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

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1 Running head: Microclimate selection and attenuation of drought stress

2

3 **Behavioural hydroregulation protects against acute effects of**  
4 **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.

## 54 **Introduction**

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

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

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

84 In many terrestrial ectotherms, behavioural trade-offs between thermoregulation and  
85 osmoregulation exist because evaporative water loss increases with body temperature and in  
86 the microclimate conditions relevant for basking (e.g., Dupoué et al., 2015b; Riddell et al.,  
87 2018). In addition, climate warming often involves concurrent changes in temperature,  
88 rainfall and moisture, especially during extreme climate events such as summer hot and dry  
89 spells that increase water loss risks (Trenberth et al., 2014; Ummenhofer & Meehl, 2017).  
90 Organisms should partially buffer the water constraints of climate change by means of  
91 behavioural hydroregulation, defined as the range of behaviours that control water gains and  
92 water losses and help individuals maintain an optimal hydration state for their activity and  
93 functional capacities (Pirtle et al., 2019; Rozen-Rechels et al., 2019). For example, reptiles  
94 living under water stress can maintain their water balance by reducing their activity (Davis &  
95 Denardo, 2010; Kearney et al., 2018) or by selecting cool (Ladyman & Bradshaw, 2003;  
96 Pintor et al., 2016) or wet microclimates (Dupoué et al., 2015b; Guillon et al., 2014; Lourdais  
97 et al., 2017).

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

104 2020). Some amphibians can reduce evaporative water loss by means of behavioural changes  
105 such as postural adjustments to reduce the cutaneous water-exchange surface, mucus  
106 production or the selection of moist microhabitats (e.g. Lillywhite et al., 1997; Wolcott &  
107 Wolcott, 2001). There is good evidence that some amphibians perform active behavioural  
108 hydroregulation to maintain an optimal hydration state independently from thermoregulation  
109 (Mitchell & Bergmann, 2016), but whether this behavioural flexibility is efficient during  
110 extreme weather events remains unknown. Behavioural data are even more elusive for dry-  
111 skin ectotherms due to difficulties disentangling hydroregulation and thermoregulation  
112 mechanisms in these organisms (Pintor et al., 2016; Rozen-Rechels et al., 2019). Dry-skin  
113 ectotherms often have a strong resistance to evaporative water loss, meaning that their  
114 hydration state changes more slowly than body temperature and that shifts caused by  
115 behavioural hydroregulation could take place over long time periods. Behavioural  
116 hydroregulation could be controlled by the internal hydration status, leading individuals to  
117 choose less desiccating microclimates as a water-saving strategy only when chronically  
118 stressed (Ladyman & Bradshaw, 2003; Rozen-Rechels et al., 2020). On the other hand,  
119 terrestrial organisms can perceive moisture gradients through hygrosensing (Chown et al.,  
120 2011) and further rely on odours or other cues to locate free standing water (Serrano-Rojas &  
121 Pašukonis, 2021; Wood et al., 2021), such that changes in air water vapour pressure  
122 conditions may also trigger rapid changes in their behaviours.

