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Running head: Microclimate selection and attenuation of drought stress

Behavioural hydroregulation protects against acute effects of drought in a dry-skinned ectotherm

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30 **Authors' contributions**

31 M.D., J.-F.L.G. and O.L. conceived the ideas and designed methodology. M.D. and O.L
32 carried all experimental procedures and collected the data. M.D. led the data analyses and
33 writing of the manuscript together with J.-F.L.G. and O.L. and all authors contributed
34 critically in result interpretation and manuscript writing. All authors gave final approval for
35 publication.

36 **Abstract**

37 During extreme climate events, behavioural thermoregulation may buffer ectotherms from
38 thermal stress and overheating. However, heatwaves are also combined with dry spells and
39 limited water availability, and how much individuals can behaviourally mitigate dehydration
40 risks through microclimate selection remains largely unknown. Herein, we investigated the
41 behavioural and physiological responses to changes in air and microhabitat humidity in a
42 terrestrial ectotherm, the asp viper (*Vipera aspis*). We exposed individuals to a simulated
43 heatwave together with water deprivation for 3 weeks, and manipulated air water vapour
44 density (wet air vs. dry air) and microclimate (wet shelter vs. dry shelter) in a two-by-two
45 factorial design. Dry air conditions led to substantial physiological dehydration and muscle
46 wasting. Vipers exposed to dry air more often used a shelter that provided a moist micro-
47 climate, which reduced dehydration and muscle wasting at the individual level. These results
48 provide the first experimental evidence that active behavioural hydroregulation can mitigate
49 specific physiological stress responses caused by a dry spell in an ectotherm. Future studies
50 investigating organismal responses to climate change should consider moisture gradient in the
51 habitat and integrate both hydroregulation and thermoregulation behaviours.

52

53 **Keywords:** behaviour; humidity gradient; hydroregulation; microclimate selection; water.

Introduction

Climate warming poses a major extinction threat to biodiversity because rising local temperatures force many terrestrial organisms to shift their range, alter their phenology, or expend more energy to maintain homeostasis (Riddell et al., 2019; Urban, 2015). These physiological and ecological effects will largely depend on potential buffering by microclimatic conditions (Pincebourde et al., 2016; Potter et al., 2013). Notably, for some terrestrial organisms, vulnerability to global warming depends less on global climate trends than on microclimate conditions at small spatial scales, such as those generated by heterogeneous vegetation and soil layers, and on behavioural strategies allowing exploitation of these microclimate conditions (Potter et al., 2013; Riddell et al., 2021). Thus, to predict ecological consequences of climate change, one should better characterise how organisms respond to microclimatic heterogeneity (Sears et al., 2019; Woods et al., 2021).

In the context of global warming, the risks of overheating are significantly reduced when an organism is capable of fast behavioural responses (Seebacher & Franklin, 2005; Terrien et al., 2011). Behavioural thermoregulation allows organisms, especially ectotherms, to exploit spatial variability in thermal conditions to maintain an optimal body temperature despite temporal fluctuations in operative temperature (Angilletta, 2009; Fuller et al., 2021). For example, many terrestrial ectotherms typically live in a complex thermal landscape where they can change their posture and coloration, seek shade, use burrows or shift their daily activity patterns to control heat gain, exploit cold and warm microclimates and ultimately avoid overheating risks (Black et al., 2019; Forget-Klein & Green, 2021; Moore et al., 2018). Given this, the most efficient thermoregulators should be better capable of maintaining homeostasis in a warming world (Huey et al., 2012; Kearney et al., 2009). Yet, climate change does not only challenge body temperature regulation but also puts a strong pressure on osmoregulation (i.e., the homeostatic maintenance of salt and body fluids balance) in

terrestrial organisms (Riddell et al., 2019; Rozen-Rechels et al., 2019). For example, avoidance of overheating in desert endotherms involves thermoregulatory costs from evaporative cooling, which implies that birds and mammals must actively seek or save water to maintain an optimal body temperature in a warmer climate (Mitchell et al., 2018; Riddell et al., 2019).

In many terrestrial ectotherms, behavioural trade-offs between thermoregulation and osmoregulation exist because evaporative water loss increases with body temperature and in the microclimate conditions relevant for basking (e.g., Dupoué et al., 2015b; Riddell et al., 2018). In addition, climate warming often involves concurrent changes in temperature, rainfall and moisture, especially during extreme climate events such as summer hot and dry spells that increase water loss risks (Trenberth et al., 2014; Ummenhofer & Meehl, 2017). Organisms should partially buffer the water constraints of climate change by means of behavioural hydroregulation, defined as the range of behaviours that control water gains and water losses and help individuals maintain an optimal hydration state for their activity and functional capacities (Pirtle et al., 2019; Rozen-Rechels et al., 2019). For example, reptiles living under water stress can maintain their water balance by reducing their activity (Davis & Denardo, 2010; Kearney et al., 2018) or by selecting cool (Ladyman & Bradshaw, 2003; Pintor et al., 2016) or wet microclimates (Dupoué et al., 2015b; Guillon et al., 2014; Lourdaïs et al., 2017).

