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ORIGINAL ARTICLE

# Female-biased sex ratios are associated with higher maternal testosterone levels in nutria (*Myocastor coypus*)

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## Abstract

Under various ecological conditions, producing a biased sex ratio may be adaptive. However, the factors that translate specific ecological conditions into internal processes remain an enigma. A potential mediator is maternal testosterone, which may reflect physical, reproductive, and social conditions. The nutria (*Myocastor coypus*) is a polygynous rodent, invasive in many parts of the world, which shows fluctuating sex ratios. Using necropsies of 82 pregnant culled nutrias, we found that in early pregnancy, offspring sex ratios are more male-biased than in later pregnancy. Since sex ratios decrease with pregnancy age, male fetuses in our study population may be terminated. In 68% of the litters, the heaviest fetus was a male, suggesting that males are the "expensive" sex. We also found that while maternal weight was not associated with testosterone, heavier females and those with lower testosterone had male-biased sex ratios. Litters of high testosterone females had female-biased sex ratios. To the best of our knowledge, this study is the first to show a negative association between maternal testosterone and male-biased sex ratios. Testosterone, through its role in reproduction, might be mediating maternal internal and external conditions by adjusting intra-uterine sex ratio.

## Significance statement

For decades, the mechanisms behind offspring sex ratios have been of interest across disciplines. Maternal testosterone has been implicated in mediating maternal condition, thus influencing secondary sex ratios. Here, we investigated the reproductive parameters of a culled nutria and integrated it with maternal hair testosterone levels to test the association between long-term testosterone and sex ratios. Our most surprising result was that high maternal testosterone levels were related with female-biased sex ratios. This is contrary to previous studies in other species and counter-intuitive. Heavier females tended to have male-biased litters. We also found that the proportionate representation of males within litters declined over the course of pregnancy. Male fetuses were usually the heaviest fetus, suggesting that they are the more "expensive" sex. We believe that our study provides new insights in this long-debated issue and will contribute to understanding the reproductive costs involved with maternal testosterone across animal models.

**Keywords** Hair testing · Invasive species · Maternal testosterone · Sex ratio · Trivers–Willard

The ubiquity of a 1:1 sex ratio has served as a classic textbook example of the stabilizing force of a negative frequency-dependent selection since raised by Fisher (1930). However, birth sex ratios may deviate from 1:1 (Clutton-Brock and Iason 1986; Booksmythe et al. 2017). Several competing, not mutually exclusive, hypotheses have been proposed to explain the adaptive value of biased birth sex ratios in chromosomal sex determination in vertebrates (e.g., Trivers and Willard 1973; Clark 1978; Silk 1983; van Schaik and Hrdy 1991; Krackow 1995; James 1996; Cameron 2004; Grant and Chamley 2010). Of these, Trivers and Willard's (TW) sex allocation theory (Trivers and Willard 1973; Leimar 1996) stands out as the leading and most-cited hypothesis (James

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2013). This theory predicts that selection should act on parents to vary their level of investment in offspring when fitness returns differ for the two sexes (Trivers and Willard 1973). Even when the two sexes have, on average, an equal reproductive value, differences in variation in reproductive success between males and females may be profound. Thus, for example, in polygamous species, high-quality males might gain higher reproductive success. Accordingly, females that can produce high-quality offspring might benefit from producing more sons. TW hypotheses can also apply for heritable sexually selected traits, where females mating with attractive mates will benefit from producing more "sexy sons" than daughters (Cameron et al. 2003). It can also apply to any other trait that is both heritable and positively correlated with reproductive success (James 2013). A second, not necessarily mutually exclusive hypothesis, which also seeks to explain the adaptive value of biased secondary (i.e., birth) sex ratios, is the local resource competition (LRC) hypothesis, which is based on sexual differences in dispersal and philopatry (Clark 1978; Silk 1983; van Schaik and Hrdy 1991). The LRC hypothesis posits that when resources are scarce and only one sex disperses, females should produce more offspring of the dispersing sex, to avoid potential future local resource competition with kin philopatric offspring (Clark 1978; Silk 1983; van Schaik and Hrdy 1991). Several empirical studies support LRC as they show that in species where one sex disperses, litters are biased towards the dispersing sex, regardless of the sexual identity of the dispersing sex (Silk, 1983). While both hypotheses lay different predictions on the evolutionary factors that maintain a biased sex ratio, both require a feedback mechanism which links the environmental condition to altering sex ratio.