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

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

139

## 140 **Material and Methods**

### 141 *Study species*

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



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

158

### 159 *Manipulation of hydric conditions and air temperature*

160 Our experimental design consisted of a strictly defined manipulation of hydric conditions  
161 independently from air temperature. We used 4 controlled environment chambers (Vötsch VP  
162 600, Balingen, Germany) allowing realistic simulations of daily fluctuations in air  
163 temperature and humidity. Asp vipers were deprived of water for three weeks, which  
164 corresponds to a moderate duration for a dry spell in natural conditions (Perkins et al., 2012).  
165 The daily temperature cycle mimicked body temperature recorded during a typical heatwave  
166 with daytime temperature of 32°C for 14h and nighttime temperature of 26°C for 5h, with  
167 temperature either gradually increasing or decreasing for the remaining 5h (Lourdais et al.,  
168 2013). We next manipulated air humidity (dry air vs. wet air) and microclimate (dry shelter  
169 vs. wet shelter) using a two-by-two factorial design. First, we compared two contrasted  
170 ambient water vapour pressure deficit (VPD) to expose individuals to either desiccant (VPD =  
171  $- 3.10 \pm 0.08$  kP<sub>a</sub> in dry air; Dupoué et al., 2015b) or humid conditions (VPD =  $- 1.43 \pm 0.08$   
172 kP<sub>a</sub> in wet air). Second, we manipulated microclimate conditions by providing either a dry or  
173 a wet shelter. Shelters consisted of small, custom made plastic containers (15 cm diameter, 11  
174 cm height; one entrance of 3 cm diameter on the side) filled with an artificial sponge and  
175 microfiber cloths. Once accustomed, vipers will readily use shelters in the laboratory. The  
176 sponge and microfiber cloths were changed every 3 days and maintained dry in the dry  
177 shelters but damp in the wet shelters in order to enhance air humidity. In dry shelters, VPD  
178 were similar to the ambient conditions and therefore offered no humidity gradient. In turn,

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

185

### 186 ***Experimental design***

187 Prior to the experiment, for the purpose of acclimation to general maintenance conditions, we  
188 housed all individuals for one week in climatic chambers using individual plastic boxes (35 x  
189 20 x 17 cm) with one dry shelter, a sheet of newspaper as a substrate and a natural day light  
190 cycle (circa 12:12). During this period, vipers had *ad-libitum* access to drinking water and  
191 were exposed to a standard daily temperature cycle (6h daytime at  $30^\circ\text{C}$ , 13h nighttime at  
192  $22^\circ\text{C}$ , and mean VPD of  $-1.20 \pm 0.10 \text{ kPa}_a$ ). Then, we randomly allocated individuals to each  
193 treatment group while ensuring a balanced sex ratio. Each individual was placed in a climatic  
194 chamber with the corresponding air humidity treatment (dry air or wet air) and we applied the  
195 following protocol over 24 days: from Day 0 to Day 2 (3 days), we provided all individuals  
196 with a dry shelter in order to quantify baseline shelter use; from Day 3 to Day 20 (18 days),  
197 we exposed each individual to its microclimate treatment (dry shelter or wet shelter) in order  
198 to quantify the combined effects of air humidity and microclimate; from Day 21 to Day 23 (3  
199 days), we provided all individuals with a wet shelter to test for change in shelter use in  
200 individuals previously exposed to dry shelters; and finally, on Day 24, we provided all  
201 individuals with water *ad-libitum* to assess post treatment water intake from drinking. We  
202 then transferred them to standard housing conditions. We did not feed the snakes in order to

203 avoid confounding effects of digestion and energy intake on body mass (Stahlschmidt et al.,  
204 2015).

205

## 206 *Variables collected and parameters measured*

### 207 *Shelter use over time*

208 Every day from Day 0 to Day 23, we recorded the position of each individual 3 times per day  
209 during daytime (9:00, 13:00 and 17:00). Individual position in the box was categorised as  
210 either using the shelter or being outside. First, we considered the cumulative shelter use from  
211 Day 0 to Day 2 (proportion of shelter use out of 9 observations) to record the baseline shelter  
212 use. To investigate responses to combined air humidity and microclimate treatments (Day 3 to  
213 Day 20), we first examined the cumulative shelter use during that period (proportion of shelter  
214 use out of 54 observations). Second, we considered more specifically changes in daily shelter  
215 use (proportion of shelter use out of the 3 daily observations) over time (Day 0 to Day 20).  
216 We also calculated an individual shelter use score over a 3-day period between Day 3 and  
217 Day 20 (total number of times inside the shelter per 9 observations, 6 repetitions) to  
218 investigate the relation between shelter use behaviour and body mass changes (recorded every  
219 3 days, see below). Finally, we also calculated cumulative shelter use from Day 21 to Day 23  
220 (proportion of shelter use out of 9 observations) to investigate wet shelter use at the end of the  
221 treatment.