A wide range of hydroregulation behaviours have been observed in the wild, from moisture harvest in desert beetles, free-standing water seeking in large mammals to soil moisture preferences in frogs (Rozen-Rechels et al., 2019; Wolcott & Wolcott, 2001). Despite that, our knowledge of the buffering capacity of these behavioural mechanisms still remains limited (Pirtle et al., 2019; Rozen-Rechels et al., 2020). Behavioural hydroregulation is most obvious in wet-skinned amphibians (Greenberg & Palen, 2021; Lertzman-Lepofsky et al.,

2020). Some amphibians can reduce evaporative water loss by means of behavioural changes such as postural adjustments to reduce the cutaneous water-exchange surface, mucus production or the selection of moist microhabitats (e.g. Lillywhite et al., 1997; Wolcott & Wolcott, 2001). There is good evidence that some amphibians perform active behavioural hydoregulation to maintain an optimal hydration state independently from thermoregulation (Mitchell & Bergmann, 2016), but whether this behavioural flexibility is efficient during extreme weather events remains unknown. Behavioural data are even more elusive for dry-skin ectotherms due to difficulties disentangling hydoregulation and thermoregulation mechanisms in these organisms (Pintor et al., 2016; Rozen-Rechels et al., 2019). Dry-skin ectotherms often have a strong resistance to evaporative water loss, meaning that their hydration state changes more slowly than body temperature and that shifts caused by behavioural hydoregulation could take place over long time periods. Behavioural hydoregulation could be controlled by the internal hydration status, leading individuals to choose less desiccating microclimates as a water-saving strategy only when chronically stressed (Ladyman & Bradshaw, 2003; Rozen-Rechels et al., 2020). On the other hand, terrestrial organisms can perceive moisture gradients through hygrosensing (Chown et al., 2011) and further rely on odours or other cues to locate free standing water (Serrano-Rojas & Pašukonis, 2021; Wood et al., 2021), such that changes in air water vapour pressure conditions may also trigger rapid changes in their behaviours.

Herein, we experimentally studied hydoregulation behaviours independently from body temperature regulation in a temperate ectotherm, the asp viper (*Vipera aspis*). We used climatic chambers to simulate weather conditions akin to those of a summer dry spell. We removed access to water and manipulated air moisture (dry versus wet air conditions) for three weeks. Water-deprived vipers were either allowed to hydoregulate by providing them with a wet shelter (with a damp substrate) or not by providing them with a dry shelter. We

examined the following hypotheses and predictions: first, water deprivation will most negatively impact hydration state in a dry air environment. We predict important mass loss and plasma osmolality increase in these conditions. Second, dehydration should induce compensatory physiological responses to mitigate water constraints. We predict, in particular, patterns of structural muscle catabolism to release bound water (Brusch et al., 2018) and a greater thirst reaction (Dezetter et al., 2021; Dupoué et al., 2014). Third, behavioural selection of wet microclimates should mitigate these effects by reducing evaporative water loss, especially when the simulated air is drier. We predict higher wet shelter use and lower physiological costs in this treatment group. The intensity of shelter use should also negatively correlate with physiological costs at the individual level.

Material and Methods

Study species

The asp viper is a typical sit-and-wait foraging and capital breeding snake distributed over southwestern Europe in sunny and warm microhabitats (Lourdais et al., 2002). This diurnal species is an active thermoregulator that maintains a high body temperature (30 to 33°C) during daily activity (Lorion et al., 2013; Lourdais et al., 2017). A functional trade-off exists between thermoregulatory needs and water loss in asp vipers: behavioural thermoregulation implies the use of warm microclimates, which increase evaporative water loss rates (Dupoué et al., 2015b; Guillon et al., 2014; Lourdais et al., 2017). Behavioural selection of warm and moist basking locations helps gravid females to mitigate the conflict between thermal needs and water loss (Lourdais et al., 2017). Access to water can be limited in natural habitats, especially during summer dry spells, which are increasing in frequency and intensity over the range distribution of this species (Spinoni et al., 2018). This capital breeder and semi-arid adapted species can tolerate long periods of fast and water deprivation, but at the cost of lower

body reserves, enhanced emaciation and physiological stress, and therefore potential reduction in future fitness (Dupoué et al., 2015a; Lorigoux et al., 2016). Herein, we used 42 non-reproductive adults (27 females and 15 males) from a captive colony originating from western France and maintained at the Centre d'Etudes Biologiques de Chizé, France.

Manipulation of hydric conditions and air temperature

Our experimental design consisted of a strictly defined manipulation of hydric conditions independently from air temperature. We used 4 controlled environment chambers (Vötsch VP 600, Balingen, Germany) allowing realistic simulations of daily fluctuations in air temperature and humidity. Asp vipers were deprived of water for three weeks, which corresponds to a moderate duration for a dry spell in natural conditions (Perkins et al., 2012). The daily temperature cycle mimicked body temperature recorded during a typical heatwave with daytime temperature of 32°C for 14h and nighttime temperature of 26°C for 5h, with temperature either gradually increasing or decreasing for the remaining 5h (Lourdais et al., 2013). We next manipulated air humidity (dry air vs. wet air) and microclimate (dry shelter vs. wet shelter) using a two-by-two factorial design. First, we compared two contrasted ambient water vapour pressure deficit (VPD) to expose individuals to either desiccant ($VPD = -3.10 \pm 0.08 \text{ kPa}$ in dry air; Dupoué et al., 2015b) or humid conditions ($VPD = -1.43 \pm 0.08 \text{ kPa}$ in wet air). Second, we manipulated microclimate conditions by providing either a dry or a wet shelter. Shelters consisted of small, custom made plastic containers (15 cm diameter, 11 cm height; one entrance of 3 cm diameter on the side) filled with an artificial sponge and microfiber cloths. Once accustomed, vipers will readily use shelters in the laboratory. The sponge and microfiber cloths were changed every 3 days and maintained dry in the dry shelters but damp in the wet shelters in order to enhance air humidity. In dry shelters, VPD were similar to the ambient conditions and therefore offered no humidity gradient. In turn,

wet shelters provided high and constant humidity ($VPD = -0.61 \pm 0.09 \text{ kPa}_a$), which simulates natural underground microhabitats that are typically moist with a stable and high humidity throughout the day. The differential between wet shelter and ambient air VPD was respectively of 2.49 kPa_a and 0.82 kPa_a for dry and wet air. Temperatures inside the shelters were similar to ambient temperatures. Note that we observed no water condensation inside the plastic shelters so drinking water was deemed extremely unlikely.

