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## **Down-regulating the stress axis: Living in the present while preparing for the future**

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1 **Down-regulating the stress axis: living in the present while preparing for**  
2 **the future**

3

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24

## 25 **Abstract**

26 The measurement of glucocorticoid (GC) hormones provides us with a window into the stress  
27 physiology of vertebrates and the adaptative responses they use to cope with predictable and  
28 unpredictable changes in the environment. Baseline GCs inform us about the metabolic  
29 demands they are subject to at that point in their yearly life-history stage, whereas GC changes  
30 (often increases) in response to acute challenges inform us on their capacity to cope with more  
31 immediate environmental challenges. However, baseline GC levels and the kinetics of GC  
32 responses to acute stressors can vary substantially among and within species, depending on  
33 individual characteristics (age, sex, condition, life-history stage). In addition, a thorough  
34 understanding of the stress status of an animal requires moving beyond the measurement of  
35 GCs alone by focusing on downstream measures of metabolic activation, such as oxidative  
36 stress. Here, we evaluated the changes in blood cortisol and oxidative stress markers in wild  
37 adult Columbian ground squirrels (*Urocitellus columbianus*), following a 30-min capture-  
38 handling stress performed in mid-late June. Measurements were taken when males were post-  
39 reproductive and preparing for hibernation and adult females were weaning litters. We found  
40 three key results. First, the time-course of GC increase was markedly slower (by an order of  
41 magnitude) than what is currently reported in the literature for most species of mammals, birds  
42 and reptiles. Second, there were marked differences in the male and female response, linked to  
43 differences in life-history stage: females close to weaning had abolished GC responses,  
44 whereas post-reproductive males did not. Third, there were mild to moderate increases in  
45 oxidative damage and decreases in oxidative defenses in response to our short-term challenge,  
46 consistent with the idea that short-term acute metabolic activation may carry physiological  
47 costs. However, these changes were not correlated to the changes in GCs, a novel result  
48 suggesting a disconnect of hormonal stress response and oxidative damage.

49

50 **Keywords:** aging, HPA axis, mammal, oxidative stress, rodent, sciurid, stress response

## 51 **Introduction**

52 The hypothalamic-pituitary-adrenal (HPA) axis is a key feature of adaptation allowing  
53 vertebrates to respond to predictable and unpredictable changes in their environment (Boonstra,  
54 2004; Boonstra et al., 2014; Crespi et al., 2013; Wingfield and Romero, 2011). In response to  
55 internal and external stressors, activation of the HPA axis ultimately results in the secretion of  
56 glucocorticoid hormones (GCs) into the blood stream. These target a wide range of tissues,  
57 regulating the expression of some 10% of the genome and control major functions related to  
58 metabolism, growth, repair, immunity, reproduction, and the management of energy balance  
59 and resource allocation (Le et al., 2005). Thus unsurprisingly, a large body of literature has  
60 focused on GCs as central mediators of the relationship between vertebrates and their  
61 environment (Boonstra, 2004; Harris, 2020; Sapolsky et al., 2000; Wingfield and Romero,  
62 2011). On one hand, studies have considered increased baseline levels as indicative of periods  
63 of increased organism workload or solicitation (Landys et al., 2006), such as during  
64 reproduction (Bonier et al., 2009, 2011; Boonstra et al., 2001b; Delehanty and Boonstra, 2011),  
65 in years or situations of high predation pressure (Boonstra, 2004; Boonstra et al., 1998; Clinchy  
66 et al., 2013), periods of low resource availability (Kitaysky et al., 2007, 1999), or times of  
67 intra- and interspecific competition (e.g. situations of peer-pressure or social challenge) (Creel  
68 et al., 2013; Lemonnier et al., 2022). On the other, studies have used acute increases in GCs in  
69 response to unpredictable environmental changes as indicative of individuals' capacity to cope  
70 with change while maintaining homeostasis (Breuner et al., 1999; Delehanty and Boonstra,  
71 2009; Sapolsky et al., 2000; Wingfield et al., 1992).

72 Whether evaluated at baseline and or when stress-induced, GCs measured in blood have  
73 several key advantages compared to other matrices (reviewed in Sheriff et al., 2011). First, GCs  
74 in blood directly reflect their production by the adrenal cortex, which is not the case when  
75 estimated from breakdown products such as fecal cortisol metabolites, for which prior

76 validation studies need to be conducted (Touma and Palme, 2005). Second, blood allows  
77 simultaneously measurement of other physiological indicators (glucose levels, free fatty acids,  
78 hematocrit, oxidative stress, immune profiles) which might vary with individual condition, life-  
79 history stage, or in response to a stressor (Boonstra et al., 1998; Clinchy et al., 2004; Davis et  
80 al., 2008; Majer et al., 2019; Viblanc et al., 2018b). Finally, using blood allows for the detailed  
81 study of the kinetics and inter-individual variation of GCs changes (onset and termination of  
82 the response) to unpredictable disturbances, providing us with a window into how GC  
83 responses may be adaptively fine-tuned to environmental stochasticity (Angelier and  
84 Wingfield, 2013).

85         A central problem in measuring GCs from blood – especially in wild vertebrates  
86 (Sheriff et al., 2011) – is being able to assess true baseline levels, since any measurement of  
87 GCs is likely to be confounded by the stress of capture and handling. Two common  
88 methodologies are typically used to deal with the problem. The first involves a hormonal-  
89 challenge: circulating GC levels are initially reduced to their minimum levels by the  
90 experimental injection of dexamethasone – a synthetic GC agonist that inhibits GC secretion  
91 through negative feedback on the HPA axis and reduction of ACTH (adrenocorticotrophic  
92 hormone) release from the pituitary – and subsequently increased to their maximal levels  
93 through the experimental injection of ACTH (Boonstra et al., 1998, 2001b; Gist and Kaplan,  
94 1976; Rich and Romero, 2005; Smith and Bubenik, 1990). However, many species are  
95 naturally resistant to dexamethasone during parts of their life cycles and/or show hypo- or  
96 hyper-GC responses to ACTH stimulation (Boonstra et al., 1998; Boonstra and McColl, 2000;  
97 Chrousos et al., 1982; Harlow et al., 1992; Keightley and Fuller, 1996; McDonald et al., 1986;  
98 Sapolsky and Altmann, 1991; Taymans et al., 1997). The second methodology uses the actual  
99 disturbance caused by capture to assess its impact on the HPA axis response (Kenagy and Place,  
100 2000; Place and Kenagy, 2000). Initial blood samples are acquired as rapidly as possible and

101 subsequent blood samples are performed at discreet time intervals on the same or different  
102 animals. The kinetics of increase in blood GCs is then examined. Whereas this approach may  
103 not capture “true” baseline GC levels, initial sampling approximates “near-baseline” levels  
104 (Romero and Reed, 2005), as indicated by significant increases from initial sampling that can  
105 be observed in subsequent time steps. However, studies that used this approach found blood  
106 GC levels to increase significantly anywhere between <1 and 5 minutes of initial sampling  
107 (Boonstra et al., 2001a; Boonstra and Singleton, 1993; Dawson and Howe, 1983; Mizrahi et  
108 al., 2001; Müller et al., 2006; Romero et al., 2008; Romero and Reed, 2005; Viblanc et al.,  
109 2018a; Wada et al., 2007), indicating that species-specific validations of the course of increase  
110 of GCs in the blood stream are required to design field protocols and interpret data.