222

### 223 *Changes in body mass and muscle condition*

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

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

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

242

#### 243 *Changes in plasma osmolality*

244 We collected blood samples on Day 0 and Day 20 to assay plasma osmolality, which  
245 measures the whole-body electrolyte and water balance (Peterson, 2002). We sampled  
246 individuals in a random order by sex and treatments and collected 150  $\mu$ L of blood through  
247 cardiocentesis, using a 1mL syringe and a heparinized 29-gauge needle. We immediately  
248 centrifuged the blood at 2000g, separated plasma from the red blood cells and stored the  
249 samples at  $-28^{\circ}\text{C}$ . Plasma osmolality ( $\pm 1$  mOsm  $\text{kg}^{-1}$ ) was measured from 10  $\mu$ L duplicates  
250 (intra-individual CV: 0.36%) using a vapour pressure osmometer (model 5500, Wescor,  
251 Logan, UT, USA). We calculated  $\Delta$ Osmolality as the difference between mean plasma  
252 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

278 manipulation, we forced a similar intercept for the four treatments in our full model. This  
279 model was built with `glmer` function of the `lme4` package (Bates et al., 2015) and fitted well  
280 with the data (overdispersion test:  $X^2 = 816.4$ , dispersion coefficient = 0.9,  $df = 896$ ,  $P =$   
281 0.92). In all cases, we performed backward elimination model selection starting from the full  
282 models to remove non-significant effects using likelihood ratio tests. We report here  $P$  values  
283 from Wald tests based on  $z$ -score of the parameter estimates of the models.

284       To analyse physiological and morphological responses, we used linear models fitted  
285 with the `lm` function (`stats` package) and univariate linear mixed effects models fitted with the  
286 `lme` function of the `nlme` package (Pinheiro et al., 2020). We performed backward elimination  
287 model selection starting from the full models after checking that the residuals of our models  
288 fulfilled a Gaussian distribution using Shapiro-Wilk tests and had homogeneous variances  
289 across treatment groups. We further explored statistical differences between treatment groups  
290 using pairwise post-hoc Tukey tests (`emmeans`, package `emmeans` ; Lenth et al., 2019).  
291 Regarding morphological and osmolality data, we first built independent linear models to  
292 confirm that there were no treatment differences in BM, TW or plasma osmolality at the  
293 beginning of the experiment. Models included the initial value as the dependent variable and  
294 air humidity, microclimate treatment, and their interaction as fixed effects. There was no  
295 difference in BM, TW, plasma osmolality among treatments before the manipulation (all  $P >$   
296 0.05, Table S1 & S2). To investigate the effects of the treatments on body mass loss over  
297 time, we used a mixed effects model for repeated measures with BM as the dependent  
298 variable, sex as a fixed factor, air humidity treatment, microclimate treatment, time (i.e. days  
299 on which individuals were weighed) and their three-way interaction as explanatory variables.  
300 Individual identity was included as a random, intercept term.

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

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

310 We also wanted to investigate the relationship between wet shelter use and mass  
311 change of snakes. To achieve this, we considered mass changes across individuals in the dry  
312 air treatment with access to wet shelter and applied the statistical methods from van de Pol  
313 and Wright, (2009). We calculated an individual-centred shelter use score at each 3-day period  
314 for each individual. We built a linear mixed effects model with the change in body mass per  
315 3-day period as the dependent variable and shelter use score together with individual-centred  
316 shelter use score as explanatory variables. Individual identity was set as a random factor.

317

## 318 **Results**

### 319 *Quantification of hydroregulation behaviour*

320 At the onset of the study (from Day 0 to Day 2), there was no difference in shelter use among  
321 treatment groups (Air humidity:  $z = -0.55$ ,  $P = 0.58$ ; Microclimate treatment:  $z = 0.55$ ,  $P =$   
322  $0.58$ ). During the cumulated manipulation period (from Day 3 to Day 20), the total proportion  
323 of shelter use was influenced by the interaction between air humidity and microclimate  
324 treatment ( $z = -2.1$ ,  $P = 0.03$ ). Supporting our prediction, shelter use was mainly observed  
325 in vipers maintained in dry air with a wet shelter, while other individuals almost never  
326 selected the shelter (Table 1). The daily shelter use gradually increased over time in the dry  
327 air and wet microclimate treatment group, while it remained stable and low in other groups

