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Non-model species deliver a non-model result: Nutria female fetuses neighboring males in utero have lower testosterone

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ABSTRACT

Neighboring fetuses may impact their siblings in various respects, depending on their in utero location and sex. The effects of the intrauterine position (IUP) are widely studied in model organisms, especially laboratory bred murine strains that are characterized by short gestations and altricial offspring. In some species, the proximity to a male fetus and its higher circulating testosterone masculinizes neighboring female fetuses. In utero testosterone exposure might be manifested as higher testosterone concentrations, which contribute to a variation in morphology, reproductive potential and behavior. In this study, we examined the influence of neighboring an opposite sex fetus on testosterone levels in a feral animal model characterized by a long gestation and precocious offspring. Using necropsies of culled nutria (*Myocastor coypus*), we accurately determined the IUP and quantified testosterone immunoreactivity in fetal hair. We found that as expected, both male and female fetuses neighboring a male in utero had longer anogenital distance. However, females adjacent to males in utero showed lower testosterone levels than male fetuses, while testosterone levels of females without a male neighbor did not differ from those of males. This surprising result suggests an alternative mode by which local exogenous steroids may modify the local fetal environment. Our study emphasizes the importance of examining known phenomena in species with different life histories, other than the traditional murine models, to enhance our understanding of the evolutionary mechanisms that are driving sexual differentiation.

1. Introduction

The fetal environment is thought to be primarily shaped by maternal conditions. In polytocous (i.e., litter-bearing) mammals, individual fetuses in the litter also influence the uteral environment via space (Ibsen, 1928), vasculature (Houtsmuller and Slob, 1990; Meisel and Ward, 1981), and resources (Labov et al., 1986). The effects of fetuses on litter mates may depend on the sex, location, and proximity of each fetus (e.g., Ryan and Vandenbergh, 2002; Ward et al., 1977; Fishman et al., 2018a). Fetal intrauterine position (IUP) reflects the proximity to fetuses of the same or opposite sex (vom Saal et al., 1990). Fetal development, sexual differentiation, and maturation can be affected by the sex of neighboring fetuses, which create local hormonal environments that might drive individual differences (Fishman et al., 2018a; Ryan and Vandenbergh, 2002). The effects of IUP on both sexes are well documented in model species (reviewed by Ryan and Vandenbergh, 2002). For example, female rodent fetuses developing between two males in utero present masculinized anatomical, physiological and behavioral features in adulthood (Ryan and Vandenbergh, 2002). These females are less attractive to males and have lower reproductive success than females that developed in utero without a male neighbor (Ryan and Vandenbergh, 2002; Zielinski et al., 1992). Most IUP-related phenotypes have been attributed to testosterone concentrations and its transfer between fetuses in utero (e.g., vom Saal and Bronson, 1980; Rohde Parfet et al., 1990). Since males are presumed to have higher circulating testosterone concentrations, fetal IUP is usually defined by its proximity to a male fetus. Two main categorization systems have been used to describe the effect of proximity. Meisel and Ward (1981) suggested a directional classification, in which the effect on a fetus is dependent on the location of opposite sex fetuses (i.e., caudally or rostrally on the uterine horn), whereby uterine vasculature is responsible for the transfer of sex steroids from one fetus to another via maternal blood flow, as seen in rats (Hernández–Tristán et al., 1999; Houtsmuller and Slob, 1990; Meisel and Ward, 1981; Richmond and Sachs, 1984). vom Saal introduced a contiguity classification, based on the proximity to a male fetus, so that a fetus without

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a male neighbor is termed 0M; 1M means that the fetus has a single male neighbor, and 2M means that there are male neighbors on both sides of the fetus, with diffusion of androgens between fetuses via amniotic fluid or blood, as seen in mice (vom Saal, 1981).

