# Sex-specific relationship between maternal and neonate cortisol in a free-ranging large mammal

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**Open science and data availability statement**

Following Kane & Amin (2023), all versions, updates and additional material are stored transparently on OSF (DOI 10.17605/OSF.IO/4YMC8). In addition, we have published the data and the script used for the analysis under CC-BY 4.0 license, also on OSF (Amin et al., 2023). These can be found via the following link: https://doi.org/10.17605/OSF.IO/WYNKE.

**Author contributions**

BA: Conceptualization, data curation, formal analysis, investigation, visualization, writing -original draft preparation; RF: Conceptualization, writing – original draft preparation, writing – review & editing; MQ: Data curation, investigation, writing – review & editing; DM: Investigation, methodology, writing – review & editing; RP: Investigation, methodology, writing – review & editing; LK: Investigation, methodology, writing – review & editing; SC: Conceptualization, funding acquisition, resources, project administration, supervision, writing – review & editing

**Abstract**

Maternal phenotypes can have long-term effects on the offspring phenotype starting at gestation. Proximate mechanisms include foetal hormone levels affected by maternal levels. Recent studies have highlighted that these maternal effects may be different between the sexes. How maternal glucocorticoid (GC) levels relate to foetal levels is, however, still not completely understood. Here we related, for the first time in a free-ranging large mammal, the fallow deer (*Dama dama*), maternal GC levels with foetal *in utero* GC levels by using novel state-of-the-art methods. We found that foetal GC levels were positively associated with maternal GC levels, but only in females. These findings highlight sex differences, which may have evolved to optimize male growth at the cost of survival.

**Keywords**

Glucocorticoids, free-ranging, fallow deer, hair-testing, faecal metabolites

54 **Introduction**

55 Parental phenotypes can be drivers of offspring variation, and in many mammalian species, these
56 parental effects are often labelled as maternal effects since it is the mother that mostly takes care of
57 the offspring. Maternal effects are seen as an adaptive way in which mothers can either fine tune
58 their offspring for the current environmental conditions (Sheriff & Love, 2013), or maximise their
59 own reproductive output (Marshall & Uller, 2007). These effects on the offspring are present from
60 the earliest stages of development and can have long lasting influence on offspring phenotype (Seckl
61 & Meaney, 2004; Weinstock, 2008).

62 Acute stress typically leads to an increase in circulating glucocorticoid (GC) levels due to
63 activation of the hypothalamic-pituitary-adrenal (HPA) axis (Sapolsky et al., 2000; Weinstock, 2008).
64 Maternal GC levels during gestation can affect offspring phenotype in many ways, including affecting
65 offspring birth weight and HPA-axis reactivity (Dantzer et al., 2013; Seckl & Holmes, 2007; Seckl &
66 Meaney, 2004) and these effects can linger until offspring are well into adulthood (e.g. 8), which is
67 also referred to as foetal programming (Seckl & Holmes, 2007). There is increasing evidence,
68 however, that there are sex differences in foetal programming (Braithwaite et al., 2018; Fishman et
69 al., 2022; Liu et al., 2001).

70 Most studies investigating hormonal maternal effects investigate offspring GC levels after
71 birth and most evidence comes from either clinical studies on humans (Gitau et al., 1998), rodents
72 (Weinstock, 2008) or birds (Groothuis et al., 2019; Jenkins et al., 2014), with a paucity of data on
73 free-ranging mammals. This is usually due to the challenges of measuring maternal and foetal
74 hormone levels during gestation in non-captive mammals. Recent developments, however, have
75 enabled measuring foetal hormonal levels post-parturition by quantifying steroids in neonate hair
76 (Amin et al., 2021; Fishman et al., 2019; Kapoor et al., 2016). Hair steroid levels represent long-term
77 levels, accumulated over the last weeks to months (Gormally & Romero, 2020), and therefore reflect
78 *in utero* integrated levels when quantified in neonates (Amin et al., 2021). Whether these levels are
79 associated with circulating maternal GC levels, however, is currently not well understood.

Here we explored, for the first time in free-ranging large mammals, the relationship between circulating maternal GC levels and offspring *in utero* accumulated GC levels. For that purpose, we collected faeces of mothers during late gestation, from which we quantified GC metabolites (Palme, 2019), and related that to their offspring hair GC levels (Amin et al., 2021), which were collected during the first days post-parturition at neonate captures. We made sure to sample females within their last five weeks of gestation, since that is when the foetus is fully covered in fur (Chapman & Chapman, 1997). To allow for sex differences in the association between maternal and foetal GC levels, we ran an additional analysis to explore differences between male and female offspring.

