



Article

Maternal cortisol level around conception is associated with offspring sex ratio in captive European wild rabbit (*Oryctolagus cuniculus*)

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ABSTRACT – The sex ratio of the offspring at birth is usually 1: 1 established by natural selection. The sex allocation model predicts that if parents have adequate resources, they would benefit from differentially allocating maternal investment to that sex having higher fitness under the particular environmental conditions. However, little is known about what mechanisms would result in biased sex ratio. One such mechanism could be the interaction of stress reactivity and progesterone levels in the mothers around conception. In the present investigation the fecal cortisol and progesterone levels at the day of conception were measured in fifteen European wild rabbit does kept in cages. The does were clustered to low and high cortisol response groups and the sex ratio of their progeny was determined. We found a significant correlation between the progesterone levels of mothers and their stress status measured at the mating. This correlation was also reflected in the sex ratio bias in their litters. Even though the litter size was not different, we found higher progesterone levels and more female offspring in the does with higher cortisol levels indicating that maternal status around conception may affect the litter sex ratio.

Keywords: sex allocation, stress, progesterone, glucocorticoids, faecal cortisol

INTRODUCTION

The hypothesis of altering sex distribution (sex allocation) is based on the benefits of adaptive manipulation for parents, i.e., natural selection favors parents by modifying their investment in male and female offspring by maximizing their parental fitness (Fisher, 1930; Charnov, 1983; Komdeur, 2012). According to Fisher's (1930) theory of equal distribution, selection does not move in the direction of either sex at the population level, as males and females have, on average, equal genetic contributions to the next generation. Hamilton (1967) was the first to point out that within a group interactions have different effects on the fitness of males and females. The balance may be biased to a sex

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that improves relatives' conditions within the group (need for helpers due to resource expansion, such as helping sex in cooperative breeding-nesting birds (Emlen *et al.*, 1986; Lessells and Avery, 1987). Trivers and Willard (1973) hypothesized that if environmental conditions affect the survival of different sexes differently, parents are able to modify the sex ratio of their offspring towards the sex resulting better fitness for them (Trivers and Willard, 1973).

Initially, the selective mortality of eggs or embryos remained the only explanation, as in vertebrates the sex is genetically determined (chromosomal), so there is little chance that the sex ratio could be affected, as the average sex ratio is evolving to 0.5 during the random meiosis (Charnov, 1983; Palmer, 2000). However, Hardy (2002) and West *et al.* (2002) suggest some specific factors that correlate with sex ratio even in species with sex chromosomes. These factors include the abundance of food available (Howe, 1977; Desfor *et al.*, 2007), the mother's condition, and social status (Trivers and Willard, 1973; Clutton-Brock *et al.*, 1982; 1984; Sheldon and West, 2004).

Studies, performed mainly in birds, have shown that the quality of males can also alter sex ratio (Weatherhead and Robertson, 1979). In addition, abnormalities in the production of X- / Y-chromosome sperm have been found in mammals, which may also be an influencing factor (Chandler *et al.*, 2007; Edwards and Cameron, 2014). The species-specific social environment can also have a strong impact, modifying the sex ratio in proportion to the benefits and / or costs (West and Sheldon, 2002; Komdeur, 2004). Similarly, there is competition between siblings, a process that can begin in the uterus, when androgens produced by males during pregnancy can have a detrimental effect on female embryos (Korsten *et al.*, 2009; Bánszegi *et al.*, 2009).

Four optional mechanisms should be highlighted that induce biased sex ratio before and during the pregnancy in the maternal body: (1) the hormone-induced conception bias (James, 2004); (2) varying mortality of embryos of different sexes due to excessive glucose metabolism (Gutiérrez-Adan *et al.*, 2006); (3) death of embryos of different sexes due to developmental asynchrony of the embryo and the uterus (Krakow, 1995a, 1997) and (4) pregnancy hormone suppression and resource deprivation (Rivers and Crawford, 1974; McMillen, 1979). Although these processes often have the opposite effect, it should be emphasized that stress has a significant effect on all of them. The association between maternal dominance and testosterone levels and the effect of glucocorticoids on glucose metabolism in rodents have been shown to cause sex allocation (Pratt and Lisk, 1989; Ryan *et al.*, 2014).