Experimental design

Prior to the experiment, for the purpose of acclimation to general maintenance conditions, we housed all individuals for one week in climatic chambers using individual plastic boxes (35 x 20 x 17 cm) with one dry shelter, a sheet of newspaper as a substrate and a natural day light cycle (circa 12:12). During this period, vipers had *ad-libitum* access to drinking water and were exposed to a standard daily temperature cycle (6h daytime at 30°C , 13h nighttime at 22°C , and mean VPD of $-1.20 \pm 0.10 \text{ kPa}_a$). Then, we randomly allocated individuals to each treatment group while ensuring a balanced sex ratio. Each individual was placed in a climatic chamber with the corresponding air humidity treatment (dry air or wet air) and we applied the following protocol over 24 days: from Day 0 to Day 2 (3 days), we provided all individuals with a dry shelter in order to quantify baseline shelter use; from Day 3 to Day 20 (18 days), we exposed each individual to its microclimate treatment (dry shelter or wet shelter) in order to quantify the combined effects of air humidity and microclimate; from Day 21 to Day 23 (3 days), we provided all individuals with a wet shelter to test for change in shelter use in individuals previously exposed to dry shelters; and finally, on Day 24, we provided all individuals with water *ad-libitum* to assess post treatment water intake from drinking. We then transferred them to standard housing conditions. We did not feed the snakes in order to

avoid confounding effects of digestion and energy intake on body mass (Stahlschmidt et al., 2015).

Variables collected and parameters measured

Shelter use over time

Every day from Day 0 to Day 23, we recorded the position of each individual 3 times per day during daytime (9:00, 13:00 and 17:00). Individual position in the box was categorised as either using the shelter or being outside. First, we considered the cumulative shelter use from Day 0 to Day 2 (proportion of shelter use out of 9 observations) to record the baseline shelter use. To investigate responses to combined air humidity and microclimate treatments (Day 3 to Day 20), we first examined the cumulative shelter use during that period (proportion of shelter use out of 54 observations). Second, we considered more specifically changes in daily shelter use (proportion of shelter use out of the 3 daily observations) over time (Day 0 to Day 20). We also calculated an individual shelter use score over a 3-day period between Day 3 and Day 20 (total number of times inside the shelter per 9 observations, 6 repetitions) to investigate the relation between shelter use behaviour and body mass changes (recorded every 3 days, see below). Finally, we also calculated cumulative shelter use from Day 21 to Day 23 (proportion of shelter use out of 9 observations) to investigate wet shelter use at the end of the treatment.

Changes in body mass and muscle condition

In the absence of food intake, body mass (BM) change provides a non-invasive proxy of the whole body hydration state in snakes (Dupoué et al., 2014). We therefore weighed (± 0.1 g) all individuals every 3 days to assess BM changes during the treatment period, and then weighed them again at the end of Day 24 to assess mass uptake and rehydration after the

manipulation. From this, we calculated total ΔBM as the difference between BM at Day 20 and at Day 0. We also analysed mass changes over each 3 days' period during the combined manipulation of air humidity and microclimate (Day 3 to Day 20, 6 repetitions per individual) to investigate the relation between individual shelter use and body mass changes during that period. Finally, post-treatment water intake was inferred from the mass difference between Day 24 and Day 23.

Bound water is a significant component of all body tissues that can be mobilised to support water demands during periods of water deprivation, especially from wet protein of muscles in reptiles (Brusch et al., 2018). In snakes, skeletal muscles can be mobilised to support water requirements (Brusch et al., 2018). To quantify skeletal muscles loss during the treatment period, we measured tail width ($\text{TW} \pm 0.01 \text{ mm}$, average from triplicates) on Day 0 and on Day 20 using an electronic pressure-sensitive specimeter (Absolute Digimatic, Mitutoyo, Japan) at the position of the sixth subcaudal scale (Lorion et al., 2016). We calculated ΔTW as the difference between mean TW at Day 20 and Day 0.

Changes in plasma osmolality

We collected blood samples on Day 0 and Day 20 to assay plasma osmolality, which measures the whole-body electrolyte and water balance (Peterson, 2002). We sampled individuals in a random order by sex and treatments and collected 150 μL of blood through cardiocentesis, using a 1mL syringe and a heparinized 29-gauge needle. We immediately centrifuged the blood at 2000g, separated plasma from the red blood cells and stored the samples at -28°C . Plasma osmolality ($\pm 1 \text{ mOsm kg}^{-1}$) was measured from 10 μL duplicates (intra-individual CV: 0.36%) using a vapour pressure osmometer (model 5500, Wescor, Logan, UT, USA). We calculated $\Delta\text{Osmolality}$ as the difference between mean plasma osmolality before and after manipulation.

