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1 **Title:** Additive effects of temperature and water availability on pregnancy in a viviparous lizard

2 **Running title:** environmental constraints and maternal costs

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13 **Key words:** pregnancy; hydration; temperature constraints; oxidative status; osmotic challenge.

14 **Summary statement:** Dehydration during pregnancy negatively affects maternal physiology but
15 has little effect on reproductive output. These effects are additive to temperature regimes and
16 therefore similar in both warmer and colder environments.

17 **Abstract:** One of the greatest current threats to biodiversity is climate change. Yet, an
18 understanding of organismal responses to fluctuations in both temperature and water availability
19 is currently lacking, especially during fundamental life-history stages such as reproduction. To
20 further explore how temperature and water availability impact maternal physiology and
21 reproductive output, we used the viviparous form of the European common lizard (*Zootoca*
22 *vivipara*) in a two-by-two factorial design manipulating both hydric and thermal conditions for
23 the first time. We collected blood samples and morphological measurements during early
24 pregnancy and post-parturition to investigate how water availability, temperature, and a
25 combination of the two influences maternal phenology, morphology, physiology and
26 reproductive output. We found, that dehydration during gestation negatively affects maternal
27 physiological condition (lower mass gain, higher tail reserve mobilization) but has little effect on
28 reproductive output. We found that these effects are mainly additive to temperature regimes with
29 a proportional increase in maternal costs in warmer environments. Our study demonstrates the
30 importance of considering combined effects of water and temperature when investigating
31 organismal responses to climate changes, especially during periods crucial for species survival
32 such as reproduction.

33 **1. Introduction**

34 Predicting how organisms will be impacted by global climate change is frequently viewed in the
35 context of thermal effects on biological and ecological processes (Huey et al., 2012; Kearney et
36 al., 2009; Nowakowski et al., 2018; Parmesan, 2006). However, it is equally important to
37 consider the effects of water availability on biodiversity because water is a fundamental resource,
38 rainfall events are forecasted to be less reliable resulting in reduced water availability for many
39 species (Marvel et al., 2019; Schlaepfer et al., 2017), and predicted changes in ambient
40 temperatures are inextricably linked to changes in precipitation (Alexander et al., 2006). For
41 example, during periods of prolonged drought, increasing temperatures and decreasing water
42 availability frequently covary (Rahmstorf and Coumou, 2011), which can have a pronounced
43 effect on hydration and survival (McLaughlin et al., 2002; Riddell et al., 2019). These combined
44 effects are especially relevant in ectotherms who rely on behavioral or physiological selection of
45 optimal body temperatures that maximize performance, survival, and reproduction (Huey, 1982;
46 Angilletta et al., 2002; Huey et al., 2012; Sinervo et al., 2010) but risk losing greater volumes of
47 water when maintaining higher body temperatures (Claussen 1967; Munsey 1972). Even though
48 dual changes in temperature and water availability are predicted across the globe, it remains
49 largely unknown if and how fundamental life-history stages, such as reproduction, will be
50 affected by these combined changes (Rozen-Rechels et al. 2019).

51 In many environments, precipitation is already unevenly distributed throughout the year
52 or water is rare, and water availability can therefore be limited during periods of reproductive
53 investment (Hao et al., 2018). Previous research has found that changes in rainfall patterns,
54 independent of temperature shifts, are currently challenging reproductive strategies (Visser and
55 Both, 2005) and can have negative impacts on entire ecosystems (McCluney et al., 2012). In
56 juxtaposition, shifts in temperature, independent of altered rainfall patterns, can drastically
57 reduce fitness (Deutsch et al., 2008) and unpredictable temperature regimes have been implicated
58 in large-scale extirpation events (Valladares et al., 2014). The negative effects of fluctuating
59 temperature and water availability may have interactive, detrimental consequences on
60 reproductive events. The physiological impacts of maternal hydration before and after pregnancy
61 have been previously explored (Nelson et al., 1989; Hanson et al., 1994; Gesquiere et al., 2008;
62 Bukovetzky et al., 2012), but far less is known about the direct consequences of hydration during
63 gestation on maternal physiology and reproductive output. Some recent studies have found that

64 reproductive females with limited access to water are able to tolerate dehydration (i.e.,
65 hyperosmolarity, Bruschi et al., 2017; Dupoué et al., 2018a) or can utilize alternative hydric
66 resources during gestation (e.g., bound water, Bruschi et al., 2018) to buffer themselves from
67 changes in water availability. However, these past studies focused on a single physiological
68 challenge, water deprivation, and it is unclear how multiple, concurrent challenges, as predicted
69 under climate change scenarios, may influence maternal physiology during reproduction. Recent
70 work has started to emphasize potential interactions between temperature and water availability
71 in ectotherms (Dupoué et al., 2020a; Rozen-Rechels et al., 2019). Yet, a combined, experimental
72 approach exploring the relationship between thermo- and hydro-regulation during reproduction is
73 currently lacking.

74 To further explore how temperature and water availability impact maternal physiology
75 and reproductive output, we used the viviparous form of the European common lizard (*Zootoca*
76 *vivipara*) in a two-by-two factorial design manipulating jointly hydric and thermal conditions for
77 the first time. We investigated how water availability (\pm water restrictions), temperature (hot or
78 cold daily cycle), or a combination of the two throughout gestation, influences maternal
79 physiology and reproductive output. Unique to our experimental design relative to most thermal
80 biology studies, we used similar water vapor deficits between temperature treatments to
81 eliminate the possibility of increased desiccation at higher temperatures, and therefore only
82 temperature was manipulated. The common lizard is wide-ranging, cold-adapted squamate that is
83 sensitive to water deprivation (Lorenzon et al., 1999; Reichling, 1957). Higher temperatures may
84 accelerate embryonic development, as it does in other viviparous lizards (Deeming, 2004; Du
85 and Shine, 2015), while simultaneously increasing metabolic and subsequent water loss rates of
86 gravid females and consequently negatively impacting maternal physiological condition if they
87 are not able to compensate with greater food intake (Dillon et al., 2010; Lourdais et al., 2017).
88 Conversely, if lower temperatures delay embryonic development, the prolonged gestational
89 period may exacerbate the effects of water restrictions and similarly reduce maternal conditions.
90 To ground truth our study in ecological relevance, we used contrasting water availability
91 treatments that mimic the natural conditions of wild populations with either limited or permanent
92 access to water (Dupoué et al. 2017; Lorenzon et al., 1999). We also selected temperature
93 treatments that reflect either warm, favorable daily conditions for embryonic development (long
94 access to preferred temperature without thermal stress) or cold, suboptimal daily conditions

95 (short access to preferred temperature), and which correspond to the thermal environments
96 experienced by this species in their typical range excluding extremely hot or cold environments.
97 We tested the hypothesis that contrasted temperature regimes and water availability will
98 differentially impact aspects of maternal physiological condition and reproductive output. We
99 used body mass and condition, tail width, plasma osmolality, oxidative stress, antioxidant
100 capacity, and food consumption as measures to evaluate maternal physiological condition. We
101 used gestation length to evaluate reproductive phenology. Finally, we used total litter size and
102 mass, and individual offspring size and mass as measures for reproductive output. We made the
103 following predictions: (1) water restrictions will negatively affect maternal traits but will not
104 impact reproductive output; (2) high temperatures will shorten pregnancy duration but negatively
105 impact maternal physiological condition; and (3) a combination of high temperature and water
106 restrictions during pregnancy will negatively affect both maternal traits and reproductive output.