The aim of the present study was to investigate whether the change in cortisol and progesterone levels are associated with biased sex ratio in European wild rabbits and, if so, in which direction. It has been hypothesized that stress during early pregnancy alters progesterone levels, and higher male embryonic death will be demonstrated similarly to hamsters (*Pratt and Lisk, 1989*).

MATERIAL AND METHODS

Ethical approval

The research was approved by the Committee on the Ethics of Animal Experiments of the Hungarian University of Agriculture and Life Sciences Kaposvári Campus (former Kaposvár University, permit number: MÁB/2-2/2019).

Animals and housing

The study was carried out on 15, one year old European Rabbit does (*Oryctolagus cuniculus*), and their first progeny (n= 76). The rabbits derived from captured wild rabbits, breed in cages for the second generation. The animals were mated by natural breeding. The tested animals had been imprinted during the first week of lactation after birth (*Pongrácz et al., 2001*) to ensure secure handling (*Bilkó et al., 2000*) by reducing their fear of humans. Does were housed individually in bicellular cages (140*60*45 cm) supplied with outside farrowing boxes (40*25*31 cm) according to the Hungarian and EU legislation in force. The cages were made of welded wire mesh, equipped with a plastic floor, hand-refillable feeders and drinkers, and hay racks on the front wire. In addition, there was a galvanized steel sheet litter tray running on rails under each cage. Water, hay, and commercial rabbit fodder was supplied ad libitum during the study. The sex of the offspring individuals was determined at the age of 60 days.

Hormone assessment

Faecal samples were collected 24 hours after the natural mating. Contamination of the faeces with urine was prevented with a mesh placed in the tray under the cage. Samples were stored at -20 °C until extraction. Cortisol and progesterone levels were measured from faeces based on their breakdown products using an RIA method according to *Benedek et al, (2021)*. After freeze-drying, the samples were ground, homogenized, and mixed thoroughly. 200 mg of dry-faeces was then placed in a glass vial and 1.6 ml 80% methanol and 200 µl

distilled water were added to extract the hormone metabolites. The vials were capped and vortexed for 30 minutes. Samples were then centrifuged (2450 rpm, 20 min, 4°C) and the supernatant was poured off and stored at -55°C. At the time of use, samples were dried in a chamber (Binder) and reconstituted with ASB buffer at a 1:1 dilution rate. The RIA method used for cortisol and progesterone, was developed for hormone determination in the plasma of food animals using tritium labelled hormones (cortisol and progesterone-1,2,6,7-3H(N)) as well as highly-specific polyclonal antibodies raised against cortisol-21-HS-BSA and 11 α OH progesterone-11HS:BSA in rabbits.

Statistical analysis

The homogeneity and normal distribution of the samples were tested and confirmed with Levene test and Shapiro Wilk test. The does were classified to two groups using k-means cluster analysis (maximum iterations: 10). The significance of the differences between the two groups in the litter size and the sex ratios was estimated by independent samples t-test. Linear regression was calculated between the faecal cortisol metabolite (FCM) values and the female sex ratio and the progesterone values and the female sex ratio. Pearson correlation was calculated between the progesterone values and the FCM values. All statistical analyses were calculated using SPSS 17.0 software.

RESULTS

The fifteen does included in the study were classified to two groups based on the FCM levels measured on the day of mating; the group with low FCM level (group L: 471.94 \pm 69.3) consisted of seven individuals and the remaining eight individuals formed the group with high FCM levels (group H: 797.25 \pm 102.7). The average litter size did not differ significantly between the two groups (*Figure 1*, group L: 4.85 \pm 1.86, group H: 5.25 \pm 1.48, $P=0.657$). The ratio of the two sexes in the total of 76 offspring individuals was equal (38 male and 38 female). However, the two treatment groups showed significantly different ($P=0.024$, $t=-2.546$, $df=13$) sex ratio (Group L: 0.619, Group H: 1.8). This was mainly due to the significant difference in the number of female individuals in the litters (*Figure 1*, mean number of female offspring per doe: group L: 1.85 \pm 1.21, group H: 3.37 \pm 1.06, $P=0.023$, $t=-2.585$, $df=13$). Although the number of the male individuals in the litters showed opposite tendency, the difference between the two groups was not statistically significant (mean number of male offspring per doe: group L: 3.00 \pm 1.15, group H: 1.87 \pm 1.24, $P=0.094$, $t=1.804$, $df=13$).