253

254 *Statistical analyses*

255 We conducted all analyses using the R software version 3.6.3 (R Core Team, 2020). To
256 investigate the effects of treatments on behavioural hydroregulation, we first built a
257 generalized linear model with a binomial error distribution and a logit link function to
258 examine cumulative shelter use (success = total number of observation when shelter was
259 used; failure = total number of observation when shelter was not used) between Day 3 to Day
260 20., We used sex, air humidity, microclimate treatment and the interaction between both
261 treatments as explanatory factors in the full model. This full model was built with *glm*
262 function of the *stats* package (R Core Team, 2020) and fitted well with the data
263 (overdispersion test: $X^2 = 43.9$, dispersion coefficient = 1.1, $df = 39$, $P = 0.27$). We used two
264 similar models to analyse the baseline shelter use before the start of the microclimate
265 manipulation (between Day 0 and Day 2) and the wet shelter use at the end of the treatment
266 (between Day 21 and Day 23). Because of significant overdispersion of the binomial
267 distribution in these two data sets, we compared treatment group using a betabinomial
268 approach (Harrison, 2015). The betabinomial model for proportions was fitted with *betabin*
269 function of the *aod* package (Lesnoff & Lancelot, 2012). We further investigated repeated
270 measures through time of daily shelter use according to treatments between Day 0 and Day 20
271 using a generalized linear mixed effects model with a binomial error distribution (success =
272 number of observations per day when shelter was used; failure = number of observation per
273 day when shelter was not used) and a logit link function. We included sex as a fixed factor,
274 and air humidity, microclimate treatment, time (number of days) and their three-way
275 interaction as explanatory variables. Individual identity was included as a random term to
276 control for repeated measures on the same individuals. Because data were skewed toward zero
277 at Day 0 and shelter use was identical between treatment groups at the beginning of the

manipulation, we forced a similar intercept for the four treatments in our full model. This model was built with glmer function of the lme4 package (Bates et al., 2015) and fitted well with the data (overdispersion test: $X^2 = 816.4$, dispersion coefficient = 0.9, df = 896, P = 0.92). In all cases, we performed backward elimination model selection starting from the full models to remove non-significant effects using likelihood ratio tests. We report here P values from Wald tests based on z-score of the parameter estimates of the models.

To analyse physiological and morphological responses, we used linear models fitted with the *lm* function (*stats* package) and univariate linear mixed effects models fitted with the *lme* function of the *nlme* package (Pinheiro et al., 2020). We performed backward elimination model selection starting from the full models after checking that the residuals of our models fulfilled a Gaussian distribution using Shapiro-Wilk tests and had homogeneous variances across treatment groups. We further explored statistical differences between treatment groups using pairwise post-hoc Tukey tests (*emmeans*, package *emmeans* ; Lenth et al., 2019). Regarding morphological and osmolality data, we first built independent linear models to confirm that there were no treatment differences in BM, TW or plasma osmolality at the beginning of the experiment. Models included the initial value as the dependent variable and air humidity, microclimate treatment, and their interaction as fixed effects. There was no difference in BM, TW, plasma osmolality among treatments before the manipulation (all P > 0.05, Table S1 & S2). To investigate the effects of the treatments on body mass loss over time, we used a mixed effects model for repeated measures with BM as the dependent variable, sex as a fixed factor, air humidity treatment, microclimate treatment, time (i.e. days on which individuals were weighed) and their three-way interaction as explanatory variables. Individual identity was included as a random, intercept term.

We next built three independent linear models to investigate the effects of the treatments on total Δ BM, Δ TW and Δ Osmolality and post-treatment Δ BM. We included air

humidity, microclimate treatment and their interaction as explanatory variables, sex as a categorical factor and the initial value as a linear covariate. We further examined whether ΔBM , ΔTW and post-treatment ΔBM were correlated with $\Delta\text{Osmolality}$ or final osmolality using linear regressions. To determine whether $\Delta\text{Osmolality}$ or final osmolality better explained physiological changes, we used the Akaike Information Criterion to compare models (*AIC*, package *stats*). We also investigated whether post-treatment ΔBM was correlated with total ΔBM using a similar model construction and comparison.

We also wanted to investigate the relationship between wet shelter use and mass change of snakes. To achieve this, we considered mass changes across individuals in the dry air treatment with access to wet shelter and applied the statistical methods from van de Pol and Wright, (2009). We calculated an individual-centred shelter use score at each 3-day period for each individual. We built a linear mixed effects model with the change in body mass per 3-day period as the dependent variable and shelter use score together with individual-centred shelter use score as explanatory variables. Individual identity was set as a random factor.

Results

Quantification of hydroregulation behaviour

At the onset of the study (from Day 0 to Day 2), there was no difference in shelter use among treatment groups (Air humidity: $z = -0.55$, $P = 0.58$; Microclimate treatment: $z = 0.55$, $P = 0.58$). During the cumulated manipulation period (from Day 3 to Day 20), the total proportion of shelter use was influenced by the interaction between air humidity and microclimate treatment ($z = -2.1$, $P = 0.03$). Supporting our prediction, shelter use was mainly observed in vipers maintained in dry air with a wet shelter, while other individuals almost never selected the shelter (Table 1). The daily shelter use gradually increased over time in the dry air and wet microclimate treatment group, while it remained stable and low in other groups

(Air humidity \times Microclimate treatment \times Time: $z = -2.1, P = 0.03$; Fig. 1a). When all individuals were provided with a wet shelter at the end of the experiment (from Day 21 to Day 23), the proportion of shelter use was then additively influenced by air humidity ($z = -3.5, P < 0.001$) and former microclimate treatments ($z = -2.4, P = 0.02$). This was due to a behavioural shift where vipers maintained in the dry microclimate treatment rapidly increased their use of the shelter when it became wet (Fig. 1a).

Physiological and morphological changes across treatments

Vipers significantly lost body mass over time during the experiment ($F_{1,290} = 1674.8, P < 0.001$, Fig. 1b), and air humidity and microclimate treatments interactively influenced BM loss (Air humidity \times Microclimate treatment \times Time: $F_{1,290} = 26.2, P < 0.001$). Vipers from the dry air and dry shelter lost BM at a much higher rate than those maintained in other conditions (Fig. 1b). In the dry air treatments, vipers with access to a wet shelter lost BM at a slower rate than those with a dry shelter. The total Δ BM over the experimental period was also influenced by the interaction between both treatment types ($F_{1,37} = 4.5, P = 0.04$; Table 1, mean Δ BM = -13.2 ± 5.5 % of initial BM).

