

24 **Abstract**

25 Stress responses have evolved to quickly and appropriately deal with environmental stressors
26 in order to secure or restore homeostasis. Since the regulation of stress hormones plays a key
27 adaptive role, the regulatory processes controlling stress hormones levels may be under high
28 selective pressure. The social environment during early life (parents and litter characteristics)
29 strongly affects ontogeny of the hypothalamic-pituitary-adrenal (HPA) axis. In cooperative
30 breeders, offspring are also confronted with helpers but whether and how variation in the helping
31 context can affect HPA axis responsiveness of offspring remains unanswered. Combining
32 dexamethasone suppression and adrenocorticotrophic hormone stimulation tests, we investigated
33 the link between the social environment and the characteristics of the HPA axis at the early
34 stages of life in wild Alpine marmots. We show that when raised in the presence of helpers,
35 marmot pups exhibit a greater capacity not only to mount, but also to turn off a stress response.
36 The capacity to mount a stress response was also higher as the pups were raised in large litters.
37 Determining impacts of such social modulation of the HPA axis functioning on individual fitness
38 would make an important contribution to our understanding of the evolution of cooperative
39 breeding.

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41

42 **1. Introduction**

43 Organisms constantly adjust to predictable environmental variations such as daily and
44 seasonal changes while needing to respond quickly and adequately to unpredictable treats
45 (Wingfield 2003, 2008). To do so, stress responses have evolved to deal rapidly and
46 proportionately with environmental stressors in order to maintain or restore a homeostatic state
47 (Monaghan and Spencer 2014). The stress response consists of a complex set of physiological
48 changes through the activation of neuronal and endocrine pathways (Sapolsky et al. 2000;
49 Wingfield 2003) where the hypothalamic-pituitary-adrenal (HPA) axis is the main orchestrator
50 by regulating the secretion and release of glucocorticoids (de Kloet et al. 2008). At baseline
51 levels, circadian fluctuations of glucocorticoids (corticosterone and cortisol) regulate
52 physiological functions and maintain energy homeostasis (McEwen and Wingfield 2003). When
53 exposed to physiological or psychological stressors, activation of the HPA axis stimulates the
54 secretion and release of glucocorticoids within minutes, mobilizing energy and preparing
55 individuals to fight or flight (Wingfield et al. 1998). While a transient increase in glucocorticoids
56 leads to short-term and immediate benefits, particularly in terms of survival (Wingfield 2008),
57 chronically elevated glucocorticoid levels carries physiological costs (Romero et al. 2009).
58 Under high glucocorticoids levels, energy is derived to an emergency state, where physiological
59 and behavioural adjustments are all directed towards immediate survival, at the expense of other
60 energy demanding functions such as growth, reproduction or body maintenance (Romero 2004).
61 Extensive literature, mostly on medical studies in humans and experimental approaches on
62 laboratory rodents, agree that maintaining high levels of glucocorticoids cause adverse effects on
63 many vital physiological functions while predisposing to multiple metabolic and immune
64 disorders and accelerating ageing (for example, see Gassen et al. 2017; Nicolaides et al. 2015).

65 In the wild, chronic elevation of glucocorticoids has been shown to have consequences on
66 individual fitness with measurable cascading effect at the population level (eg. Boonstra et al.
67 1998; Dulude-de Broin et al. 2020). Hence, regulatory mechanisms of the stress response which
68 control both the ability to mount a stress response (the initial response phase to challenge) and
69 the mechanisms contributing to restore baseline glucocorticoid levels (the recovery phase),
70 should play a key adaptive role (Crespi et al. 2013; Taff and Vitousek 2016) and thus under high
71 selective pressure (MacDougall-Shackleton et al. 2013; Bonier and Martin 2016).

72 The stress response is driven by the activation of the HPA axis, which results in the
73 hypothalamus releases of corticotropin-releasing hormone (CRH), a neuropeptide that stimulate
74 the secretion of adrenocorticotrophic hormone (ACTH) by the anterior pituitary gland. ACTH is
75 then transported through bloodstream and stimulates the systemic release of glucocorticoids by
76 the adrenal cortex (Figure 1a). The adrenocortical reactivity to ACTH is a major determinant of
77 glucocorticoid elevation in blood and therefore controls the rate of the increase in energy demand
78 and changes in resources allocation (i.e. emergency life-history stage) during the stress challenge
79 (Wingfield et al. 1998). Conversely, the primary inhibitory control of HPA axis involves
80 glucocorticoid-mediated negative feedback on the release of CRH and ACTH, so that the
81 glucocorticoid levels return to baseline once the threat is dealt with. The reactivity of the adrenal
82 to ACTH, as well as the effectiveness of negative glucocorticoid feedback, are plastic
83 physiological traits whose expression aims to optimize the intensity and duration of stress
84 responses in a given context (Angelier and Wingfield 2013). Although evidences that the
85 reactivity of HPA traduces into to fitness benefits remains mixed (see Breuner et al. 2008), it is
86 likely to be under high selective pressure (Bonier and Martin 2016).

