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

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Data availability statement

All data used in this manuscript will be made available on OSF or Zenodo at acceptance, and is already available for the review process as supplementary material. We commit to providing the DOI of the dataset in this section in the final version of the manuscript.

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

53 **Introduction**

54 Parental phenotypes can be drivers of offspring variation, and in many mammalian species, these
55 parental effects are often labelled as maternal effects since it is the mother that mostly takes care of
56 the offspring. Maternal effects are seen as an adaptive way in which mothers can either fine tune
57 their offspring for the current environmental conditions [1], or maximise their own reproductive
58 output [2]. These effects on the offspring are present from the earliest stages of development and
59 can have long lasting influence on offspring phenotype [3,4].

60 Acute stress typically leads to an increase in circulating glucocorticoid (GC) levels due to
61 activation of the hypothalamic-pituitary-adrenal (HPA) axis [4,5]. Maternal GC levels during gestation
62 can affect offspring phenotype in many ways, including affecting offspring birth weight and HPA-axis
63 reactivity [3,6,7] and these effects can linger until offspring are well into adulthood (e.g. 8), which is
64 also referred to as foetal programming [6]. There is increasing evidence, however, that there are sex
65 differences in foetal programming [8–10].

66 Most studies investigating hormonal maternal effects investigate offspring GC levels after
67 birth and most evidence comes from either clinical studies on humans [11], rodents [4] or birds
68 [12,13], with a paucity of data on free-ranging mammals. This is usually due to the challenges of
69 measuring maternal and foetal hormone levels during gestation in non-captive mammals. Recent
70 developments, however, have enabled measuring foetal hormonal levels post-parturition by
71 quantifying steroids in neonate hair [14–16]. Hair steroid levels represent long-term levels,
72 accumulated over the last weeks to months [17], and therefore reflect *in utero* integrated levels
73 when quantified in neonates [16]. Whether these levels are associated with circulating maternal GC
74 levels, however, is currently not well understood.

75 Here we explored, for the first time in free-ranging large mammals, the relationship
76 between circulating maternal GC levels and offspring *in utero* accumulated GC levels. For that
77 purpose, we collected faeces of mothers during late gestation, from which we quantified GC
78 metabolites [18], and related that to their offspring hair GC levels [16], 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 [19]. 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 17th century, with a population size of about 600 individuals in late summer, after fawn births [20]. 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 [19,21]. 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

105 cortisol metabolites in the samples were quantified [18]. We added 5 mL of methanol (80%) to
106 weighed aliquots (0.5 g) of homogenized faecal samples, after which the samples were shaken and
107 centrifuged as previously described [22]. FCMs were analysed with an 11-oxoaetiocholanolone
108 enzyme immunoassay (for details see Möstl et al., 2002 [23]), which has already been validated for
109 fallow deer [24].
110
111 *Neonate hair collection and hair GC level quantification*
112 Neonate hair samples were collected during the fawn captures in June 2020. Fawns were routinely
113 captured with fishing nets, as part of the long-term management of the population. During handling,
114 we collected physiological and behavioural data (see Amin et al., 2021 [16] for full details on the
115 capture protocol), as well as hair samples (>100 mg) from the belly of the fawn using an electric
116 trimmer (Wahl model 9639; Wahl Clipper Corporation). We extracted cortisol levels from fawn hair
117 using a standardized protocol for hair-testing (e.g. 15,25), by using commercial enzyme
118 immunoassay (EIA; Salimetrics; Ann Arbor, MI, USA, item no. 1-3002) kits. Full details on these
119 extractions and validations are described in Amin et al., 2021 [16] and in the supplementary material
120 (S1).
121
122 *Mother-fawn pairing*
123 Mother-fawn pairs were based on field observations with data collection starting in July, when
124 young fawns were making their first entrances into the female herds. For details regarding the
125 pairing, see Griffin et al., 2022 [20] and Supplementary S1.
126
127 *Sample sizes*
128 We collected and analysed a total of 164 faecal samples of 99 different pregnant does. For
129 individuals with multiple values, we took the mean of these values to create a dataset that had one
130 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 [26].

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[27]) 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 [28].

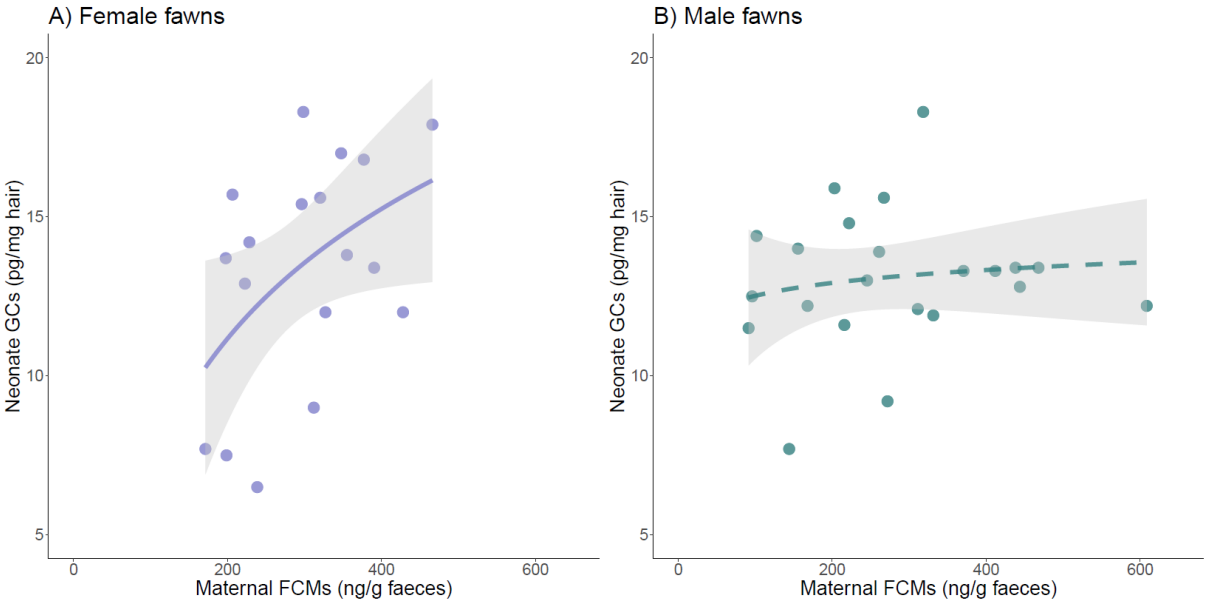
Finally, we decided to run an additional model *post-hoc*, including both sexes in the same model, to further investigate whether the slopes of our models were different between male and female offspring. We ran a linear model with neonate GC as the response variable. As explanatory