328 (Air humidity  $\times$  Microclimate treatment  $\times$  Time:  $z = -2.1, P = 0.03$ ; Fig. 1a). When all  
329 individuals were provided with a wet shelter at the end of the experiment (from Day 21 to  
330 Day 23), the proportion of shelter use was then additively influenced by air humidity ( $z =$   
331  $-3.5, P < 0.001$ ) and former microclimate treatments ( $z = -2.4, P = 0.02$ ). This was due to  
332 a behavioural shift where vipers maintained in the dry microclimate treatment rapidly  
333 increased their use of the shelter when it became wet (Fig. 1a).

334

### 335 *Physiological and morphological changes across treatments*

336 Vipers significantly lost body mass over time during the experiment ( $F_{1,290} = 1674.8, P <$   
337  $0.001$ , Fig. 1b), and air humidity and microclimate treatments interactively influenced BM  
338 loss (Air humidity  $\times$  Microclimate treatment  $\times$  Time:  $F_{1,290} = 26.2, P < 0.001$ ). Vipers from  
339 the dry air and dry shelter lost BM at a much higher rate than those maintained in other  
340 conditions (Fig. 1b). In the dry air treatments, vipers with access to a wet shelter lost BM at a  
341 slower rate than those with a dry shelter. The total  $\Delta$ BM over the experimental period was  
342 also influenced by the interaction between both treatment types ( $F_{1,37} = 4.5, P = 0.04$ ; Table  
343 1, mean  $\Delta$ BM =  $-13.2 \pm 5.5$  % of initial BM).

344 Plasma osmolality increased in all groups indicating physiological dehydration, and  
345 the increase in osmolality was influenced by sex ( $F_{1,37} = 23.7, P < 0.001$ ), air humidity  
346 treatment ( $F_{1,37} = 15.5, P < 0.001$ ) but only marginally by microclimate treatment ( $F_{1,37} =$   
347  $2.9, P = 0.09$ ). Individuals had a higher increase in osmolality in dry conditions, and tended  
348 to have a lower increase in osmolality when a wet shelter was available (Table 1). However,  
349 one male from the dry air and wet microclimate treatment almost never used the wet shelter  
350 (shelter use score of 10%). Excluding this individual, we found that  $\Delta$ Osmolality was  
351 significantly influenced by the interaction between air humidity and microclimate treatments  
352 ( $F_{1,35} = 6.6, P = 0.01$ ) and by sex ( $F_{1,35} = 23.5, P < 0.001$ ). Increase in plasma osmolality



353 was highest in vipers maintained in dry air with a dry shelter and lowest and similar in the  
354 other three groups (Table 1). Overall, males had a higher increase in plasma osmolality than  
355 females. Tail width change  $\Delta TW$  was additively influenced by air humidity ( $F_{1,38} = 5.7, P =$   
356  $0.02$ , Fig. 2a) and microclimate treatments ( $F_{1,38} = 5.6, P = 0.02$ , Fig. 2b). Vipers had  
357 increased TW loss in dry air conditions and when they only had access to a dry shelter (Table  
358 1; Fig. 2a).

359

#### 360 *Benefits of hydroregulation and post-treatment water intake*

361 Focusing on the dry air and wet shelter treatment, we found that body mass loss was  
362 negatively correlated with the individual-centred shelter use score ( $F_{1,65} = 26.1, P < 0.001$ ).  
363 Higher behavioural selection of the wet shelter led to reduced body mass loss (Fig. 3). Post-  
364 treatment water intake was additively influenced by air humidity ( $F_{1,37} = 32.5, P <$   
365  $0.001$ , Fig. 2c) and microclimate treatments ( $F_{1,37} = 8.7, P = 0.005$ , Fig. 2D). Vipers exposed  
366 to dry air conditions and vipers with access to a dry shelter drank more on Day 24 (Table 1;  
367 Fig. 2c).