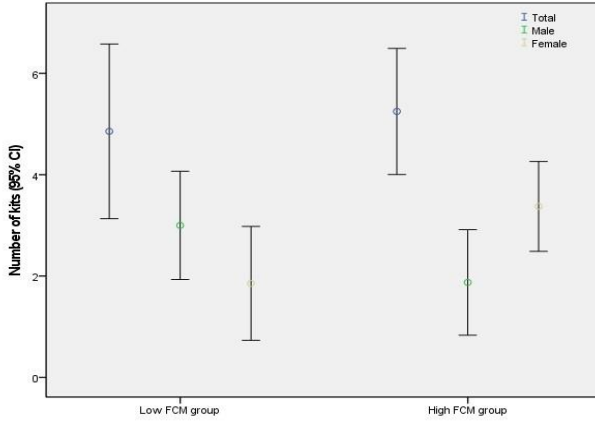


Figure 1. Distribution of the number of the total, male and female kits in the two groups. The differences are significant only in the number of female kits ($P=0.023$, t-test).

The linear regression between the FCM values measured on the day of conception and the ratio of the female offspring in the litters (female ratio = $0.001 \cdot \text{FCM} - 0.024$, Figure 2.) was significant ($F=6.997$, $P=0.020$, $r^2=0.350$). The female individuals have higher ratio in the litters of does with higher cortisol response.

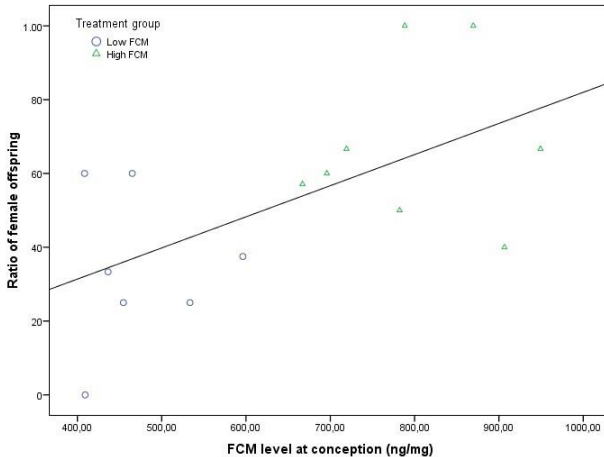


Figure 2. The linear regression between the FCM values measured on the day of conception and the ratio of the female offspring in the litters ($F=6.997$, $P=0.020$, $r^2=0.350$).

The progesterone hormone levels showed similar regression with the ratio of female offspring like FCM level. The linear regression between the two variables (female ratio= $0.001 \cdot P + 0.086$, Figure 3.) was significant ($F=7.884$, $P=0.015$, $r^2=0.378$). The faecal progesterone and cortisol metabolite levels showed a positive, moderate correlation (Pearson correlation, $r=0.626$, $P=0.013$, $N=15$).

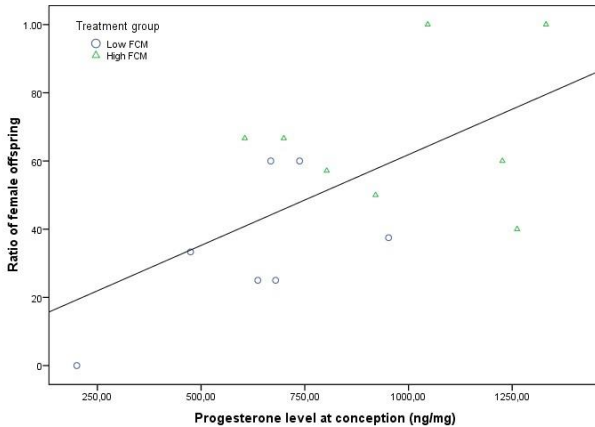


Figure 3. The linear regression between the faecal progesterone values measured on the day of conception and the ratio of the female offspring in the litters ($F=7.884$, $P=0.015$, $r^2=0.378$).