Females are considered to be more sensitive to IUP effects than males (Ryan and Vandenbergh, 2002). However, most research on the association between IUP and fetal testosterone was conducted on a few model species, mostly lab and domesticated animals (Ryan and Vandenbergh, 2002), and showed diverse findings, which might represent different mechanisms. In mice, 2M female fetuses had higher circulating and amniotic fluid testosterone concentrations than 0M females (vom Saal et al., 1990; vom Saal and Bronson, 1980). In gerbils, circulating testosterone levels were higher in both 2M male and female fetuses, in comparison to 0M males and females (Clark et al., 1991). In rats, Houtsmuller et al. (1995) found that circulating testosterone levels were higher in 2M females than in 0M females, while Hernández–Tristán et al. (1999) did not, although male fetuses had higher testosterone concentrations than females. In pigs, males had higher circulating testosterone concentration than females, but they were not related to IUP (Wise and Christenson, 1992). Yet, it is possible that IUP classification systems that are suitable for certain species or mechanisms are not useful for others, requiring alternative approaches.

During specific stages of gestation, the effect of testosterone transfer between fetuses (Even et al., 1992) can influence the distance between the anus and genitalia (i.e., anogenital distance; AGD) through its role in perineal tissue elongation (Hotchkiss and Vandenbergh, 2005; vom Saal and Bronson, 1980). This anatomical manifestation can be hindered by antiandrogen (e.g., flutamide) administration (Clemens et al., 1978). As such, AGD has been used as a biomarker for androgen exposure in utero when verifying IUP via a caesarian section [e.g., mice (Hotchkiss and Vandenbergh, 2005; Jubilan and Nyby, 1992), rats (Hernández–Tristán et al., 1999)]. However, AGD has also been extrapolated into systems where IUP was not verified, and used as an indication of IUP postnatally (e.g., Correa et al., 2013). The AGD had been associated with morphological, physiological, behavioral and reproductive traits in mice (Hotchkiss and Vandenbergh, 2005), rats (Tobet et al., 1982; Zehr et al., 2001), pigs (Drickamer et al., 1999), rabbits (Banszegi et al., 2012), gerbils (Clark et al., 1990) and degus (Correa et al., 2013). In mice, 0M females have shorter AGD than 2M females (Ryan and Vandenbergh, 2002). In rats, females located caudally to males in utero (Hernández–Tristán et al., 1999; Houtsmuller and Slob, 1990; Meisel and Ward, 1981; Richmond and Sachs, 1984), as well as 2M females (Tobet et al., 1982) have longer AGD. In gerbils, 2M males have longer AGD than 0M males, while no differences were found in females (Clark et al., 1990), suggesting that female gerbil fetuses have a lower sensitivity to testosterone. Conversely, female rats and mice show higher androgen sensitivity than males (Ryan and Vandenbergh, 2002). An association between IUP and AGD was not found in all of the mouse and rat strains. For example, in CF-1 mice no relationship was found (Jubilan and Nyby, 1992; Nagao et al., 2004; Simon and Cologer-Clifford, 1991). Similarly, no association was found between IUP and AGD, and testosterone concentrations were not related to either IUP or AGD in Sprague-Dawley rats at the end of gestation (Hotchkiss et al., 2007).

In this study, we investigated the association between integrated testosterone levels over the last trimester of pregnancy and neighboring an opposite sex fetus in utero in the feral nutria (*Myocastor coypus*). This polygynous rodent is characterized by a long gestation (127–138 days) and precocious offspring. Thus, fetuses cope with the mechanical and hormonal pressures that their neighbors exert in utero for several months, while they develop. Nutria reproduction includes post gestational estrus, large litters, and receptivity year-round, making it a successful invasive species (considered one of the world's 100 worst; ISSG, 2013). We previously found that females with higher testosterone levels produce female-biased litters (Fishman et al., 2018b). Males seem to be the more 'expensive' sex in the last stages of pregnancy in terms of morphological development (i.e., length and weight gain), and more vulnerable, as sex ratios decline throughout the pregnancy (Fishman et al., 2018b). Severe intrauterine growth retardation was 4 times more likely in male fetuses than in females (Fishman et al., 2018b). In the last stages of pregnancy, fetuses in litters with equal sex ratios (i.e., the highest sex heterogeneity), had the highest cortisol levels (Fishman et al., 2018a), which is the main glucocorticoid produced by the nutria adrenal glands (Callard and Leathem, 1969; Wilson et al., 1964). Fetuses neighboring an opposite sex fetus also had longer trunks, regardless of sex (Fishman et al., 2018a), which might imply better lung development (Flint, 1906). Here, we explored whether females who are next to males in utero are impacted via higher testosterone levels and longer AGDs, and whether their relationship can be predicted by IUP.