107

108 **2. Materials and methods**

109 *2.1 Study species*

110 *Zootoca vivipara* is a small-bodied lizard species (adult snout-vent length, SVL 50-75 mm) in
111 the family Lacertidae with the widest geographic range of all terrestrial reptiles (Dely and
112 Böhme, 1984). The species is predominately found in cooler, mesic habitats with permanent
113 access to water across northern Eurasia and occurs in both viviparous and oviparous forms. At
114 our study sites, all females were viviparous. As lecithotrophic viviparous species, the majority of
115 maternal energetic investment occurs during vitellogenesis (Blackburn, 2015). After ovulation
116 embryos remain enveloped in a thin membrane throughout gestation (Heulin, 1990; Panigel,
117 1956), which supports important maternal water transfers (Dauphin-Villemant et al. 1986) and
118 also calcium, and some micronutrients to the developing embryos (Stewart, 1992; van Dyke and
119 Beaupre, 2012). The young of viviparous females are usually born in the membranes and hatch
120 within 1–2 hours of parturition (Lorenzon et al., 2001). Females emerge from over-wintering in
121 late April to early May, with mating and fertilization occurring shortly thereafter (Bleu et al.,
122 2013). Parturition occurs from late June through July and females typically give birth to 6
123 neonates (range 1-12) depending on their body size (Dupoué et al., 2018a; Foucart et al. 2014).

124

125 *2.2 Experimental design*

126 Females used for this study were captured in the first week of June 2018 from five different sites
127 on the Plateau de Millevache (Limousin, France) separated by a maximum distance of 30 km
128 (Table S1). Gravid females (N = 97) were captured by hand and reproductive status was
129 confirmed using abdominal palpation. Non-reproductive females were immediately released at
130 the site of capture. Within 72 h of capture, all females were moved to the Centre d'Etudes
131 Biologiques de Chizé, France. Mass and SVL of lizards were recorded, after which all visible
132 ectoparasites were removed with forceps and females were treated with an anti-parasite spray of
133 Frontline (Merial Inc. Duluth, GA, USA). Females were then housed individually with an
134 12L:12D light regimen in opaque containers (30 × 18 × 10 cm) filled with approximately 1 cm of
135 soil and two different shelters and provided *ad libitum* access to food (*Acheta domestica*) and
136 water. During the 8 h light cycle, a subsurface heating element was provided below one end of
137 each cage to create a thermal gradient (20° – 40°C). All females were held in these conditions for
138 ca. seven days prior to the start of the experiment after which an initial blood sample and
139 scanned image were collected (see below).

140 The experiment was performed from 15-June until parturition for all females so as to
141 mimic stable and consistent differences in temperature and water availability throughout the
142 majority of gestation. Pregnant females maintain stable body temperatures during the day (range:
143 29° – 33°C) by selecting appropriate micro-climates (Le Galliard et al., 2003; Dupoué et al.
144 2017, 2018b). We assigned lizards of similar mean body size to one of two water regimens with
145 permanent access to water or a water restriction (hereafter referred to as wet and dry,
146 respectively) following similar protocols used in previous studies (Dupoué et al., 2018a;
147 Lorenzon et al. 1999). Females in the wet group (n = 49) were provided with water *ad libitum*
148 and water was sprayed twice per day (morning and afternoon) on one wall of each container so
149 that drops could be used for drinking up to 1 h after spraying. Females assigned to the dry group
150 (n = 48) were only provided with water once per day (morning) by similarly spraying one wall of
151 each container. Lizards were further divided into one of two temperature regimens, hereafter
152 called “cold” or “hot”. Each day of the experiment, females in the cold group (n = 49) were held
153 overnight (17h00 to 9h00; 16 hours) at 15°C, during a portion of the day (11h00 to 15h00; 4
154 hours) at 31°C, and spent 4 hours where temperatures were either gradually increasing or
155 decreasing to avoid abrupt changes. Females in the hot group (n = 48) were held overnight
156 (19h00 to 07h00, 12 hours) at 22°C instead of 15°C, during a majority of the day (09h00 to

157 17h00, 8 hours) at 31°C, and similarly were held for the remaining four hours with temperatures
158 either gradually increasing or decreasing (Fig. 1). Final treatment groups of the factorial design
159 were thus hot and dry (n = 24), hot and wet (n = 24), cold and dry (n = 24), or cold and wet (n =
160 25). Stable temperatures were maintained by housing females of similar treatment groups in
161 climatic chambers (Vötsch VP 600, Balingen, Germany). The relative humidity in each chamber
162 was adjusted depending on the temperature so that the water vapor deficit remained constant
163 around 1 kPa (equivalent to 60% RH at 20°C).

164 During the study, each female was provided with live crickets ($400 \pm 20\text{mg}$) every other
165 day which were dusted with vitamin powder containing calcium and vitamin D3. During
166 feedings any remaining crickets were removed and counted in order to quantify the total number
167 and ratio of crickets consumed (i.e., number of crickets consumed / crickets offered) throughout
168 the study. Females were weighed once per week and each cage was visually inspected twice per
169 day for parturition. After parturition, females and their litter (total mass of living and stillborn
170 offspring) were weighed, and a final blood sample and scanned image were collected. Offspring
171 were classified as either living or stillborn in order to quantify the proportion of stillborn (i.e.,
172 number of stillborn / total offspring) for each female. Living neonates were also individually
173 weighed and scanned. Females and neonates were placed into separate individual containers,
174 provided a thermal gradient (20° – 40°C), and *ad libitum* access to food and water, before being
175 released at the maternal site of capture.

176

177 2.3 Blood sample collection and determination of plasma osmolality and oxidative status

178 We used 20 μL hematocrit tubes to collect a $\sim 50 \mu\text{L}$ blood sample from the postorbital sinus of
179 all reproductive females. Total time for capture, restraint, and blood collection did not exceed 3
180 min. We immediately centrifuged the blood samples at 4,000 g for 5 min at 20°C to separate
181 plasma from blood cells, and then aliquoted plasma ($\sim 20 \mu\text{L}$) into separate vials and froze at -
182 30°C until we used them to measure plasma osmolality and oxidative status. We determined
183 plasma osmolality using a vapor pressure osmometer ($\pm 3 \text{ mOsm kg}^{-1}$; model 5100C; Wescor
184 Inc., Logan, Utah, USA). To allow for duplicate readings, plasma samples were diluted (1:1) in
185 reptile Ringer's solution (300 mOsm kg^{-1}) following methods from Secor et al. (1994). Prior to
186 use, we calibrated the osmometer using sealed osmolality standards (100, 290, and 1000 mOsm
187 kg^{-1}) in accordance with factory recommendations. We ran samples in triplicate and used 290

188 mOsm kg⁻¹ standards to check the osmometer for variation after every sample. If the standard
189 varied more than the limits of the osmometer (± 3 mOsm kg⁻¹), the osmometer was recalibrated.
190 If it continued to vary more than the limits, the osmometer head was cleaned and the machine
191 was recalibrated before continuing duplicate analysis beginning with the last sample prior to
192 calibration/cleaning to verify correct measurement of the sample.