87 Both the abiotic and biotic components of the environment can affect individual stress
88 response (Creel et al. 2013; Grace and Anderson 2018). Experimental approach shows that when
89 experienced early in life, environmental conditions strongly affect the ontogeny of the HPA axis,
90 resulting in long-term effects on many physiological and neural functions (McMillen and
91 Robinson 2005), and contributing greatly to a phenomenon referred as “developmental
92 programming” (Seckl 2004). For instance, in rats, offspring that received extensive maternal care
93 in early life showed reduced adrenal reactivity to acute stress, but improved efficacy of
94 glucocorticoids negative-feedback in adulthood (Liu et al. 1997).

95 The social environment early in life is known to have an impact on glucocorticoids levels
96 although most of our knowledge in this field comes from experimental approaches. For example,
97 social deprivation (social isolation: (Malkesman et al. 2006); deprivation of mother and siblings:
98 (Avishai-Eliner et al. 1995)) or social crowding experienced early in life (Bugajski et al. 1993)
99 have been shown to cause chronic stress in laboratory rodents. Under natural conditions,
100 investigations on the effects of the social environment in the early life have showed that litter
101 size and composition affected circulating glucocorticoid of rat and rabbit pups (Hudson et al.
102 2011). However, in social species, offspring interact not only with their parents or siblings, but
103 also with other members of their social groups. In particular, in cooperative breeders, animals
104 live in kin-based family groups where it is not only the parents who care for the young (Brown
105 1987), but also non-breeding individuals called helpers (Keller and Reeve 1994). The presence
106 of helpers early in life has major short- and long-term effects on offspring’s phenotypes and
107 fitness (*e.g.* Berger et al 2018; Hammers et al 2019), but the underlying physiological
108 mechanisms are not yet understood. In this study, we tested the hypothesis that the presence of
109 helpers could modulate the HPA responsiveness of the offspring they care for.

110 In order to investigate whether the social environment (litter characteristics and helping
111 context) experienced early in life shapes the individual HPA axis responsiveness, we
112 implemented hormonal challenge protocols in the wild Alpine marmot (*Marmota marmota*).
113 Alpine marmots are territorial cooperatively breeding ground-dwelling squirrels that live in
114 family groups of 2 to 16 individuals composed of a dominant pair and sexually mature and
115 immature subordinates (Allainé 2000). Only the dominant pair reproduces once a year.
116 Subordinates of both sexes delay dispersal and forgo their own reproduction beyond sexual
117 maturity (Cohas et al. 2006). However, only subordinate males are considered as helpers, notably
118 because of their crucial role in thermoregulation which drastically improves the survival of
119 juveniles during hibernation (Arnold 1988; Allainé and Theuriau 2004) but also because of their
120 role in the thermoregulation of the pups at night and in the construction of nests. Mating takes
121 place in the second half of April, gestation lasts 35 days, the females then give birth to altricial
122 pups and nurse them for 40 days. The pups remain in their natal underground burrows (i.e. in a
123 buffered environment offering protection from climatic fluctuations and predators) until
124 weaning, from the last week of June to mid-July, at about 40 days of age. During the whole
125 nursing period, the impacts of group characteristics on these naïve individuals are thus expected
126 to be prominent. We previously showed (1) that litter sex composition and size affect male
127 juvenile Alpine marmot survival and probability to become dominant for both sexes (Dupont et
128 al. 2015), and (2) that the number of helpers during early life has long-term consequences such
129 as increased longevity of females (Berger et al. 2015) or alteration of actuarial senescence
130 pattern in both sexes (Berger et al. 2018). However, whether litter size, sex-ratio and helpers can
131 impact pups physiological traits remains to be investigated. From these previous studies, we
132 predict that the pups benefiting from helpers early in life should present increased HPA axis

133 responsiveness with enhanced capacity to rapidly mount and to turn off a stress response. We
134 also expect the litter size and sex-ratio to affect the HPA axis responsiveness.

135

136 **2. Materials and Methods**

137 (a) Field methods

138 The study took place in La Grande Sassièrè Nature Reserve (French Alps, 45°29'N, 65°90'E)
139 where a population of wild Alpine marmots (*Marmota marmota*) is extensively monitored since
140 1990. As part of a long-term capture-mark-recapture protocol, marmots are captured annually
141 from mid-April to mid-July using two-door live-capture traps (see Cohas et al. 2006). Once a
142 year, captured individuals are tranquilized by an intramuscular injection of tiletamine and
143 zolazepam (Zolétil 100, 0.1 ml.kg⁻¹, Virbac, France), sexed, aged, and their social status
144 (dominant or subordinate) is determined by examining sexual characteristics (scrotum for males
145 and teats for females). Social status is further confirmed by behavioural observations. Each
146 animal is individually marked with a microchip (Trovan, Germany) inserted subcutaneously and
147 a numbered ear-tag. Dominant individuals also received a coloured plastic ear-tag.

148 Fieldwork work was undertaken after the issuance of permit number AP n82010/121 by
149 the Prefecture of Savoie. All the procedures were approved by the ethical committee of the
150 University Claude Bernard Lyon 1 (n8BH2012-92 V1, 2017012500169084 v1).

