1   **Maternal glucocorticoids promote offspring growth without inducing oxidative stress or**

2   **shortening telomeres in wild red squirrels**

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19   **Running title:** Maternal glucocorticoids, growth, and oxidative stress

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21   **Summary Statement:** We show that experimental increases in glucocorticoids in breeding

22   female North American red squirrels affects offspring postnatal growth but not levels of

23   oxidative damage and antioxidants or telomere lengths.

24

25   **Abstract**

26   Elevations in glucocorticoid levels (GCs) in breeding females may induce adaptive shifts in

27   offspring life histories. Offspring produced by mothers with elevated GCs may be better

28   prepared to face harsh environments where a faster pace of life is beneficial. We examined how

29   experimentally elevated GCs in pregnant or lactating North American red squirrels

30   (*Tamiasciurus hudsonicus*) affected offspring postnatal growth, structural size, oxidative stress

31   levels (two antioxidants and oxidative protein damage) in three different tissues (blood, heart,

32   liver), and liver telomere lengths. We predicted that offspring from mothers treated with GCs

33   would grow faster but would also have higher levels of oxidative stress and shorter telomeres,

34   which may predict reduced longevity. Offspring from mothers treated with GCs during

35   pregnancy were 8.3% lighter around birth but grew (in body mass) 17.0% faster than those from

36   controls, whereas offspring from mothers treated with GCs during lactation grew 34.8% slower

37   than those from controls and did not differ in body mass around birth. Treating mothers with

38   GCs during pregnancy or lactation did not alter the oxidative stress levels or telomere lengths of

39   their offspring. Fast-growing offspring from any of the treatment groups did not have higher

40   oxidative stress levels or shorter telomere lengths, indicating that offspring that grew faster early

41   in life did not exhibit oxidative costs after this period of growth. Our results indicate that

42   elevations in maternal GCs may induce plasticity in offspring growth without long-term

43   oxidative costs to the offspring that might result in a shortened lifespan.

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45   Keywords: Developmental plasticity, Life history, Maternal effects, Stress

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56   **Introduction**

57   Parents can have long-lasting impacts on their offspring across a diversity of taxa. These

58   parental effects have drawn substantial interest because they suggest that parental characteristics

59   or the parental environment itself could induce adaptive shifts in offspring traits that prepare

60   them for specific environments (i.e., adaptive transgenerational phenotypic plasticity: Mousseau

61   and Fox, 1998; Agrawal et al., 1999; Galloway and Etterson, 2007; Dantzer et al., 2013).

62   Furthermore, changes in maternal hormone levels, especially glucocorticoids (GCs), are widely

63   suspected to act as a mediator of transgenerational phenotypic plasticity in vertebrates (Love and

64   Williams, 2008; Sheriff et al., 2017).

65   GCs are metabolic hormones released by the hypothalamic-pituitary-adrenal (HPA) axis

66   (Sapolsky et al., 2000), in response to a variety of ecologically salient cues. In mammals, studies

67   in laboratory animals (Barbazanges et al., 1996; Harris and Seckl, 2011), 10) and in humans

68   (Weinstock, 2005; Lupien et al., 2009) show that elevated maternal GCs can generate stable

69   individual differences in offspring physiology and behaviour through the transfer of maternally-

70   derived GCs to offspring across the placenta (Barbazanges et al., 1996; Lupien et al., 2009), in

71   milk (Zarrow et al., 1970; Casolini et al., 1997), or through changes in maternal behaviour

72   (Herrenkohl and Whitney, 1976; Brummelte and Galea, 2010; Nephew and Bridges, 2011).

73   Maternal GCs could also act as an internal cue for offspring to modify their own development

74   (Nettle et al., 2013). Regardless of the pathway, there is much evidence that changes in maternal

75   GCs mediate parental effects whose influence may even persist across generations via epigenetic

76   mechanisms (Weaver et al., 2004).

77   Some of these changes in offspring characteristics that are caused by elevations in

78   maternal GCs are suspected to reflect adaptive plasticity in offspring life history traits, such as

79   modifying the trade-off between early life growth and lifespan (Monaghan and Haussmann,

80   2015; Berghänel et al., 2017). Elevated maternal GCs may induce a "faster" life history strategy

81   whereby offspring produced by mothers with elevated GCs grow or develop faster (Dantzer et

82   al., 2013; Berghänel et al., 2016). Such adjustments in the "pace of life" may be adaptive, as fast

83   postnatal growth or a quicker developmental time may be beneficial when the risk of extrinsic

84   mortality is heightened (Reznick et al., 1990; Stearns et al., 2000), which may coincide with

85   elevated maternal GCs. However, this increased investment in postnatal growth is expected to

86   carry costs for offspring longevity whereby fast-growing individuals exhibit a shortened lifespan

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87   (Metcalfe and Monaghan, 2001; Lee et al., 2013; Lemaître et al., 2015; Monaghan and Ozanne,

88   2018).

89   Identifying if maternal stress induces a trade-off between growth and lifespan in wild

90   animals is needed, as any costs of increased exposure to maternal GCs may be masked by benign

91   environmental conditions in the laboratory. Accurately documenting lifespan in wild animals

92   remains challenging in many species, and the stochastic nature of mortality in wild animals may

93   obscure any mechanistic costs of increased exposure to maternal GCs for offspring.

94   Consequently, one way to examine if elevated maternal GCs induce a trade-off between growth

95   and longevity in offspring is to examine how it affects the possible underlying mechanisms of

96   reduced longevity or physiological correlates that may predict a shortened lifespan. The free-

97   radical theory of aging (Beckman and Ames, 1998) provides one framework to examine the

98   mechanisms by which maternal GCs may induce this trade-off between offspring growth and

99   longevity. Reactive oxygen species (ROS) produced during aerobic respiration can have

100   damaging effects on cells (Beckman and Ames, 1998; Halliwell and Gutteridge, 2015). ROS

101   production may be elevated by increased aerobic respiration due to enhanced investment in

102   growth or reproduction (Fletcher et al., 2012; Blount et al., 2016; Smith et al., 2016; Monaghan

103   and Ozanne, 2018) or by increased GCs (Kotrschal et al., 2007; You et al., 2009; Costantini et

104   al., 2011; Herborn et al., 2014). Antioxidants produced by individuals (enzymatic antioxidants

105   such as superoxide dismutase) as well as those antioxidants from the external environment (non-

106   enzymatic antioxidants in the diet) can lessen the impact of ROS production (Selman et al.,

107   2012). An important type of oxidative damage occurs to the protective ends of chromosomes,

108   called telomeres. Telomeres are the repetitive DNA sequences that occur at the ends of eukaryote

109   chromosomes whose length is shortened during each cell division (Harley et al., 1990; Aubert

110   and Lansdorp, 2008), and may also be reduced by the increased production of ROS (von

111   Zglinicki, 2002; Houben et al., 2008; Reichert and Stier, 2017; Monaghan and Ozanne, 2018).

112   Cells with a reduced telomere length become senescent, stop dividing, and undergo apoptosis

113   unless the enzyme telomerase, or another elongation process, is produced to elongate the

114   telomeres (Bodnar et al., 1998; Rudolph et al., 1999). Telomere length or rate of loss has been

115   found to be predictive of the mortality risk of individuals (Cawthon et al., 2003; Haussmann et

116   al., 2005; Bize et al., 2009; Salomons et al., 2009; Boonekamp et al., 2014; Munoz-Lorente et al.,

117   2019), though the strength of this relationship may vary among taxa and in relation to other life

118   history traits. Furthermore, avian or mammalian species with longer lifespans have been shown

119   to exhibit slower age-specific rates of telomere loss (Haussmann et al., 2003; Dantzer and

120   Fletcher, 2015; Sudyka et al., 2016; Tricola et al., 2018).

121   Elevated exposure to maternal GCs may, therefore, induce a life history trade-off

122   between offspring growth and longevity, because offspring may experience elevated oxidative

123   damage, decreased antioxidant levels, and/or shortened telomeres, either due to oxidative stress

124   or increased cell division associated with elevated growth (Shalev, 2012; Haussmann and

125   Heidinger, 2015; Monaghan and Haussmann, 2015; Shalev and Belsky, 2016; Monaghan and

126   Haussmann, 2018). Previous studies across taxa show that elevated exposure to maternal GCs

127   can shorten telomere lengths in offspring (Entringer et al., 2011; Haussmann et al., 2012;

128   Herborn et al., 2014; Marchetto et al., 2016) or increase their rate of attrition as they age

129   (Haussmann and Heidinger, 2015), which could cause or be associated with a shortened lifespan.

130   For example, experimental studies in captive and wild birds show that offspring that had

131   exogenous GCs added to their eggs or were administered GCs during chick growth had a

132   heightened physiological stress response, higher levels of oxidative stress, and shorter telomeres

133   early in life (Haussmann et al., 2012; Herborn et al., 2014). Despite much interest in this topic,

134   few studies in wild animals have examined if experimental elevations in the GCs of breeding

135   females impact the oxidative state of offspring or explicitly tested the prediction that elevations

136   in the GCs of breeding females increase early life growth. Additionally, few studies have tested

137   whether elevated GCs in breeding females or fast early life growth comes at some cost by

138   promoting oxidative stress and shortening telomeres in offspring.

139   We tested the hypothesis that elevations in maternal GCs would promote a faster life

140   history strategy in offspring of wild North American red squirrels (*Tamiasciurus hudsonicus*).

141   We treated females with GCs using a protocol that allowed us to increase circulating GCs within

142   a physiologically-relevant range (van Kesteren et al., 2019). We treated females with GCs either

143   during pregnancy or lactation to assess if the timing of exposure to maternal GCs influenced

144   their effects on offspring. Other than for offspring growth in body mass, we did not have strong *a*

145   *priori* expectations of how the timing of elevated maternal GCs would differentially impact

146   offspring because elevated maternal GCs during pregnancy or lactation can impact offspring

147   through the same pathways: direct transfer of maternal GCs to offspring across the placenta or

148   through milk ("programming"), altering maternal behaviour, or affecting offspring behaviour

149   (see references above). However, based upon our previous study (Dantzer et al., 2013), we

150   predicted that offspring produced by mothers treated with GCs during pregnancy would grow

151   faster in body mass. We did not have an *a priori* expectation for how treating mothers with GCs

152   during lactation would impact offspring growth in body mass, though results from a previous

153   study suggested that it should reduce growth (Nephew and Bridges, 2011).

