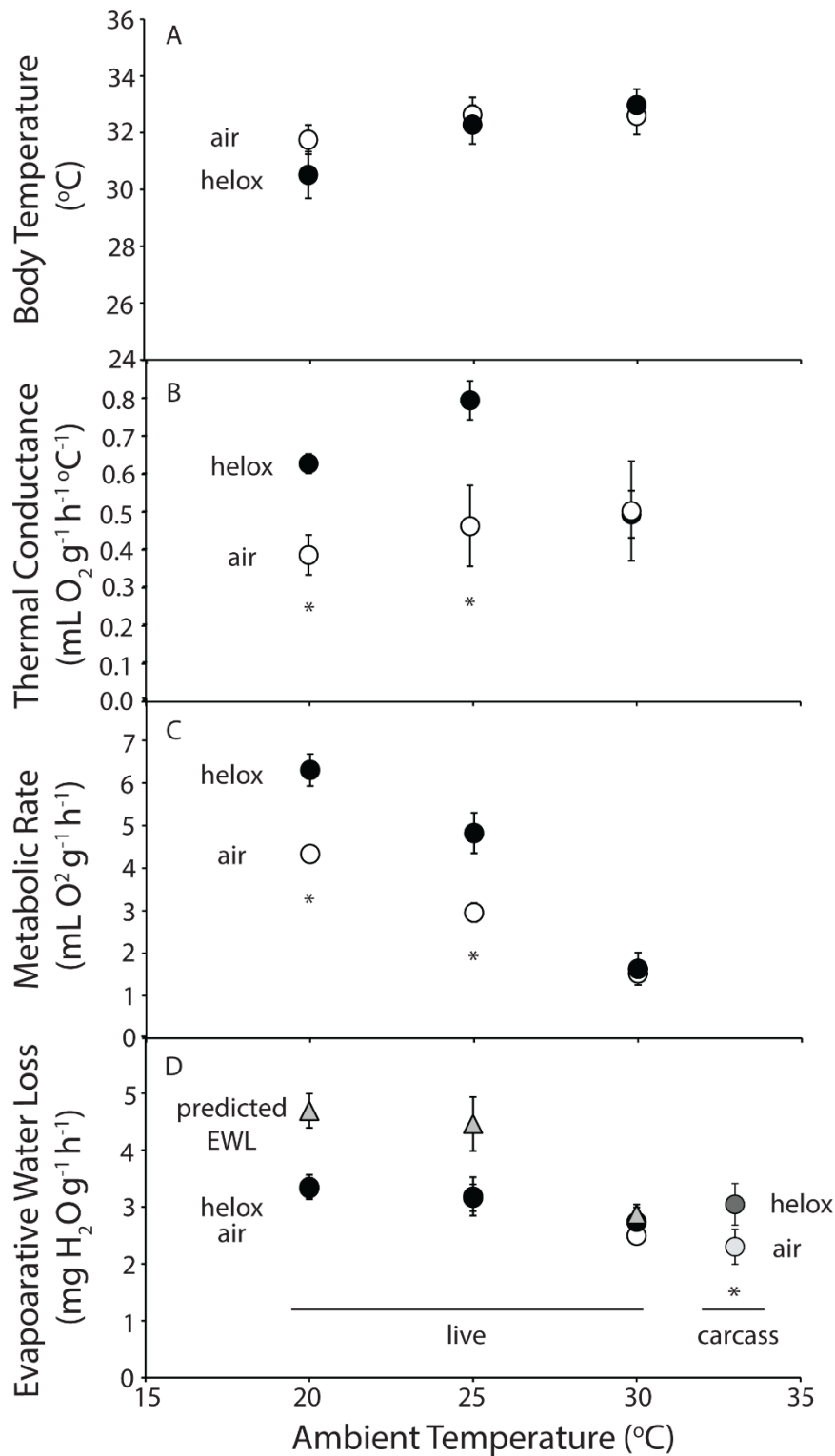


Figure 2. Body temperature (A), metabolic rate (B), wet thermal conductance (C) and evaporative water loss (EWL; D) for live ningauis at ambient temperatures of 20, 25 and 30 °C, and ningauis carcasses at  $T_a = 33^\circ\text{C}$  in helox (dark circles) and air (light circles). Expected



rates of EWL in helox, calculated assuming 50:50 REWL/CEWL partitioning and the measured diffusive (1.3x) and respiratory (1.5x) effects of helox for ningauis are shown with grey triangles. Values are mean  $\pm$  SE (N = 7, n = 6-7). There was a significant effect of ambient temperature for all variables except wet thermal conductance. \* indicates a significant effect of helox compared to air at a particular T<sub>a</sub>.