## Methods

### *Study population*

We conducted this study in Phoenix Park, a 7.07 $km^2$ urban park located in Dublin, Ireland. There is a resident population of free-ranging fallow deer that has been introduced in the 17$^{th}$ century, with a population size of about 600 individuals in late summer, after fawn births (Griffin et al., 2022). Most births occur between early and late June of each year. Fallow deer are a hider species and fawns remain hidden, usually in tall grass or understory vegetation, away from the main doe herd during the first weeks of life after which they are brought into the doe herd by their mothers (Chapman & Chapman, 1997; Ciuti et al., 2006). Fawns are occasionally predated upon by red foxes (*Vulpes vulpes*), the only natural predator in the park, and domestic dogs who are brought into the park by public visitors.

### *Faeces collection and analysis of maternal faecal cortisol metabolites (FCMs)*

Faecal samples were collected between 8 AM and 3 PM, from May 19th until May 29th 2020, when the does were in late gestation. Groups of deer were observed from a distance of 50 meters using a spotting scope to identify individuals (>80% of the population is identifiable via unique colour coded ear-tags). Fresh faecal samples were collected within a minute of defecation and immediately stored in zip locked bags. These were kept in a cooler bag until they could be stored in a freezer at -20˚C, which was always within a few hours. Samples (see *Sample sizes*-section below) were kept frozen on dry ice during transportation to the University of Veterinary Medicine (Vienna, Austria), where cortisol metabolites in the samples were quantified (Palme, 2019). We added 5 mL of methanol (80%) to weighed aliquots (0.5 g) of homogenized faecal samples, after which the samples were shaken and centrifuged as previously described (Palme et al., 2013). FCMs were analysed with an 11-oxoaetiocholanolone enzyme immunoassay (for details see Möstl et al., 2002), which has already been validated for fallow deer (Konjević et al., 2011).

*Neonate hair collection and hair GC level quantification*

Neonate hair samples were collected during the fawn captures in June 2020. Fawns were routinely captured with fishing nets, as part of the long-term management of the population. During handling, we collected physiological and behavioural data (see Amin et al., 2021 (Amin et al., 2021) for full details on the capture protocol), as well as hair samples (>100 mg) from the belly of the fawn using an electric trimmer (Wahl model 9639; Wahl Clipper Corporation). We extracted cortisol levels from fawn hair using a standardized protocol for hair-testing (e.g. 15,25), by using commercial enzyme immunoassay (EIA; Salimetrics; Ann Arbor, MI, USA, item no. 1-3002) kits. Full details on these extractions and validations are described in Amin et al., 2021 and in the supplementary material (S1).

*Mother-fawn pairing*

Mother-fawn pairs were based on field observations with data collection starting in July, when young fawns were making their first entrances into the female herds. For details regarding the pairing, see Griffin et al., 2022 and Supplementary S1.

*Sample sizes*

We collected and analysed a total of 164 faecal samples of 99 different pregnant does. For individuals with multiple values, we took the mean of these values to create a dataset that had one estimate for each individual. Out of these, we were able to pair 41 does with their fawns through the mother-fawn observations during summer. We removed one extreme outlier from the male neonate cortisol dataset (value: 29.5 pg/mg hair; range of other values: 7.7-18.3 pg/mg hair). Running our analysis with and without this outlier revealed that removing this outlier did not affect the conclusions of our analysis. This led to our final sample size of 40 fawns (18 females and 22 males) paired with their mothers.

*Statistical analysis*

All analyses were performed in RStudio (Version 1.3.1093) using R version 4.0.2 (R Core Team, 2020). We first checked whether maternal or neonatal GC levels differed between the offspring sexes by performing a t-test, considering unequal variances.

To investigate the relationship between neonate hair GC and maternal FCMs, we ran a linear model for each fawn sex separately. In both models, we had the neonate hair GC as the response variable and the maternal FCMs as the explanatory variable. Maternal FCM levels were log-transformed to improve model fit, since these suffered from a slight positive skew. Model assumptions were checked with the *DHARMa*-package (Version 0.4.3; Hartig, 2021) and were successfully met. Statistical inferences were made based on the estimate and the associated 95% Confidence Interval from the models. During the preliminary analysis, we included the number of days between the collection of mothers' faeces and the day of fawn birth in our models. This was to

account for potential variation in FCM levels as a function of gestational day of collection (i.e. mid May vs late May when closer to parturition). This additional predictor had no clear effect and made our models worse (higher AIC); we therefore decided not to include it. We plotted our results using the *ggplot2*-package (Wickham, 2016).