154   We measured offspring postnatal growth in body mass prior to weaning (~1 to 25 d of

155   age) and subsequently obtained measures of oxidative stress when pups were weaned (~70 d of

156   age). In three tissues (liver, heart muscle, and blood) collected from weaned offspring, we

157   measured one enzymatic antioxidant (superoxide dismutase), one type of non-enzymatic

158   antioxidant (total antioxidant capacity), and one type of oxidative damage (protein damage

159   measured via protein carbonyls). We used multiple tissues because other studies have

160   highlighted how experimental manipulations can have tissue-specific effects (Garratt et al.,

161   2012). To assess the cumulative impact of elevated maternal GCs on the oxidative state of

162   offspring and how offspring growth impacted telomere lengths, we also measured telomere

163   lengths in DNA from the liver. We focused on liver telomere lengths because the liver is a

164   mitotically active tissue, produces growth hormones, and previous studies have documented a

165   reduction in telomere length with faster growth (Monaghan and Ozanne, 2018), suggesting that

166   the liver would be a good tissue to investigate any oxidative costs of growth. Although we only

167   measured telomere lengths in one tissue, previous studies indicate that telomere lengths

168   measured in one somatic tissue are strongly correlated with those in others (Friedrich et al.,

169   2000). Because our estimate of offspring growth from ~1 to 25 d of age was temporally

170   separated from when we obtained our measures of oxidative stress and telomere lengths (when

171   offspring were weaned [~70 d]), we were able to assess whether there were persistent or

172   cumulative oxidative costs to fast growth.

173   We predicted that offspring from mothers treated with GCs during pregnancy would

174   grow quicker in body mass after birth, but would experience more oxidative stress (manifested as

175   a reduction in antioxidants and an increase in oxidative damage) and decreased telomere length,

176   which would be a result of increased oxidative stress or increased cell division associated with

177   faster growth. Because we have previously found that female red squirrels can ameliorate the

178   trade-off between offspring number and growth (Dantzer et al., 2013; Westrick et al., 2019

179   preprint), we examined if elevated maternal GCs altered the trade-off between litter size and

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180   offspring growth or structural (skeletal) size. Previous studies highlight that elevated maternal

181   GCs can impact offspring birth weight (Berghänel et al., 2017) so we also examined the

182   treatment effects on the first measure of body mass. Because early life exposure to GCs may

183   modify the direction and strength of the association between two variables (Careau et al., 2014;

184   Merill and Grindstaff, 2018), we also examined if increases in maternal GCs affected the

185   expected negative relationship between offspring growth and oxidative stress state (Smith et al.,

186   2016) by assessing the statistical interaction between offspring growth and maternal treatment.

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188   **Materials and Methods**

189   *Study area & measuring offspring growth*

190   We conducted this study as a part of a long-term study of red squirrels in the Yukon,

191   Canada that takes place on the traditional territory of the Champagne and Aishihik First Nations.

192   Squirrels in our study population were all marked individually with unique ear tags and

193   combinations of coloured wire threaded through the ear tags (McAdam et al., 2007). Females in

194   our study population usually produce one litter in the spring, and rarely produce more than one

195   litter of offspring to weaning per year (Boutin et al., 2006). Adult females were captured and

196   handled every ~3 to 21 d to assess reproductive status through abdominal palpation and nipple

197   condition. Pups were retrieved from the nest two times. The first nest entry occurred immediately

198   after parturition and the second nest entry occurred when pups were approximately 25 d of age.

199   At both nest entries, pups were briefly removed from their nest, sexed, and weighed to the

200   nearest 0.01 g using a portable balance. At the first nest entry, we marked them uniquely by

201   obtaining a small ear biopsy (for later paternity analyses) and then we permanently marked pups

202   at the second nest entry with unique metal ear tags. Because offspring growth in body mass

203   during this period of time is approximately linear (McAdam et al., 2002), we estimated offspring

204   growth as the change in body mass from the first to second nest entry divided by the total

205   number of days elapsed between the two measures of body mass. Red squirrels usually first

206   emerge from their nest around 30-35 d old and are usually weaned around 70 d old, such that this

207   period of growth from ~1-25 d of age represents a period in which offspring are only consuming

208   milk from their mother. At the second nest entry, we measured zygomatic arch width and right

209   hind foot length to the nearest 1 mm using digital callipers or a ruler, respectively. Because we

210   only obtained these morphological measures at the second nest entry (when pups were ~25 d

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211 old), we were not able to measure the change in offspring structural size as we did for growth.

212 All of our procedures were approved by the Animal Care and Use Committee at the University

213 of Michigan (PRO00007805).

214   *Maternal treatments*

215   We used four separate treatment groups to assess the effects of elevated maternal GCs on

216 offspring over four different years (2012, 2015-2017), although in 2012 we only collected

217 growth in body mass data. Individual adult females were treated with GCs either during

218 pregnancy or lactation ("Pregnancy GCs" and "Lactation GCs"), whereas other females were

219 treated as controls during pregnancy or lactation ("Pregnancy Controls" or "Lactation Controls").

220 We increased maternal GCs either during pregnancy or lactation using an established

221 experimental protocol (Dantzer et al., 2013; van Kesteren et al., 2019). Briefly, we treated

222 females in the Pregnancy GCs (n = 44 total litters from 43 unique females) and Lactation GCs (n

223 = 18 litters from 17 unique females) treatment groups with exogenous cortisol (hydrocortisone,

224 Sigma H4001) dissolved in peanut butter and wheat germ mixture (8 g of peanut butter, 2 g of

225 wheat germ). Females in the Pregnancy Control (n = 31 total litters from 32 unique females) or

226 Lactation Control (n = 17 litters from 16 unique females) treatment groups were fed the same

227 amount of peanut butter and wheat germ mixture but lacking the hydrocortisone. GC treatments

228 were prepared by dissolving hydrocortisone in 1 mL of 100% ethanol and then 5 mL of 100%

229 peanut oil before allowing the emulsion to sit overnight so that the ethanol could evaporate. The

230 following morning, the hydrocortisone emulsion was thoroughly mixed with the appropriate

231 amount of peanut butter and wheat germ, weighed out into individual dosages (~10 g each),

232 placed into an individual container, and then frozen at -20 °C until provisioning to the treated

233 squirrels.

234   Each day during the treatment period, we placed individual dosages into a bucket that

235 was hung ~7-10 m off the ground on the centre of the squirrel's territory. Squirrels defend these

236 buckets from all other conspecifics and heterospecifics (van Kesteren et al., 2019), so we can,

237 therefore, be confident that the squirrels that were given these treatments were consuming them.

238 The dosage of hydrocortisone varied among some of the treatment groups but we have

239 previously shown that either 3, 6, 8, or 12 mg of hydrocortisone per day significantly elevates

240 baseline plasma cortisol and faecal glucocorticoid metabolite levels but within a physiologically-

241 relevant range (Dantzer et al., 2013; van Kesteren et al., 2019). Females in the Pregnancy GCs

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242   treatment group were provisioned either with 3 mg (n = 4 litters), 6 mg (n = 6 litters), 8 mg (n =

243   26 litters), or 12 mg (9 litters) of hydrocortisone whereas females in the Lactation GCs treatment

244   groups were provisioned either with 8 mg per day (n = 10 litters) or 12 mg per day (n = 8 litters).

245   Although the dosage administered to females treated during pregnancy or lactation varied, we

246   grouped those administered GCs in the same treatment group regardless of dosage (i.e.,

247   Pregnancy GCs contained females administered 3-12 mg of hydrocortisone per day, Lactation

248   GCs contained females administered 8 or 12 mg of hydrocortisone per day). We did this for three

249   reasons. First, we have previously shown that females provisioned with 6, 8, or 12 mg of

250   hydrocortisone per day did not differ in their faecal glucocorticoid metabolite levels (FGM),

251   although there were non-significant trends where squirrels fed higher dosages of hydrocortisone

252   had higher FGM (van Kesteren et al., 2019). Second, in preliminary analyses of the data

253   presented here and in a previous study (Dantzer et al., 2013), we found that the effects of

254   different GC dosages (3, 6, 8, and 12 mg per day) that were provided to pregnant females on

255   offspring growth were in the same direction (increased postnatal growth: Table S1). For females

256   treated with Lactation GCs (8 or 12 mg per day), we also found that the effects on offspring

257   growth were in the same direction (decreased postnatal growth: Table S1). Finally, our sample

258   sizes in some of the treatment groups (3 and 6 mg per day in the Pregnancy GCs treatment

259   group) were too small to assess whether there were statistical differences among the different

260   dosage groups.

261   We aimed to treat females in the pregnancy treatments from approximately 20 d after

262   conception until 5 d after birth (or 20-40 d post-conception as red squirrels on average have a

263   ~35 d gestation period), whereas we actually treated females in the pregnancy GCs treatment

264   group from 24.2 ± 0.9 d (mean $\pm$ SE) to 39.0 ± 0.6 d post-conception (mean $\pm$ SE treatment

265   duration: 14.9 d $\pm$ 0.8 d) and females in the pregnancy control treatment group from 20.4 ± 0.8 d

266   to 38.6 ± 0.5 d (treatment duration: 18.2 ± 0.8 d). We aimed to treat females in the lactation

267   treatments from approximately 5 d after parturition until 15 d post-parturition, whereas we

268   actually treated females in the Lactation GCs treatment group from 5.4 ± 0.5 d (mean $\pm$ SE) to

269   14.5 ± 0.6 d post-conception (mean $\pm$ SE treatment duration: 9.1 d $\pm$ 0.1 d) and females in the

270   Lactation Control treatment group from 4.9 ± 0.4 d to 14.1 ± 0.5 d (treatment duration: 9.2 ± 0.5

271   d). Given a ~35 d gestation period and a ~70 d lactation period in this population, our pregnancy

272   treatments corresponded to treating females during the last trimester of gestation and into the

273   first few days of lactation, whereas our lactation treatments corresponded to early lactation

274   before offspring begin to feed independently (they typically leave the nest on their own for the

275   first time at ~35 d). Note that this means that the lactation treatments occurred during a time

276   when the offspring would not be able to consume any of the treatments themselves so any effects

277   on offspring were likely due to the maternal phenotype.