Plasma osmolality increased in all groups indicating physiological dehydration, and the increase in osmolality was influenced by sex ($F_{1,37} = 23.7, P < 0.001$), air humidity treatment ($F_{1,37} = 15.5, P < 0.001$) but only marginally by microclimate treatment ($F_{1,37} = 2.9, P = 0.09$). Individuals had a higher increase in osmolality in dry conditions, and tended to have a lower increase in osmolality when a wet shelter was available (Table 1). However, one male from the dry air and wet microclimate treatment almost never used the wet shelter (shelter use score of 10%). Excluding this individual, we found that Δ Osmolality was significantly influenced by the interaction between air humidity and microclimate treatments ($F_{1,35} = 6.6, P = 0.01$) and by sex ($F_{1,35} = 23.5, P < 0.001$). Increase in plasma osmolality

was highest in vipers maintained in dry air with a dry shelter and lowest and similar in the other three groups (Table 1). Overall, males had a higher increase in plasma osmolality than females. Tail width change ΔTW was additively influenced by air humidity ($F_{1,38} = 5.7, P = 0.02$, Fig. 2a) and microclimate treatments ($F_{1,38} = 5.6, P = 0.02$, Fig. 2b). Vipers had increased TW loss in dry air conditions and when they only had access to a dry shelter (Table 1; Fig. 2a).

Benefits of hydroregulation and post-treatment water intake

Focusing on the dry air and wet shelter treatment, we found that body mass loss was negatively correlated with the individual-centred shelter use score ($F_{1,65} = 26.1, P < 0.001$). Higher behavioural selection of the wet shelter led to reduced body mass loss (Fig. 3). Post-treatment water intake was additively influenced by air humidity ($F_{1,37} = 32.5, P < 0.001$, Fig. 2c) and microclimate treatments ($F_{1,37} = 8.7, P = 0.005$, Fig. 2D). Vipers exposed to dry air conditions and vipers with access to a dry shelter drank more on Day 24 (Table 1; Fig. 2c).

Correlations between physiological responses

Overall, BM loss was positively correlated with both $\Delta Osmolality$ ($F_{1,39} = 55.9, P < 0.001$, Fig. S1a) and final osmolality ($F_{1,39} = 4.99, P < 0.001$), but most influenced by $\Delta Osmolality$ (Table S8). TW loss was also positively correlated with both $\Delta Osmolality$ ($F_{1,39} = 17.9, P < 0.001$, Fig. S1b) and final osmolality ($F_{1,39} = 19.2, P < 0.001$; Fig. S6) with similar strength of correlation (Table S8). Post-treatment water intake was positively correlated with $\Delta Osmolality$ ($F_{1,39} = 66.0, P < 0.001$, Fig. S1c), final osmolality ($F_{1,39} = 80.0, P < 0.001$) and ΔBM ($F_{1,39} = 34.3, P < 0.001$), but most influenced by final osmolality (Table S8).

Discussion

We provide the first experimental evidence that behavioural hydoregulation can mitigate physiological consequences of a simulated dry spell in a terrestrial ectotherm. Wet shelters were used more frequently when individuals were exposed to dry air conditions. This pattern was gradual, reaching 75% of the time by the end of the manipulation, compared to less than 10% in other treatment groups. This demonstrates microclimate selection based on water vapour gradient independently from temperature, thus providing unambiguous evidence of behavioural hygroregulation *sensu stricto* (Mitchell & Bergmann, 2016; Wolcott & Wolcott, 2001). This behaviour allowed individuals to attenuate mass loss and dehydration risks in more desiccating conditions.

Concurrent changes in morphology, plasma osmolality and post-treatment drinking behaviour all indicated that more desiccating air led to more severe physiological dehydration. Body mass loss throughout the treatment period was higher in individuals exposed to dry air and these individuals also had the greatest increase in plasma osmolality. Muscle loss and post-treatment water intake were greater in individuals exposed to dry air and both traits were correlated with physiological dehydration, here assessed by final osmolality values. Therefore, dry air conditions increased muscle catabolism, probably in order to release the bound water that makes up a significant portion of skeletal muscles (Brusch et al., 2018) and resulted in increased thirst levels (Edwards et al., 2021; Lillywhite et al., 2019). Dehydration-induced muscle catabolism can be beneficial to support water needs and reduce the immediate survival risks associated with hyperosmolality in vertebrates (Brusch et al., 2018; Gerson & Guglielmo, 2011; McCue et al., 2017). However, such compensatory mechanisms directly alter structural muscles and associated locomotor capacities (Lorion et al., 2016; Lourda et al., 2004). It is well-known that dehydration can impair locomotor performance as

demonstrated in amphibians and some lizards (Greenberg & Palen, 2021; Anderson & Andrade, 2017; Wilson et al., 1989). Although we did not measure behavioural capacities or functional properties such as locomotion or foraging, the high levels of dehydration and muscle mobilization observed in some of our snakes are most likely associated with impaired locomotor abilities. Altogether, these results indicate that a more desiccating air in the absence of free standing water caused greater dehydration, likely through increased evaporative water loss (Dupoué et al., 2015b). Such hygrometric constraints are likely even more prominent at high ambient temperatures because desiccation risks rise exponentially with temperature (increased magnitude of VPD, see Riddell et al., 2019) and may be even stronger during key life history events when thermal requirements are elevated such as gestation in female vipers (see Dupoué et al., 2015b; Stahlschmidt et al., 2011).