2. Methods

2.1. Sample collection and IUP recording

We collected nutrias that were routinely culled by the authorities at the Agamon Hula Park, Israel. Permits are not needed for collecting carcasses because nutrias are an invasive species, not protected by Israeli laws. A total of 153 culled females were collected, of which 117 (76%) were pregnant. Our sample for this study was composed of 316 fetuses, belonging to 58 litters at a pregnancy stage over 82 days. The average litter size was 5.6 fetuses. Twenty-two females whose pregnancy stage was 111–138 days had fetuses with sufficient amount of hair to allow testosterone quantification (114 fetuses: 55 males and 59 females). Estimation of pregnancy stage followed Newson's formula (Newson, 1966): Estimated age = $43.69 + 14.27 * \sqrt[3]{\text{fetal weight}}$, cross-validated with multiple fetal morphometric measurements (Fishman et al., 2018a) presented by Felipe and Masson (2008) and Sone et al. (2008).

Upon dissection, the IUP for each fetus was noted by the uterus horn (left or right) and its location relative to the ovary. The IUP of 2 fetuses from the same litter could not be determined since the mother was not intact. Fetuses were removed and weighed using an electronic balance to the nearest 0.01 mg (Precisa, Switzerland, BJ610C, d = 0.01 g). Two IUP schemes were used:

1. Proximity to opposite sex fetus (Fishman et al., 2018a): fetuses that had a neighboring fetus of the opposite sex on either or both sides were termed P1, while fetuses that had no neighbor of the opposite sex were termed P0.
2. Contiguity scheme (vom Saal, 1981): Fetuses located between two males were termed 2M, between one male 1M, and not adjacent to any male 0M.

2.2. AGD measurement and sexing

AGD was measured using an electronic digital caliper (accuracy of 0.02 mm and resolution of 0.01 mm). Though using AGD for sexing is widely used in the nutria (e.g., Sone et al., 2008; Gale R. Willner et al., 1979), fetuses were sexed based on both internal and external morphology, validated using molecular tools. We validated AGD by internal examination of 10 male fetuses and 12 female fetuses. The AGD index (AGDi) was calculated by dividing fetal AGD length by fetal weight (Hotchkiss and Vandenbergh, 2005). For molecular validation, we used published primers for the Sry gene (Garcia-Meunier et al., 2001), which is only expressed in males. We used the housekeeping gene 12S as a positive control, and an adult female as a negative control (for details see Fishman et al., 2018b).

2.3. Steroid measurements

Hair follicles start to appear on nutria fetuses at 85–90 days of the gestation. First on the skull and dorsal surface of the body, and then on

the dorsal and ventral surfaces of the head, body, and tail by 100–105 days of gestation (Felipe and Masson, 2008; Sone et al., 2008). However, most litters did not have the sufficient amount of hair for testosterone quantification until approximately 111 day of gestation.