278   Some females in the Pregnancy GCs (n = 3 litters) or Pregnancy Control (n = 2 litters)

279   treatment groups aborted their litters prior to the first nest entry (no females in the lactation

280   treatments aborted their litters prior to the first nest entry). Some females lost their litters (likely

281   due to nest predation: Studd et al., 2015) after the first nest entry but before the second nest entry

282   when we could obtain the second measure of pup body mass to estimate their growth and the

283   only measures of offspring morphology, thereby reducing our sample sizes to estimate the

284   treatment effects on the first measure of offspring body mass, postnatal growth and body size

285   (sample sizes shown in Tables 2-4, S1-S5). Below we show that there was no evidence that the

286   treatments had differential effects on litter survival from the first to second nest entry (see

287   Results).

288   *Tissue sample collection*

289   Pups are weaned when they are approximately ~70 d of age and generally stay on their

290   natal territory until dispersal soon after (Larsen and Boutin, 1994). When juvenile squirrels were

291   ~70 d of age, they were euthanized and tissues (liver and cardiac muscle) were immediately

292   removed, rinsed with PBS buffer, snap frozen on dry ice, and then stored in liquid nitrogen or in

293   a -80 °C freezer until analysis. Trunk blood was collected through decapitation and then

294   centrifuged at 10,000 g for 10 min at room temperature to separate plasma and red blood cells.

295   *Haematocrit*

296   We measured packed red blood cell volume (haematocrit) as a measure of body condition

297   as some (though not all) previous studies have found that higher haematocrit levels correspond to

298   better body condition or improved reproductive performance, at least in some studies of wild

299   birds (Breuner et al., 2013; Minias, 2015; Fronstin et al., 2016). Before pups were euthanized, we

300   collected a blood sample from the hind nail into a heparinized capillary tube. Haematocrit was

301   quantified using a micro-capillary reader after centrifuging blood samples at 10,000 g for 10 min

302   at room temperature.

303   *Protein carbonyls*

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304   We measured oxidative damage to proteins (Monaghan et al., 2009) using the protein

305   carbonyl colorimetric kit by Cayman Chemical (Ann Arbor, USA). Briefly, ~200 mg of cardiac

306   muscle or liver were homogenized in ~1000 µL of 50mM MES buffer containing 1mM EDTA

307   using a sonicator, and then centrifuged at 10,000 g for 15 min at 4 °C. The protein concentration

308   of tissue homogenate supernatant and plasma samples was measured prior to the assay using a

309   Biotek Take3 protocol (Biotek, Vermont, USA) and samples were diluted in PBS buffer to give a

310   protein range between 1-10 mg/ml, as recommended by the manufacturer. The average intra-

311   assay CV for samples for plasma, heart, or liver were 1.2%, 2.8%, and 1.6%, respectively. Inter-

312   assay CVs for a red squirrel pooled sample run on repeat assays for plasma (n = 7 assays), heart

313   (n = 6), or liver (n = 7) were 3.5%, 9.0%, and 8.3%, respectively. We also ran a positive control

314   (oxidized bovine serum albumin) in two different assays and the inter-assay CV was 3.1%.

315   *Superoxide dismutase*

316   We obtained one measure of the levels of enzymatic antioxidants (Monaghan et al., 2009)

317   by quantifying levels of superoxide dismutase (SOD) using the SOD kit from Cayman Chemical.

318   SOD was expressed as units/mg/ml protein (quantified using a Biotek Take3 protocol). Red

319   blood cells were lysed as per the manufacturer's protocol. The average intra-assay CV for

320   samples for RBCs, heart, or liver were 2.3%, 4.7%, and 3.1%, respectively. Inter-assay CVs for a

321   red squirrel pooled sample run on repeat assays for RBCs (n = 10 assays), heart (n = 2), or liver

322   (n = 4) were 16.9%, 4.6%, and 6.5%, respectively.

323   *Total antioxidant capacity*

324   We obtained one measure of the levels of non-enzymatic antioxidants (Monaghan et al.,

325   2009) by quantifying total antioxidant capacity (TAC) using the TAC kit from Cayman

326   Chemical. Plasma was diluted in assay buffer and assayed according to the manufacturer's

327   protocol. Liver and cardiac muscle (~47 mg) were separately homogenized in 250 µL PBS using

328   a sonicator and the supernatant was diluted in assay buffer and used in the assay. The average

329   intra-assay CVs for samples for plasma, heart, or liver were 4.7%, 3.2%, and 4.7%, respectively.

330   The inter-assay CVs for standards run on all the plates for plasma (n = 5 assays) was 15.8%

331   whereas the inter-assay CV for a red squirrel pooled sample run on repeat for heart (n = 8) or

332   liver (n = 2) were 15.4% and 5.4%, respectively.

333   *Telomeres*

334   Liver telomere lengths were measured using the telomere restriction fragment (TRF)

335   assay following established methods (Haussmann and Mauck, 2008). Briefly, 2 to 10 g slices of

336   liver tissue were homogenized in cell lysis solution and proteinase K (Qiagen, Germantown,

337   USA). DNA was extracted from the liver homogenates and resuspended in buffer. The

338   resuspended DNA was restriction digested with 15 U of HinfI, 75U of HaeIII and 40U of RsaI

339   (New England BioLabs, Ipswich, USA) at 37 °C. DNA was then separated using pulsed field

340   electrophoresis at 14˚C for 19 hours followed by in-gel hybridization overnight at 37˚ C with a

341   radioactively labeled telomere-specific oligo (CCCTAA)4. Hybridized gels were placed on a

342   phosphorscreen (Amersham Biosciences, Buckinghamshire, UK), which was scanned on a

343   Typhoon Imager (Amersham Biosciences). Densitometry in ImageJ (v. 1.51s) was used to

344   determine the position and the strength of the radioactive signal in each of the lanes compared

345   with the molecular marker (Quick-Load1 kb DNA Extend DNA Ladder; New England BioLabs)

346   to calculate telomere lengths for each sample. Inter-gel variation was accounted for by

347   calculating the mean TRF length of standard samples run on each gel.

348   *Statistical analyses*

349   We assessed the effects of maternal treatments on litter survival from the first to second

350   nest entry (proportion of pups present at both first and second nest entries) and litter size and

351   litter sex ratio (proportion of litter composed of males) as recorded at the first and second nest

352   entry using generalized linear mixed-effects models (GLMMs: litter survival and litter sex ratio,

353   using binomial errors) or a linear mixed-effect model (LMM: litter size). Each of these separate

354   models contained maternal treatment, birth date, and year as fixed effects. Models for lactation

355   and pregnancy treatments were run separately. There was one litter from a Pregnancy Control

356   treatment female where her litter size at the second nest entry was greater than the at the first,

357   likely because we missed a pup in the nest at the first nest entry, and we therefore excluded this

358   litter from our analyses of the effects of the treatments on litter survival, litter size, and litter sex

359   ratio. We confirmed that none of the GLMMs were overdispersed as all dispersion parameters

360   were <1.

361   We assessed the effects of maternal treatments on offspring body mass at the first nest

362   entry soon after parturition (birth mass), growth in postnatal body mass, and a single measure of

363   size using separate LMMs for pregnancy and lactation treatments. Each of the six LMMs (one

364   model for birth mass, one model for growth, and one model for size for pregnancy treatments;

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365   one model for birth mass, one model for growth, and one model for size for lactation treatments)

366   included a fixed effect for maternal treatment and covariates (sex, year [categorical variable],

367   birth date, litter size) that could impact offspring birth mass, growth, or size. We included a two-

368   way interaction term between treatment and litter size to identify if elevations in maternal GCs

369   altered the trade-off between litter size and offspring birth mass, growth, or size, as shown

370   previously for offspring growth (Dantzer et al., 2013). We included a two-way interaction

371   between treatment and sex to assess if the treatments had sex-specific effects on birth mass,

372   growth, and size, as documented in other species (Dantzer et al., 2019). In our model for the

373   effects of the treatments on offspring birth mass, we included a fixed effect for age of the pups to

374   control for the impact of age on body mass. We used a principal component analysis (PCA)

375   using a covariance matrix in the R package ade4 (version 1.7-13, Dray and Dufour, 2007) to

376   generate a composite score of offspring size. The first principal component axis (PC1, hereafter

377   "size") explained 69.9% of the variation in offspring size as measured by zygomatic arch width

378   and hind foot length. Both zygomatic arch width (0.71) and hind foot length (0.71) loaded

379   positively on PC1, indicating that larger PC1 scores corresponded to offspring with longer hind

380   feet and wider crania.

381   Oxidative stress reflects an imbalance between antioxidants and the production of ROS

382   that can damage proteins, lipids, or DNA (Monaghan et al., 2009). Consequently, the effects of

383   our treatments on measures of antioxidants should not be viewed in absence of their effects on

384   our measures of oxidative damage (Costantini and Verhulst, 2009). We used a PCA to create a

385   composite variable that reflected the oxidative state of an offspring. The PCA was composed of

386   the two antioxidants (SOD, TAC) and one measure of oxidative damage (PCC). We conducted a

387   separate PCA (using a correlation matrix) for each tissue type using the package ade4. For some

388   individuals, we were missing measures of TAC (heart: n = 3; plasma: n = 2) or PCC (heart: n =

389   2; plasma: n = 4) so we substituted average values for the PCA.

390   Low scores for Blood PC2, Heart PC2, or Liver PC1 corresponded to squirrels that were

391   exhibiting oxidative stress as they represented lower levels of the two antioxidants (SOD, TAC)

392   for blood and liver tissue or just one antioxidant (SOD) for heart tissue and, for heart tissue,

393   higher levels of protein damage (PCC, Table 1). We used these composite variables describing

394   oxidative state of each tissue, haematocrit, or telomere length as the response variables in

395   separate LMMs. Each of these LMMs contained a fixed effect for maternal treatment and

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396   offspring sex, year [categorical variable], birth date, and litter size. Because offspring growth

397   may impact oxidative stress levels or telomere lengths (Monaghan and Ozanne, 2018), we

398   included a two-way interaction term between treatment and offspring growth to examine if

399   mothers with elevated maternal GCs exhibited an altered relationship between growth and the

400   response variable (Careau et al., 2014; Merill and Grindstaff, 2018). Due to smaller sample sizes

401   for these variables, we did not include an interaction between sex and treatment. In the model to

402   assess treatment effects on telomere lengths, we also included a fixed effect for the oxidative

403   stress levels in the liver (Liver PC1).