193 In order to examine oxidative status, we performed two plasma-based assays within five
194 months of blood collection. To evaluate the activity of organic hyperoxides (an index of
195 oxidative damage; Costantini, 2016), we measured the concentration of reactive oxidative
196 metabolites (ROMs) using d-ROMs colorimetric kits (MC003, Diacron International, Italy).
197 Antioxidant capacity (OXY) was also assessed to determine the non-enzymatic ability of diluted
198 female plasma samples (1:100) to neutralize an oxidant attack from hypochlorous acid
199 (Constantini, 2011) using OXY-absorbent test kits (MC435, Diacron International, Italy). For all
200 assays, we used a pooled plasma sample of randomly selected females (n = 8) three times in each
201 96-well plate to measure coefficients of variation in ROMs (intra-plate: 2.8%; inter-plate: 6.0%)
202 and OXY (intra-plate: 3.4%; inter-plate: 13.8%).

203

204 *2.4 Scanned images*

205 Prior to the experimental manipulation and just after parturition, the ventral surface of all
206 females was scanned at 600 dots per inch using a flatbed scanner (Hewlett-Packard Co., ScanJet
207 3670). From these images, we measured tail width at the 7th sub-caudal scales using Inkscape
208 (v.0.92.3). We also scanned each neonate just after parturition to accurately measure their SVL.
209 Animals were gently restrained by hand behind the neck and the tip of the tail during image
210 collection.

211

212 *2.5 Statistical analyses*

213 Before testing the effect of water availability and temperature on maternal traits and reproductive
214 output, we identified a set of explanatory variables to model data from reproductive females.
215 Larger females typically emerge from overwintering, ovulate, and females with earlier ovulation
216 dates give birth earlier in the year (Bauwens and Verheyen, 1985). Therefore, we included
217 parameters on relative body size (individual SVL minus mean SVL of all females) and relative
218 duration of treatment (individual time in treatment minus mean time in treatment of females

219 within respective treatments). We did not directly manipulate litter size or mass (i.e.,
220 reproductive effort) and therefore included a variable describing individual differences in
221 reproductive effort (standardized residuals from a linear regression of litter mass against SVL) to
222 control for potential allocation differences between females (Dupoué et al., 2020b). We also
223 explored potential interactions between reproductive effort and treatments to test for different
224 allocation trade-offs depending on temperature and water availability. However, these
225 interactions were never significant (all $p > 0.05$) and therefore removed from all models. Starting
226 with a full model, we used stepwise removal of insignificant variables (Arnold, 2010; Zuur et al.,
227 2010).

228 We used linear models to examine the effect of water availability (wet or dry) and
229 temperature (hot or cold) on mass and tail width changes and changes in plasma osmolality in
230 reproductive females throughout the experiment. We used temperature, water availability, and
231 their interaction as fixed effects, and relative body size and reproductive effort as covariates. To
232 model the duration that females were in the experiment and differences in litter size and mass we
233 used models with only relative body size as a covariate. Similarly, we used linear models to
234 compare differences in initial and final OXY and only used relative duration as a covariate.
235 Models used to investigate differences in initial and final ROMs, included that same fixed effects
236 and interactions but did not include any covariates. We used general linear models for ratio and
237 proportion data (crickets consumed and number of stillborn) with temperature, water availability,
238 and their interaction as fixed factors. For ratio of crickets consumed, we used relative body size,
239 relative duration, and reproductive effort as covariates, and for proportion of stillborn, only
240 relative duration was used as a covariate. For offspring data (mass and SVL), we used linear
241 mixed-effect models with maternal treatments (temperature, water, and their interaction) as fixed
242 factors, SVL or mass as covariates, and maternal identity as a random factor to control for non-
243 independence among offspring from the same litter. All analyses were performed using R
244 software (R Development Core Team, version 3.5.2) using the packages ‘nlme’, ‘lattice’, ‘car’,
245 ‘lsmeans’, and ‘MASS’ (Fox and Weisberg, 2011; Pinheiro et al., 2018; Russell, 2016; Sarkar,
246 2008; Venables and Ripley, 2002). Data are presented as mean \pm SEM and differences were
247 accepted as significant at the level of $p < 0.05$.

248

249

250 3. Results

251 3.1 Impact on gestation length and female morphology

252 When comparing the duration that females were in their experimental treatments (time until
253 parturition), we found a significant main effect of temperature regime ($F_{1,92} = 172.04, p < 0.001$),
254 but no main effect of water availability or interaction between the two kinds of treatment ($p >$
255 0.05). Regardless of water availability, reproductive females exposed to hot temperatures gave
256 birth sooner in the study compared to those exposed to cold temperatures. Comparing duration
257 against the relative body size of females further revealed a significant interaction between
258 relative body size and treatment ($F_{1,93} = 13.31, p < 0.001$). The relationship with relative body
259 size was more pronounced in reproductive females exposed to cold ($\beta = -1.68, SE = 0.24, R^2_{adj} =$
260 0.497) compared to hot temperatures ($\beta = -0.73, SE = 0.10, R^2_{adj} = 0.499$; Fig. 2).

261 Females at the beginning of the experiment had similar body mass between treatments
262 ($F_{3,93} = 0.16, p = 0.93$; Table S2). Mass change during the experiment (pre-parturition mass –
263 initial mass) was additively influenced by water availability ($F_{1,91} = 8.39, p = 0.004$) and
264 temperature regime ($F_{1,91} = 32.48, p < 0.001$), but we found no interaction between temperature
265 and water availability ($F_{1,91} = 0.82, p = 0.366$). Females exposed to hot and wet conditions gained
266 less mass during gestation compared to all other treatment groups (Fig. 3C). Similarly, post-
267 partum body condition (body mass adjusted to the mean body size of females: $F_{1,91} = 186.75, p <$
268 0.001) was influenced by water availability ($F_{1,91} = 4.12, p = 0.045$) and temperature treatment
269 ($F_{1,91} = 17.57, p < 0.001$), with females exposed to hot and dry conditions having the lowest post-
270 parturition body condition ($3.21 \pm 0.12g$) and females exposed to cold and wet conditions having
271 the highest post-parturition body condition ($3.73 \pm 0.17g$). We found no interaction between
272 temperature and water availability for post-parturition body condition ($F_{1,91} < 0.01, p = 0.94$).

273 There was no initial difference in tail width among the treatment groups ($F_{3,93} = 0.11, p =$
274 0.95 ; Table S2). Most females had reductions in tail width during the experiment and these
275 decreases were additively influenced by water availability ($F_{1,91} = 17.23, p < 0.001$) and
276 temperature treatment ($F_{1,91} = 5.75, p = 0.018$), with females exposed to hot and dry conditions
277 losing the most tail width and females exposed to cold and wet conditions losing the least tail
278 width (Fig. 3A). We found no interaction between temperature and water availability ($F_{1,91} =$
279 $0.49, p = 0.486$).