Supporting our initial predictions, preferential wet shelter use buffered the effects of dry air conditions on physiological dehydration. When a wet shelter was available, individuals had on average a lower body mass loss especially in dry air, tended to have a lower increase in plasma osmolality and had a lower post-treatment water intake. Interestingly, when we excluded one viper whose behaviour was not responsive to wet shelter availability, the influence of a wet microclimate on osmolality and body mass changes was only significant under the dry air condition. Individuals with access to a wet shelter also had lower muscle loss rates compared to those with a dry shelter, which suggests that they had less of a need to catabolize muscle protein. In addition, within the dry air and wet shelter treatment, the more a viper used the shelter, the less body mass it lost. Behavioural selection of a moist microclimate thus allowed vipers to attenuate dehydration risks and specific associated morphological responses. Altogether, snakes that had access to a wet shelter could thus mitigate the direct performance costs possibly induced by hyperosmolality (Greenberg &

Palen, 2021; Anderson & Andrade, 2017), and also minimised the indirect performance costs of muscle catabolism (Brusch et al., 2018; Lorigoux et al., 2016).

We also found significant differences among individuals in their wet shelter use irrespective of treatment conditions and sex suggesting wide variation in hydoregulation behaviours likely exist. Within natural populations, such variation might translate in individual differences in physiological sensitivity to extreme climate events. Since flexible hydoregulation behaviours buffer physiological consequences of desiccating air, they should be beneficial for populations facing more frequent and intense dry spells, such as those located at the warm edge of the distribution. At the end of the experiment, most vipers that had been housed with a dry shelter immediately selected the wet shelter at a frequency similar to that of vipers that were maintained with a wet shelter and dry air for several days. This sudden shift suggests that these snakes had reached a significant dehydration threshold, which induced an immediate behavioural response once a wet microclimate was available.

Hygrosensing and behavioural hydoregulation have been previously observed in dry-skinned ectotherms both in the field (Guillon et al., 2014; Lourdais et al., 2017) and in laboratory conditions (Dupoué et al., 2015b; Stahlschmidt & Denardo, 2010; Stahlschmidt et al., 2011). Our findings extend these earlier observations and highlight that microclimate selection within a “hygrometric landscape” caused by spatial heterogeneity in humidity promotes water balance regulation and minimises the costs of dehydration. We posit that such behavioural adjustments are frequent in terrestrial ectotherms and call for a better characterisation of microclimate moisture gradients in the context of climate change (Pincebourde et al., 2016; Woods et al., 2021). Our results indicate that the use of moist microhabitats may allow ectotherms to avoid desiccation during extreme climate events, similar to the key role of behavioural thermoregulation in limiting exposure to acute thermal extremes and overheating (Huey & Tewksbury, 2009; Kearney et al., 2009). For example,

452 following the classic terminology for thermoregulation, some species may actively
453 “hygroregulate” while others may behave as “hygroconformer”. The former may efficiently
454 seek microclimates minimising the deviance to optimal moisture (Moore et al., 2018; Székely
455 et al., 2018; Lourdais et al., 2017), which would allow individuals to maintain their hydration
456 state, performance and activity (Greenberg & Palen, 2021; Anderson & Andrade, 2017).
457 However, their behavioural capacity to do so ultimately depends on habitat “hygrometric
458 quality”. This emphasizes the critical importance of the availability of moist microhabitats for
459 dry-skinned ectotherms, which can decrease with anthropogenic disturbances (Gorissen et al.,
460 2017) or climate change (Dundas et al., 2021). In addition, we posit that a behavioural
461 capacity to maintain optimal hygrometric conditions may dampen long-term exposure to
462 dehydration risks along gradients of climate or habitat aridity. This may reduce selection on
463 physiological tolerance to dehydration, similar to the concept of the “Bogert effect” for
464 thermoregulatory behaviour (Muñoz, 2021). More generally, the occurrence of behavioural
465 hygroregulation may have implications for our understanding of the evolution of ecological
466 niches of organisms (Encarnación-Luévano et al., 2021; Farallo et al., 2020; Woods et al.,
467 2021).

468 Understanding and predicting organismal responses to climate change is currently a
469 major challenge in ecology and conservation (Kearney et al., 2018; Riddell et al., 2019). Our
470 experimental study demonstrates that hygrometric constraints and behavioural
471 hydoregulation capacity must be considered in this context. Recent individual-based models
472 have used energy and heat budgets to predict fine-scale movements within microhabitats in
473 response to thermal microclimate conditions (Malishev et al., 2018; Sears et al., 2016), but
474 those approaches often overlooked water constraints. Future mechanistic models should
475 include the effects of air moisture and behavioural hydoregulation on the water budget of
476 organisms (see Huang et al., 2020; Peterman & Semlitsch, 2014). One challenge to this

approach is that, in contrast to behavioural thermoregulation where individuals generally immediately adjust their behavioural activity in response to well-known temperature thresholds or thermal performance curves (Angilletta, 2009), behavioural hygroregulation likely changes more gradually in response to a shift in individual hydration state. The physiological costs of desiccant conditions should also vary among species depending on their resistance to water loss, thus influencing the timing of behavioural changes. For example, some dry-skinned ectotherms may adjust their hydroregulation behaviour over days or weeks, as shown here, while most wet-skinned ectotherms may adjust over minutes or hours (Peterman & Semlitsch, 2014; Székely et al., 2018). In addition, empirical data suggest that the performance costs of dehydration increase abruptly only after a significant dehydration threshold is reached in amphibians (Cheuvront & Kenefick, 2014; Greenberg & Palen, 2021). Such nonlinear, threshold effects should trigger more or less rapid behavioural adjustment depending on the shape of hydric performance response curve (Edwards et al., 2021; Lillywhite et al., 2019). Future studies should therefore examine the strength and shape of the relationships between dehydration and performance capacities for which we currently lack data (Rozen-Rechels et al., 2019).