404   All analyses were conducted in R (version 3.5.2, R Development Core Team, 2013) using

405   lme4 (version 1.1-18-1, Bates et al., 2015) and *P*-values for fixed effects were estimated using

406   lmerTest (version 3.0-1, Kuznetsova et al., 2017). Continuous predictor variables were

407   standardized (mean of 0, SD of 1) with birth date, litter size, and growth being standardized

408   within each grid-year-treatment combination. Assumptions of homoscedasticity, normality of

409   residuals for our LMMs, and a lack of high leverage observations were confirmed using

410   diagnostic plots (Zuur et al., 2010). As we only had one estimate of birth mass, growth, size,

411   oxidative stress, or telomere lengths per individual pup, we did not include random intercept

412   terms for individual identity in any of the models described above. Whenever we had repeated

413   observations of the same litter (e.g., multiple estimates of offspring growth from different pups

414   within the same litter), we included a random intercept term for litter identity. However, we did

415   not include a random intercept in some models (shown in electronic supplementary materials)

416   due to model convergence issues that were likely the result of having only a few observations of

417   multiple pups being measured within one litter or for having a relatively small number of litters.

418   As we had few observations of the same females across years for most of our response variables

419   (sample sizes shown above), we did not include a random intercept for maternal identity as this

420   was usually redundant with litter identity. Random effects were not included in our models of the

421   effects of the treatments on litter characteristics (survival, size, sex ratio) as we only had a few

422   females observed across multiple years. We estimated variance inflation factors (VIFs) from our

423   models to assess multicollinearity among the predictor variables (Zuur et al., 2010) and VIFs

424   indicated that multicollinearity was not an issue in these models (all VIF < 3 except if included

425   in an interaction or a multi-level categorical variable).

426

14

427 **Results**

428 *Effects of treatments on litter survival, litter size, & litter sex ratio*

429   There was no evidence that treating mothers with GCs during pregnancy or lactation

430 caused litter failure or altered litter size or litter sex ratio compared to the controls. For those

431 mothers producing offspring until at least the first nest entry (occurring soon after pups were

432 born), the proportion of the litter that survived from the first to the second nest entry did not

433 differ between mothers treated with GCs during pregnancy (n = 42 litters, 53.8 ± 7% of total

434 pups survived from the first to second nest entry) and the pregnancy (time-matched) controls (n

435 = 29 litters, 63 ± 8%, z = -0.66, P = 0.51), nor between mothers treated with GCs during lactation

436 (n = 18 litters, 79.6 ± 8% pups survived) and the lactation (time-matched) controls (n = 17 litters,

437 72.6 ± 9.5% pups survived, z = -0.63, P = 0.53).

438   Litter size did not differ between mothers treated with GCs during pregnancy or the

439 controls at the first nest entry (Pregnancy GCs: n = 42 litters, 3.07 ± 0.14 pups, range = 1-5 pups;

440 Pregnancy Controls: n = 31 litters, 2.9 ± 0.17 pups, range = 1-5 pups, effect of treatment, t_{52} = -

441 0.19, P = 0.85) nor at the second nest entry (Pregnancy GCs: n = 23 litters, 2.75 ± 0.24 pups,

442 range = 1-5 pups; Pregnancy Controls: n = 20 litters, 2.70 ± 0.16 pups, range = 1-4 pups, effect

443 of treatment, t_{37} = 0.11, P = 0.92). Litter size also did not differ between mothers treated with

444 GCs during lactation or the controls at the first nest entry (Lactation GCs: n = 18 litters, 2.67 ±

445 0.2 pups, range = 1-4 pups; Lactation Controls: n = 17 litters, 2.94 ± 0.13 pups, range = 2-4 pups,

446 effect of treatment, t_{30} = -1.11, P = 0.27), nor at the second nest entry (Lactation GCs: n = 16

447 litters, 2.25 ± 0.2 pups, range = 1-3 pups; Lactation Controls: n = 14 litters, 2.57 ± 0.2 pups,

448 range = 1-4 pup, effect of treatment, t_{25} = -1.31, P = 0.20).

449   The litter sex ratio (proportion of males) at the first nest entry did not differ between

450 mothers treated with GCs during pregnancy or the controls (Pregnancy GCs: n = 42 litters, 53 ±

451 4% males; Pregnancy Controls: n = 29 litters, 40.9 ± 5% males, effect of treatment: z = 0.57, P =

452 0.57), nor at the second nest entry (Pregnancy GCs: n = 23 litters, 59.3 ± 7% males; Pregnancy

453 Controls: n = 19 litters, 46.5 ± 7% males, effect of treatment: z = 0.26, P = 0.80). Similarly, the

454 litter sex ratio at the first nest entry did not differ between mothers treated with GCs during

455 lactation or the controls (Lactation GCs: n = 18 litters, 52.8 ± 7.2% males; Lactation Controls: n

456 = 17 litters, 53.4 ± 7.9% males, effect of treatment: z = 0.09, P = 0.92) nor at the second nest

457   entry (Lactation GCs: n = 16 litters, 58.3 ± 8.7% males; Lactation Controls: n = 14 litters, 64.9 ±

458   9.3% males, effect of treatment: $z = -0.27$, $P = 0.79$).

459   *Effects of treating pregnant females with GCs on offspring growth, oxidative stress, and telomere*

460   *lengths*

461   Offspring from mothers treated with GCs during pregnancy grew 17.0% faster ($t_{41.4} =$

462   3.07, $P = 0.004$, Table 2A, Fig. 1A) but were not larger in structural size (Table 2B, Fig. 1C),

463   and did not differ in body condition (as reflected in their haematocrit levels: Table S2A) than

464   those produced by control mothers. After correcting for the effects of age on the first body mass

465   we recorded when pups were first weighed ("birth mass") as well as other variables (Table 3A),

466   offspring from mothers treated with GCs during pregnancy were 8.3% smaller than those from

467   control mothers ($t_{59.8} = -2.51$, $P = 0.015$, Table 3A), suggesting that these pups exhibited catch-

468   up growth after being born at a smaller body mass. There was no indication that the treatments

469   had sex-specific effects on offspring growth or size (Table 2). There was also no indication of a

470   trade-off between litter size and offspring growth or size during the years that we studied (Table

471   2) nor was there any evidence that treating mothers with GCs during pregnancy altered the

472   relationship between litter size and growth rate, as indicated by the lack of significant

473   interactions between treatment and litter size for offspring growth and size (Table 2). Because

474   the treatments had no significant effects on litter size or litter sex ratio (see above), the effects of

475   the treatments on offspring growth were not simply due to a reduction in litter size.

476   Offspring from mothers treated with GCs during pregnancy did not have higher oxidative

477   stress levels in the blood, liver, or heart (Table S3, Fig. 2) and they also did not have shorter

478   telomere lengths (Table S4, Fig. 3). Offspring from mothers treated with GCs during pregnancy

479   or provided with supplemental food during pregnancy (controls) that grew faster did not have

480   higher oxidative stress levels in blood, heart, or liver nor did they have shorter telomere lengths

481   (Tables S3-S4). There was no indication that growth or its interaction with maternal treatment

482   impacted oxidative stress levels (Table S3) or liver telomere lengths (Table S4) in offspring from

483   females treated during pregnancy.

484   *Effects of treating lactating females with GCs on offspring growth, oxidative stress, and telomere*

485   *lengths*

486   Offspring from mothers treated with GCs during lactation grew 34.8% slower ($t_{27} = -$

487   2.08, $P = 0.047$, Table 4A, Fig. 1B), but were not significantly smaller in structural size (Table

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488   4B, Fig. 1D), and did not differ in body condition (as reflected in haematocrit levels: Table S2B)

489   or first body mass (Table 3B) from those of pups from control mothers. There was no indication

490   that the treatments had sex-specific effects on offspring growth or size, as reflected in the lack of

491   significant sex by treatment interactions (Table S4). There was also no indication of a trade-off

492   between litter size and offspring growth or size (Table S4) and no evidence that treating females

493   with GCs during lactation altered the relationship between litter size and growth rate, as

494   indicated by the lack of significant interactions between treatment and litter size for offspring

495   growth and size (Table S4). Because the treatments had no significant effects on litter size or

496   litter sex ratio (see above), the effects of the treatments on offspring growth were not simply due

497   to an increase in litter size.

498   Offspring from mothers treated with GCs during lactation did not have higher oxidative

499   stress levels than those from control mothers in any of the three tissues (blood, heart, liver: Table

500   S5, Fig. 2B, 2D, 2F) and they also did not have shorter telomere lengths (Table S4, Fig. 3B).

501   Offspring from mothers treated with GCs during lactation or provided with supplemental food

502   during lactation (controls) that grew faster did not have higher oxidative stress levels in blood,

503   heart, or liver nor did they have shorter telomere lengths (Table S4). There was also no

504   indication that growth or its interaction with maternal treatment impacted oxidative stress levels

505   (Table S5) or liver telomere lengths (Table S4) in offspring from females treated during

506   lactation.

507

508   **Discussion**

509   Mothers treated with GCs during pregnancy produced offspring that were lighter around

510   birth but then exhibited significantly faster postnatal growth prior to weaning, whereas mothers

511   treated with GCs during lactation tended to produce slower growing offspring that were of

512   similar mass at birth. There were no treatment effects on offspring structural size, indicating that

513   while offspring from mothers treated with GCs gained mass at a different rate than the controls,

514   the treatments did not influence skeletal size. However, we only obtained one measure of

515   structural size when offspring were ~25 d of age, and therefore did not quantify any treatment

516   effects in the rate of change in structural size as we did for body mass. Our results differ from a

517   recent literature analysis across mammals showing that offspring from mothers experiencing late

518   gestational stress grew more slowly before weaning (Berghänel et al., 2017). One explanation for

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519   the difference between this previous literature analysis and our results is that elevated GCs

520   simply modulated the trade-off between offspring quantity and quality (e.g., producing small

521   litters of fast-growing offspring) or ameliorated the trade-off between offspring quantity and

522   quality (e.g., lessening the effect of increased litter size on the growth rate of each individual

523   offspring: Stearns, 1992). However, this is unlikely because we found no treatment effects on

524   litter size nor on the trade-off between litter size and growth rate. Thus, somehow females treated

525   with GCs during pregnancy produced fast growing offspring without merely reducing their litter

526   sizes, though it is notable that in the years in which we conducted this study, we also did not

527   document a trade-off between litter size and offspring growth in any of the GC-treated or control

528   females, as we have found previously (Dantzer et al., 2013). It is possible that we did not find

529   evidence of a trade-off between litter size and offspring growth in the present study because both

530   the GC-treated and control females were fed supplemental food and a previous study showed that

531   *ad libitum* food-supplementation ameliorates this trade-off in red squirrels (Dantzer et al., 2013).