111 We applied this approach to a 30-min acute capture stress protocol in a wild mammal,  
112 the Columbian ground squirrel (*Urocitellus columbianus*). Columbian ground squirrels are  
113 sciurid rodents that hibernate approximately 8-9 months of the year, and exhibit a brief period  
114 of activity (from mid- to late-April until late-July to mid-August) during which reproduction,  
115 molt and fattening for subsequent hibernation occur (Dobson et al., 1992; Dobson and Murie,  
116 1987). We sampled animals during the month of June after the mating period had passed, during  
117 which males were post-reproductive and females were weaning litters. Our study had three  
118 objectives.

119 First, we aimed to characterize the kinetics of ground squirrel plasma GC response to  
120 acute stress. Specifically, we wished to determine the time-course over which plasma GC  
121 increased following an acute stress, and the timing at which GCs differed from near-baseline  
122 levels. In addition to total cortisol levels (the main GC in Columbian ground squirrel; Bosson  
123 et al., 2009), we measured changes in corticosteroid binding globulin (CBG) and estimated  
124 changes in free cortisol levels. About 90% of blood GCs are bound to CBG in vertebrates

125 (Delehanty et al., 2020, 2015; Desantis et al., 2013) and only free GCs are thought to be  
126 biologically active (Breuner et al., 2013; Perogamvros et al., 2012).

127         Second, we wished to explore inter-individual variation in GC responses in regards to  
128 individual sex, mass, and age. We expected to find sex and mass-specific differences in baseline  
129 and capture-induced GCs levels, given the different energy requirements of post-reproductive  
130 males and lactating females at that time. Females around weaning were expected to sustain  
131 higher circulating levels of GCs due to the high energy demands of reproduction at that time  
132 (Crespi et al., 2013; Haase et al., 2016; Kenagy and Place, 2000), the requirements of GCs for  
133 offspring development (Edwards et al., 2021; Edwards and Boonstra, 2018), or on the contrary,  
134 might have buffered high levels of GCs to limit offspring exposure to GCs (Brunton et al.,  
135 2008; Thoman et al., 1970). In addition, several studies have shown GC levels to increase with  
136 age (including in fecal signatures of Columbian ground squirrels; Sosa et al., 2020), either as a  
137 reflection of increased reproductive and metabolic demands (Crespi et al., 2013), or as a  
138 reflection of a senescing HPA axis and a deterioration of negative feedback control (Gupta and  
139 Morley, 2014; Sapolsky and Altmann, 1991).

140         Third, we moved beyond the HPA-axis alone in characterizing the consequences of an  
141 acute capture on stress physiology. For this, we focused on oxidative stress, which arises as a  
142 consequence of the imbalance between pro-oxidant molecules (mostly due to the by-production  
143 of reactive oxygen species (ROS) during mitochondrial respiration; Metcalfe and Alonso-  
144 Alvarez, 2010) and the body's antioxidant defenses. Interactions among the HPA-axis, the  
145 mitochondria, and ROS (Du et al., 2009; Manoli et al., 2007; Picard et al., 2014) make oxidative  
146 stress an appealing candidate mechanism to consider in addition to GC responses in the study  
147 of vertebrate "stress status" and "stress responses" to changing environments. Increased  
148 oxidative stress has been associated with increased GCs in vertebrates at baseline levels  
149 (Costantini et al., 2011), and GCs have been shown to down-regulate antioxidant defenses in



150 some studies (Briehl and Baker, 1996; McIntosh et al., 1998). However, while some studies  
151 have considered the dynamics of oxidative stress in relation to acute challenges in vertebrates  
152 (Djordjevic et al., 2010; Haussmann et al., 2012; Huber et al., 2017; Jacobs et al., 2021;  
153 Nadeem et al., 2006), only a handful of such studies have been conducted in the wild (Cohen  
154 et al., 2007; Costantini et al., 2012; Stier et al., 2019), primarily on birds, and results have been  
155 conflicting. Thus, we considered markers of plasma oxidative damage and antioxidant defense,  
156 and their relation to the GC response, to further our understanding of the time course of changes  
157 in oxidative stress that occur during responses to acute challenges in wild mammals.

158

## 159 **Methods**

### 160 *Study population*

161 We studied Columbian ground squirrel responses to acute stress in a population intensively  
162 monitored from 1992 to the present in Sheep River Provincial Park, Alberta, Canada (50° 38'  
163 10.73" N; 114° 39' 56.52" W; 1524 m; 2.3 ha). Ground squirrels were marked with numbered  
164 metal ear tags (National Band and Tag Company, Newport, KY) when weaned (or at first  
165 capture for immigrants) and a unique black mark on the pelage using human hair dye (Clairol®  
166 Hydrience, Clairol Inc., New York, USA) was applied annually to allow individual recognition  
167 in the field. The entire population was trapped annually using peanut butter bait and live-traps  
168 (chipmunk size, Tomahawk Live Trap, Hazelhurst, WI, USA), first at spring emergence (in  
169 April), and monitored subsequently throughout the breeding season until weaning of the young  
170 in late-June to early July.

171

### 172 *Capture and standardized stress protocol*

173 During June 2022, we subjected 23 ground squirrels (12 males, 11 females) to a standardized  
174 capture stress protocol. All males were post-reproductive and females were weaning litters. All

175 means below are given  $\pm 1$  S.E. Males in our study were aged  $2.6 \pm 0.2$  years ([min – max] =  
176 [2 – 4]), whereas females averaged  $6.3 \pm 0.9$  years ([2 – 10]).

177 Individuals were identified from a distance in the field using binoculars, and a single  
178 baited trap was deployed on the focal animal's typical home range close to the burrow opening  
179 where it was seen entering upon approach of the experimenter. We then waited for the  
180 individual to exit the burrow and enter the trap. As soon as the trap door shut, we started a  
181 stopwatch, and timed the duration taken to acquire the initial blood sample, always under 3  
182 minutes ( $2.11 \pm 0.07$  min; [1.67 – 2.67]; N = 22). The animal was then loosely hand-held,  
183 secured in a denim-cloth bag that covered its eyes, during the 30-minute duration of the stress  
184 protocol. Subsequent blood samples were acquired at 5 ( $5.79 \pm 0.09$ ; [5.15 – 6.92]; N = 22), 10  
185 ( $10.82 \pm 0.11$ ; [9.92 – 12.18]; N = 21), 20 ( $20.79 \pm 0.14$ ; [19.63 – 22.40]; N = 23) and 30 ( $31.90$   
186  $\pm 0.43$ ; [30.30 – 38.25]; N = 22) minutes following capture. For simplicity, we refer to those  
187 timings as T<sub>3</sub>, T<sub>5</sub>, T<sub>10</sub>, T<sub>20</sub> and T<sub>30</sub> time points. We acquired blood (100  $\mu$ L or less each time)  
188 from the saphenous veins of the animal (both legs) using a 27G needle fitted to an 0.5 mL  
189 heparinized syringe. Not all bleeds were successful, explaining the slight variation in sample  
190 sizes. Sampling was carried out throughout the day between 10:00 and 20:00 and balanced  
191 between males (9:54 – 18:49) and females (10:29 – 19:58).