In conclusion, our study emphasizes the importance of air humidity, as well as behavioural hydroregulation, when investigating the effects of climate change on ectotherms. Microhabitat quality and heterogeneity appear to be essential to support behavioural responses and mitigate deleterious effects of extreme weather events. However, current habitat simplifications associated with human activities are likely to compromise these important components of microhabitat (Guiller et al., 2022).

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Declarations

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Conflict of interest

The authors declare no competing or financial interests.

Ethics approval

All experimentation procedures with animals listed in this study were approved by an independent ethical committee (Apafis#23238-2019121010574211_V3).

Consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

Data and associated material are available in a public repository at
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Code availability

Not applicable

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779 **Table 1.** Summary statistics of the traits measured on asp vipers from each treatment group. Values written in *italic* are from the Dry air & Wet
780 shelter group when excluding the individual that almost never selected the shelter (see details in main text). Different superscript letters are
781 used to show significant differences between treatment groups for each variable.

Variables	Experimental treatments				
	Wet air		Dry air		
	Wet shelter (n=10)	Dry shelter (n=11)	Wet shelter (n=11)		Dry shelter (n=10)
	Mean (± SE)	Mean (± SE)	Mean (± SE)		Mean (± SE)
Cumulative shelter use (%)	7.96 (4.64) ^A	4.71 (3.48) ^A	58.58 (6.90) ^B	63.33 (5.53) ^B	10.92 (3.57) ^A
Δ Body mass – Day20 (g)	-15.31 (1.23) ^A	-14.37 (0.93) ^A	-18.73 (1.05) ^{AB}	-18.26 (1.04) ^A	-22.77 (1.49) ^B
Δ Plasma osmolality (mOsm.kg ⁻¹)	30.15 (4.17) ^A	31.45 (5.12) ^A	45.50 (8.96) ^{AB}	38.55 (6.26) ^A	68.60 (11.87) ^B
Δ Tail width (mm)	-0.21 (0.09) ^A	-0.48 (0.13) ^{AB}	-0.46 (0.11) ^{AB}	-0.43 (0.12) ^{AB}	-0.81 (0.15) ^B
Post-treatment water intake (g)	10.19 (1.01) ^A	12.94 (1.19) ^{AB}	17.63 (1.62) ^{BC}	16.63 (1.40) ^{BC}	21.84 (1.66) ^C

Figure captions

Figure 1. Effects of experimental treatments on behavioural hydoregulation and body mass loss. (a) Changes in daily shelter use (proportion of shelter use out of the 3 daily observations) over time in each treatment group. Points are mean values with error bars representing ± 1 SE. The predictions of the best logistic regression model (curves) were fitted with the data from Day 0 to Day 20, together with their 95% confidence intervals (shaded background). Vipers had access to either a dry or a wet shelter and were maintained in dry or wet air from Day3 to Day20. All individuals had access to a dry shelter from Day0 to Day2, and to a wet shelter from Day21 to Day23. (b) Average body mass loss (g) loss through time in each treatment group. All individuals were deprived of drinking water from Day0 to Day 23. On Day24, all individuals were provided with drinking water *ad-libitum*. Error bars represent ± 1 SE.

Figure 2. Additive effects of air humidity and microclimate treatment on changes in tail width (mm) throughout the experiment (a; b) and on post-treatment water intake (g) following access to drinking water on Day24 (c; d). Error bars represent ± 1 SE.

Figure 3. Correlation between body mass change (g) and individual-centred shelter use score per 3-day period within the dry air and wet shelter treatment group (6 repetitions per viper from Day 3 to Day 20 of the manipulation). The individual-centred score quantifies intraindividual variation in shelter use (positive values indicate stronger shelter use than the mean score of the individual). Drawing credits: Sydney Hope.

Figure 1.