532   GCs are metabolic hormones that have well-known effects on partitioning resources dedicated to

533   survival vs. reproduction within-individuals (Wingfield et al., 1998). Here, it is possible that GCs

534   also play a role in the partitioning of resources between mothers and offspring where they

535   increase investment in offspring (increasing postnatal growth rates without a reduction in litter

536   size) perhaps at the expense of maternal energy stores.

537   We did not find support for the hypothesis that elevated maternal GCs during pregnancy

538   or lactation or increased offspring growth caused an elevation in oxidative stress levels or

539   shortened telomere lengths in offspring. This differs from previous studies that have found that

540   offspring growth is positively correlated with oxidative stress levels or inversely correlated with

541   offspring telomere lengths (Haussmann and Heidinger, 2015; Monaghan and Hausssmann, 2015;

542   Monaghan and Ozanne, 2018). This is surprising and requires explanation. First, we measured

543   oxidative stress levels in offspring when they were weaned (~70 d of age) whereas we treated

544   their mothers with GCs either during pregnancy or early lactation. Thus, it is possible that the

545   offspring in our study experienced elevated oxidative stress levels but these effects had

546   disappeared by weaning. A second possibility is an artefact associated with selective

547   disappearance of poor-quality individuals from those mothers treated with GCs during

548   pregnancy, such as slow growing individuals with short telomeres dying before we could obtain

549   our measures of oxidative stress and telomere lengths. This is unlikely as we observed no

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550   treatment effects on litter size or the reduction in litter size from the first to second nest entry.

551   Finally, the fact that mothers treated with GCs did not produce offspring with elevated oxidative

552   stress levels or shorter telomeres may have been because of the effects of maternally-derived

553   GCs on offspring telomerase levels, an enzyme that is capable of rebuilding telomeres or

554   buffering them from attrition (Blackburn, 2005). We did not measure telomerase levels, but a

555   previous study showed that long-term exposure of laboratory rats to unpredictable stressors

556   increased the production of telomerase (Beery et al., 2012). Thus, a testable prediction for future

557   studies is that treating mothers with GCs promotes increases in telomerase or enzymatic

558   antioxidant production that has protective effects on offspring. This would be consistent with

559   predictions from the oxidative shielding hypothesis (Blount et al., 2016) that proposes that

560   females may reduce their own levels of oxidative damage (perhaps by upregulation of enzymatic

561   antioxidants) to mitigate their detrimental influence on offspring.

562   Our results indicate that elevated GCs can impact maternal investment in the current

563   litter. Females experiencing elevated GCs during pregnancy increased their investment in the

564   current litter (as indicated by an increase in postnatal growth) whereas females experiencing

565   elevated GCs during lactation tended to reduce their investment in the current litter, the latter

566   possibly being because mothers spent less time interacting or nursing their offspring, as in a

567   previous study in laboratory rats (Nephew and Bridges, 2011). Alternatively, the reduction in

568   offspring growth when mothers were treated with GCs during lactation could have been because

569   milk quality or content was altered. Life history theory predicts that such changes in maternal

570   investment in offspring could alter the survival or future reproduction of mothers (Stearns, 1992)

571   or increase their oxidative stress levels (Monaghan and Haussmann, 2006; Blount et al., 2016).

572   However, our previous work in red squirrels shows that their food caching nature results in

573   unexpected patterns with respect to the potential costs of reproductive investment. Red squirrels

574   can elevate reproductive output (i.e., producing a second litter or larger litters) in anticipation of

575   increased future food abundance (Boutin et al., 2006), or produce faster growing offspring when

576   the fitness payoffs warrant increased investment in the current litter (Dantzer et al., 2013). They

577   seem to be able to do this without additional access to food except the food that they already

578   have stored from the previous autumn (Humphries and Boutin, 2000; Boutin et al., 2006;

579   Dantzer et al., 2013). Thus far, survival costs for this increased reproductive investment in the

580   current litter exhibited by female red squirrels seem to be small or absent. Female red squirrels

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581   with increased reproductive effort do expend more energy (Humphries and Boutin, 2000;

582   Fletcher et al., 2012) and experience increased oxidative protein damage (Fletcher et al., 2012)

583   but we have not yet documented substantive survival costs for females that increase their

584   reproductive output (Humphries and Boutin, 2000; Descamps et al., 2009). We have not yet

585   quantified any oxidative or survival costs to mothers who were treated with GCs during

586   pregnancy and who on average produced faster growing offspring. Unless females upregulate the

587   production of protective enzymatic antioxidants or telomerase (Blount et al., 2016), it seems

588   likely that females with elevated GCs would experience increased oxidative damage due to their

589   elevated reproductive investment, or because of the elevated levels of GCs that they experience.

590   For example, previous studies in red squirrels (Fletcher et al., 2012) and other species (Blount et

591   al., 2016) highlight that increased reproductive investment or increased exposure to GCs

592   (Kotrschal et al., 2007; You et al., 2009; Costantini et al., 2011) may elevate oxidative damage in

593   breeding females with elevated GCs during reproduction.

594   Although it is not known whether increased antioxidants, reduced oxidative damage, or

595   elongated telomeres actually cause an increase in longevity (Simons, 2015; but see Munoz-

596   Lorente et al. 2019), our results suggest that fast growing offspring or those from mothers treated

597   with GCs during pregnancy or lactation would not experience a reduction in lifespan. Our results

598   and our previous study in red squirrels (Dantzer et al., 2013) show that maternal GCs during

599   pregnancy or lactation can induce plasticity in offspring growth and that this plasticity should be

600   adaptive for the fluctuating environments experienced by Yukon red squirrels (Dantzer et al.,

601   2013). Specifically, when population density is elevated, females have elevated GCs and fast

602   offspring growth increases offspring survival (Dantzer et al., 2013). We have shown here that

603   females with elevated GCs during pregnancy produce faster growing offspring, whereas females

604   with elevated GCs during lactation produce slower growing offspring. This suggests that

605   plasticity in GC levels during female reproduction (e.g., females have elevated GCs during

606   pregnancy but exert strong negative feedback on further GC production during lactation) would

607   maximize female reproductive success when density is high. However, we did not find support

608   for the hypothesis that elevated maternal GCs induce a faster pace of life where offspring grow

609   faster and are more competitive early in life but this comes at some oxidative cost that may

610   predict a shortened lifespan. Future studies should assess oxidative stress using an even broader

611   array of measures than the few measures we used here, and will of course need to assess if

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612   elevated maternal GCs actually impact offspring lifespan. Our results to date indicate that the

613   increases in maternal GCs during pregnancy in response to population density result in an

614   adaptive maternal effect on offspring postnatal growth and do not pose a developmental

615   constraint on offspring postnatal growth.

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622

623   **Competing Interests:** None

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630   **Data Availability:** All data files are available on FigShare from the first author

631   (https://figshare.com/account/home#/projects/71585). All other requests for data or analysis code

632   will be fulfilled by the first author.

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Table 1. Results from principal component analyses to derive axes of variation of oxidative stress state in weaned pups. For each principal component shown, high values correspond to lower oxidative stress levels as they reflect samples with low levels of two (Blood PC2, Liver PC1) or one (Heart PC2) antioxidants (total antioxidant capacity, superoxide dismutase) and for heart tissue, higher levels of oxidative protein damage (protein carbonyls).

Measurement	Tissue		
	Blood PC2	**Heart PC2**	**Liver PC1**
Total Antioxidant Capacity	-0.57	-0.0007	-0.70
Superoxide Dismutase	-0.82	-0.71	-0.70
Protein Carbonyls	0.03	0.71	0.09
Prop. Variance Explained	34.3%	34.3%	38.5%

864 **Table 2. Effects of treating female red squirrels with glucocorticoids (GCs) during**
865 **pregnancy on offspring postnatal growth (A) and structural size (B).** Offspring growth is the
866 linear change in body mass from ~1 d to ~25 d of age. Offspring size is a composite variable
867 where high scores of PC1 correspond to offspring (one estimate of size obtained at ~25 d of age)
868 with larger zygomatic arch widths and longer hind foot lengths. Models contained random
869 intercept term for litter identity (growth model: $\sigma^2 = 0.07$; size model: $\sigma^2 = 0.29$).
870

(A)

Offspring trait	Variable	b	SE	t	df	P-value
Growth	**Intercept (2012, Control, Female)**	**1.81**	**0.13**	**14.2**	**35.6**	**<0.0001**
	Year (2015)	-0.09	0.22	-0.42	34.5	0.67
	Year (2016)	**-0.58**	**0.13**	**-4.3**	**34.2**	**<0.0001**
	Year (2017)	**-0.74**	**0.13**	**-5.8**	**34.2**	**<0.0001**
	Sex (Male)	**0.074**	**0.03**	**2.10**	**72.6**	**0.04**
	Birth date	-0.001	0.05	-0.03	34.6	0.98
	Litter size	0.06	0.09	0.67	37.0	0.50
	Treatment (GCs)	**0.28**	**0.09**	**3.07**	**41.4**	**0.004**
	Treatment (GCs) x Sex (Male)	-0.08	0.05	-1.64	73.8	0.11
	Treatment (GCs) x Litter size	-0.08	0.11	-0.68	36.7	0.50

Results based upon 114 offspring from 43 litters across 4 years

(B)

Offspring trait	Variable	b	SE	t	df	P-value
Size (PC1)	Intercept (2015, Control, Female)	0.14	0.41	0.35	31.9	0.72
	Year (2016)	**-1.14**	**0.43**	**-2.65**	**28.9**	**0.013**
	Year (2017)	-0.17	0.43	-0.39	29.4	0.70
	Sex (Male)	0.27	0.16	1.69	59.6	0.09
	Birth date	0.14	0.11	1.25	25.9	0.22
	Litter size	0.02	0.21	0.11	33.2	0.92
	Treatment (GCs)	0.10	0.25	0.39	44.8	0.70
	Treatment (GCs) x Sex (Male)	-0.10	0.22	-0.42	60.8	0.67
	Treatment (GCs) x Litter size	-0.21	0.27	-0.77	32.2	0.44