As environmental or social conditions change, parents are expected to re-adjust their efforts and invest in the more "profitable" sex (West and Sheldon 2002). In mammals, the prenatal and often the postnatal investments in offspring are mostly maternal. Consequently, maternal body condition or social status might predict the direction of sex-biased investment according to the costs and benefits of raising each sex (Clark 1978; Correa et al. 2011). Meta-analysis showed that mammalian mothers in good condition at the time of conception tend to have male-biased litters (Cameron 2004; but also see Hewison and Gaillard 1999; Brown 2001; Sheldon and West 2004), and suggests that facultative sex ratio adjustments might occur around the time of implantation (Cameron 2004). Mothers can vary their sex-biased investment by producing unequal numbers of male and female offspring (Johnson and Ritchie 2002), adjusting litter size (e.g., by abortion (Gosling 1986; James 2015) or infanticide (Beery and Zucker 2012)), via sex selective lactational investment (Moses et al. 1995), and/or adjusting the quality of the sexes produced (Laaksonen et al. 2004; Love and Williams 2008).

Regardless of the adaptive value in producing biased sex ratios, the factors that mediate the maternal condition, which alter in utero sex ratios, remain a mystery. In other words, the question of how the reproductive system "knows" to produce more sons or daughters with respect to maternal and environmental condition remains open. Among the proximate mechanisms that have been suggested to influence sex allocation are maternal glucose levels (e.g., Cameron 2004), glucocorticoids (e.g., Love et al. 2005; Navara 2010), and testosterone (e.g., Grant 2007). Steroid hormones are known to influence various aspects of embryonic development, including sex (Adkins-Regan et al. 1995; James 1996; Dufty et al. 2002; Grant and Chamley 2010). For example, maternal glucocorticoids have been shown to be linked with sex ratios (e.g., Love et al. 2005; Ryan et al. 2011, 2014). The steroid hormone testosterone is an androgen involved in numerous physiological processes, including neuronal growth and function, muscle and bone development, immune function, and spermatogenesis in males (Staub and De Beer 1997; Muehlenbein and Bribiescas 2005; Moore et al. 2011). A few mammalian studies showed male-biased sex ratios following maternal testosterone elevation (Grant and Irwin 2005; Shargal et al. 2008; Helle and Laaksonen 2008; Grant et al. 2011; but also see Diez et al. 2009; French et al. 2010; Banszegi et al. 2012; Ryan et al. 2014). Given that elevated maternal androgens are transferred to the young via the placenta and yolk and have direct observable phenotypic effects on developing embryos and postnatal offspring, these hormones are excellent candidates to play a key role in sex-biased investment in vertebrates (Navara 2013).

Adaptive sex ratio biases at birth are not common in most mammalian orders (Hewison and Gaillard 1999). However, the nutria (*Myocastor coypus*) shows fluctuating sex ratios across groups and years (Gosling 1983). The nutria mating system is polygynous (Gosling and Baker 1989; Guichón et al. 2003; Túnez et al. 2009), with larger males that are socially dominant (Guichón et al. 2003; Túnez et al. 2009). The variance in reproductive success between males and the level of polygyny can become extremely high so that a single large male can dominate two adjacent groups (Túnez et al. 2009). Differential maternal investment in the sexes can be seen as early as lactation, when males spend more time suckling from the highest yielding teat and females from the lowest yielding teat (Gosling et al. 1984). Males also grow faster than females throughout lactation and weaning (Gosling et al. 1984). In nutrias, females in good condition may selectively abort small litters that are predominantly female yet retain large litters and small predominantly male litters (Gosling 1986).

Reproduction in nutrias includes relatively early sexual maturity (i.e., at 4 month of age), year round breeding (Leblanc 1994), post-partum estrus, and large litters reaching 13 pups (Newson 1966). All of these make this species a successful invader worldwide (Carter and Leonard 2002). The nutria was introduced to Israel in the 1950s. Their damage to canal banks have led to an intensive culling program in the

Hula Valley, in Northern Israel, from which we collected maternal hair samples, and conducted necropsies to quantify sex ratios in utero. We predicted that high maternal testosterone will be associated with better female condition and male-biased litters (Grant 2007). Given that female condition has been shown to be associated with testosterone in several species (e.g., Bouissou 1978; Grant et al. 2011) and that better condition mothers have male-biased litters (Clutton-Brock et al. 1984; Sheldon and West 2004), the main hypothesis of this study was that maternal testosterone is a significant mechanism that mediates female reproduction through facultative litter sex ratio adjustment (James 1989; Grant 2007).