368

#### 369 *Correlations between physiological responses*

370 Overall, BM loss was positively correlated with both  $\Delta Osmolality$  ( $F_{1,39} = 55.9, P < 0.001$ ,  
371 Fig. S1a) and final osmolality ( $F_{1,39} = 4.99, P < 0.001$ ), but most influenced by  $\Delta Osmolality$   
372 (Table S8). TW loss was also positively correlated with both  $\Delta Osmolality$  ( $F_{1,39} = 17.9, P <$   
373  $0.001$ , Fig. S1b) and final osmolality ( $F_{1,39} = 19.2, P < 0.001$ ; Fig. S6) with similar strength  
374 of correlation (Table S8). Post-treatment water intake was positively correlated with  
375  $\Delta Osmolality$  ( $F_{1,39} = 66.0, P < 0.001$ , Fig. S1c), final osmolality ( $F_{1,39} = 80.0, P < 0.001$ )  
376 and  $\Delta BM$  ( $F_{1,39} = 34.3, P < 0.001$ ), but most influenced by final osmolality (Table S8).

377

378 **Discussion**

379 We provide the first experimental evidence that behavioural hydroregulation can mitigate  
380 physiological consequences of a simulated dry spell in a terrestrial ectotherm. Wet shelters  
381 were used more frequently when individuals were exposed to dry air conditions. This pattern  
382 was gradual, reaching 75% of the time by the end of the manipulation, compared to less than  
383 10% in other treatment groups. This demonstrates microclimate selection based on water  
384 vapour gradient independently from temperature, thus providing unambiguous evidence of  
385 behavioural hygroregulation *sensu stricto* (Mitchell & Bergmann, 2016; Wolcott & Wolcott,  
386 2001). This behaviour allowed individuals to attenuate mass loss and dehydration risks in  
387 more desiccating conditions.

388

389 Concurrent changes in morphology, plasma osmolality and post-treatment drinking behaviour  
390 all indicated that more desiccating air led to more severe physiological dehydration. Body  
391 mass loss throughout the treatment period was higher in individuals exposed to dry air and  
392 these individuals also had the greatest increase in plasma osmolality. Muscle loss and post-  
393 treatment water intake were greater in individuals exposed to dry air and both traits were  
394 correlated with physiological dehydration, here assessed by final osmolality values.

395 Therefore, dry air conditions increased muscle catabolism, probably in order to release the  
396 bound water that makes up a significant portion of skeletal muscles (Brusch et al., 2018) and  
397 resulted in increased thirst levels (Edwards et al., 2021; Lillywhite et al., 2019). Dehydration-  
398 induced muscle catabolism can be beneficial to support water needs and reduce the immediate  
399 survival risks associated with hyperosmolality in vertebrates (Brusch et al., 2018; Gerson &  
400 Guglielmo, 2011; McCue et al., 2017). However, such compensatory mechanisms directly  
401 alter structural muscles and associated locomotor capacities (Lorioux et al., 2016; Lourdais et  
402 al., 2004). It is well-known that dehydration can impair locomotor performance as

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

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

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

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

440         Hygrosensing and behavioural hydroregulation have been previously observed in dry-  
441 skinned ectotherms both in the field (Guillon et al., 2014; Lourdais et al., 2017) and in  
442 laboratory conditions (Dupoué et al., 2015b; Stahlschmidt & Denardo, 2010; Stahlschmidt et  
443 al., 2011). Our findings extend these earlier observations and highlight that microclimate  
444 selection within a “hygrometric landscape” caused by spatial heterogeneity in humidity  
445 promotes water balance regulation and minimises the costs of dehydration. We posit that such  
446 behavioural adjustments are frequent in terrestrial ectotherms and call for a better  
447 characterisation of microclimate moisture gradients in the context of climate change  
448 (Pincebourde et al., 2016; Woods et al., 2021). Our results indicate that the use of moist  
449 microhabitats may allow ectotherms to avoid desiccation during extreme climate events,  
450 similar to the key role of behavioural thermoregulation in limiting exposure to acute thermal  
451 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 hydroregulation 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 hydroregulation on the water budget of  
476 organisms (see Huang et al., 2020; Peterman & Semlitsch, 2014). One challenge to this