280

281 *3.2. Changes in blood parameters*

282 Plasma osmolality change during the experiment was additively influenced by water availability
283 ($F_{1,74} = 16.26, p < 0.001$) and temperature treatment ($F_{1,74} = 7.62, p = 0.007$), with females
284 exposed to hot and dry conditions having the largest increases in plasma osmolality
285 ($308 \pm 2 \text{mOsm kg}^{-1}$ to $323 \pm 3 \text{mOsm kg}^{-1}$ for mean initial and final values, respectively), females in
286 hot and wet, and in cold and dry conditions having intermediary increases in plasma osmolality
287 ($305 \pm 2 \text{mOsm kg}^{-1}$ to $314 \pm 2 \text{mOsm kg}^{-1}$ and $311 \pm 2 \text{mOsm kg}^{-1}$ to $318 \pm 1 \text{mOsm kg}^{-1}$), and females
288 exposed to cold and wet conditions having decreased plasma osmolality ($323 \pm 3 \text{mOsm kg}^{-1}$ to
289 $315 \pm 3 \text{mOsm kg}^{-1}$, Fig. 3B). We found no interaction between temperature and water availability
290 ($F_{1,74} = 2.04, p = 0.157$).

291 There was no initial difference in oxidative stress (ROMs; $F_{3,93} = 0.40, p = 0.75$) or non-
292 enzymatic antioxidant capacity (OXY; $F_{3,93} = 1.32, p = 0.27$) among the treatment groups. After
293 parturition, females in dry conditions throughout pregnancy had significantly higher ROMs ($F_{1,93}$
294 $= 8.34, p = 0.005$), but we found no significant interaction or main effect of temperature regime
295 ($p > 0.05$; Fig. 4A). When examining OXY values after parturition, we found a significant
296 interaction between temperature and water availability ($F_{1,92} = 6.39, p = 0.013$). Within the hot
297 treatments, females in wet conditions had higher OXY values compared to those in dry. In
298 juxtaposition, females exposed to cold and wet treatments had slightly lower OXY values
299 compared to those in dry (Fig. 4B).

300

301 *3.3 Impact on food intake*

302 When evaluating the ratio of crickets consumed during the same period, we found a significant
303 main effect of water availability ($F_{1,94} = 4.16, p = 0.044$) and temperature regime ($F_{1,95} = 36.43, p$
304 < 0.001) but no interaction between the two ($F_{1,90} = 2.76, p = 0.100$). A Tukey's HSD post-doc
305 test revealed that females in the hot temperature regimes ate a greater ratio of crickets than those
306 in the cold regimes ($p < 0.01$) regardless of water regime, and within each temperature regime,
307 females in wet conditions consumed a higher proportion of crickets although not significantly so
308 ($p > 0.05$; Fig. 5).

309

310 *3.4 Impact on reproductive output*

311 We did not detect any significant differences in litter size ($F_{3,93} = 0.49, p = 0.688$), total litter
312 mass ($F_{3,92} = 0.49, p = 0.688$), individual offspring mass ($F_{3,92} = 1.08, p = 0.363$) or snout-vent
313 length ($F_{3,92} = 0.35, p = 0.786$) among the treatment groups. When comparing the proportion of
314 stillborn offspring among treatment groups, we found a significant effect of temperature ($F_{1,95} =$
315 $6.69, p = 0.011$), where females exposed to cold temperatures had three times more stillborn
316 compared to females exposed to hot temperatures, irrespective of water regime ($p < 0.05$; Table
317 1).

318

319 **4. Discussion**

320 Understanding how animals cope with both altered temperature regimes and restricted resources,
321 such as water, will enable us to better predict how they might be affected by anticipated climate
322 change scenarios. This is especially important because in many environments climate warming
323 induces concurrent changes in mean temperature and water availability as well as in the
324 occurrence of extreme weather events (Fischer and Knutti, 2015; Mann et al., 2017; Rahmstorf
325 and Coumou, 2011). Ours is the first study to explore the independent and combined effects of
326 water availability and thermal conditions during gestation in a vertebrate. Our results regarding
327 water availability are mostly consistent with previous work in squamate reptiles: mild
328 dehydration during gestation negatively affects maternal physiology but has little effect on
329 reproductive output (Dupoué et al., 2015, Lourdais et al., 2015). For all traits except anti-oxidant
330 capacity of females, we also found that these effects are additive to temperature regimes and
331 therefore similar in both warmer and colder environments despite very significant effects of
332 thermal conditions on the length of gestation and maternal physiology. Overall, our study adds to
333 a growing body of evidence that water is a fundamental currency used during reproduction. Our
334 study also emphasizes the importance of considering multiple, concurrent physiological
335 challenges when investigating organismal responses to climate changes, especially during
336 fundamental life-history stages such as reproduction.

337 We found that temperature but not water availability affected gestation length, with
338 higher temperatures resulting in a shorter period of pregnancy (Fig. 2). In both temperature
339 regimes smaller females had a longer duration of pregnancy, although this size-dependent
340 gestation length was far more pronounced in the cold treatments. Maternal body size is often an
341 important factor influencing reproductive output and timing (Blueweiss et al., 1978; Díaz et al.,

2012; Iverson et al., 1997; Kiefer et al., 2008), including in this species (Bauwens and Verheyen, 1985). Higher temperatures in our study appear to have accelerated gestation and partially outweighed the size-dependent timing of pregnancy suggesting that small females caught-up for initial differences in the timing of reproduction when provided with warmer environmental conditions. These results are in accordance with previous research showing that higher maternal temperatures result in shorter gestation length and faster embryonic development both in oviparous and viviparous squamates (Dubey and Shine, 2011; Foucart et al., 2018; Loricou et al., 2012, 2013a). They also confirm comparative findings among wild populations showing that climate warming induces phenological acceleration (e.g., shorter gestation time and lower variance in laying date) and significant demographic changes (Massot et al., 2017; Rutschmann et al., 2016).

Previous field and laboratory studies further suggested that increased gestational temperatures can also impact reproductive output, offspring phenotypes, and future fitness (Chamaille-Jammes et al., 2006; Loricou et al. 2013b; Lourdais et al 2004; Ma et al., 2014; Marquis et al., 2008). Although we did not investigate effects on future reproduction and survival, we detected no significant differences in total litter size and mass, and offspring metrics between any of the experimental groups but found that females had a poorer embryonic success in the cold treatment. In ectotherms, embryonic metabolism is temperature-dependent and mothers often behaviorally thermoregulate to maintain optimal temperatures for embryonic development (Farmer, 2003; Gillooly et al., 2002; Loricou et al. 2013a; Lourdais et al., 2013; Mettouris et al., 2017). Extreme temperatures are often associated with decreased reproductive success in ectotherms because they compromise behavioral thermoregulation and may increase physiological stress and decrease performance when they cannot be avoided behaviorally (Clusella-Trullas et al., 2011; Sinervo et al., 2010; Wang et al., 2016). In our study, females were not allowed to thermoregulate but had longer access time to preferred temperature (31°C) in the hot than in the cold treatment. The hot treatment temperature (31°C) was within the range of preferred temperature during gestation and was not meant to induce thermal stress or heat-related embryonic mortality (Le Galliard et al., 2003). Thus, embryonic success was maximized in hot conditions probably because gravid females were given better opportunities to maintain their preferred body temperatures during the prolonged daytime period (Foucart et al., 2018; Le Galliard et al., 2010; Le Henanff et al., 2013; Loricou et al., 2012, 2013a). While females in the

373 cold treatment were provided access to preferred temperatures for 4 hr each day, that window
374 does not appear to have been large enough to effectively maintain embryonic development in
375 these cold-adapted lizards, resulting in a greater proportion of stillborn as seen in wild
376 populations (Le Galliard et al. 2010).