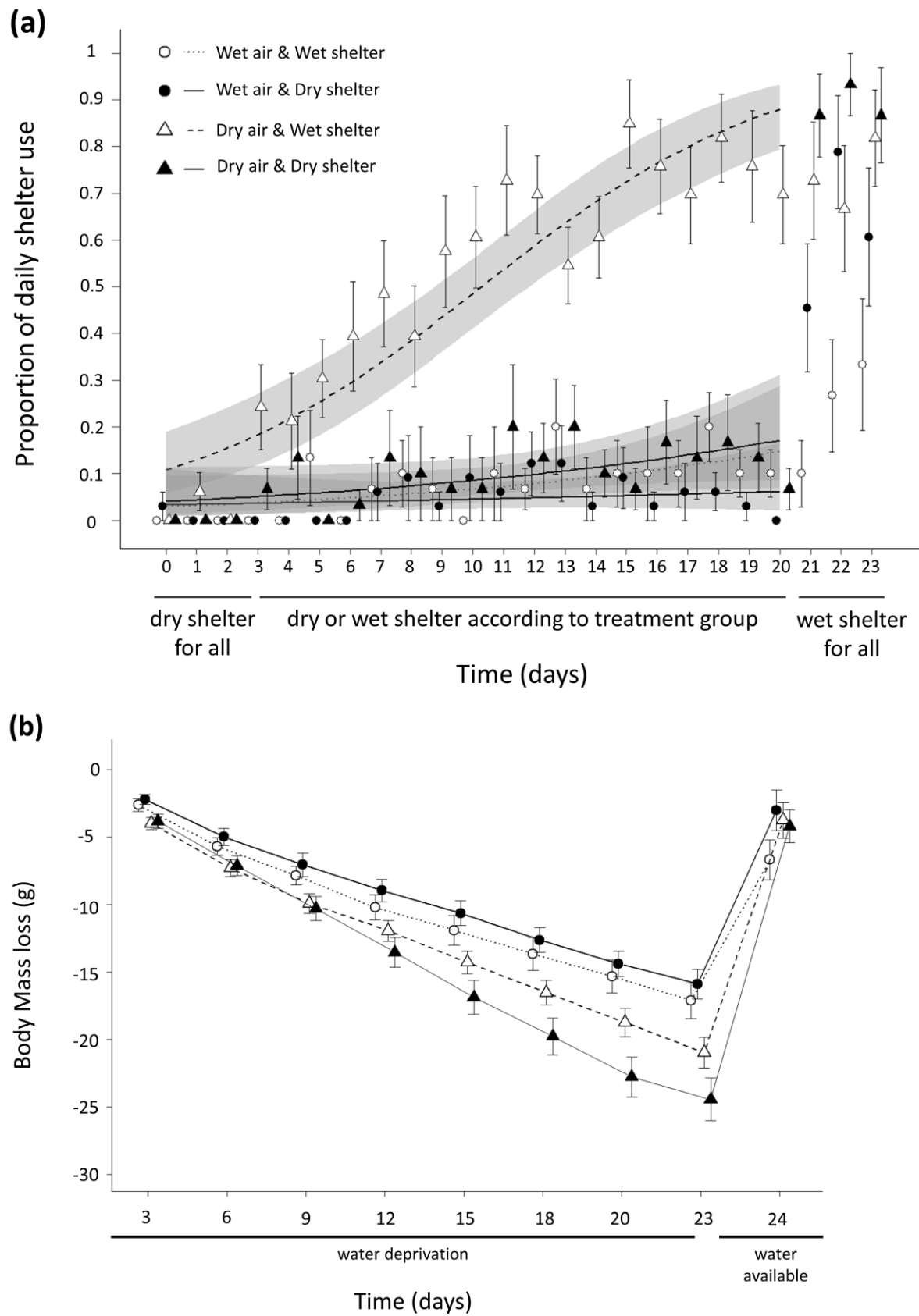


Figure 2.

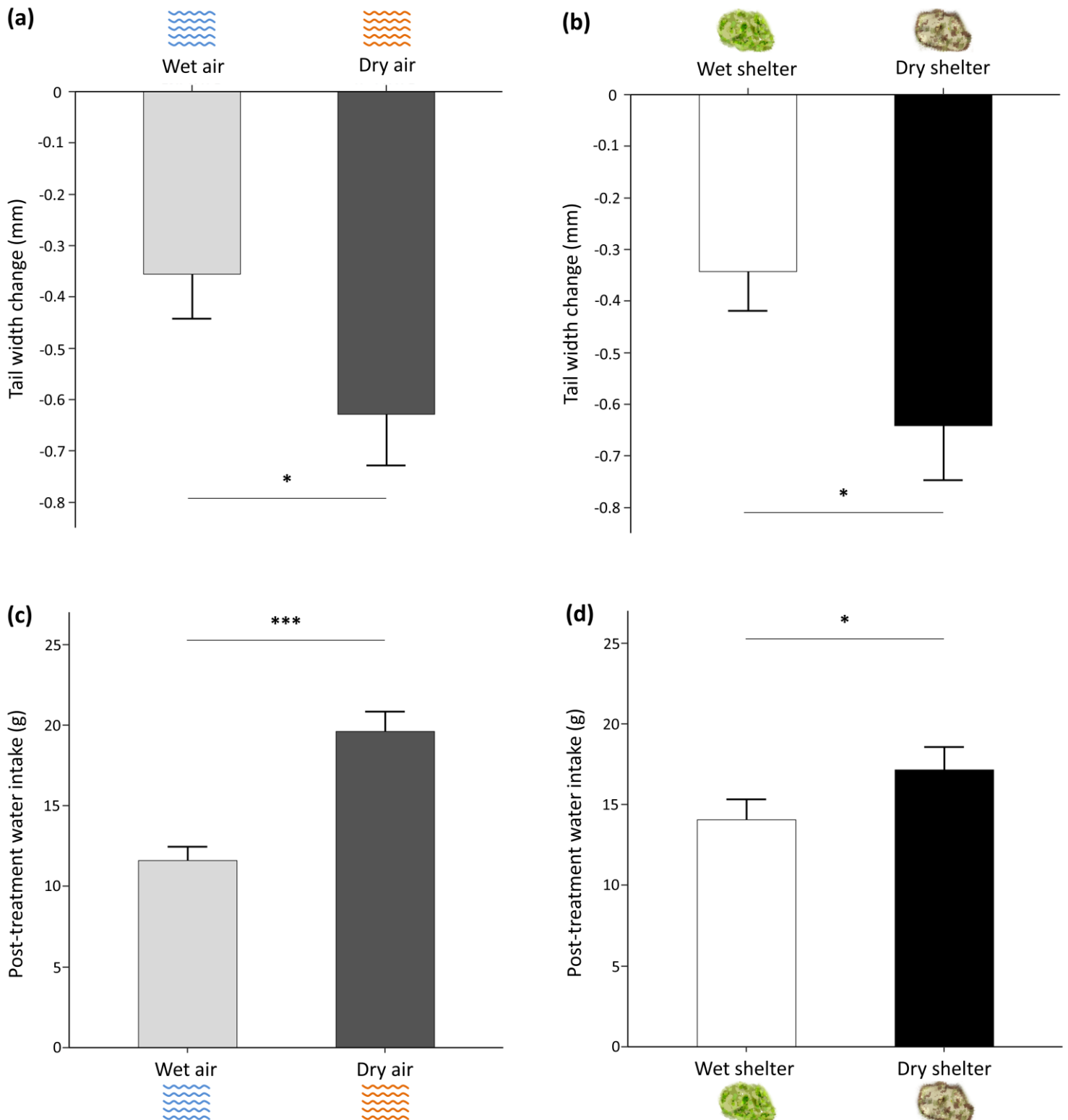


Figure 3.