Testosterone was extracted and immunoreactivity was quantified using a protocol that was developed for wildlife, and validated for nutria (e.g., Fishman et al., 2018a; Fishman et al., 2018b; Koren et al., 2002). Hair was collected by shaving the entire fetus, and was washed twice with water for 3 min, and twice with isopropanol for 3 min, to remove external contaminants. Following sonication and an overnight extraction with methanol at 50 °C, the extract was dried down, reconstituted, and quantified using a commercial ELISA kit (Salimetrics Europe, Newmarket, UK) for testosterone. The manufacturer reported antibody cross-reactivity of 36.4% with dihydrotestosterone, 21.02% with 19-nortestosterone, 1.9% with 11-hydroxytestosterone, 1.157% with androstenedione, and < 0.49% with all other steroids. Kits were validated for nutria fetal hair by showing linearity (5–40 mg hair) and parallelism between serially diluted hair extracts (representing 5–40 mg of hair) and kit standards (slope covariance P = 0.91). Intra-assay CV was 1.96% for six repeats on the same plate. Inter-assay CV was 8.12% across four plates. Recovery was 100.67%, quantified by comparing hair samples spiked with a known testosterone amount to unspiked samples.

2.4. Statistical analysis

Fetal hair testosterone levels were log transformed to achieve normal distribution. We included maternal identity as a random factor in all tests that included individual fetuses, to account for the fetal uterine environment. Linear mixed models were used to examine fetal testosterone levels, where sex and proximity to an opposite sex fetus (P0 or P1) were the model effects. Fetal testosterone was also measured in relation to pregnancy stage and maternal testosterone levels. In order to examine the effect of IUP by contiguity on AGDi, we used sex and contiguity (i.e., 2M, 1M and 0M) as the model effects. Testosterone immunoreactivity was the model effect in examining the effect of testosterone on AGDi. In order to examine the possible effects of sex ratio and litter size on hair testosterone, these parameters were used as the model effects. Model fitting and post-hoc analysis were done in JMP (version 12, SAS Inc.).

3. Results

Fetal hair testosterone levels were not related to estimated pregnancy stage, nor to maternal testosterone levels. We found no association between testosterone levels and litter sex ratios or litter sizes. Differences between mothers' testosterone levels were significant (Wald P = 0.0075). Overall, considering litter differences (maternal ID included as a random factor), male fetuses had significantly higher hair testosterone levels than females. However, both male and female fetuses that were next to a fetus of the opposite sex had lower testosterone levels than fetuses that were next to the same sex (Sex: $F_{1,\ 89} = 12.74$, P = 0.0006; Proximity: $F_{1,\ 94} = 5.24$, P = 0.02). We found that females that were next to males (P1 females) had the lowest testosterone levels in the litter, but not significantly lower than females that were not next to males in utero (P0 females; Tukey post hoc comparison; Fig. 1, Table 1). P0 female testosterone did not differ from males. Males did not differ in their testosterone levels, regardless of the sex of their neighbor (Fig. 1). In a complimentary statistical approach, we constructed a model examining the differences in fetal testosterone levels between the four groups (male P0, male P1, female P0, female P1; $F_{3,91.39} = 5.37$, P < 0.0019). Here, the Tukey post hoc analysis showed a significant between-group difference only between P1 females and males (both P1 and P0 males; Table 1). This analysis enabled us to compare each group to the average levels, and assess its relative significant increase or decrease, using the parameter estimates. Parameter estimates revealed that female fetuses neighboring males in utero (P1 females) showed a significant relative reduction in testosterone level that was not seen in females that were not next to males (P0 females). In males, those that did not neighbor a female in utero (P0 males) showed a significant increase in testosterone levels, unlike males that were next to females (P1 males; see Fig. 1, Table 1).