377 Higher temperatures and dry conditions resulted in additively higher tail width loss (Fig.
378 3A) and lower total body mass change during gestation (Fig. 3C). Tail reserves are a very
379 important site for storing accumulated resources in many species of lizards (reviewed in
380 Bateman and Fleming, 2009). However, our study is the first to demonstrate the negative impact
381 of dehydration, enhanced by higher temperatures, on tail morphology. The tails of *Z. vivipara* are
382 composed of both lipids and proteins (Avery, 1974), and increased muscle catabolism at this site
383 may release bound water to offset physiologically damaging levels of dehydration (Brusch et al.
384 2018). Additionally, altered tail reserves likely explain lower body mass after parturition in
385 females exposed to hot and dry treatments. A decreased maternal body condition after
386 reproduction has been shown to challenge future survival due to increased starvation or predation
387 risks if food is not readily available or if weaker mothers are less able to avoid predation (Bleu et
388 al., 2013). These females also had higher plasma osmolality (Fig. 3B) suggesting that both high
389 temperatures and water shortage challenge hydric state during pregnancy and lead to
390 dehydration. Surprisingly, we did not detect an interactive effect. That is, gravid females exposed
391 to hot and dry conditions did not have significantly higher plasma osmolality compared to
392 females who were exposed to cold and wet or hot and wet conditions (Fig. 3B). Females may
393 have increased muscle catabolism to release bound water and support some of the water demands
394 of reproduction (Brusch et al., 2018) as females in dry conditions had higher tail width loss
395 compared to females in wet (Fig. 3A). Alternatively, females may have behaviorally adjusted
396 their behavior to maximize drinking when water was available in the morning as has been shown
397 in this species (Rozen-Rechels et al., 2020).

398 In addition to being more dehydrated, females in dry conditions throughout pregnancy
399 also had higher markers of oxidative damage after parturition, regardless of temperature
400 treatment (Fig. 4A). Unlike all the other dependent variables measured in our study, we found a
401 significant interaction between temperature and water availability on non-enzymatic antioxidant
402 levels. Dehydrated females in the hot treatment had the lowest non-enzymatic antioxidant levels
403 (Fig. 4B) which, coupled with high levels of oxidative damage, equates overall to the highest

404 levels of oxidative stress. These results suggest that the production of potentially damaging
405 oxygen species is linked to hydric resources and it appears that females were able to protect
406 themselves with increased antioxidant production, as shown previously (Dupoué et al., 2020a;
407 Stier et al., 2017). These results further complement recent comparative findings between wild
408 populations, showing that warmer climates may be associated with lower OXY levels in
409 pregnant *Z. vivipara* (Dupoué et al., 2020b), although as demonstrated here, thermal impacts
410 were conditioned by water resources. Importantly, the magnitude of alterations in oxidative
411 status during pregnancy can have serious and delayed consequences on offspring mortality rate,
412 which will need further examinations (Dupoué et al., 2020a). Our study did not explicitly
413 measure such reproductive trade-offs however, and females in the hot treatments were exposed
414 to water restrictions for ~3 weeks, half that of females from the cold treatments. It is worth
415 noting that kinetic changes in oxidative stress are not stable during gravidity (Kouyoumdjian et
416 al., 2019; Speakman and Garratt, 2014), so this may partly explain observed differences post-
417 parturition. Additionally, antioxidants serve a myriad of functions and their production is
418 frequently temporarily mismatched with the creation of reactive oxygen species over acute
419 timescales (Costantini, 2019; Halliwell and Gutteridge, 2015). Females in the hot treatments may
420 not have had enough time or hydric resources to balance their oxidative status and a mechanistic
421 understanding of these results is beyond the scope of this study but deserves further exploration.

422 When free-standing water is limited, some organisms are able to rely almost entirely on
423 metabolic (as a by-product of metabolism) or dietary (that present in food) water (Karasov 1983;
424 Nagy and Gruchacz, 1994; Ostrowski et al. 2002). Because our experimental manipulations
425 began after vitellogenesis, which is considered the main energy allocation period in this species
426 (Foucart et al. 2014), and because metabolism is relatively low in squamate reptiles (Andrews
427 and Pough, 1985), metabolic water was likely negligible (Shoemaker and Nagy, 1977).

428 Additionally, temperature and metabolism are tightly linked in ectotherms (Dillon et al., 2010),
429 but females in the hot treatments, regardless of water availability, had no significant differences
430 in plasma osmolality changes (Fig. 3B), which further supports that metabolic water was
431 negligible in our study. This still leaves the possibility that females in our experiment were able
432 to partially compensate the hydric demands of reproduction through increased food consumption
433 and dietary intake of water. Females in hot treatments ate a greater ratio of crickets (Fig. 5). If
434 dietary water was an important component of hydration, we might expect lizards consuming a

435 greater ratio of crickets to have decreased plasma osmolality, which was not the case in our
436 study. Because we did not detect a significant difference in plasma osmolality change between
437 females in hot and wet treatments and cold and dry treatments, it is difficult to conclude if
438 females were gaining any hydric benefit from food consumption. Overall, these results may
439 suggest that free-standing water is the most important source during pregnancy and essential for
440 maintaining maternal hydration. Especially considering that previous research has found no net
441 osmotic advantage from eating in non-reproductive squamates (Lillywhite, 2017; Murphy and
442 DeNardo, 2019; Wright et al., 2013). Higher temperatures still had an important effect on food
443 consumption, and females with *ad libitum* access to water consumed marginally higher ratios of
444 crickets, indicating a much more complex relationship between the temperature, water
445 availability, and food consumption that merits future investigation.

446 Previous studies examining limited hydric resources during periods of reproductive
447 investment have similarly found that mothers are burdened with the majority of water imbalance
448 in favor of embryonic hydration (Brusch et al., 2018; Dupoué et al., 2015, 2018a). While
449 developing embryos often depend on maintaining water balance for survival (Cagle et al., 1993,
450 Warner and Andrews, 2002), water imbalance is not necessarily physiologically detrimental in
451 all cases. For example, immune enhancement as the result of dehydration has been documented
452 in multiple taxa (Brusch and DeNardo, 2017; Hoang, 2001; Moeller et al., 2013) including
453 mothers and embryos during conflicts for limited hydric resources (Brusch and DeNardo, 2019;
454 Brusch et al., 2017). Future studies should focus on the interplay between osmolality and
455 physiological functions in both mothers and embryos, especially considering that previous
456 explorations of more cryptic physiological traits have elucidated that both mothers and embryos
457 are impacted by dehydration (Brusch et al., 2019).