192 Following the 30-min capture, we recorded the mass of the animal to the nearest g  
193 (using a Pesola spring-slide scale; Pesola Ag, Baar, Switzerland), before releasing the animals.  
194 Males averaged  $590.8 \pm 17.9$  g ([485 – 695]), and females averaged  $523.2 \pm 15.3$  g ([460 –  
195 600]).

196

### 197 *Cortisol measurements*

198 Individuals' HPA response to the standardized capture were evaluated by measuring plasma  
199 levels of total cortisol, corticosteroid-binding globulin (CBG) and free cortisol. Total cortisol

200 levels were measured by radioimmunoassay (RIA) using a commercially available kit  
201 (ImmuChem™ Coated Tube Cortisol <sup>125</sup>I RIA Kit; MP Biomedicals, LLC, Orangeburg, NY,  
202 USA). Details on the method for Columbian ground squirrels are provided in Roth et al. (2022).  
203 Samples were run together and the mean intra-assay coefficient of variation was < 5%.

204 Corticosteroid-binding globulin (CBG) was measured for each sample as the maximum  
205 corticosteroid-binding capacity (MCBC) with the saturated ligand method described in  
206 McDonald et al. (1981) (see Delehanty and Boonstra, 2009; Roth et al., 2022; for details on  
207 ground squirrels). The intra- and inter-assay coefficients of variation based on the pools were  
208 7.58% and 6.81%, respectively.

209 Free cortisol was calculated based on the measurement of MCBC in each sample, the  
210 CBG equilibrium dissociation constant (Kd), and a value for albumin. For this calculation we  
211 used the formula of Barsano and Baumann (1989). Cortisol is bound with high affinity to CBG  
212 (a protein specific for binding cortisol) and low affinity to albumin (Westphal 1986, Peters  
213 1996, Perogamvros et al. 2012). Cortisol affinity is the major determinant of the bioavailable,  
214 free fraction. Though albumin has high capacity for cortisol and other blood components, it is  
215 thus nonspecific for them, acting as a temporary reservoir and, in the case of cortisol, rapidly  
216 releases it. We used a Kd value of 5.1 nM calculated for Columbian ground squirrels  
217 (Delehanty et al., 2015). We assumed the albumin concentration and proportion of cortisol  
218 bound to albumin were comparable to the values calculated for Arctic ground squirrels (2.54 g  
219 albumin/100 mL plasma; proportion bound = 0.19; Boonstra and McColl, 2000).

220

### 221 *Oxidative stress measurements*

222 Individuals' oxidative stress response to the standardized capture was assessed through global  
223 measures of oxidative damage (d-ROMs test) and antioxidant defences (OXY adsorbent test)

224 occurring in plasma (Diacron International, Grosseto, Italy), as previously done in this species  
225 (for details see Roth et al., 2019; Viblanc et al., 2018b). All sample measurements were  
226 duplicates. Intra-plate variation was 4.37% and 5.58% for ROMs and OXY, respectively. Inter-  
227 plate variation based on a standard sample repeated over plates was 8.81% and 13.17% for  
228 ROMs and OXY, respectively.

229

### 230 *Statistical analyses*

231 All analyses were run in R v 4.1.2 (R Core Team; R Foundation for Statistical Computing,  
232 2021). First, we tested whether total cortisol, MCBC and free cortisol at baseline levels (< 3  
233 min - T<sub>3</sub>) (dependent variables in separate linear models, LM) differed with individual sex, age  
234 and mass (independent covariates). We then tested whether individual baseline levels (T<sub>3</sub>) of  
235 total cortisol, MCBC and free cortisol, affected the magnitude of the HPA response calculated  
236 as the difference between T<sub>30</sub> and T<sub>3</sub> levels ( $\Delta_{T_{30}-T_3}$ ), or as the area under (or above) the curve  
237 (AUC) calculated from T<sub>3</sub> for each response (see Online Supplementary Materials), using  
238 linear models.

239 Second, we analyzed the kinetics of all HPA measures (total cortisol, MCBC, free  
240 cortisol) and oxidative stress (ROM and OXY) responses to capture stress using Linear Mixed  
241 Models (LMMs, 'lme4' package v.1.1-27.1 in R; Bates et al., 2015). We specified total cortisol,  
242 MCBC, free cortisol, ROM and OXY levels as dependent variables in separate LMMs, and  
243 sampling timing (T<sub>3</sub>, T<sub>5</sub>, T<sub>10</sub>, T<sub>20</sub>, T<sub>30</sub> minutes) as the independent factor of interest. We  
244 included individual sex, age, and mass at capture as covariates in the models, to account for  
245 potential condition and age effects on the HPA and oxidative stress responses to acute stress.  
246 We also included the interactions between *sex\*sampling timing*, *mass\*sampling timing*, and  
247 *age\*sampling timing* to test for potentially different kinetics in physiological responses to acute  
248 capture stress, but removed these from the final models if not significant. Significant contrasts

249 among time points were evaluated using post-hoc Tukey Honest Significant Difference (HSD)  
250 tests ('multcomp' package v.1.4-18 in R; Hothorn et al., 2008), and percentage point differences  
251 (magnitude of effect sizes) between time points are given based on the least-square means  
252 obtained for each separate time point using the 'lsmeans' package v.2.30-0 in R (Lenth, 2016).

253 Thirdly, we tested the correlations ( $r$ ; Pearson correlation test) between HPA (total  
254 cortisol, MCBC, free cortisol) and oxidative (ROM, OXY) responses focusing on the  
255 correlations between baseline ( $T_3$ ) levels on one hand, and correlations between AUCs on the  
256 other.

257 Model residuals inspection using Q-Q plots ('fitdistrplus' package v.1.1-6 in R;  
258 Delignette-Muller and Dutang, 2015) indicated no substantial departures from normality.  
259 Sample sizes varied depending on success at drawing blood or depending on laboratory  
260 analyses (minimum volume), and are indicated for each analysis as the total number of data  
261 points ( $n_{\text{obs}}$ ) and the total number of individuals concerned ( $N_{\text{ID}}$ ). Values presented in text are  
262 means  $\pm$  1 SE unless otherwise indicated.