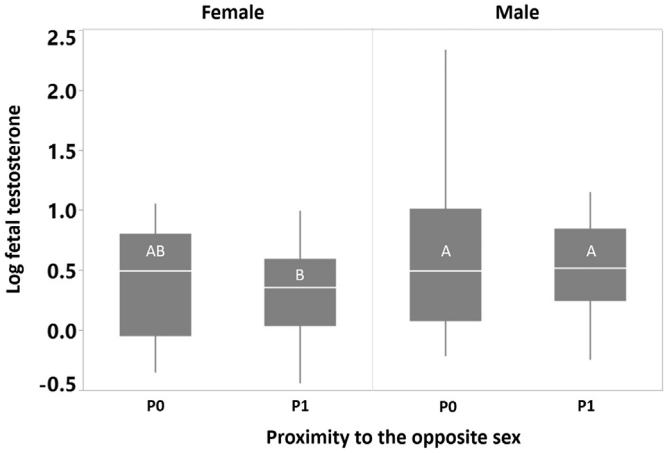

Fig. 1. Box plot of log transformed fetal testosterone levels (originally pg/mg hair) by sex and proximity to opposite sex fetus. P0 – no proximity to an opposite sex fetus, P1 – proximity to an opposite sex fetus. Letters denote statistically significant differences based on a Tukey post hoc comparison.

Table 1
Parameter estimates for the four groups constructing the model. The assumption is that each parameter does not differ from 0. Significant estimates and values are marked in bold. The between groups Tukey post hoc comparison is presented in the form of a 'connecting letters' report.

Group	Estimate	Std error	df	t ratio	P value	Tukey post hoc
Male P0	**0.187549**	0.068567	98.7	2.74	**0.0074**	A
Male P1	0.0231028	0.047202	88.89	0.49	0.6257	A
Female P0	−0.036025	0.057881	93.67	−0.62	0.5352	AB
Female P1	**−0.174627**	0.048146	88.9	−3.63	**0.0005**	B

Male AGDi was significantly longer than female AGDi ($F_{1,\ 101} = 636.97$, N = 117, P < 0.0001). In addition, in our sample of 316 fetuses whose pregnancy stage > 82 days, in which AGD could be accurately measured, AGDi differed with IUP (Sex: $F_{1,\ 261} = 1559.54$, N = 316, < 0.0001; IUP $F_{1,\ 261} = 3.33$, N = 316, P = 0.037). Males and females neighboring a male fetus had longer AGDi than those not neighboring a male. No association was found between testosterone levels and AGDi.

4. Discussion

In this study we show that neighboring the opposite sex in utero has a significant effect on fetal testosterone levels. However, opposite to findings in rodent model species, female nutrias neighboring males in utero did not show an increase in testosterone levels. On the contrary, they showed a reduction in testosterone immunoreactivity. While between-group differences in testosterone levels among nutria females that were next to males and those that were not neighboring males were not statistically significant, the whole model significance was driven by a negative effect in P1 females and a positive effect in P0 males (see Table 1). Surprisingly, both males and females that were not neighboring males in utero (P0 females and P1 males) had similar testosterone levels. Prior research on rodents showed that females adjacent to males in utero had higher testosterone and suffered fitness consequences (Ryan and Vandenbergh, 2002). Although we cannot assess long-term fitness in culled nutria, we previously found evidence that

testosterone may impose reproductive consequences on females, as females with higher testosterone levels had female biased litters (Fishman et al., 2018b), as opposed to other mammalian species (e.g., Grant, 2007; Grant et al., 2011; Grant and Irwin, 2005; Helle and Laaksonen, 2008; Shargal et al., 2008). We also found that nutria male offspring were generally larger and grew faster than females (Catalano et al., 1995; Fishman et al., 2018b; Gosling et al., 1984), but were also more vulnerable (Fishman et al., 2018b). However, we did not detect a trade-off between sex ratio and litter size (Fishman et al., 2018b). Male nutrias may demand more nutrients to support their accelerated growth (Gosling et al., 1984), and contribute to the crowding effect, which was associated with fetal size (i.e., weight and length), but not with cortisol levels (Fishman et al., 2018a). Nutria litters that contained equal amounts of fetuses from both sexes had longer trunks and higher cortisol levels, possibly facilitating lung development and maturation (Fishman et al., 2018a).