Results based upon 88 offspring from 34 litters across 3 years

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873 **Table 3 Effects of treating female red squirrels with different dosages of glucocorticoids**
874 **(GCs) during (A) pregnancy or (B) lactation on the first body mass measure of offspring**
875 **("birth mass").** Offspring body mass was recorded soon after birth. Models contained random
876 intercept term for litter identity (pregnancy model: $\sigma^2 = 2.3$; lactation model: $\sigma^2 = 0.83$).
877

(A)

Treatment Period	Variable	b	SE	t	df	P-value
Pregnancy	**Intercept (2012, Control, Female)**	**14.64**	**0.50**	**29.4**	**53.1**	**<0.0001**
	Age of pup	**4.81**	**0.22**	**21.8**	**49.0**	**<0.0001**
	Year (2015)	0.04	1.1	0.04	48.7	0.97
	Year (2016)	-0.13	0.60	-0.22	47.9	0.83
	Year (2017)	0.59	0.52	1.14	48.8	0.26
	Sex (Male)	0.32	0.23	1.35	117.8	0.18
	Birth date	-0.20	0.24	-0.83	47.9	0.41
	Litter size	-0.35	0.50	-0.70	53.4	0.49
	Treatment (GCs)	**-1.22**	**0.49**	**-2.51**	**59.8**	**0.015**
	Treatment (GCs) x Sex (Male)	0.42	0.31	1.33	119.4	0.18
	Treatment (GCs) x Litter size	0.59	0.57	1.03	52.6	0.31

Results based upon 168 offspring from 57 litters across 4 years

(B)

Treatment Period	Variable	b	SE	t	df	P-value
Lactation	**Intercept (2015, Control, Female)**	**13.9**	**0.47**	**29.5**	**37.9**	**<0.0001**
	Age of pup	**5.02**	**0.23**	**23.2**	**22.2**	**<0.0001**
	Year (2016)	0.43	0.81	0.53	19.9	0.59
	Year (2017)	0.76	0.43	1.75	21.6	0.09
	Sex (Male)	0.13	0.36	0.36	49.3	0.72
	Birth date	0.13	0.24	0.54	22.1	0.59
	Litter size	0.29	0.39	0.75	22.5	0.46
	Treatment (GCs)	0.20	0.51	0.39	42.8	0.70
	Treatment (GCs) x Sex (Male)	0.26	0.47	0.55	49.5	0.58
	Treatment (GCs) x Litter size	-0.22	0.47	-0.48	24.0	0.64

Results based upon 73 offspring from 30 litters across 3 years

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880 **Table 4. Effects of treating female red squirrels with GCs during lactation on offspring**
881 **postnatal growth (A) and structural (skeletal) size (B).** Offspring growth is the linear change
882 in body mass from ~1 d to ~25 d of age. Offspring size is a composite variable where high scores
883 of PC1 correspond to offspring (~25 d of age) with larger zygomatic arch widths and longer hind
884 foot lengths. Models contained random intercept term for litter identity (growth model: $\sigma^2 =$
885 0.19; size model: $\sigma^2 = 0.69$).
886

(A)

Offspring trait	Variable	b	SE	*t*	df	*P*-value
Growth	**Intercept (2015, Control, Female)**	**1.88**	**0.17**	**10.9**	**25.8**	**<0.0001**
	Year (2016)	-0.12	0.35	-0.34	22.2	0.74
	Year (2017)	-0.29	0.18	-1.57	22.6	0.13
	Sex (Male)	-0.007	0.06	-0.11	41.04	0.91
	Birth date	**0.29**	**0.10**	**2.97**	**22.6**	**0.007**
	Litter size	0.09	0.16	0.55	22.8	0.59
	Treatment (GCs)	**-0.37**	**0.18**	**-2.08**	**27.0**	**0.047**
	Treatment (GCs) x Sex (Male)	0.09	0.08	1.06	41.2	0.30
	Treatment (GCs) x Litter size	0.07	0.19	0.38	23.0	0.71

Results based upon 72 offspring from 30 litters across 3 years

(B)

Offspring trait	Variable	b	SE	*t*	df	*P*-value
Size (PC1)	Intercept (2015, Control, Female)	0.14	0.36	0.40	43.8	0.69
	Year (2016)	0.04	0.56	0.07	18.8	0.94
	Year (2017)	**0.70**	**0.31**	**2.29**	**23.2**	**0.03**
	Sex (Male)	0.22	0.29	0.76	46.1	0.45
	Birth date	**0.56**	**0.16**	**3.37**	**22.6**	**0.003**
	Litter size	-0.10	0.28	-0.37	25.3	0.71
	Treatment (GCs)	-0.56	0.37	-1.51	44.1	0.14
	Treatment (GCs) x Sex (Male)	-0.02	0.37	-0.06	45.0	0.95
	Treatment (GCs) x Litter size	0.64	0.33	1.90	25.6	0.068

887 Results based upon 67 offspring from 30 litters across 3 years
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889   **Figure 1. Effects of treating pregnant or lactating female red squirrels with glucocorticoids**

890   **(GCs) on offspring postnatal growth and structural size. A)** Offspring from females treated

891   with GCs (n = 62 pups) during pregnancy grew significantly faster than those from controls (n =

892   52, $t_{41.4}$ = 3.07, P = 0.004). **B)** Offspring from females treated with GCs during lactation (n = 36)

893   grew significantly slower than those from controls (n = 36, t_{27} = –2.08, P = 0.047). **C & D)**

894   Offspring from mothers treated with GCs either during pregnancy (n = 46) or lactation (n = 35)

895   did not differ in structural size compared to those from controls (pregnancy: n = 42; lactation: n

896   = 32, Tables 2 & 4). Offspring growth was measured as the change in body mass from ~1 to ~25

897   d of age. Offspring size is a composite variable where high scores of PC1 correspond to

898   offspring with larger zygomatic arch widths and longer hind foot lengths when pups were ~25 d

899   of age. Results in Tables 2 & 4. Upper and lower hinges correspond to the first and third

900   quartiles while white diamonds indicate means and horizontal line indicate medians.

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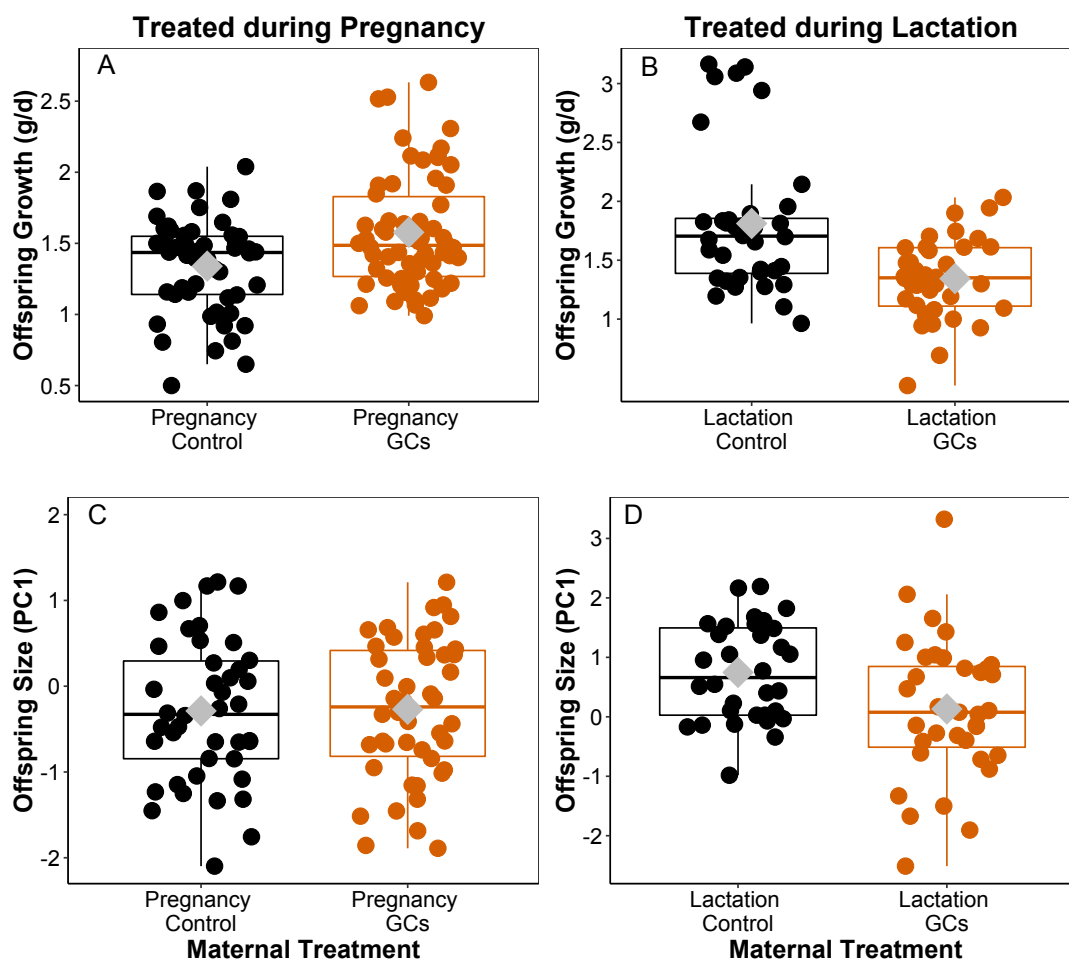
Figure 2. Effects of treating pregnant or lactating female red squirrels with glucocorticoids (GCs) on oxidative stress levels in blood, liver, and heart tissue from weaned juvenile red squirrels. There were no significant treatment effects on oxidative stress levels in the blood (**A, B**), heart (**C, D**), or liver (**E, F**) for offspring produced by mothers treated with GCs during pregnancy (n = 20 pups) or lactation (n = 13) compared with the controls (pregnancy: n = 21; lactation: n = 14, statistical results in Tables S3 & S5). Values on y-axes reflect a composite variable generated by separate principal component analyses for blood, heart, and liver tissue where high scores correspond to low levels of one or two of the antioxidants (TAC, SOD) and, for heart, higher levels of protein damage (PCC; see Table 1). Note differences between y-axes among the panels.
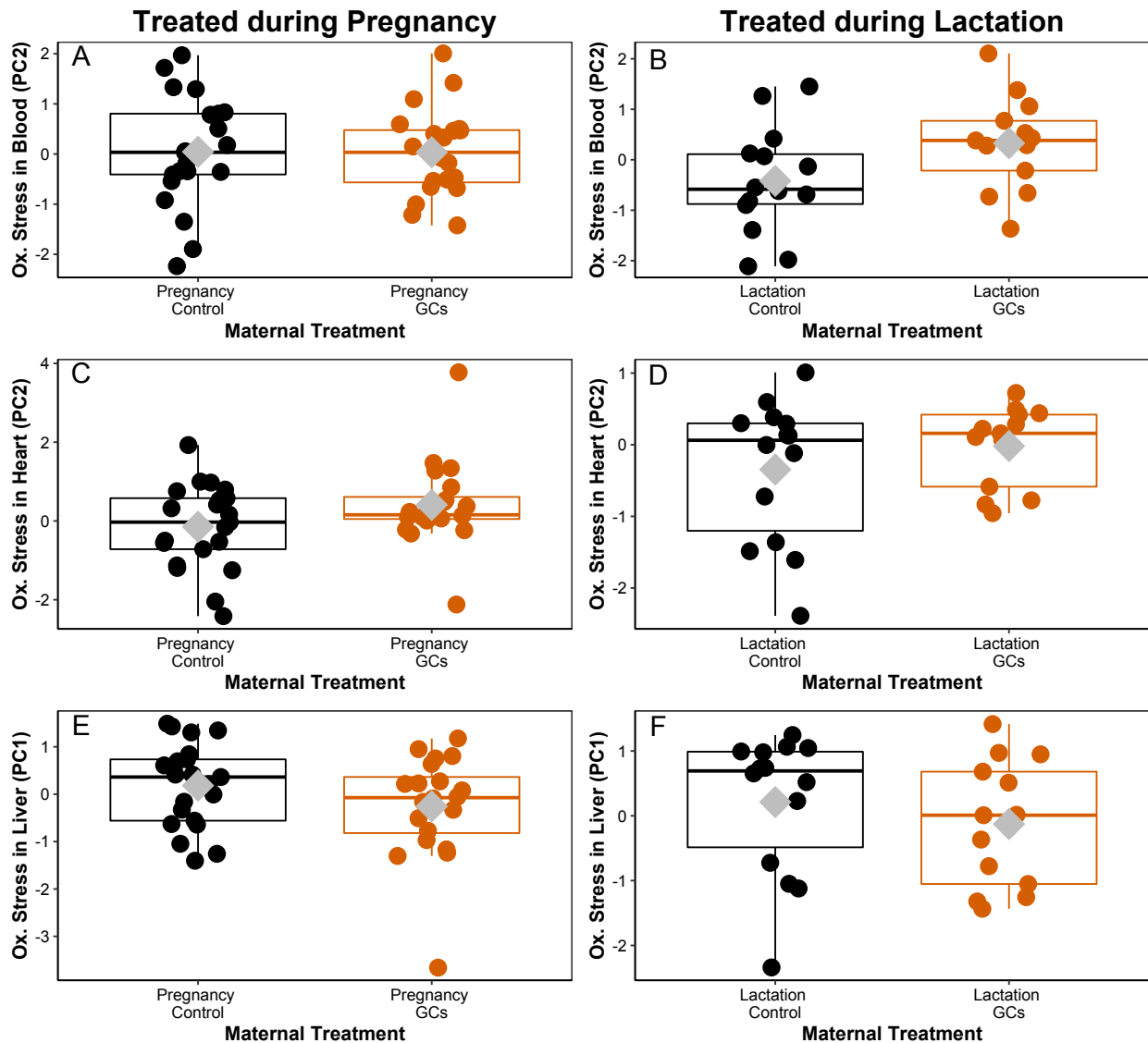
914   **Figure 3. Effects of treating pregnant or lactating female red squirrels with glucocorticoids**