263

## 264 ***Ethics***

265 Authorizations for conducting research and collecting samples in Sheep River  
266 Provincial Park were obtained from Alberta Environment and Parks and Alberta Tourism,  
267 Parks, and Recreation. Animal care was carried out in accordance with Auburn University  
268 IACUC protocols, with additional approvals from the University of Calgary.

269

## 270 **Results**

### 271 ***Baseline cortisol and relationship to cortisol increase***

272 Average baseline ( $T_3$ ) total cortisol levels, MCBC and free cortisol levels were not  
273 affected by individual age or mass (LMs; all  $F < 1.76$ ; all  $P > 0.20$ ). However, whereas total

274 cortisol levels did not differ between males and females ( $F_{1,17} = 1.12$ ;  $P = 0.305$ ; Fig. 1A)  
275 MCBC levels in males were 43% higher than females ( $F_{1,17} = 2.55$ ;  $P = 0.021$ ; Fig. 1B). This  
276 resulted in free cortisol levels being 103% higher in females ( $F_{1,17} = 6.19$ ;  $P = 0.023$ ; Fig. 1C).

277 Baseline levels of total cortisol and MCBC were negatively related to the  $\Delta_{T_{30}-T_3}$   
278 responses (LM; total cortisol: slope =  $-0.72 \pm 0.21$ ;  $F_{1,19} = 11.68$ ;  $P = 0.003$ ; MCBC: slope = -  
279  $0.37 \pm 0.15$ ;  $F_{1,18} = 6.17$ ;  $P = 0.023$ ) and overall AUC responses (total cortisol: slope =  $-0.72 \pm$   
280  $2.49$ ;  $F_{1,19} = 14.07$ ;  $P = 0.001$ ; MCBC: slope =  $-0.97 \pm 0.40$ ;  $F_{1,19} = -2.41$ ;  $P = 0.026$ ; see Online  
281 Supplementary Materials). Baseline levels of free cortisol did not significantly affect the  
282  $\Delta_{T_{30}-T_3}$  (slope =  $-0.51 \pm 0.31$ ;  $F_{1,18} = 2.72$ ;  $P = 0.117$ ) response, but unsurprisingly were  
283 significantly related to the overall AUC (slope =  $-2.03 \pm 0.79$ ;  $F_{1,19} = 6.55$ ;  $P = 0.019$ ),  
284 suggesting that animals with higher baseline levels expressed less of a cortisol response over  
285 the entire 30-minute period.

286

### 287 *HPA activity in response to capture*

288 Males and females differed markedly in their total cortisol increases in response to  
289 capture (LMM; *time x sex*:  $F_{4,78.20} = 13.88$ ;  $P < 0.0001$ ;  $n_{\text{obs}} = 109$ ;  $N_{\text{ID}} = 23$ ; Fig 2A), but  
290 neither was influenced by individual age ( $F_{1,18.52} = 0.30$ ;  $P = 0.59$ ) nor mass ( $F_{1,18.58} = 0.42$ ;  $P$   
291  $= 0.52$ ). The increase in total cortisol levels was not significantly different from baseline ( $T_3$ )  
292 values before 10 min in males and 20 min in females (see Tukey HSD tests; Fig 2A), and  
293 appeared to plateau as soon as 10 minutes in males and 20 minutes in females. In males, total  
294 cortisol reached maximal values (149% over baseline) after 20 minutes of capture. In contrast,  
295 in females, maximal values (31% over baseline) were reached after 30 minutes.

296 MCBC showed no significant change over the 30-minute capture, handling, and  
297 bleeding challenge in males and females (LMM; *time*:  $F_{4,73.93} = 0.57$ ;  $P = 0.684$ ; *time x sex*:  
298  $F_{4,73.93} = 0.25$ ;  $P = 0.906$ ;  $n_{\text{obs}} = 107$ ;  $N_{\text{ID}} = 23$ ; Fig 2B), nor was it influenced by age ( $F_{1,15.25} =$

299 2.19;  $P = 0.160$ ) or mass ( $F_{1,15.40} = 2.08$ ;  $P = 0.169$ ). Males, on average, had markedly higher  
300 (+ 46%) MCBC levels than females ( $F_{1,15.24} = 13.69$ ;  $P = 0.002$ ; Fig 2B).

301 As a result, the kinetics of plasma free cortisol closely followed that of plasma total  
302 cortisol, but the difference between males and females was markedly less for free than for total  
303 cortisol levels (LMM; *time x sex*:  $F_{4,75.34} = 6.21$ ;  $P = 0.0002$ ;  $n_{\text{obs}} = 107$ ;  $N_{\text{ID}} = 23$ ; Fig 2C).  
304 Again, it was not influenced by age ( $F_{1,17.63} = 0.00$ ;  $P = 0.964$ ) or mass ( $F_{1,17.72} = 0.03$ ;  $P =$   
305  $0.874$ ). In males, free cortisol levels reached 720% over baseline levels at T<sub>30</sub>: the increase in  
306 free cortisol could be detected at T<sub>10</sub> and plateaued as of 20 minutes post-capture (Tukey HSD;  
307 Fig 2C). In females, the increase in free cortisol levels reached 80% over baseline values after  
308 30 minutes, and were only different from baseline values at T<sub>30</sub> (levels at T<sub>20</sub> and T<sub>30</sub>, were not  
309 different; Tukey HSD; Fig 2C). Although average responses indicated a general increase in  
310 cortisol levels with different dynamics in males and females, there were marked inter-  
311 individual differences in total cortisol, MCBC and free cortisol responses to capture, but those  
312 did not appear to be explained by age or mass (see Online Supplementary Materials).

313

#### 314 ***Oxidative stress response to captures***

315 Changes in reactive oxygen metabolites (ROM) exhibited similar kinetics in males and  
316 females (LMM; *time x sex*:  $F_{4,69.68} = 0.50$ ;  $P = 0.734$ ,  $n_{\text{obs}} = 108$ ;  $N_{\text{ID}} = 23$ ). When interactions  
317 were removed from the model, ROM increased slightly over the 30-minute capture, but only  
318 T<sub>20</sub> levels differed from T<sub>3</sub> levels (Tukey HSD;  $z = 2.86$ ;  $P = 0.035$ ) and marginally from T<sub>10</sub>  
319 levels (Tukey HSD;  $z = 2.76$ ;  $P = 0.046$ ) (Fig 3A), being 13.3 and 12.7 % higher. This appeared  
320 to be due to a few individuals showing a pattern of increased ROM levels at T<sub>20</sub> (see Online  
321 Supplementary Materials). ROM levels were not significantly affected by sex ( $F_{1,18.73} = 0.03$ ;  
322  $P = 0.859$ ), mass ( $F_{1,18.86} = 0.98$ ;  $P = 0.334$ ), but tended to decrease with age (LMM; *age*:

323 estimate =  $-4.30 \pm 2.26$ ;  $F_{1,18.70} = 3.62$ ;  $P = 0.072$ ). The effect of age became significant if sex  
324 was removed from the model (estimate =  $-4.58 \pm 1.57$ ;  $F_{1,19.84} = 8.47$ ;  $P = 0.009$ , Fig 4).