We measured the testosterone immunoreactivity that was integrated throughout the second and third trimester of pregnancy, in fetal hair. Whereas quantifying steroids in blood or amniotic fluids provide the momentary concentrations and reflect short-term trends, hair-testing represents longer time frames, which may be more informative for assessing the accumulated effects of in utero testosterone exposure. The length of in utero testosterone exposure is expected to play a significant role in fetal development. Relative to rodent models, nutria pregnancy is especially long (i.e., approximately 140 days). A prolonged chronic exposure to males throughout the extended pregnancy may cause female nutrias developing next to males in utero (P1) a desensitization or a metabolic reaction that may not occur in P0 females. Mechanisms that reduce female sensitivity or exposure to excessive testosterone may include lowering endogenous testosterone production via hypothalamus-pituitary-gonadal axis down regulation, decreasing androgen receptor sensitivity (Pariante and Miller, 2001; Rosewicz et al., 1988), and regulating the levels of enzymes in the androgen metabolism pathway (e.g., Nonneman et al., 1992). Similar to glucocorticoids, where clearance rates and metabolism can vary between the sexes (El Hani et al., 1980; Glenister and Yates, 1961), androgen clearance and metabolism may also be different for males and females (Baum et al., 1988). Although mechanisms for testosterone depletion via monopolization, magnetizing, or shifting affinities are unknown, these may explain our unusual findings of females next to males in utero having lower testosterone levels.

Higher testosterone exposure at specific developmental stages may cause female masculinization in some species (Ryan and Vandenbergh, 2002). In the nutria, both males and P1 females had longer AGDi than P0 females, congruent with the published literature [e.g., 2M males and females had longer AGDi (Clark et al., 1990; Drickamer et al., 1999; Ryan and Vandenbergh, 2002; Tobet et al., 1982)]. In swine, which like nutria have a relatively long gestation (i.e., an average of 114 days), the presence of male fetuses affected female AGD (Drickamer et al., 1999). In addition, female testosterone in the last stages of gestation was not influenced by the proximity to males (Wise and Christenson, 1992). In the nutria, we did not find an association between hair testosterone and AGDi. However, similar to the aforementioned swine study, we measured fetal testosterone levels during late pregnancy ($>$ 85–90 days of gestation), well after the external genitalia were fully differentiated ($<$ 70 days of gestation; Willner et al., 1979). Perhaps circulating or amniotic testosterone concentrations measured during the first trimester of gestation would have been associated with AGDi.

Most of the studies that tested the association between IUP, AGD, and testosterone, were in laboratory-bred model systems, with short pregnancies [19–22 days (Clancy et al., 2001)], principally rats and mice (Ryan and Vandenbergh, 2002). Contradictory results in the literature may reflect species, strains, pregnancy stages, and matrix variability (Nagao et al., 2004). For example, in swine, no effect of IUP was found on AGD (Rohde Parfet et al., 1990). Our results suggest that traditional murine models may not be representative of systems where gestation is longer or pups are precocious, especially since mice and rats' sexual differentiation continues postnatally (Baum et al., 1988; vom Saal, 1981; Wallen and Baum, 2002). Studying the effects of intrauterine sex steroids on multiple processes in additional species is crucial since human sex steroid production is unlike the traditional murine models (Barrett and Swan, 2015). For example, the ambiguous relationship between prenatal stress and female reproductive development may be explained by the lack of adrenal androgen production in murine species (Barrett and Swan, 2015). Guinea pigs, which are related to nutrias (Huchon and Douzery, 2001), produce adrenal androgens and show a relationship between prenatal stress and reproductive effects (Barrett and Swan, 2015). Like the nutria, guinea pigs also have a long gestation period relative to its size [$>$ 2 months (Goy et al., 1957)]. Thus, we suggest that it may be valuable to study the endocrinological aspects of human pregnancy in alternative species, in addition to the traditional murine models. Humans and the feral nutria share relatively long pregnancies, vast natural diversities, and cortisol as the main glucocorticoid produced by their adrenals. While the benefits of investigating a classic lab model species are clear, this study shows how species with various life histories can widen our perspectives to the diversity of evolutionary selected mechanisms. This is especially important when investigating the mechanisms driving key life history events such as sexual differentiation, fetal development, sexual maturation, and reproductive success.

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