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

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

499

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505

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515

#### 516 **Conflict of interest**

517 The authors declare no competing or financial interests.  
518

#### 519 **Ethics approval**

520 All experimentation procedures with animals listed in this study were approved by an  
521 independent ethical committee (Apafig#23238-2019121010574211\_V3).  
522

#### 523 **Consent to participate**

524 Not applicable  
525

526 **Consent for publication**

527 Not applicable

528

529 **Availability of data and material**

530 Data and associated material are available in a public repository at

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532

533 **Code availability**

534 Not applicable

535

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778

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 ( $\pm$ SE)	Mean ( $\pm$ SE)	Mean ( $\pm$ SE)	Mean ( $\pm$ SE)	
Cumulative shelter use (%)	7.96 (4.64) <sup>A</sup>	4.71 (3.48) <sup>A</sup>	58.58 (6.90) <sup>B</sup>	63.33 (5.53) <sup>B</sup>	10.92 (3.57) <sup>A</sup>
$\Delta$ Body mass – Day20 (g)	-15.31 (1.23) <sup>A</sup>	-14.37 (0.93) <sup>A</sup>	-18.73 (1.05) <sup>AB</sup>	-18.26 (1.04) <sup>A</sup>	-22.77 (1.49) <sup>B</sup>
$\Delta$ Plasma osmolality (mOsm.kg <sup>-1</sup> )	30.15 (4.17) <sup>A</sup>	31.45 (5.12) <sup>A</sup>	45.50 (8.96) <sup>AB</sup>	38.55 (6.26) <sup>A</sup>	68.60 (11.87) <sup>B</sup>
$\Delta$ Tail width (mm)	-0.21 (0.09) <sup>A</sup>	-0.48 (0.13) <sup>AB</sup>	-0.46 (0.11) <sup>AB</sup>	-0.43 (0.12) <sup>AB</sup>	-0.81 (0.15) <sup>B</sup>
Post-treatment water intake (g)	10.19 (1.01) <sup>A</sup>	12.94 (1.19) <sup>AB</sup>	17.63 (1.62) <sup>BC</sup>	16.63 (1.40) <sup>BC</sup>	21.84 (1.66) <sup>C</sup>