325 Changes in total antioxidant capacity (OXY) did not differ between males and females  
326 (LMM; *time x sex*:  $F_{4,69.39} = 1.11$ ;  $P = 0.358$ ,  $n_{\text{obs}} = 108$ ;  $N_{\text{ID}} = 23$ ). When interactions were  
327 removed from the model, OXY varied marginally over the 30-min capture (LMM;  $F_{4,81.86} =$   
328  $2.18$ ;  $P = 0.079$ ;  $n_{\text{obs}} = 104$ ;  $N_{\text{ID}} = 23$ ) with a 7% decrease in OXY levels between T<sub>3</sub> and T<sub>5</sub>  
329 (Tukey HSD;  $z = -2.91$ ;  $P = 0.029$ ; Fig 3B). OXY levels were not affected by sex ( $F_{1,18.56} =$   
330  $1.88$ ;  $P = 0.187$ ), mass ( $F_{1,18.67} = 0.61$ ;  $P = 0.444$ ), or age ( $F_{1,18.54} = 2.30$ ;  $P = 0.147$ ), and  
331 removing those variables from the model did not change the results. There were marked inter-  
332 individual differences in ROM and OXY responses to capture at an individual level, but that  
333 did not appear to be explained by age or mass (See Online Supplementary Materials).

334

### 335 *Correlations between oxidative stress and cortisol responses to captures*

336 At baseline (T<sub>3</sub>) levels, ROM were not related to total ( $r = 0.19$ ,  $t = -0.85$ ,  $df = 19$ ,  $P = 0.408$ )  
337 or free ( $r = -0.16$ ,  $t = -0.69$ ,  $df = 19$ ,  $P = 0.498$ ) cortisol levels, but were positively related to  
338 MCBC levels ( $r = 0.60$ ,  $t = 3.27$ ,  $df = 19$ ,  $P = 0.004$ ). OXY were not related to total cortisol ( $r$   
339  $= 0.19$ ,  $t = 0.86$ ,  $df = 19$ ,  $P = 0.401$ ), or MCBC ( $r = -0.21$ ,  $t = -0.95$ ,  $df = 19$ ,  $P = 0.357$ ) levels  
340 at T<sub>3</sub>, but were positively related to free cortisol levels ( $r = 0.46$ ,  $t = 2.25$ ,  $df = 19$ ,  $P = 0.037$ ).

341 Because individual HPA and oxidative stress responses varied widely among  
342 individuals, the areas under the curves (AUC) calculated for total cortisol ( $303.62 \pm 66.73$ , [-  
343  $243.46 - 879.30$ ], MCBC ( $-30.20 \pm 26.50$ , [- $270.40 - 290.24$ ], free cortisol ( $78.41 \pm 18.48$ , [-  
344  $59.48 - 248.56$ ]), ROM ( $47.26 \pm 16.19$ , [- $142.96 - 169.53$ ]), and OXY ( $-66.43 \pm 25.89$ , [-  
345  $237.09 - 135.11$ ]) were either positive (significant increase), negative (significant decrease), or  
346 null (no significant change). ROM AUCs were not related to total cortisol ( $r = -0.02$ ,  $t = -0.06$ ,  
347  $df = 18$ ,  $P = 0.949$ ), MCBC ( $r = -0.20$ ,  $t = -0.87$ ,  $df = 18$ ,  $P = 0.396$ ) or free cortisol ( $r = -0.12$ ,



348  $t = -0.52, df = 18, P = 0.610$ ) AUCs. Similarly, OXY AUCs were not related to total cortisol ( $r$   
349  $= 0.31, t = 1.39, df = 18, P = 0.182$ ); MCBC ( $r = -0.145, t = -0.62, P = 0.542$ ), or free cortisol  
350 ( $r = 0.29, t = 1.82, df = 18, P = 0.086$ ) AUCs.

351

## 352 **Discussion**

353 Our initial objective was to determine the kinetics of HPA responses to acute stress and time  
354 thresholds below which cortisol levels could be considered near baseline (*i.e.*, unstressed)  
355 values in this species. Studies have typically found blood GC levels to increase significantly  
356 anywhere between <1 and 5 minutes of initial sampling (Boonstra et al., 2001a; Boonstra and  
357 Singleton, 1993; Hare et al., 2014; Romero and Reed, 2005), suggesting that initial sampling  
358 under 3 minutes may be a safe guideline (Romero and Reed, 2005). In Columbian ground  
359 squirrels, free plasma cortisol levels did not increase substantially in magnitude before at least  
360 5 minutes, only becoming significantly different from baseline ( $T_3$ ) levels at 10 minutes at the  
361 earliest (males) and 30 minutes at the latest (females). These results show that, in mammals,  
362 species-, sex- and breeding-stage-specific validations are required to correctly identify  
363 maximal times for obtaining near-baseline cortisol levels in the field: in our case 3 minutes  
364 may not necessarily be required, if trapping the animal is complicated by field conditions.

365 The HPA axis response to acute capture stress differed markedly between the sexes.  
366 First, baseline ( $T_3$ ) (all within 3 min of capture) total cortisol, MCBC and free cortisol were,  
367 respectively, higher, lower, and higher, in females than males. Higher baseline free cortisol  
368 (103% higher than males) suggests that female metabolic demands in mid-late June were higher  
369 than males. The same pattern was found in Arctic ground squirrels, *Urocitellus parryii*  
370 (Boonstra et al., 2001a). This makes sense as females were nearing the end of lactation, the  
371 period of highest energy demand for small mammals (Speakman, 2008). High free cortisol  
372 levels would ensure continuous mobilization of energy stores (Viblanco et al., 2018a) to meet

373 higher metabolic demands around the time of weaning (for cortisol-metabolic rate associations,  
374 see Haase et al., 2016; for higher metabolic rates with reproductive investment in Columbian  
375 ground squirrels, see Skibieli et al., 2013), and may be critical for offspring programming and  
376 development (Dantzer et al., 2013; Edwards et al., 2021; Edwards and Boonstra, 2018). In  
377 contrast, males had 43% higher MCBC levels than females, resulting in free cortisol levels  
378 being very low ( $3.32 \pm 1.52$  ng/mL). Thus, the male HPA axis appeared geared towards minimal  
379 glucocorticoid secretion and energy savings at a period of time when reproduction has passed,  
380 aggression subsided (Murie and Harris, 1988), and stocking-up to survive the pending winter  
381 hibernation is important. MCBC did not appear to vary significantly over the 30-min capture,  
382 which is consistent with observations that in many species CBG levels respond to acute stress  
383 over the course of hours, rather than minutes (Delehanty & Boonstra 2009, 2011; Fletcher &  
384 Boonstra 2006; Edwards et al. 2022).