458 Warmer and drier environmental conditions may challenge reproductive modes in several
459 ways and these lines of research deserve further attention. Future studies should continue to
460 consider the interaction between temperature and water availability and further explore the
461 impacts they have on maternal physiology, reproductive output, and offspring physiology. In
462 particular, developmental temperatures and hydric conditions outside optimal ranges, which may
463 become more frequent under expected climate change scenarios, may lead to increased
464 embryonic mortality (Gibbons et al., 1983; Madsen et al., 2006; Packard et al. 1977; Semlitsch
465 1987; Sperry and Weatherhead, 2008). Therefore, considering both hydro- and thermoregulation

466 under joint conditions of thermal and hydric stress will be essential for understanding
467 ectothermic responses to predicted shifts in temperature and water availability (Rozen-Rechels et
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469

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483

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485

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488

489 **5. References**

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801

802 **Table 1** – Details of reproductive output from female *Zootoca vivipara* held in either hot and dry
803 (HD), hot and wet (HW), cold and dry (CD), or cold and wet (CW) conditions throughout
804 pregnancy (mean \pm SEM).

Treatment	Litter mass (g)	Offspring mass (mg)	Offspring SVL (mm)	Proportion stillborn
HD	1.15 \pm 0.09	17.33 \pm 0.17	19.51 \pm 0.08	0.03 \pm 0.02
HW	1.10 \pm 0.09	17.02 \pm 0.18	19.63 \pm 0.09	0.07 \pm 0.03
CD	1.17 \pm 0.09	16.93 \pm 0.19	19.50 \pm 0.11	0.11 \pm 0.03
CW	1.09 \pm 0.09	17.38 \pm 0.18	19.59 \pm 0.08	0.19 \pm 0.05

813 Litter mass represents the total mass of living and stillborn offspring from each female.

814 Offspring mass and snout-vent-length (SVL) is only for living neonates from each female.

815 Proportion of stillborn represents the number of stillborn divided by the total litter size (living
816 and stillborn).

817 **Figure legends**

818 **Figure 1 – Hourly temperature regimens of reproductive female *Zootoca vivipara* held in**
819 **climatic chambers.** Lizards were exposed to stable ($\pm 0.1^\circ\text{C}$) hot (closed triangles; $n = 48$) or
820 cold (open circles, $n = 49$) temperature treatments during pregnancy.

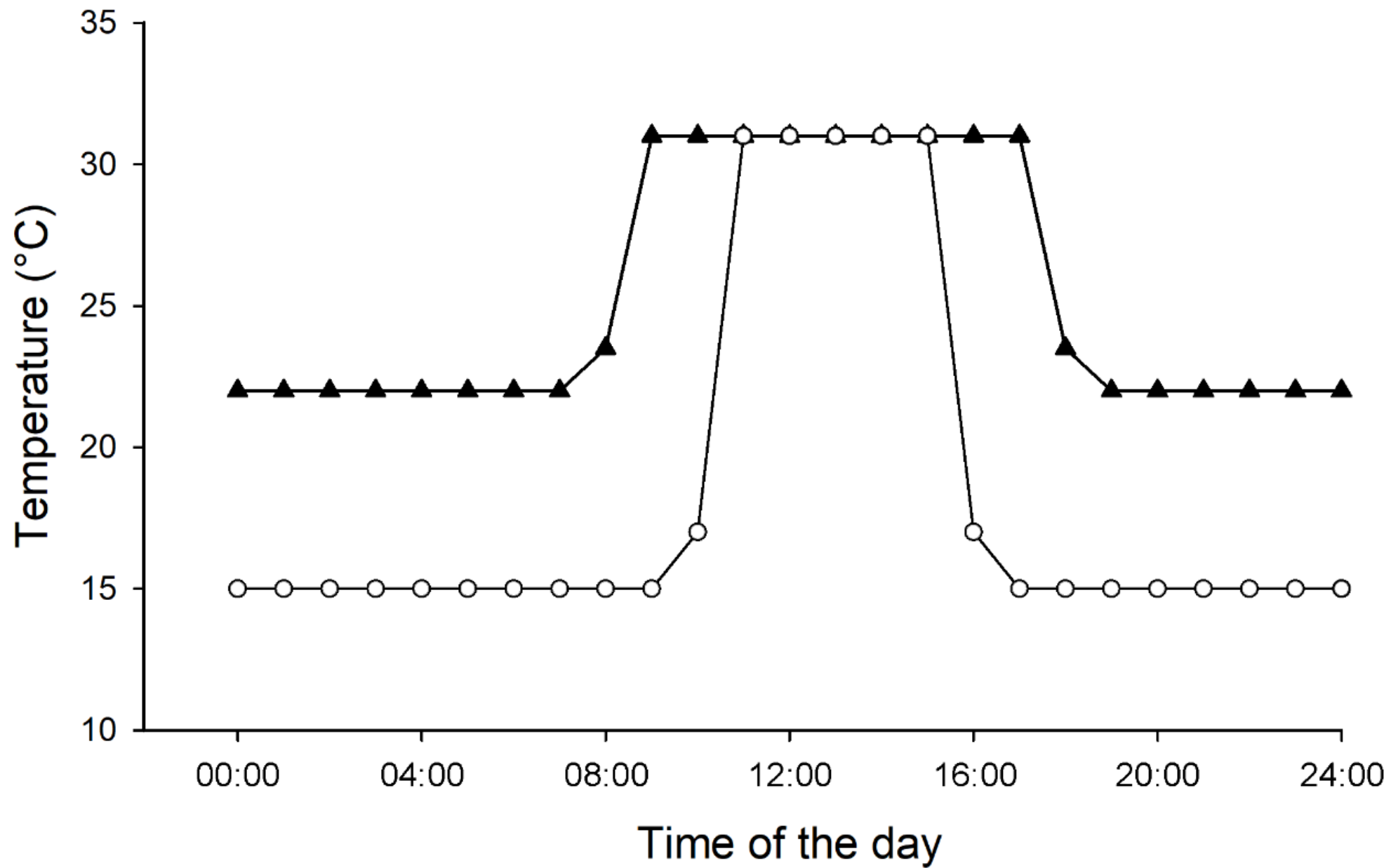
821
822 **Figure 2 – Relationships between body size and gestation length in temperature treatments.**
823 Duration of experimental regimen (number of days from beginning of the experiment to
824 parturition) and snout-vent length (mm) measured in reproductive female *Zootoca vivipara*
825 exposed to hot (open circles, dashed line; $n = 48$) or cold (closed circles, solid line; $n = 49$)
826 temperature regimes during pregnancy. A line of best fit is included for each significant
827 relationship ($p < 0.05$).