151

152 (b) Characterization of the social environment

153 We observed each family group on average 1h per day for a minimum of 30h per year
154 with sessions being randomly distributed during the activity period. Marmots were observed
155 from a distance of 80-200 m using 10x50 binoculars and 20x60 monocular telescopes. Ear-tags

156 allowed us to distinguish the sex and social status of individuals and their size allowed us to
157 categorize them as yearlings, 2-year-olds or adults. Combining these observations with mark-
158 recapture data, we defined the number of helpers as the number of male subordinates aged 1 year
159 and older present in a family group from the end of mating to the emergence of the pups. The
160 number of helpers (median = 1.50, range = 0-4) was not manipulated, but we benefited from the
161 natural intergroup variability in the number of helpers in this population. Among the 45 pups
162 studied, 12 were raised in absence of helpers in their family group.

163 From additional daily observations conducted during the whole activity period, we
164 recorded the date of the pups' first emergence from the natal burrow and the litter size. All pups
165 of a given litter emerge together. We defined the litter size (median = 4.00, range = 2-6) as the
166 total number of pups emerging from the same natal burrow. Combining observations with the
167 capture of the pups, we characterized the composition of the litter by the litter sex-ratio (median
168 = 0.75, range = 0.25-1), the litter sex-ratio being measured as the number of males in a litter
169 divided by the litter size. Sibship was further confirmed by parentage analyses (Cohas et al.
170 2006).

171

172 (c) Corticosterone assay

173 In vertebrates, two adrenal glucocorticoids are secreted: cortisol and corticosterone.
174 While these two hormones are believed to play a role in the stress response of vertebrates, one is
175 generally predominant and the cortisol to corticosterone ratio is varies considerably depending
176 on the species (Koren et al. 2012). In rodents, it is generally accepted that the dominant
177 glucocorticoid is corticosterone (Cockrem, 2013), and this hormone or its derivatives has been
178 preferred to cortisol in literature on marmot species (eg. Monclus et al. 2011; Petelle et al. 2017;

179 Blumstein et al. 2018; Price et al. 2018; Pinho et al. 2019). However, there is no consensus on
180 this point since other authors have shown higher cortisol than corticosterone concentrations in
181 yellow-bellied marmot plasma (Kastner et al. 1977). Here, we measured total (bound and
182 unbound) corticosterone in plasma using commercial enzyme immuno-assay kits (Cayman
183 Chemicals Corticosterone EIA kits n°501320). The assays were carried out in microplates coated
184 with mouse anti-rabbit IgG and relied on the competition between corticosterone and a
185 corticosterone-acetylcholinesterase for a limited amount of corticosterone antiserum.
186 Corticosterone-acetylcholinesterase complexed with the IgG colours the Ellman's reagent in
187 yellow, the intensity of which is measured spectrophotometrically at 412nm (Xenius, Safas
188 Instrument) and is inversely proportional to corticosterone in the sample. This assay has been
189 validated for rodents (see manufacturer's instructions), nevertheless, we checked the specificity
190 of the corticosterone assay by verifying that the hormone levels measured in a series of plasma
191 diluted in assay buffer (from 1:5 to 1:20) were parallel to the dilution series of the hormonal
192 standards. Final assays were performed in duplicate with a volume of 50 μ L of plasma diluted
193 1:10. The mean intra-assay coefficient of variation that was calculated for each sample was
194 6.8%. The corticosterone concentrations were calculated against a standard curve and expressed
195 as pg.mL^{-1} .

196

197 (d) Characterization of the stress axis

198 In 2015, 45 pups from 13 family groups were captured by hand between their first and
199 third day of emergence from their natal burrow (0 to 2 days after emergence). At emergence,
200 pups are weaned and beyond the stress hyporesponsive period, which is known to be in the first
201 half of the period between birth and weaning in rodents (Levine 1994). Immediately upon

202 capture, the pups were transferred to a nearby field laboratory in a burlap bag, before being
203 tranquilized and tagged as described above. To limit potential confounding effects of
204 environmental variability, the experiments were all conducted the same year in family groups
205 occupying valley and south facing territories. Given the size of the overall study site (~0.3 km²)
206 and the low range of altitude (~50m), all studied territories were expected to face very similar
207 climatic conditions.

208 To assess measures of pup's stress axis responsiveness and resulting corticosterone levels
209 we recorded complementary measures. Firstly, we applied a dexamethasone suppression test
210 (Boonstra et al., 1998). Dexamethasone is a synthetic steroid molecule that exerts a negative
211 feedback on the hypothalamus and the anterior pituitary gland inhibiting the secretion of CRH
212 and ACTH thus suppressing glucocorticoids release by the adrenal cortex (Fig 1a). Therefore,
213 measuring the rate of plasma corticosterone disappearance following dexamethasone
214 administration $\left(\frac{CORT\ at\ dexamethasone\ injection - suppressed\ CORT\ level}{time\ at\ ACTH\ injection - time\ at\ dexamethasone\ injection}\right)$, hereafter referred as
215 "adrenal suppression by dexamethasone") provides an effective measure of the efficacy of the
216 negative feedback regulation. In other words, it measures the ability of the HPA to recover from
217 initial stimulation and to return to baseline level. A low efficiency of negative feedback indicates
218 a reduced ability to shut-off the release of glucocorticoids, exposing individuals to the adverse
219 effects of prolonged stress. Such an effect has been observed repeatedly in wild and captive
220 vertebrate populations subjected to persistent stress (see Dickens and Romero 2013 for review).
221 Corticosterone concentration measured two hours post-dexamethasone injection (hereafter
222 referred as "suppressed corticosterone level") assumes effective inhibition of endogenous CRH
223 and ACTH secretion. Secondly, we performed an ACTH stimulation test (Boonstra et al., 1998).
224 Following exogenous ACTH administration, the appearance of the corticosterone in the blood