Finally, we decided to run an additional model *post-hoc*, including both sexes in the same model, to further investigate whether the slopes of our models were different between male and female offspring. We ran a linear model with neonate GC as the response variable. As explanatory variables, we included maternal FCMs (log transformed), fawn sex and the interaction between both these explanatory variables.

**Results**

Considerable variation was found in both the individual maternal FCM levels (range: 91-608 ng/g faeces) as well as the neonate hair GC levels (range: 6.50-18.30 pg/mg hair). We found no clear sex differences, neither for mean maternal FCM levels (t-test; t = 0.56, 95% CI [-51.99, 91.50], p = 0.58) nor mean neonate hair GC levels (t-test; t = 0.26, 95% CI [-1.74, 2.25], p = 0.80).

We found that female hair cortisol was positively associated with maternal FCMs (LM: β = 5.88, 95% CI [0.20, 11.57], p = 0.04, N = 18, $R^2_{adjusted}$ = 0.18; Fig. 1A). For males, however, there was no clear association between both measures (LM: β = 0.59, 95% CI [-1.32, 2.49], p = 0.53, N = 22, $R^2_{adjusted}$ = -0.03; Fig. 1B). Our post-hoc model confirmed that there was indeed an interaction between fawn sex and maternal levels, where the slope between maternal and foetal levels was lower in male offspring compared to female offspring (LM: β = -5.30, 95% CI [-10.39, -0.20], p = 0.04, N = 40, $R^2_{adjusted}$ = 0.10).

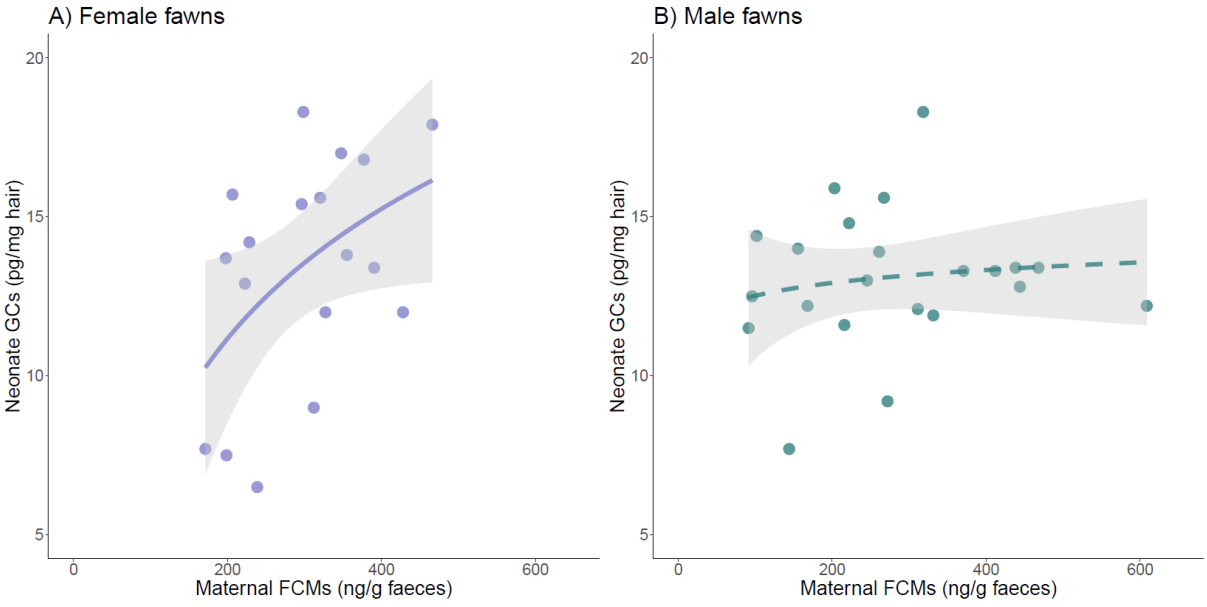


**Figure 1:** The relationship between maternal faecal cortisol metabolites (FCMs) and neonate hair GCs, for female (A) and male (B) offspring. Trendlines and their 95% confidence intervals are fitted in each plot. Solid trendline indicates a statistically meaningful relationship.

184 **Discussion**

185 In this study we found that maternal FCM levels were positively related to foetal GC levels, but only
186 in females. In male offspring, we found no clear relationship between maternal and foetal GC levels.
187 These findings suggest that maternal GC levels may affect male offspring less then female offspring.
188 We discuss here how, from an evolutionary point of view, sex-specific mechanisms may have
189 evolved to optimize life-history trade-offs.