915   **(GCs) on mean liver telomere lengths in weaned offspring. A & B)** There were no significant

916   treatment effects on liver telomere lengths for offspring produced by mothers treated with GCs

917   during pregnancy (n = 20 pups) or lactation (n = 13) compared with the controls (pregnancy: n =

918   21; lactation: n = 14, statistical results in Table S4).
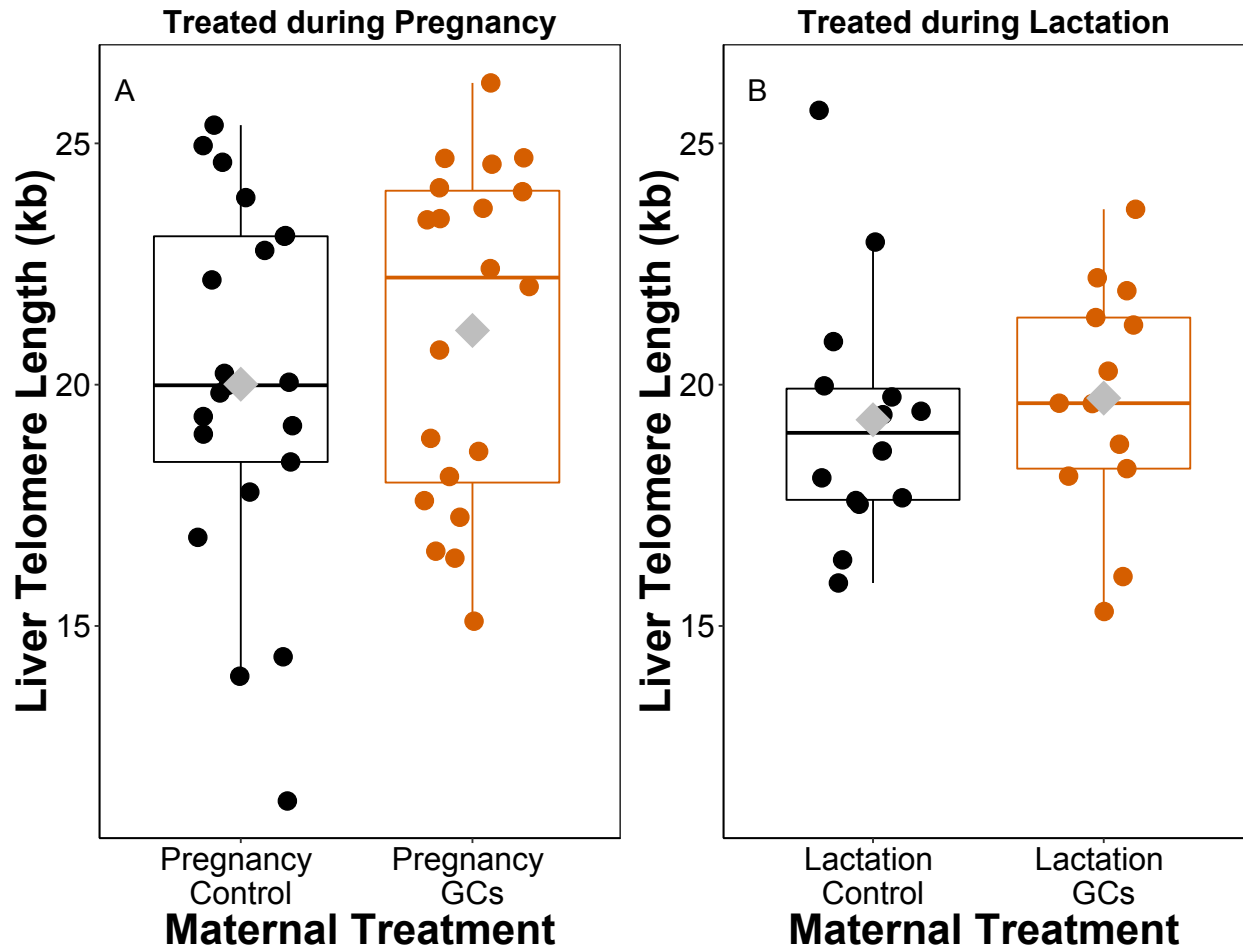
919

1   ***Electronic Supplementary Material***

2

3   **Maternal glucocorticoids promote offspring growth without inducing oxidative stress or**

4   **shortening telomeres in wild red squirrels**

5

6   Ben Dantzer[1,2], Freya van Kesteren[1], Sarah E. Westrick[1], Stan Boutin[3], Andrew G. McAdam[4],

7   Jeffrey E. Lane[5], Robert Gillespie[6], Ariana Majer[7], Mark Haussmann[7], Pat Monaghan[6]

8

9   **Supplementary Results**

10

11 **Table S1. Preliminary analyses for effects of treating female red squirrels with different**
12 **dosages of glucocorticoids (GCs) during (A) pregnancy or (B) lactation on offspring**
13 **postnatal growth.** Offspring growth is the linear change in body mass from ~1 d to ~25 d of
14 age. The dosages of GCs are listed below with 0 mg (pregnancy control treatment, n = 52 pups;
15 lactation control treatment, n = 36 pups) as the reference value. Models contained random
16 intercept term for litter identity (pregnancy model: $\sigma^2 = 0.059$; lactation model: $\sigma^2 = 0.23$). Note
17 that these results were used to justify our decision of lumping the different pregnancy GCs
18 treatment groups (with different dosages of GCs) together.