225 $\left(\frac{\text{stimulated } \text{CORT level} - \text{suppressed } \text{CORT level}}{\text{time at stimulated } \text{CORT level} - \text{time at ACTH injection}} \right)$, hereafter referred as “adrenal reactivity to
226 ACTH”) measures the efficiency of the adrenal cortex to produce and release stress hormones
227 (Fig 1a). In turn, this test informs directly on the reactivity of the adrenal gland and on its
228 ability to initiate glucocorticoids-dependant adaptive stress responses (Boonstra et al. 1998), a
229 faster glucocorticoid release after ACTH stimulation indicating a greater physiological capacity
230 to orchestrate an effective response to a perceived stressor. The corticosterone concentration
231 measured one hour after ACTH injection (hereafter referred to as “stimulated corticosterone
232 level”) reflects the secretory activity of the adrenal gland during that time under ACTH
233 stimulation.

234 Each pup thus underwent three successive blood sampling (Fig 1b) of 200µl consisting in
235 a small puncture of the great saphenous vein with a 0.5 x 16mm sterile needle (Terumo, Europe).
236 Blood was collected in heparinized capillary tubes (Microvette, Sarstedt, Germany). Immediately
237 upon the first blood sampling, pups received an intramuscular injection of dexamethasone
238 sodium phosphate (D0720000, Sigma-Aldrich, France, 0.4 mg.kg⁻¹). A second blood sample was
239 taken two hours (mean±SD = 1:59±0:01, range = 1:55-2:03) after dexamethasone administration
240 and was immediately followed by an intramuscular injection of ACTH (Sigma-Aldrich, France,
241 4.0 IU.kg⁻¹). A third blood sample was collected one hour (mean±SD = 0:59±0:01, range = 0:51-
242 1:08) after ACTH injection. The times between dexamethasone or ACTH injections and blood
243 sampling were determined from a preliminary trial on 5 individuals for whom multiple blood
244 samples were taken at different time interval after injections (30 min, 1h, 2h, 3h). For all
245 individuals, two hours were required to obtain a marked decrease in plasma corticosterone. For 3
246 of the 5 individuals tested, peak corticosterone level was already reached and corticosterone
247 concentration decreased two hours after ACTH injections; we thus set the blood sampling at 1

248 hour after the ACTH injection in or protocol. All blood samples were centrifuged for 5 min at
249 3000×g and the plasma stored in the field at -20°C. At the end of the procedure, pups were
250 released at their natal burrow. They were observed on a daily basis for 3 days; all individuals
251 were reintegrated to their family group without any adverse effects being observed.

252

253 (e) Statistical analyses

254 To test whether social environment affects corticosterone levels and HPA axis
255 responsiveness of marmot pups, the suppressed and the stimulated corticosterone levels as well
256 as the adrenal suppression by dexamethasone and the reactivity of the adrenal gland to ACTH
257 were entered as dependent variables respectively in two generalized linear mixed models with a
258 logarithm link and a variance given by a gamma distribution and in two linear mixed models
259 (LMM). To test for an effect of litter characteristics, the litter size and sex-ratio were entered as
260 explanatory variables. To test for an effect of the helping context, the number of helpers was
261 entered as an explanatory variable. Additionally, the sex and the age (in day since emergence
262 from the natal burrow) of the pup were entered as potential confounding explanatory variables.
263 To control both for pseudo-replication arising from measures done for several pups of the same
264 litters sharing a common environment (*e.g.* genetic background, maternal effects, territory
265 quality), we entered a variable “litter” as a random intercept. Agreeing with our hypotheses, only
266 additive effects of all the fixed explanatory variables were considered in our models. Although
267 inspection of the residuals from models including only previous effects did not show any
268 evidence of potential interaction between the considered explanatory variables, to assure the
269 robustness of the results obtained from these previous models, we further constructed models

270 with all 2-way interactions. Interactions were removed if not significant following a backward
271 procedure. Both approaches gave the exact same results.

272 Statistical analyses were performed with R 3.3.1 (R Core Team, 2014). The function
273 “lmer” in the package “lme4” (Bates et al. 2015) was used to fit GLMMs (Venables and
274 Ripley 2002) and the package “lmerTest” (Kuznetsova et al. 2016) used to calculate parameter-
275 specific *p*-values based on Satterthwaite's approximations. We set the level of significance to $\alpha =$
276 0.05 and parameter estimates are given as mean \pm SE.