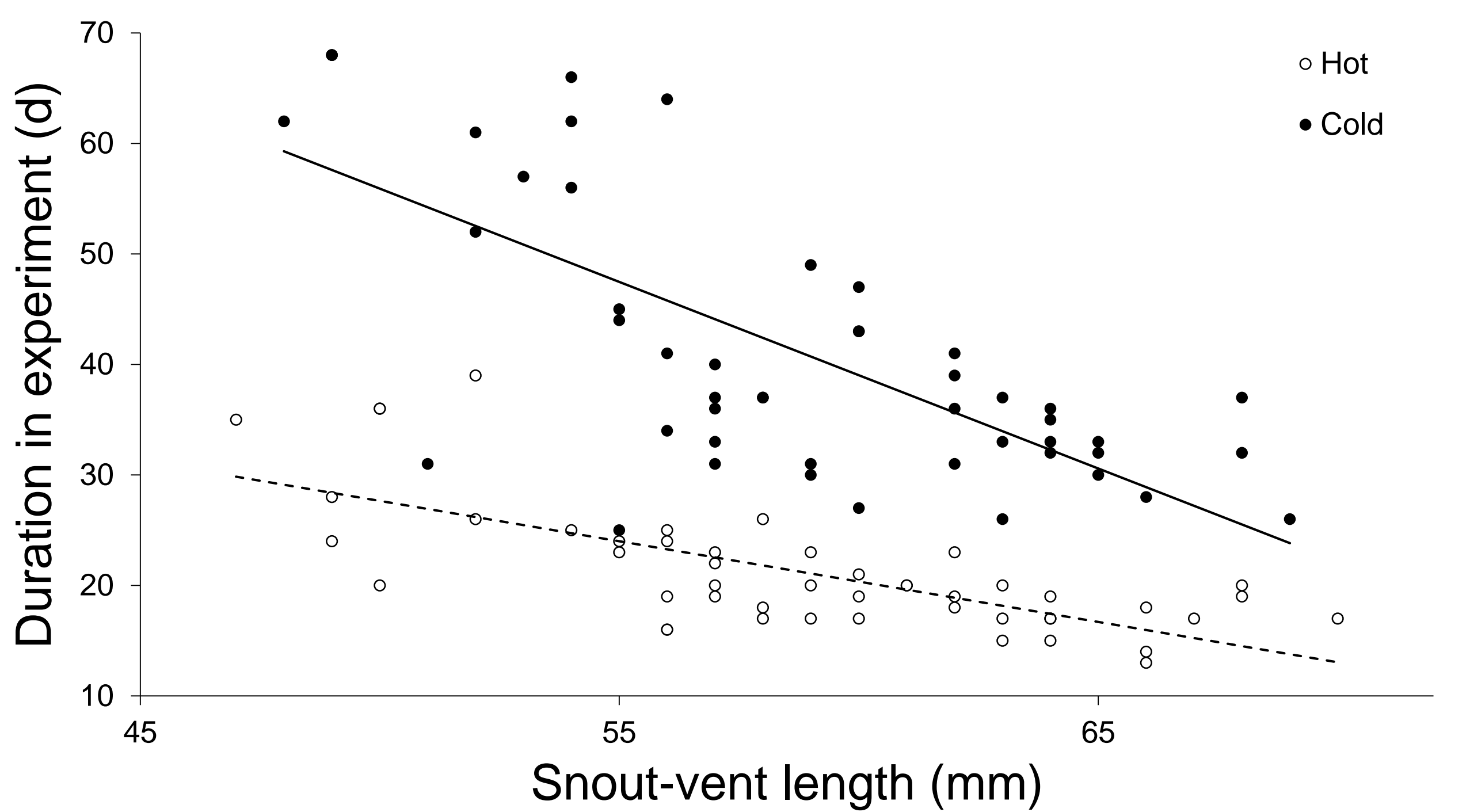
828
829 **Figure 3 – Average morphological and physiological changes in reproductive female**
830 ***Zootoca vivipara*.** Change in tail width (A), plasma osmolality (B), and body mass (C) measured
831 from beginning of the experiment to parturition for lizards held in either hot and dry (HD; $n =$
832 24), hot and wet (HW; $n = 24$), cold and dry (CD; $n = 24$), or cold and wet (CW; $n = 25$)
833 conditions throughout pregnancy. Error bars represent ± 1 SEM. Different letters indicate
834 significant differences among groups ($p < 0.05$; Tukey's HSD post-hoc test).