DISCUSSION

The causes of sex ratio bias are complex. Even if focusing only on the two major endocrine axes, the maternal glucose level related to the hypothalamic pituitary adrenal gland (glucocorticoids) and the testosterone and progesterone level related to the gonadal (gonadal) axis in the hypothalamic pituitary gland should be mentioned. Changes in maternal glucose levels in the uterine environment immediately after conception may affect sex ratio proven by experiments (Larson *et al.*, 2001; Cameron, 2004). Higher glucose levels favoured male embryos (Rosenfeld and Roberts, 2004). Elevated testosterone levels in females due to environmental stress would also be a possible explanation for reinterpreting the atypical sex ratios experienced so far, making the seemingly contradictory results compatible (Grant 2007). Several studies have found that a change in non-adaptive sex ratio associated with maternal adrenal hormones

(James, 2006) has described the connection between pre-conception testosterone levels in the mammalian female's follicles and the sex of the embryo (Grant and Irwin, 2005).

As for wild rabbits, our results show that the amount of FCM during mating, which indicates the stress sensitivity of the individuals, is related to the progesterone metabolite values measured in the faeces. In case of rabbits, it has been shown, that they respond quite differently to stressful situations, which is indicated by remarkable differences in blood serum and glucocorticoid metabolites (Cabezas *et al.*, 2007). Similar to processes in humans, in rabbits the adrenal gland produces significant amounts of progesterone in addition to the ovary, as adrenocorticotrophic hormone regulates progesterone production in the adrenal gland (Fajer *et al.*, 1971). In several species (*Bos taurus*, *Zalophus californianus*, *Rattus rattus*), progesterone secretion in stressed females has been reported to be as strong in the adrenal gland as in the ovary and a positive correlation has been found between cortisol and progesterone levels. (Fajer *et al.*, 1971). In case of wild rabbits, a positive correlation was also found between cortisol and progesterone values measured on the day of parturition (Benedek *et al.*, 2021), which can be explained with a common metabolic pathway, as some progesterone is released into the bloodstream before it is converted to cortisol (Beaulieu-McCoy *et al.*, 2017).

Furthermore, we found that the number of female offspring among the offspring of high-stress mothers was significantly higher than that of non-stressed mothers.

Furthermore, we found that the number of female offspring among the does exhibit relatively high cortisol values was significantly higher than that of does showing relatively low cortisol values.

The cortico-adrenal stress response can be elicited by external stress (Teixeira *et al.*, 2007) and internal anxiety (Breazile, 1988). There may be several outcomes, one of which may be low progesterone production or altered maternal progesterone sensitivity (Albrecht *et al.*, 1978). But the opposite has also been described in pregnant baboons, where progesterone hormone levels were associated with cortisol levels, and with greater stress, progesterone hormone levels became higher (Albrecht *et al.*, 1978). Cortisol has a higher affinity for uterine and placental receptors than progesterone, thereby inhibiting its recognition. Cortisol inhibits uterine blood flow, capillary permeability, thereby inhibiting implantation (Coubrough, 1985), thus stress disrupts and impairs the synchronization between the uterus and the embryo. In hamsters, stress around implantation has been shown to result in higher mortality in males than in female embryos (Pratt and Lisk, 1989), however, progesterone

levels were reduced in this species. In human terms, the mortality of the male embryo was also higher under stress (*Obel et al., 2007; Jongbloet, 2004*), and the growth rate of the male foetus was higher in the early stages of pregnancy, which may explain the differences in differentiation and vulnerability (*Henriksen et al., 1995*). Stress, or internal anxiety activates the hypothalamic-pituitary-adrenal axis, and cortisol can cause more severe damage to the foetus, so stress may contribute to the recently observed sex ratio bias in humans (*Marcus et al., 1998*).

The results described herein provide a small step closer to understanding the processes discussed above.

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