277

278 **3. Results**

279 The average suppressed and stimulated corticosterone levels were $1837 \pm 877(\text{SD})$ and
280 $4080 \pm 1703(\text{SD})$ pg.ml^{-1} respectively (See Supplementary Information). The average adrenal
281 suppression by dexamethasone and adrenal gland reactivity to ACTH were -9.42 ± 10.70 (SD)
282 $\text{pg.ml}^{-1}.\text{min}^{-1}$ and $37.37 \pm 19.81(\text{SD})$ $\text{pg.ml}^{-1}.\text{min}^{-1}$ respectively (see supplementary material).
283 Adrenal reactivity to ACTH, adrenal suppression and the suppressed and stimulated
284 corticosterone levels showed some degree of correlation (Table 1).

285 The suppressed and the stimulated corticosterone levels of the pups were not affected by
286 the number of helpers in their family groups or the litter sex-ratio (Table 2). The stimulated
287 corticosterone level was significantly affected by the litter size but not the suppressed
288 corticosterone level (Table 2).

289 Both the adrenal suppression by dexamethasone (negative feedback, Fig 2a) and the
290 adrenal gland reactivity to ACTH (Fig 2b) increased with the number of helpers (Table 2). The
291 litter sex-ratio had no effect on both components of the HPA responsiveness but the litter size
292 positively affected the adrenal gland reactivity to ACTH in pups (Table 2, Fig 3).

293

294 **4. Discussion**

295 We showed that marmot pups raised in naturally contrasting social contexts display
296 different sensitivity profiles of the HPA axis. An increase in litter size has led to a greater
297 reactivity to mount a stress response and higher corticosterone level after stimulation by
298 exogenous ACTH. While the helping context did not impact the corticosterone levels after
299 stimulation by ACTH or inhibition by dexamethasone, the number of helpers significantly
300 affected the pup's HPA axis responsiveness: the presence of helpers triggered a higher reactivity
301 of the adrenal gland to ACTH and a higher hypothalamo-pituitary sensitivity to inhibition by
302 dexamethasone. These data indicate that, when raised in the presence of helpers, marmot pups
303 thus exhibit a greater capacity not only to mount but also to turn off a stress response. Although
304 the four measured physiological traits are not totally independent and showed some degree of
305 correlations, they seemed to have been shaped by different evolutionary pressures linked to the
306 social context.

307 We found that litter size but not litter sex-ratio had a significant impact on marmot pups'
308 adrenal reactivity to ACTH and stimulated corticosterone levels. Such effect is congruent with
309 extensive literature showing variation in hormones levels linked to litter size. For instance, in
310 small laboratory mammals (Fey and Trillmich 2008; Roedel et al. 2010), pups from large litters
311 exhibit higher baseline glucocorticoids levels than pups from small litters due to increased
312 competition during lactation, particularly in species where the number of offspring exceeds the
313 number of teats. In contrast, the lack of sex-ratio effect is somehow surprising. Indeed, the sex
314 composition of litters has been shown to influence the stress hormone levels of offspring
315 (Benhaiem et al. 2013; Blanco et al. 2006). Both hormonal infusion during pregnancy and

316 competition between siblings had been advanced to modulate the stress hormone levels (Blanco
317 et al. 2006; Hudson et al. 2011; Benhaiem et al. 2013). Regarding the lack of effect of litter size
318 and sex-ratio on adrenal suppression and suppressed corticosterone levels, comparison with the
319 literature is not possible as no study to date have investigated the impact of litter characteristics
320 on adrenal negative feedback.

321 As predicted, the HPA characteristics depended on the number of helpers present in a
322 family group. This could arise through direct effects on pups. In Alpine marmots, as in other
323 cooperative breeders, helpers provide important social support to offspring through babysitting
324 (Clutton-Brock et al. 2000), food provisioning (Brotherton et al. 2001; Raihani and Ridley 2008),
325 grouping or social thermoregulation (Arnold 1988). Hence, the beneficial effect of helping can
326 be expected to allow pups to invest more in somatic functions and to accelerate the ontogeny of
327 physiological traits such as the HPA axis. If there is no evidence that helping influence the age at
328 emergence from the natal burrow, helping behaviours clearly enhance offspring phenotypic
329 quality, notably growth and body mass (Clutton-Brock et al. 2001; Hodge 2005). Despite nothing
330 is known about an alteration of the HPA axis functioning linked to help received directly as pups
331 in cooperatively breeding species, the enhanced adrenal reactivity and feedback efficiency we
332 observed in Alpine marmot pups could arise as benefits of help directed toward pups on HPA
333 axis ontogeny and rate of maturation. Similarly, social support in humans (Uchino et al. 1996)
334 and affiliative behaviours in other mammalian species (Hennessy et al. 2009; Tuchscherer et al.
335 2016) have been shown to affect HPA axis positively.v

336 Effect of helpers could also be indirect, for example by affecting the hormonal status of
337 the mother. Indeed, maternal effects are a major determinant of offspring HPA axis profile and
338 previous findings on vertebrates suggest that maternally-induced stress can cause significant

339 variation in the responsiveness of an offspring's HPA axis involving both pre- (Moisiadis and
340 Matthews 2014) and postnatal developmental mechanisms (Liu et al. 1997; Macri and Wuerbel
341 2006). Helping context has been shown to modify both maternal condition and physiology
342 leading to change in maternal allocation in birds (Emlen et al. 1986; Hatchwell 1999; Paquet et
343 al. 2013; Dixit et al. 2017). In mammals, a reduced number of helpers raises hormone stress
344 levels of the mothers (Cameron 2004). Effects of helpers in modifying maternal glucocorticoids
345 levels could thus affect the functioning of the HPA axis in pups.