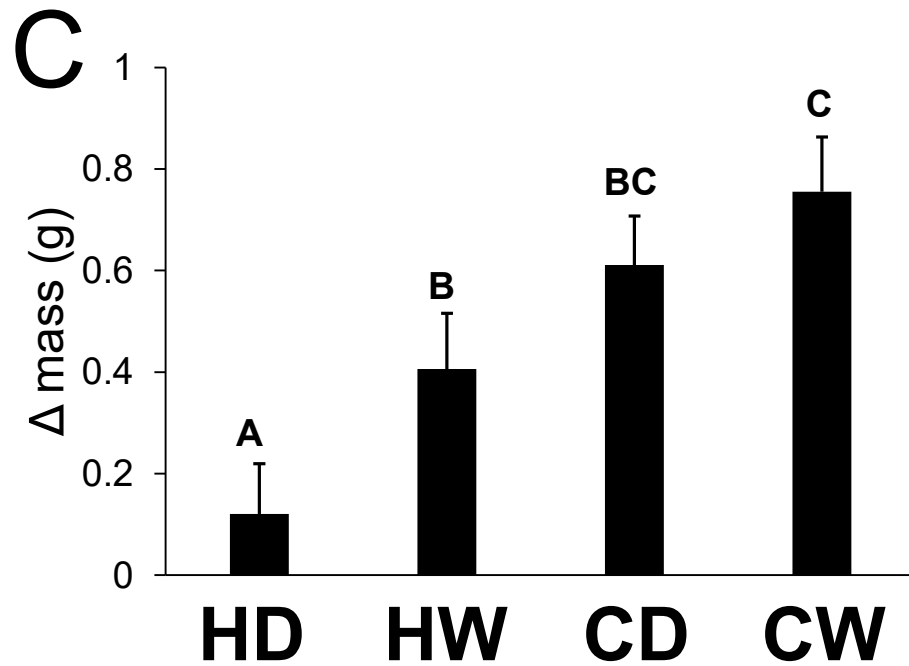
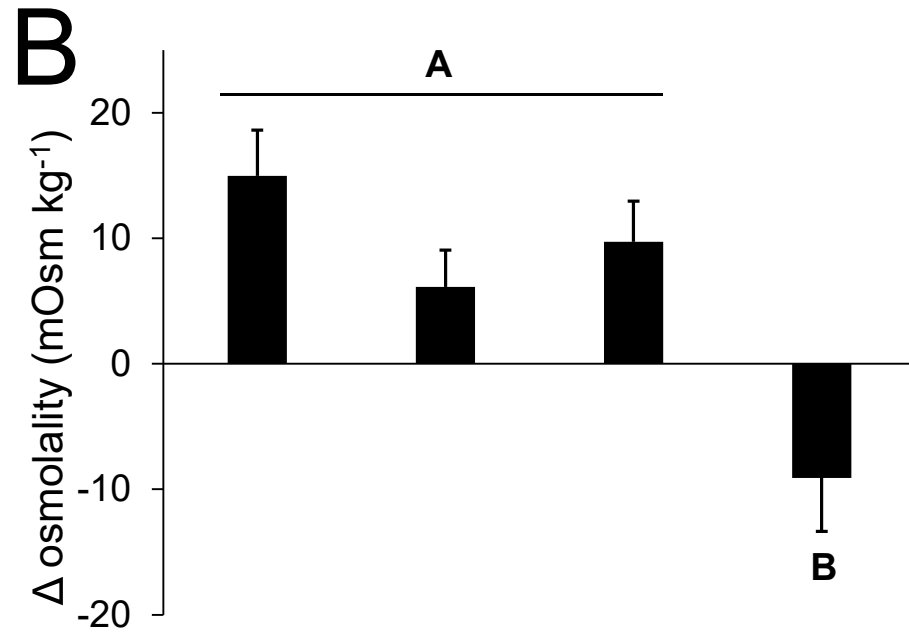
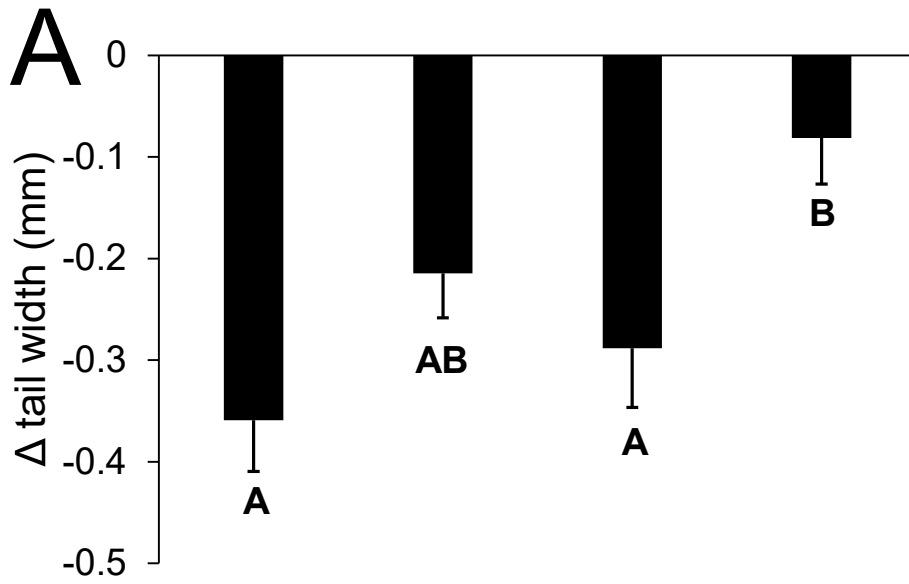
835
836 **Figure 4 – Average markers of oxidative status in post-reproductive female *Zootoca***
837 ***vivipara*.** Oxidative damage [ROMs; A] and non-enzymatic antioxidant capacity [OXY; B])
838 measured in lizards held in either hot and dry (HD; $n = 24$), hot and wet (HW; $n = 24$), cold and
839 dry (CD; $n = 24$), or cold and wet (CW; $n = 25$) conditions throughout pregnancy. Error bars
840 represent ± 1 SEM. Oxidative damage was significantly higher ($p < 0.05$) in females held without
841 water regardless of temperature. Different letters indicate significant differences among groups
842 (Tukey's HSD post-hoc test). There was a significant temperature by water availability
843 interaction effect ($p < 0.05$) on non-enzymatic antioxidant capacity.

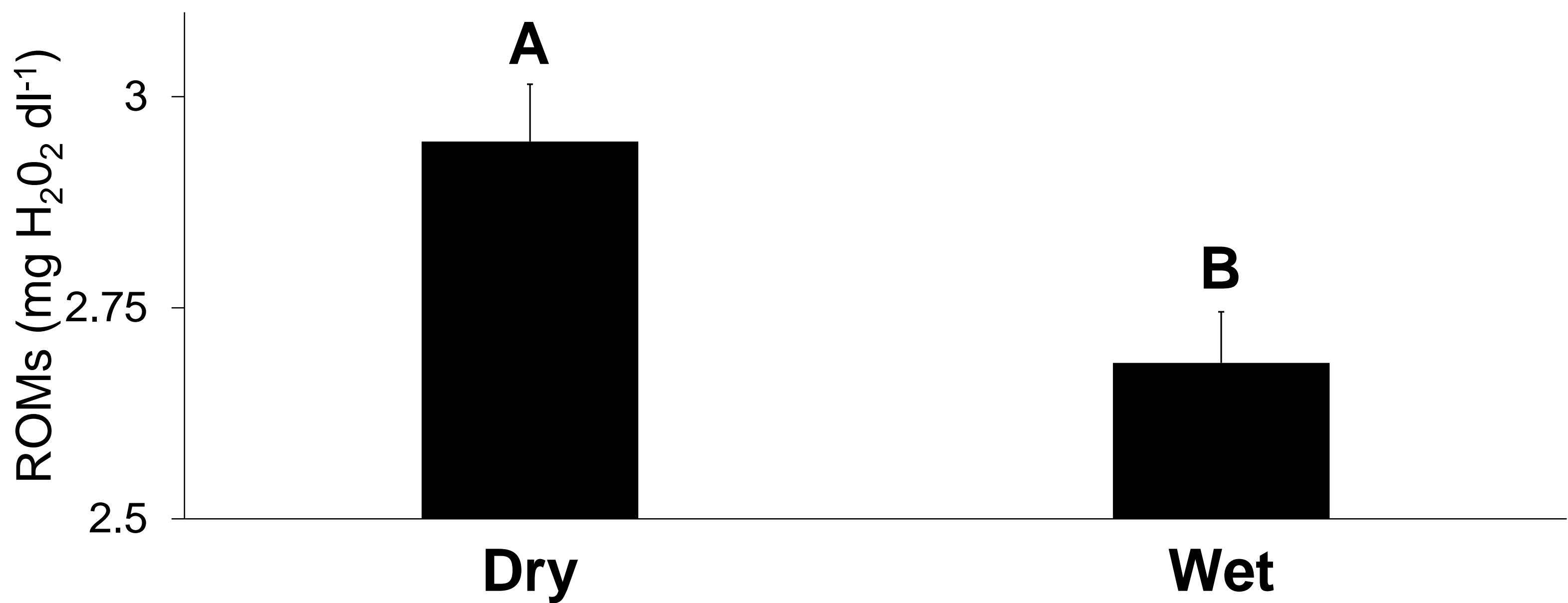
844
845 **Figure 5 – Average ratio of crickets consumed by reproductive female *Zootoca vivipara*.**
846 Lizards were held in either hot and dry (HD; $n = 24$), hot and wet (HW; $n = 24$), cold and dry
847 (CD; $n = 24$), or cold and wet (CW; $n = 25$) conditions throughout pregnancy. Error bars

848 represent ± 1 SEM. Different letters indicate significant differences among groups ($p < 0.05$;
849 Tukey's HSD post-hoc test).







A**B**