385         The HPA response to acute stress was marked in males, but virtually non-existent in  
386 females. One explanation is that HPA responses to stressors in females were suppressed to favor  
387 reproduction close to the period of weaning. Attenuated HPA responses to acute stress may be  
388 selected to avoid increased energy investments in self-maintenance at a time when reproductive  
389 demands are high, which would entail favoring reproduction over survival. Seasonal mammals  
390 with short active seasons have been suggested to resist acute stress in favor of reproduction  
391 (Boonstra et al., 2001b; Wingfield and Sapolsky, 2003). For instance, in Arctic ground  
392 squirrels, female levels of free cortisol were found to be low during pregnancy and lactation,  
393 though this study investigated baseline rather than cortisol responses to acute stressors  
394 (Edwards and Boonstra, 2016). Preventing high levels of cortisol in the milk of lactating  
395 females would safeguard their nursing of offspring from potentially negative, programming  
396 effects of cortisol at an important time in their development (Stead et al., 2022).

397 A high commitment to offspring is similarly found in several bird species suggested to  
398 fine-tune acute stress responses according to the reproductive value of their brood, down-  
399 regulating stress responses when brood value is high (Bókony et al., 2009; Lendvai et al.,  
400 2007; Viblanc et al., 2016). However, this explanation is somewhat at odds with previous  
401 findings of female Columbian ground squirrels favoring self-maintenance over reproduction  
402 (e.g. skipping breeding when spring condition is low; Rubach et al., 2016), and suggests that  
403 the trade-off between investments in current and future reproduction is complex and may  
404 depend on the time within the breeding season at which it is measured. Further, baseline free  
405 cortisol and MCBC levels, but not total cortisol levels, were negatively and significantly related  
406 to the magnitude of cortisol responses in our study, suggesting that individuals with higher  
407 baseline levels mounted lower acute stress responses. Given that females maintained higher  
408 baseline cortisol levels than males, it is perhaps not surprising that the responses they mounted  
409 to acute captures were lower than males. At a very basic level, cortisol increases appetitive  
410 behavior (Dallman et al., 1993), at a stage where energy demands on mothers are highest.

411 Studies on oxidative responses to acute stress vary, with some studies showing either  
412 an upregulation in antioxidant enzymatic activity with no change (or slight decreases) in  
413 oxidative damage markers (Stier et al., 2019), or increased oxidative damage and decreased  
414 antioxidant defenses (Hausmann et al., 2012; Majer et al., 2019; Treidel et al., 2013). Our  
415 results appear to conform to the latter pattern, though caution is suggested by the small effect  
416 sizes that we detected. ROM levels appeared to increase moderately (some 13%) after 20  
417 minutes of capture, and OXY levels decreased by 7% within the first minutes of capture.

418 ROM levels and age were negatively related in our study. This was unexpected, since  
419 most of senescence theory rests on the free-radical theory of ageing (Beckman and Ames, 1998;  
420 Kirkwood and Kowald, 2012), with increasing accumulating damage as organisms age (but see  
421 (Speakman and Selman, 2011), and our previous studies on oxidative stress dynamics failed to

422 document age effects on ROM (Viblanco et al., 2018b). However, the effect was small and only  
423 significant when other variables were removed from the model, and should be considered with  
424 caution. Interestingly, we did not detect a signature of HPA axis deterioration with age in our  
425 study. This is consistent with a recent comparative study across five ground-squirrel species,  
426 concluding that a functional, integrated stress axis is a prerequisite for life that does not undergo  
427 senescence (Delehanty and Boonstra, 2023). However, this explanation cannot account for the  
428 age-related increase in fecal cortisol metabolites we found in a previous study of Columbian  
429 ground squirrels (Sosa et al., 2020). This suggests that fecal and plasma GCs do not always tell  
430 the same story (Sheriff et al., 2010), even when species-specific validations of hormone levels  
431 are conducted in both matrices as is the case in our species (Bosson et al., 2009).

432         While a previous study on Japanese quail (*Coturnix japonica*) showed causal effects of  
433 experimentally increased glucocorticoids on oxidative stress (increase in ROM levels; Majer  
434 et al. 2019), there was no evidence in our study that increased cortisol during captures was  
435 associated with increased ROM or decreased OXY levels. We found positive correlations  
436 between ROM and MCBC levels ( $r = 0.60$ ) and between OXY and free cortisol levels at  
437 baseline values ( $r = 0.46$ ), which contrast with studies documenting higher ROM levels with  
438 increased baseline glucocorticoids (Costantini et al., 2011), though many of these studies  
439 examined oxidative damage in response to experimentally increased – and not naturally  
440 occurring – levels of glucocorticoids. Perhaps one explanation to our results is that the  
441 increased levels of MCBC with ROM occur as a response to buffer the effects of elevated  
442 circulating GCs on oxidative stress (Spiers et al. 2015), which would be consistent with the  
443 observation that OXY levels also increased with free cortisol levels. However, this would not  
444 explain why we found no relationship between ROM levels and total cortisol. Alternatively,  
445 hepatic synthesis of CBG may itself be a cause of increased oxidative stress, which remains to  
446 be tested. Thus, while there is an intuitive appeal in considering the relationship between the

447 glucocorticoid response and the oxidative stress response to acute stress, it is clear that the link  
448 between these is not straightforward. Future studies should aim at better understanding the  
449 interplay between the HPA and oxidative stress, especially since these relationships may be  
450 bidirectional, oxidative stress in the brain possibly being an early trigger of HPA activation (see  
451 Lemonnier et al., 2022; Li and Xia, 2020).

452       Taken together, our results highlight the critical need to determine and interpret the time  
453 course of changes in total cortisol, MCBC and free cortisol in other mammals, since responses  
454 are likely to vary widely depending on species-specific ecology, life history and previous  
455 experience with capture. For instance, long-term studies such as ours may also have shaped  
456 ground squirrel HPA responses to hand-held captures through habituation (Cyr and Romero,  
457 2009). Where validation studies or the acquisition of samples close to capture can not be  
458 performed (e.g. capture of larger mammals in winter or far the the site of processing), a  
459 standardization approach should be taken, or alternative tissues considered (Sheriff et al.,  
460 2011). In any case, a proper interpretation of data will require knowledge on the biological state  
461 of the monitored individuals (e.g., age, sex, breeding status).

462

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483

484

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814 **Figure captions**

815 **Fig. 1.** Sex differences in plasma baseline (sampled under 3 minutes) levels (ng/ml) of (A) total  
816 cortisol, (B) maximum corticosteroid binding globulin (MCBC) and (C) free cortisol in  
817 Columbian ground squirrels.