346 Finally, while the litter and the characteristics of the social group affected the reactivity
347 of the pituitary-adrenal system of the pups, little or no effect was observed on the corticosterone
348 levels after stimulation by ACTH or inhibition by dexamethasone. These results are in line with
349 the previously stated idea that static measures of stress hormone levels do not always allow
350 capturing information on overall endocrine functions. For instance, glucocorticoid value
351 measured at the peak of secretion not only poses the problem of whether the peak has been
352 properly identified, but is may be a poor indicator of the integrated stress response over time
353 (Romero 2004).

354 Our approach also has limitations. Our protocol requires individuals to be tranquilized
355 and constrained for several hours, which is hardly feasible for many wild species in their natural
356 environment. The potential effect of the tranquilizing drug (Zoletil), which consisted in a
357 combination of tiletamine (a dissociative anesthetic) and zolazepam (a benzodiazepine) should
358 also be considered. Dissociative anesthetics have little or no adverse effect on the HPA axis
359 functioning, however, but data on humans showed that benzodiazepines may compromise
360 adrenocortical steroidogenesis in a dose-dependent manner (Besnier et al. 2016). Although the
361 effect of zolazepam on HPA has yet to be demonstrated in wildlife, particularly at the dose used,

362 and the doses were standardized per g of body weight to allow comparisons between individuals,
363 it cannot be formally excluded that the tranquilization impacted our results. While our protocol
364 allowed to evaluate the propensity of the adrenal gland to release of glucocorticoids into the
365 bloodstream, there are other regulatory mechanisms that modulate the amplitude and the duration
366 of the stress response. These include those affecting the pharmacokinetics of glucocorticoids
367 (e.g. hepatic glucocorticoids metabolism, plasma clearance and excretion (McKay and Cidlowski
368 2003), and the concentration of binding proteins (Breuner and Orchinik 2002). Applying a
369 holistic approach has become a necessary need to better understand the adaptive value of the
370 responses to stress (Rey 2020). Hence, further investigations should focus on whether the social
371 context impact other regulatory mechanisms such as concentration of glucocorticoids bindings
372 proteins or the fraction of free hormone that is available for uptake by tissues. Further studies are
373 also needed to clarify how modulation of the reactivity of the HPA axis by the social context
374 translates into fitness and by which mechanisms (Breuner et al. 2008).

375 At this stage, we know that early social environment has major repercussions on an
376 individual's fitness (Beckerman et al. 2002; Lindström 1999). In Alpine marmot, help received
377 during early life modulates female lifetime reproductive success (Berger et al. 2015) and both
378 male and female actuarial senescence (Berger et al. 2018). Moreover, litter sex composition
379 affects male juvenile survival and both male and female probabilities of reaching dominant status
380 (Dupont et al. 2015). These very same social factors were the ones we found to modulate the
381 reactivity of the HPA. Whether these factors, and more particularly helpers in cooperative
382 breeders, could influence such long-lasting effects through an early programming of the HPA
383 axis remain to be investigated and the following questions need to be answered: to what extent

384 social programming of the HPA axis occur and why? To what extent these induced changes are
385 reversible? Are these changes adaptive or do they reflect potential constraints?

386

387 **Acknowledgements**

388 We thank all the students and field assistants in marmot catching. We are grateful to the
389 municipality of Tignes for the use of the Santel chalet.

390

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Table 1 Pearson coefficients of correlation between the different corticosterone levels measured and both adrenal reactivity to ACTH and adrenal suppression by dexamethasone. Significant correlations are in bold ($p < 0.05$).

	Suppressed corticosterone level	Stimulated corticosterone level	Adrenal suppression by dexamethasone	Adrenal reactivity to ACTH
Corticosterone at dexamethasone injection	0.65 95% CI [0.43;0.79] t = 5.54 N = 45 p < 0.01	0.68 95% CI [0.48;0.82] t = 6.01 N = 43 p < 0.01	-0.85 95% CI [-0.92;-0.74] t = 10.67 N = 45 p < 0.01	0.51 95% CI [0.24;0.70] t = 3.75 N = 43 p < 0.01
Suppressed corticosterone level		0.76 95% CI [0.59;0.87] t = 7.55 N = 43 p < 0.01	-0.15 95% CI [-0.43;0.16] t = 1.00 N = 45 p = 0.33	0.38 95% CI [0.08; 0.62] t = 2.63 N = 43 p = 0.01
Stimulated corticosterone level			-0.38 95% CI [-0.62;-0.09] t = 2.67 N = 43 p = 0.01	0.89 95% CI [0.79;0.94] t = 12.29 N = 43 p < 0.01
Adrenal suppression by dexamethasone				-0.41 95% CI [-0.63;-0.11] t = 2.84 N = 43 p < 0.01

Table 2 Effect of the litter size and composition as well as of the number of helpers on HPA responsiveness and plasma corticosterone levels of Alpine marmot pups subjected to dexamethasone suppression and ACTH stimulation tests. Significant effects are in bold ($p < 0.05$).