190 Like many other species, fallow deer have a skewed mating distribution (McElligott et al.,
191 2001; Moore et al., 1995). While females tend to produce one fawn per year (Chapman & Chapman,
192 1997), male reproduction skew is steep. Only a small proportion of the males reproduces, with the
193 majority having no or very little offspring (Ciuti et al., 2011; McElligott et al., 2001; Moore et al.,
194 1995). Therefore, there may be a strong selection for males to prioritize growth over survival, since
195 low quality sons will end up costing resources, with no fitness benefits in the long run. A recent
196 study has shown that male birthweights in our populations are indeed higher than female
197 birthweights (Griffin et al., 2023).

198 GC levels play a key role during gestation and have major effects on offspring phenotype
199 early in life. In addition to being crucial for organ development during the late stages of pregnancy
200 (Kitterman et al., 1981; Liggins, 1994), GCs play a role in fighting inflammation (Auphan et al., 1995)
201 and thereby help to keep the foetus vital. At the same time, high GC levels may restrict growth
202 (Edwards et al., 1996; Meakin et al., 2021; Seckl & Holmes, 2007). As males tend to prioritize growth
203 (Meakin et al., 2021), it may be that there would be sex differences in the mechanisms that evolved.
204 For example, maternal GC contribution may be limited in male foetuses to prevent growth
205 retardation, at the cost of prenatal survival. This may contribute in some degree to why males
206 generally face higher prenatal mortality rates (Desportes et al., 1994; Eriksson et al., 2010; Kruuk et
207 al., 1999).

208 The mammalian placenta likely plays a crucial role in mediating these sex differences.
209 Previous studies, in rodents and humans, have shown sex differences in the structure and activity of

210 the placenta (Meakin et al., 2021; Murphy et al., 2003; O'Connell et al., 2013; Rosenfeld, 2015;
211 Wieczorek et al., 2019). There is also evidence that maternal GCs may transport more easily through
212 female placentas than males' (Wieczorek et al., 2019). This may be, for instance, through different
213 levels of expression and activity of enzymes or transporters that mitigate different maternal GC level
214 spill over between males and females.

215 One of the key enzymes, 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), inactivates
216 cortisol to cortisone (Edwards et al., 1996; Tomlinson & Stewart, 2001). Placental 11β-HSD has been
217 frequently shown to be positively related to birthweight (Edwards et al., 1996; Meakin et al., 2021;
218 Seckl et al., 2000; Seckl & Meaney, 2004), thus likely playing a role in preventing growth restriction.
219 Previous studies have shown that male foetuses have higher expression or activity of 11β-HSD2 in
220 response to maternal GC surges (Murphy et al., 2003; Wieczorek et al., 2019). Similarly in ovine,
221 male foetuses increased 11β-HSD2 in response to maternal dexamethasone administration, whereas
222 female foetuses did not (Braun et al., 2009). This may explain why a different ovine study also found
223 that, following an antenatal GC treatment, growth restriction was lower in males than in females
224 (Miller et al., 2012). In addition, male mice were shown to also have increased ATP-binding cassette
225 transporters, which mediate GC efflux toward maternal circulation (Wieczorek et al., 2019),
226 indicating that there are different mechanisms through which males may be able to limit maternal
227 GC levels (Montano et al., 1993). This altogether may explain why females may have higher foetal
228 survival (Meakin et al., 2021), whereas males may be larger and faster growing.

229 We acknowledge that there are some shortcomings to this study. We report patterns taken
230 over only one year, whereas ecological patterns may vary between years. Furthermore, we have
231 modest sample sizes (due to the difficulty of the design), which restrict us in doing more elaborate
232 analyses. Nevertheless, as one of the first studies quantifying the relationship between maternal and
233 foetal GC levels non-invasively, we do provide novel insights into how these fundamental
234 relationships may function in a free-ranging large mammal population. It is important to note that

235 the nature of the study described here is exploratory, meaning that it generates hypotheses that can
236 be tested in the future.

237 To conclude, we explored in this study the relationship between maternal and foetal GC
238 levels in a free-ranging population of fallow deer. Our findings suggest that there are sex differences
239 in the underlying evolutionary processes, which may have optimized growth at the cost of survival
240 for male offspring, whereas the opposite is true for female offspring. Most of the existing literature
241 uses the rodent or human model, with very little existing studies on other taxa. Although some
242 features of the placenta may be conserved, there are also evolutionary differences between species
243 (Fowden, 2003; Rosenfeld, 2015). Non-model species may provide different results (Fishman et al.,
244 2019), through which we can gain a better fundamental understanding of evolutionary mechanisms.
245 By studying free-ranging fallow deer, this study therefore also provides novel insights into possible
246 sex-specific maternal effects in a non-model species, and provides a method that can be viable for
247 other populations as well, enabling the study of patterns rarely studied before in the wild.

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