818

819 **Fig. 2.** Kinetics of plasma levels (ng/ml) of (A) total cortisol, (B) maximum corticosteroid  
820 binding globulin (MCBC) and (C) free cortisol over a 30-minute capture stress in Columbian  
821 ground squirrels. Sampling time points were under 3 minutes (mean  $\pm$  s.e. =  $2.11 \pm 0.07$ ), at 5  
822 ( $5.79 \pm 0.09$ ), 10 ( $10.82 \pm 0.11$ ), 20 ( $20.79 \pm 0.14$ ) and 30 ( $31.90 \pm 0.43$ ) minutes. Boxplots  
823 show the median (bold line) and first and third quartiles. Raw data points are overlaid. Boxplots  
824 not sharing the same letter are significantly different at  $P < 0.05$  (Tukey HSD). The letters  
825 compare timepoints for males (uppercase) and females (lowercase).

826

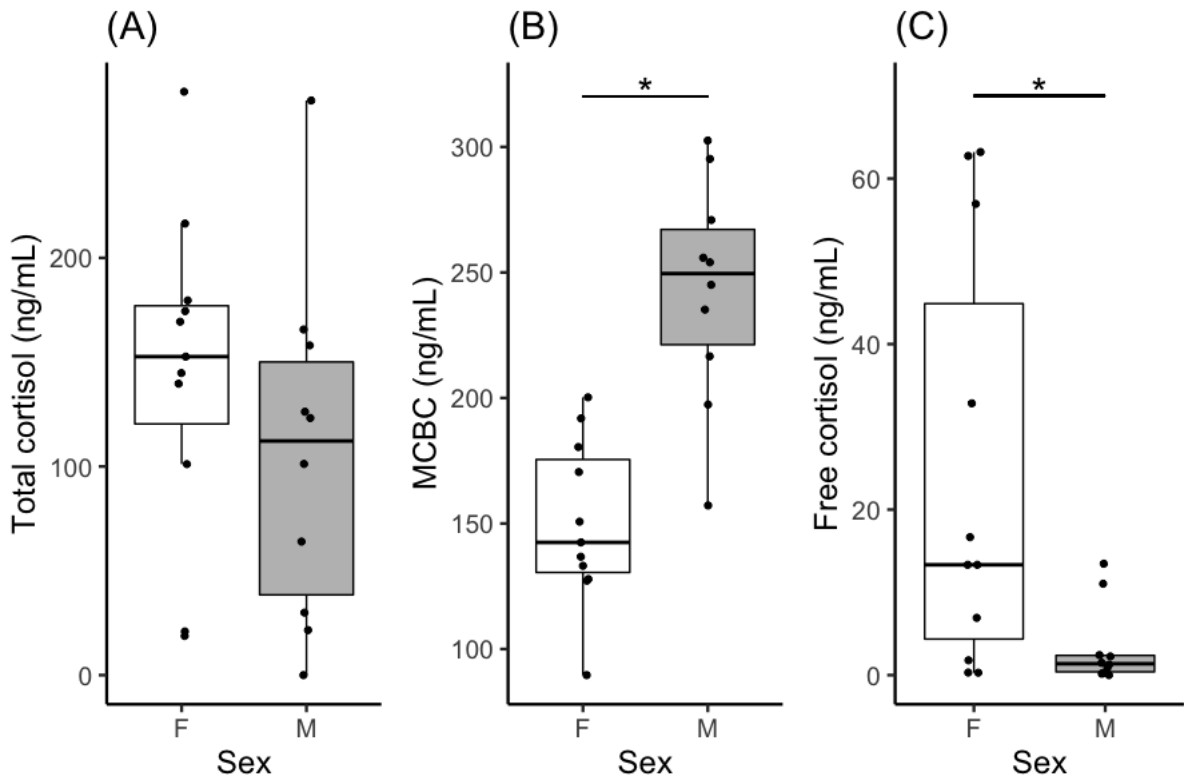
827 **Fig. 3.** Kinetics of plasma levels of (A) reactive oxygen metabolites (ROM; mg H<sub>2</sub>O<sub>2</sub>/dL), and  
828 (B) total antioxidant capacity (OXY;  $\mu$ mol HCL/mL) over a 30-minute capture stress in  
829 Columbian ground squirrels. Sampling time points were under 3 minutes (mean  $\pm$  s.e. =  $2.11 \pm$   
830  $0.07$ ), at 5 ( $5.79 \pm 0.09$ ), 10 ( $10.82 \pm 0.11$ ), 20 ( $20.79 \pm 0.14$ ) and 30 ( $31.90 \pm 0.43$ ) minutes.  
831 Box plots show the median (bold line) and first and third quartiles. Raw data points are overlaid.  
832 Significantly differences between boxplots at  $P < 0.05$  (Tukey HSD) are indicated by an  
833 asterisk.

834

835 **Fig. 4.** Relationship between reactive oxygen metabolites (ROM; mg H<sub>2</sub>O<sub>2</sub> /dL) levels and  
836 individual age. The predicted line and 95% CI of a linear mixed model with sampling timing,  
837 age, and mass at capture specified as independent variables, and ID as a random factor, are  
838 given (see Results). Note that overlapping data points appear darker.