Fixed effects	Adrenal suppression by dexamethasone N = 45			Suppressed corticosterone level N = 45			Adrenal reactivity to ACTH N = 43			Stimulated corticosterone level N = 43		
	Estimate ± SE	t value	p-value	Estimate ± SE	t value	p-value	Estimate ± SE	t value	p-value	Estimate ± SE	t value	p-value
Intercept	8.01 ± 10.92	0.73	0.48	1329 ± 1055	1.26	0.23	-10.05 ± 13.93	0.72	0.47	-370 ± 1335	0.23	0.78
Age	0.44 ± 2.52	0.18	0.87	260 ± 217	1.20	0.24	-6.27 ± 4.20	1.49	0.24	-302 ± 402	0.75	0.46
Sex (male)	0.74 ± 2.97	0.25	0.81	-95 ± 247	0.39	0.70	-0.96 ± 5.55	0.17	0.86	-20 ± 532	0.04	0.97
Sex-ratio	-7.97 ± 9.36	0.85	0.41	1206 ± 911	1.32	0.21	17.98 ± 12.48	1.44	0.16	1973 ± 1196	1.65	0.11
Litter size	1.94 ± 2.33	0.83	0.42	-16 ± 224	0.07	0.84	6.65 ± 3.08	2.16	0.04	623 ± 296	2.11	0.04
Number of helpers	-2.98 ± 1.29	2.31	0.04	11 ± 126	0.09	0.93	5.50 ± 1.63	3.36	0.002	276 ± 157	1.76	0.09

Figure captions

Figure 1 (a) Schematic representation of the stress response through the hypothalamic-pituitary-adrenal (HPA) axis. The stressor may be environmental, physical or psychological. It causes secretion of corticotropin-releasing hormone (CRH) by the hypothalamus. Under CRH stimulation, the pituitary gland releases adrenocorticotrophic hormone (ACTH) into the bloodstream that in turn stimulates glucocorticoid secretion by the adrenal gland. Glucocorticoids retroact at the hypothalamus and pituitary gland levels inhibiting CRH and ACTH release. Following HPA axis stimulation, if the negative feedback mechanism works efficiently, plasma stress hormones rapidly returns to baseline level. (b) Illustration of the experimental protocol. Plasma glucocorticoid concentration rises following capture. Injection of dexamethasone leads to the suppression of the glucocorticoid secretion by the adrenal gland. The rate of dexamethasone-induced decrease of plasma glucocorticoid probes the efficiency of the negative feedback and the ability of the HPA axis to return to baseline level. The injection of ACTH, on the other hand, triggers a strong transient increase in plasma glucocorticoid concentration, an effect that reveals the reactivity of the adrenal gland.

Figure 2 Effects of the number of helpers (a) on the adrenal suppression by dexamethasone (measured as plasma corticosterone disappearance following dexamethasone administration) and (b) on the adrenal reactivity to ACTH (measured as plasma corticosterone appearance following ACTH administration) of Alpine marmot pups. Black dots represent residuals (a) of the adrenal suppression by dexamethasone or of (b) adrenal reactivity to ACTH per number of helpers after controlling for other effects. Line represents the model predictions (black) and their associated standard errors (dotted).

Figure 3 Effects of the litter size on the adrenal reactivity to ACTH of Alpine marmot pups. Black dots represent residual adrenal reactivity to ACTH after controlling for the effects of the number of helpers. Line represents the model predictions (black) and their associated standard errors (dotted).