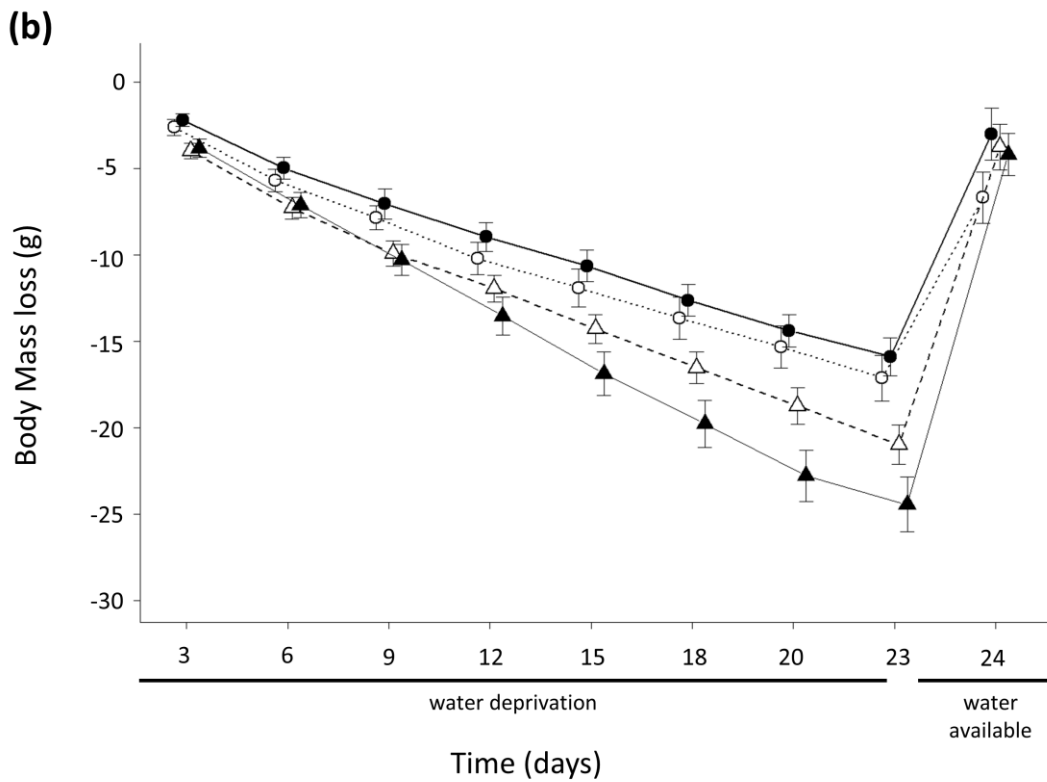
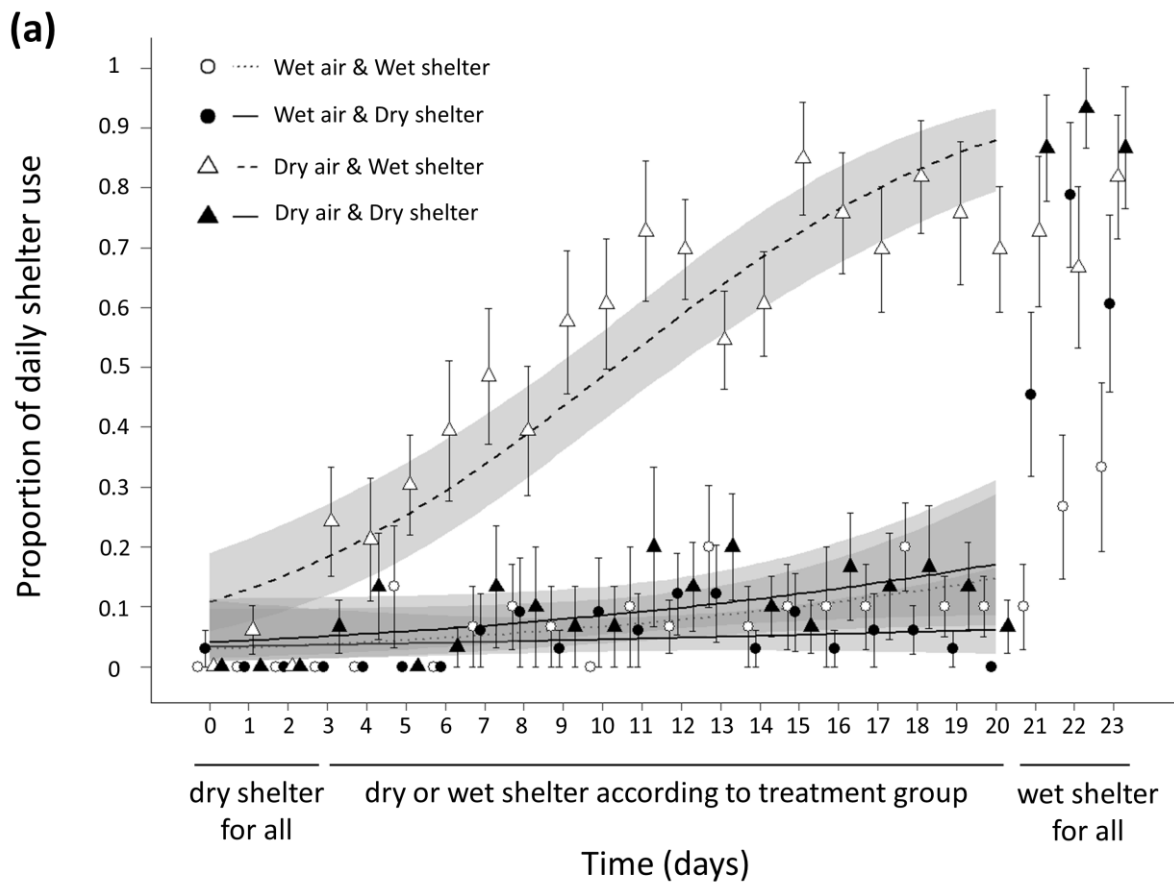
## Figure captions