(A)

Treatment Period	Variable	b	SE	t	df	P-value
Pregnancy	**Intercept (2012, Female, 0 mg)**	**1.56**	**0.17**	**9.3**	**32.0**	**<0.0001**
	Year (2015)	0.06	0.21	0.31	32.2	0.76
	Year (2016)	-0.26	0.18	-1.41	31.7	0.17
	Year (2017)	**-0.41**	**0.18**	**-2.28**	**31.6**	**0.03**
	Sex (Male)	0.037	0.02	1.45	74.5	0.15
	Birth date	-0.001	0.05	-0.01	32.8	0.99
	Litter size	0.035	0.05	0.72	34.2	0.48
	Treatment (GC dosage)					
	3 mg GCs (n = 9 pups)	0.37	0.24	1.52	30.9	0.14
	6 mg GCs (n = 7 pups)	**0.68**	**0.20**	**3.45**	**32.4**	**0.001**
	8 mg GCs (n = 45 pups)	0.12	0.09	1.40	33.1	0.17
	12 mg GCs (n = 1 pup)	**0.91**	**0.31**	**2.95**	**36.9**	**0.005**

Results based upon 114 offspring from 43 litters across 4 years

(B)

Treatment Period	Variable	b	SE	t	df	P-value
Lactation	**Intercept (2015, 0 mg, Female)**	**1.96**	**0.19**	**9.9**	**23.7**	**<0.0001**
	Year (2016)	-0.12	0.34	-0.35	22.1	0.73
	Year (2017)	-0.49	0.25	-1.94	22.5	0.065
	Sex (Male)	0.04	0.04	1.10	42.5	0.27
	Birth date	**0.25**	**0.10**	**2.46**	**22.8**	**0.021**
	Litter size	0.15	0.09	1.53	23.1	0.14
	Treatment (GCs)					
	8 mg GCs (n = 24 pups)	-0.16	0.22	-0.74	22.1	0.47
	12 mg GCs (n = 13 pups)	-0.55	0.27	-1.99	23.1	0.058

Results based upon 72 offspring from 30 litters across 3 years

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20 **Table S2. Effects of treating female red squirrels with GCs during (A) pregnancy or (B)**
21 **lactation on offspring haematocrit levels (packed red blood cell volume) collected from**
22 **weaned offspring**. Results for the pregnancy model contained random intercept term for litter
23 identity (pregnancy: $\sigma^2 = 18.8$) whereas results for the lactation model are from a general linear
24 model.
25

(A)

Treatment Period	Variable	b	SE	t	df	P-value
Pregnancy	**Intercept (2015, Control, Female)**	**46.6**	**3.3**	**14.2**	**18.5**	**<0.0001**
	Year (2016)	-4.93	3.5	-1.42	17.0	0.17
	Year (2017)	-4.78	3.3	-1.45	16.5	0.17
	Sex (Male)	1.88	1.1	1.75	16.2	0.10
	Birth date	-0.39	1.2	-0.31	18.4	0.76
	Growth	-1.14	1.3	-0.88	29.9	0.38
	Litter size	-0.75	1.2	-0.62	17.4	0.54
	Treatment (GCs)	-0.45	2.1	-0.21	18.8	0.84
	Treatment (GCs) x Growth	1.85	2.2	0.85	29.7	0.40

Results based upon 39 offspring from 25 litters across 3 years

(B)

Treatment Period	Variable	b	SE	t	df	P-value
Lactation	**Intercept (2015, Control, Female)**	**44.5**	**2.3**	**19.4**	**15**	**<0.0001**
	Year (2016)	**-6.35**	**2.9**	**-2.17**	**15**	**0.046**
	Year (2017)	-4.16	2.3	-1.77	15	0.096
	Sex (Male)	**5.27**	**2.0**	**2.57**	**15**	**0.021**
	Birth date	**3.99**	**1.8**	**2.20**	**15**	**0.04**
	Growth	-2.13	1.7	-1.23	15	0.24
	Litter size	2.76	1.9	1.46	15	0.16
	Treatment (GCs)	2.27	2.3	0.98	15	0.34
	Treatment (GCs) x Growth	2.56	2.3	1.09	15	0.29

Results based upon 24 offspring from 16 litters across 3 years

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29 **Table S3. Effects of treating pregnant red squirrels with GCs on oxidative stress levels in**
30 **(A) blood, (B) heart, and (C) liver tissue from weaned offspring.** High PC scores correspond
31 to low levels of antioxidants and, for heart, higher levels of protein damage (see Table 1).
32 Models for blood and liver tissues contained random intercept term for litter ID (blood: $\sigma^2 =$
33 0.32; liver: $\sigma^2 = 0.04$).
34

(A)

Offspring trait	Variable	b	SE	t	df	P-value
Blood PC2	Intercept (2015, Control, Female)	-0.61	0.53	-1.14	14.0	0.27
	Year (2016)	0.44	0.57	0.76	13.8	0.46
	Year (2017)	0.32	0.54	0.59	13.6	0.56
	Sex (Male)	**0.58**	**0.28**	**2.06**	**23.8**	**0.050**
	Birth date	**-0.50**	**0.22**	**-2.29**	**18.9**	**0.034**
	Growth	-0.15	0.25	-0.62	23.5	0.54
	Litter size	-0.37	0.21	-1.79	17.4	0.09
	Treatment (GCs)	0.21	0.37	0.57	16.6	0.57
	Treatment (GCs) x Growth	-0.57	0.42	-1.33	21.0	0.20

(B)

Offspring trait	Variable	b	SE	t	df	P-value
Heart PC2	Intercept (2015, Control, Female)	-0.69	0.51	-1.34	32	0.19
	Year (2016)	0.99	0.55	1.78	32	0.084
	Year (2017)	0.62	0.52	1.19	32	0.24
	Sex (Male)	-0.09	0.34	-0.26	32	0.79
	Birth date	0.34	0.22	1.52	32	0.14
	Growth	-0.003	0.25	-0.01	32	0.99
	Litter size	0.41	0.21	1.94	32	0.06
	Treatment (GCs)	0.27	0.37	0.73	32	0.47
	Treatment (GCs) x Growth	0.39	0.43	0.91	32	0.37

(C)

Offspring trait	Variable	b	SE	t	df	P-value
Liver PC1	**Intercept (2015, Control, Female)**	**-1.02**	**0.38**	**-2.71**	**12.4**	**0.018**
	Year (2016)	0.60	0.41	1.48	12.1	0.16
	Year (2017)	**1.76**	**0.38**	**4.63**	**12.1**	**0.0006**
	Sex (Male)	0.02	0.24	0.09	28.7	0.93
	Birth date	0.04	0.16	0.25	18.0	0.81
	Growth	-0.21	0.18	-1.14	18.6	0.27
	Litter size	0.09	0.15	0.57	17.3	0.57
	Treatment (GCs)	-0.28	0.27	-1.02	14.6	0.32
	Treatment (GCs) x Growth	-0.07	0.31	-0.24	16.3	0.81

35 Results from 41 offspring from 26 litters over 3 years.

36 **Table S4. Effects of treating female red squirrels with GCs during (A) pregnancy or (B)**
37 **lactation on liver telomere lengths (kb) of weaned offspring.** Telomeres measured in DNA
38 from liver tissue using the TRF method. Models contained random intercept term for litter
39 identity (pregnancy: $\sigma^2 = 0.96$; lactation: $\sigma^2 = 6.7$).
40

(A)

Treatment Period	Variable	b	SE	*t*	df	*P*-value
Pregnancy	**Intercept (2015, Control, Female)**	**20.59**	**1.40**	**14.7**	**15.5**	**<0.0001**
	Year (2016)	**3.69**	**1.41**	**2.61**	**13.0**	**0.022**
	Year (2017)	-3.07	1.63	-1.88	19.0	0.076
	Sex (Male)	0.33	0.76	0.43	26.4	0.67
	Birth date	0.54	0.54	1.01	18.2	0.32
	Growth	-0.30	0.62	-0.49	20.7	0.63
	Litter size	-0.39	0.51	-0.76	17.7	0.45
	Liver PC1	0.23	0.57	0.41	30.8	0.69
	Treatment (GCs)	0.42	0.92	0.45	15.5	0.65
	Treatment (GCs) x Growth	1.18	1.04	1.14	17.5	0.27

Results based upon 41 offspring from 26 litters across 3 years

(B)

Treatment Period	Variable	b	SE	t	df	*P*-value
Lactation	**Intercept (2015, Control, Female)**	**19.03**	**1.97**	**9.67**	**14.9**	**<0.0001**
	Year (2016)	2.88	2.61	1.10	11.5	0.29
	Year (2017)	0.29	2.68	0.11	15.8	0.91
	Sex (Male)	0.12	1.04	0.11	11.1	0.91
	Birth date	-0.09	1.24	-0.07	12.1	0.94
	Growth	-0.08	0.86	-0.09	16.6	0.93
	Litter size	0.57	1.52	0.37	11.9	0.71
	Liver PC1	-0.41	0.85	-0.48	16.2	0.64
	Treatment (GCs)	0.52	1.49	0.35	11.3	0.73
	Treatment (GCs) x Growth	1.03	1.17	0.88	14.1	0.39

Results based upon 27 offspring from 18 litters across 3 years

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53 **Table S5. Effects of treating lactating red squirrels with GCs on oxidative stress levels in**
54 **(A) blood, (B) heart, and (C) liver tissue from weaned offspring.** High PC scores correspond
55 to low levels of antioxidants and, in heart tissue, higher levels of protein damage (Table 1). The
56 model for liver contained a random intercept term for litter identity (liver: $\sigma^2 = 0.33$).
57

(A)

Offspring trait	Variable	b	SE	*t*	df	*P*-value
Blood PC2	Intercept (2015, Control, Female)	0.21	0.52	0.40	18	0.69
	Year (2016)	-0.16	0.73	-0.22	18	0.83
	Year (2017)	-0.56	0.57	-0.99	18	0.34
	Sex (Male)	-0.53	0.49	-1.06	18	0.30
	Birth date	-0.23	0.39	-0.59	18	0.56
	Growth	0.39	0.33	1.17	18	0.26
	Litter size	-0.42	0.46	-0.92	18	0.37
	Treatment (GCs)	0.44	0.44	1.01	18	0.32
	Treatment (GCs) x Growth	-0.29	0.47	-0.62	18	0.54

(B)

Offspring trait	Variable	b	SE	t	df	*P*-value
Heart PC2	**Intercept (2015, Control, Female)**	**-1.14**	**0.41**	**-2.78**	**18**	**0.012**
	Year (2016)	0.55	0.57	0.97	18	0.35
	Year (2017)	0.84	0.44	1.87	18	0.08
	Sex (Male)	0.36	0.39	0.92	18	0.37
	Birth date	0.23	0.31	0.76	18	0.46
	Growth	-0.09	0.26	-0.36	18	0.72
	Litter size	0.27	0.36	0.74	18	0.47
	Treatment (GCs)	0.58	0.34	1.68	18	0.11
	Treatment (GCs) x Growth	-0.16	0.37	-0.44	18	0.66

(C)

Offspring trait	Variable	b	SE	t	df	*P*-value
Liver PC1	**Intercept (2015, Control, Female)**	**-1.35**	**0.40**	**-3.38**	**11.9**	**0.005**
	Year (2016)	0.71	0.62	1.14	9.6	0.28
	Year (2017)	**2.24**	**0.47**	**4.81**	**9.9**	**0.0007**
	Sex (Male)	0.12	0.31	0.40	14.1	0.69
	Birth date	-0.18	0.31	-0.59	11.4	0.57
	Growth	-0.29	0.23	-1.25	17.6	0.22
	Litter size	-0.35	0.37	-0.93	10.7	0.37
	Treatment (GCs)	-0.12	0.37	-0.32	10.1	0.75
	Treatment (GCs) x Growth	-0.13	0.33	-0.40	17.9	0.69

58 Results from 27 offspring from 18 litters over 3 years.
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