Figure 1

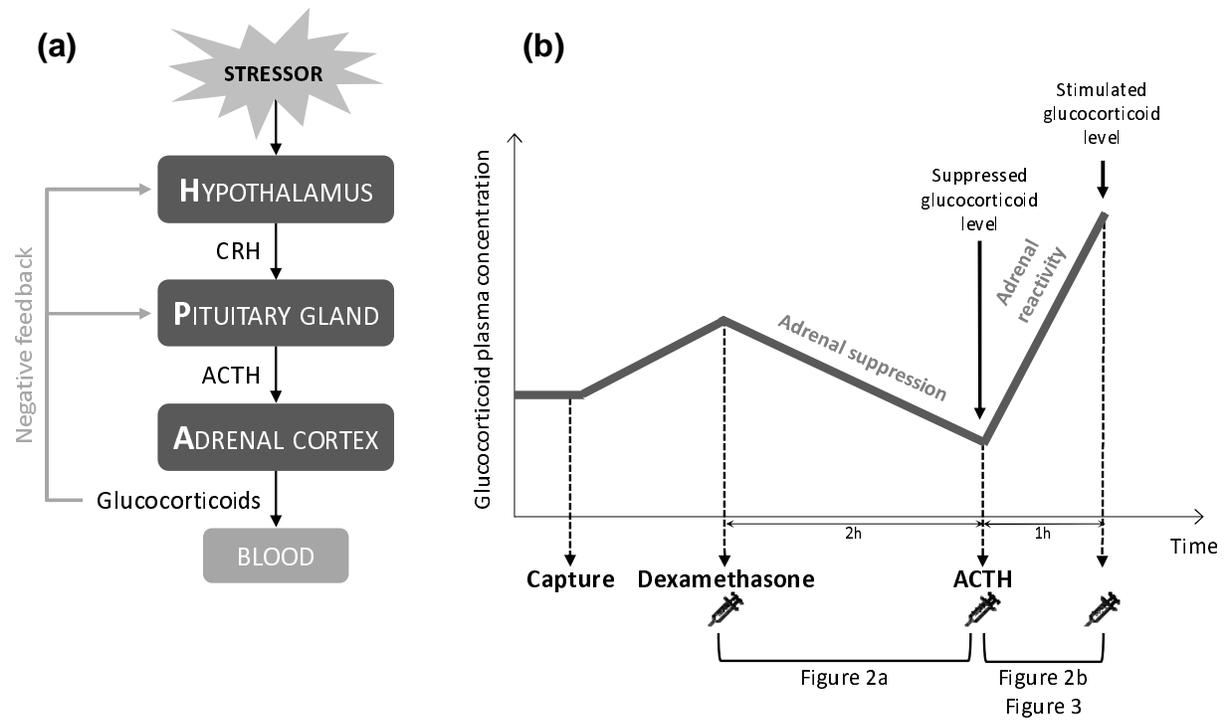


Figure 2

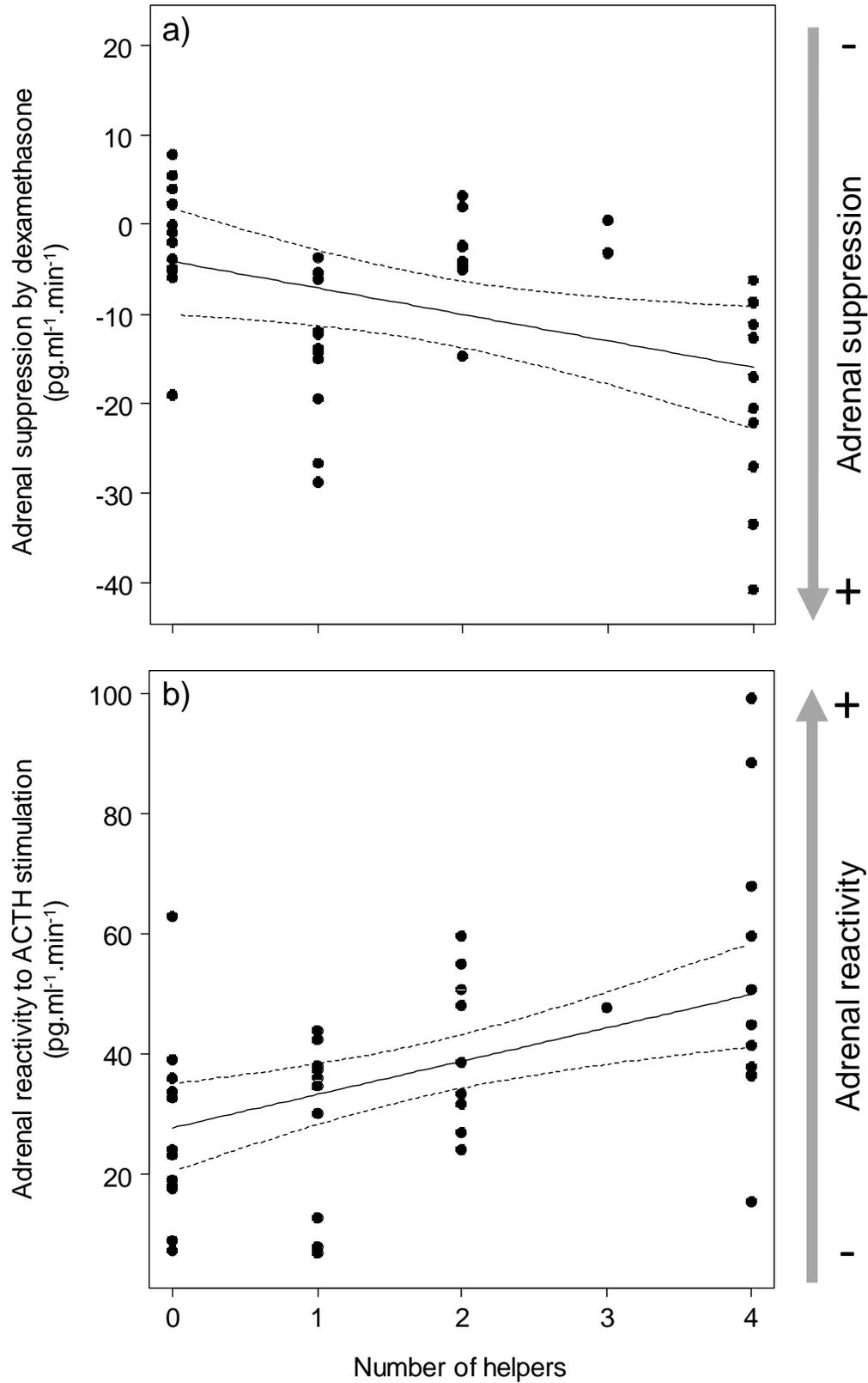


Figure 3