**Figure 1.** Effects of experimental treatments on behavioural hydroregulation 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  $\pm 1$  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  $\pm 1$  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  $\pm 1$  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.**

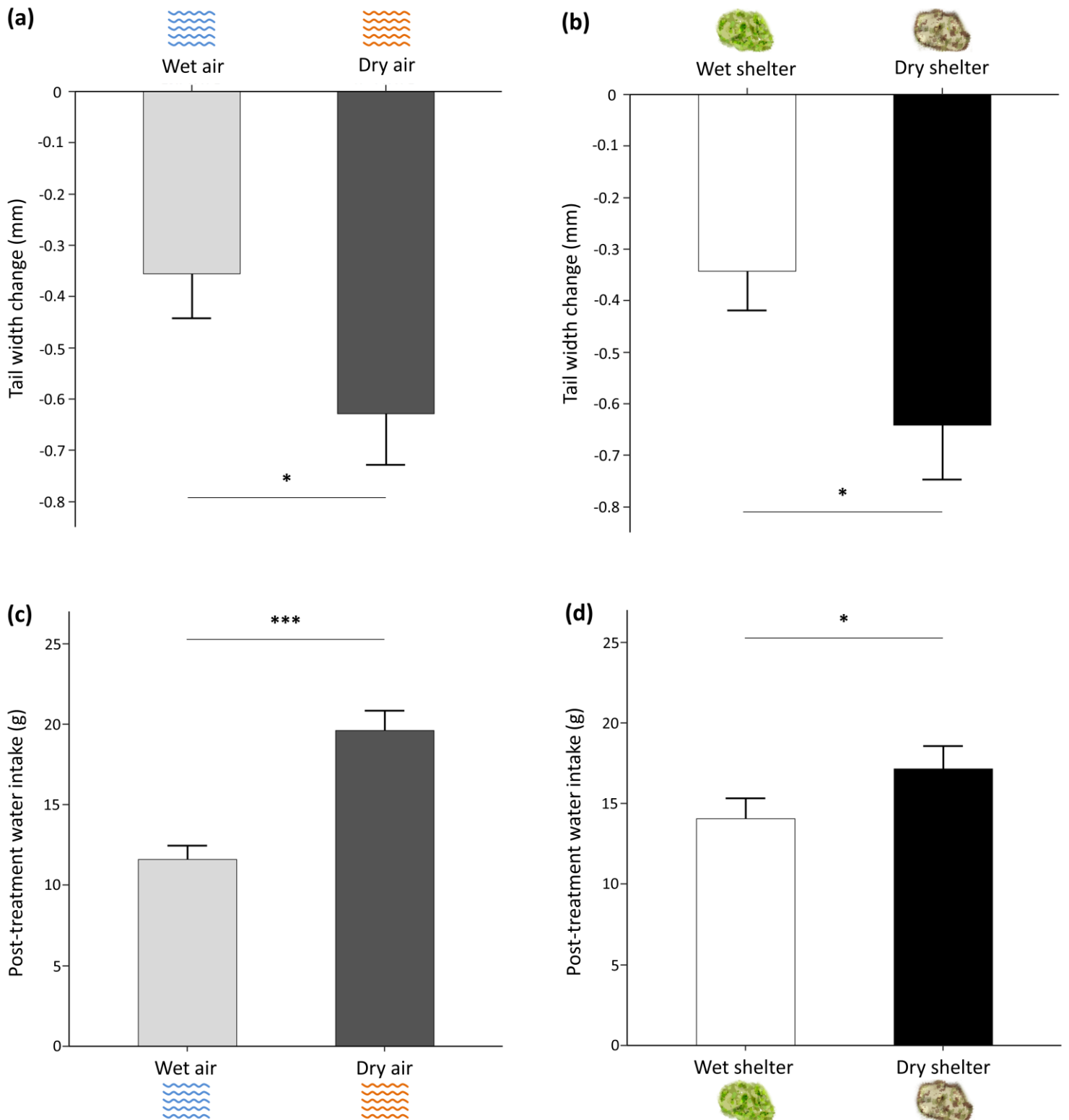


Figure 3.