839 **Figures**

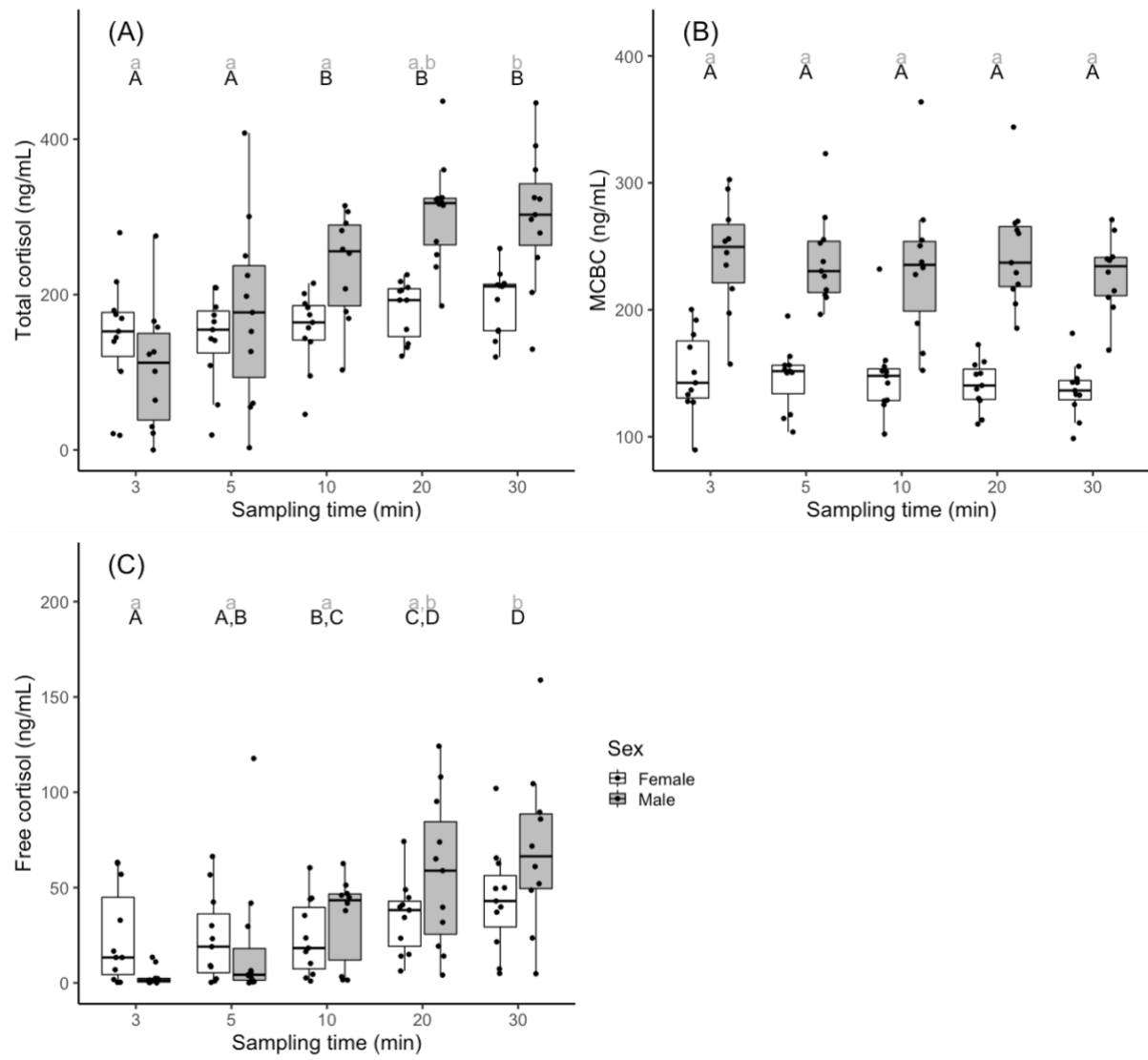
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842 **Fig. 1**

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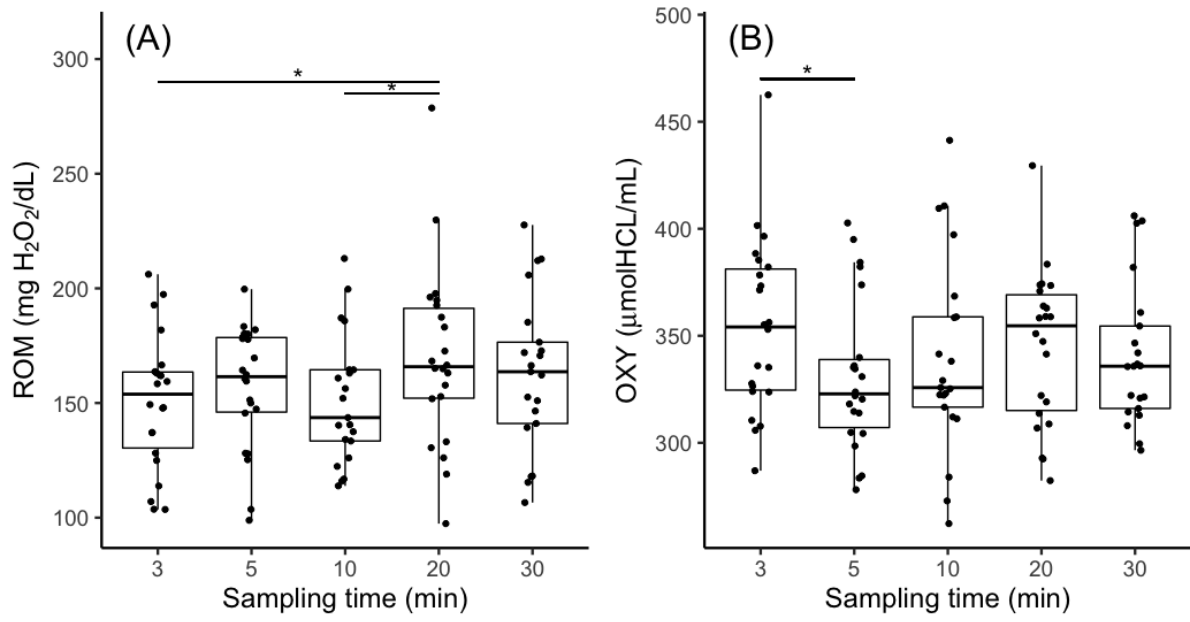


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845 **Fig. 2**

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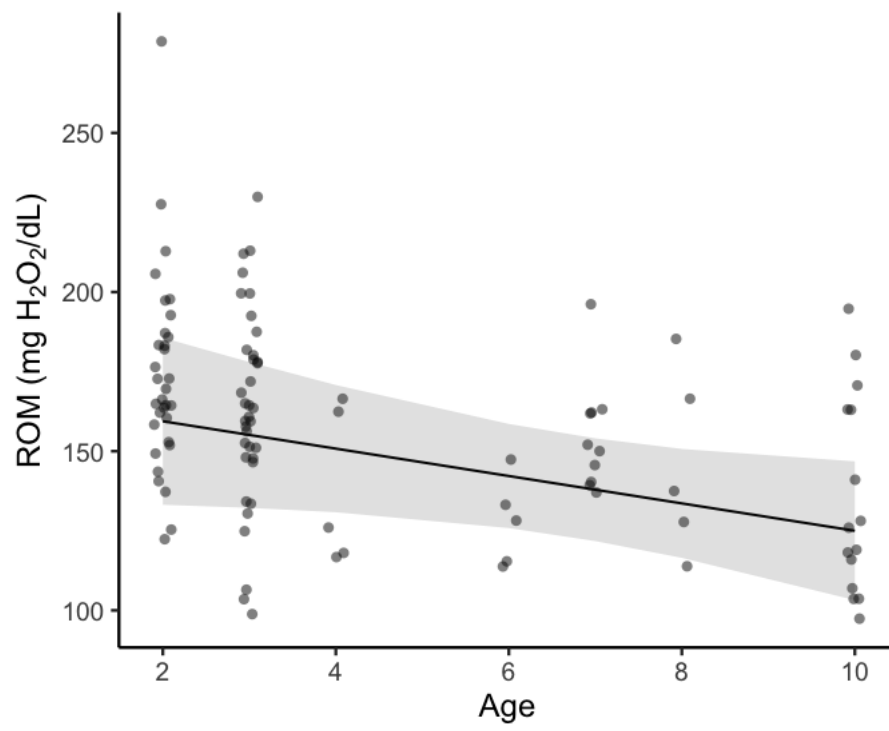
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849 **Fig. 3**

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853 **Fig. 4**

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