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).



173

174 **Figure 1:** The relationship between maternal faecal cortisol metabolites (FCMs) and neonate hair

175 GCs, for female (A) and male (B) offspring. Trendlines and their 95% confidence intervals are fitted in

176 each plot. Solid trendline indicates a statistically meaningful relationship.

177 **Discussion**

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

183 Like many other species, fallow deer have a skewed mating distribution [29,30]. While
184 females tend to produce one fawn per year [19], male reproduction skew is steep. Only a small
185 proportion of the males reproduces, with the majority having no or very little offspring [29–31].
186 Therefore, there may be a strong selection for males to prioritize growth over survival, since low
187 quality sons will end up costing resources, with no fitness benefits in the long run. A recent study has
188 shown that male birthweights in our populations are indeed higher than female birthweights [32].

189 GC levels play a key role during gestation and have major effects on offspring phenotype
190 early in life. In addition to being crucial for organ development during the late stages of pregnancy
191 [33,34], GCs play a role in fighting inflammation [35] and thereby help to keep the foetus vital. At the
192 same time, high GC levels may restrict growth [6,36,37]. As males tend to prioritize growth [36], it
193 may be that there would be sex differences in the mechanisms that evolved. For example, maternal
194 GC contribution may be limited in male foetuses to prevent growth retardation, at the cost of
195 prenatal survival. This may contribute in some degree to why males generally face higher prenatal
196 mortality rates [38–40].

197 The mammalian placenta likely plays a crucial role in mediating these sex differences.
198 Previous studies, in rodents and humans, have shown sex differences in the structure and activity of
199 the placenta [36,41–44]. There is also evidence that maternal GCs may transport more easily
200 through female placentas than males' [43]. This may be, for instance, through different levels of
201 expression and activity of enzymes or transporters that mitigate different maternal GC level spill
202 over between males and females.

203 One of the key enzymes, 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), inactivates
204 cortisol to cortisone [37,45]. Placental 11β-HSD has been frequently shown to be positively related
205 to birthweight [3,36,37,46], thus likely playing a role in preventing growth restriction. Previous
206 studies have shown that male foetuses have higher expression or activity of 11β-HSD2 in response to
207 maternal GC surges [41,43]. Similarly in ovine, male foetuses increased 11β-HSD2 in response to
208 maternal dexamethasone administration, whereas female foetuses did not [47]. This may explain
209 why a different ovine study also found that, following an antenatal GC treatment, growth restriction
210 was lower in males than in females [48]. In addition, male mice were shown to also have increased
211 ATP-binding cassette transporters, which mediate GC efflux toward maternal circulation [43],
212 indicating that there are different mechanisms through which males may be able to limit maternal
213 GC levels [49]. This altogether may explain why females may have higher foetal survival [36],
214 whereas males may be larger and faster growing.
215 We acknowledge that there are some shortcomings to this study. We report patterns taken
216 over only one year, whereas ecological patterns may vary between years. Furthermore, we have
217 modest sample sizes (due to the difficulty of the design), which restrict us in doing more elaborate
218 analyses. Nevertheless, as one of the first studies quantifying the relationship between maternal and
219 foetal GC levels non-invasively, we do provide novel insights into how these fundamental
220 relationships may function in a free-ranging large mammal population. It is important to note that
221 the nature of the study described here is exploratory, meaning that it generates hypotheses that can
222 be tested in the future.
223 To conclude, we explored in this study the relationship between maternal and foetal GC
224 levels in a free-ranging population of fallow deer. Our findings suggest that there are sex differences
225 in the underlying evolutionary processes, which may have optimized growth at the cost of survival
226 for male offspring, whereas the opposite is true for female offspring. Most of the existing literature
227 uses the rodent or human model, with very little existing studies on other taxa. Although some
228 features of the placenta may be conserved, there are also evolutionary differences between species

[44,50]. Non-model species may provide different results [15], through which we can gain a better fundamental understanding of evolutionary mechanisms. By studying free-ranging fallow deer, this study therefore also provides novel insights into possible sex-specific maternal effects in a non-model species, and provides a method that can be viable for other populations as well, enabling the study of patterns rarely studied before in the wild.

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