## Materials and methods

### Sample collection

All animals were collected in the Agamon Hula Park, as a secondary use of eight culling efforts in 2013–2015. A total of 153 females were dissected, of which 76% (117) were pregnant. However, we included only 82 pregnant nutrias (i.e., gestation age of 50–138 days, where fetuses showed differentiation of corporal regions) and their fetuses ($n =$ 461; 250 males, 211 females). All females were in good physical condition, without mange or bruising.

Pregnant females were weighed using a spring scale (Pesola, Switzerland, 10 Kg capacity, 100 g division). Fetuses were weighed using an electronic balance to the nearest 0.01 mg (Precisa, Switzerland, BJ610C, $d = 0.01$ g). Morphometric measurements were collected using a standard measuring tape. We measured total nutria length, length from nose to tail base, shoulders to tail base in mothers, and crown to rump in fetuses. One mother was physically damaged during the culling. While we were able to accurately measure weight in the mother and eight fetuses, length measurements could not be accurately recorded.

### Offspring sex ratio

Fetuses were mostly sexed based on external morphology. We measured the anogenital distance (AGD; Correa and Frugone 2013), which has been historically used as a proxy for early androgen exposure since testosterone is responsible for the elongation of the perineal tissue (vom Saal and Bronson 1980). Though nutria fetal sex is clearly visible by external examination, we followed Willner et al. (1979) validation of AGD via internal examination of 10 male and 12 female fetuses. This method is widely used in nutrias (e.g., Sone et al. 2008). We calculated an AGD index by dividing fetal AGD length by fetal weight (Hotchkiss and Vandenbergh 2005). We found that AGD was significantly longer in male fetuses (AGD index, $t_{241} = 14.8$; $P < 0.001$) and used it thereafter. In addition, we used published primers for the *Sry* gene (Garcia-Meunier et al. 2001), which is only expressed in males, for sexing fetuses that were < 11 weeks old. Before this gestation age, the length of the AGD is less than 3.5 mm in males and 2 mm in females and is thus unreliable. The molecular method was validated using four adult males, four adult females, two male fetuses, and three female fetuses, whose internal and external genitalia were examined. The housekeeping gene 12S was used as a positive control, and an adult female was used as a negative control.

### Steroid measurements

Hair testosterone and cortisol were extracted and quantified using a protocol that was developed for wildlife and applied to multiple species (e.g., Koren et al. 2002; Bryan et al. 2015; Arnon et al. 2016). Hair is an ideal medium for studying the long-term effects of stable social, physiological, nutritional, and environmental conditions, baseline levels, and chronic stress. In addition, hair is easy to store and is stable for long periods (i.e., thousands of years; Webb et al. 2010; Wilson et al. 2013). The main advantage of this approach is that hair provides a long-term record of steroid hormone concentrations integrated over the period of hair growth, reflecting the average individual baseline and disregarding the acute stress of culling. Hair testing is especially suitable for measuring testosterone, which has been shown to be highly repeatable between subjects over weeks and months, using a test–retest design (Dabbs 1990; Sellers et al. 2007; Liening et al. 2010; While et al. 2010).

Studies show that nutrias have two juvenile molts, yet after the age of 7 months, hair growth becomes constant (Nabozny et al. 2015). In our study population, it takes mature females 6–8 weeks to regrow full-length hair after it is fully shaven. Thus, hair samples of culled pregnant females used in our study likely reflect integrated circulating steroid levels from the first and second trimester of pregnancy. Hair was sampled by shaving an area of 2 $\mathrm{cm}^2$ on each side of the upper thigh. Hair was washed twice with water for 3 min, and twice with isopropanol for 3 min, to remove external contaminants. Steroids were extracted overnight with methanol following sonication and incubation at 50 °C and quantified using commercial ELISA kits (Salimetrics Europe, Newmarket, UK). For more details on the extraction protocol, see Koren et al. (2002), Klein et al. (2004), and Arnon et al. (2016). 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 less than 0.49% with all other steroids. For cortisol, reported antibody cross-reactivity is 19.2% with dexamethasone, and less than 0.568% with all other steroids. Kits were validated for nutria hair by showing linearity (5–50 mg hair for testosterone and 0.5–10 mg hair for cortisol) and parallelism between serially diluted hair extracts (representing 5–50 mg for testosterone and 0.5–10 mg for cortisol) and kit standards (slope covariance $P = 0.68$ for testosterone and $P = 0.21$ for cortisol). For testosterone, intra-assay CV was 1.96% for six repeats on the same plate and 6.61% for cortisol. Inter-assay CV was 4.62% across three plates for testosterone and 8.17% for cortisol. Recovery was studied by spiking hair samples with a known steroid amount and was calculated as 100.67% for testosterone and 90.93% for cortisol.

## Statistical analysis

We estimated pregnancy age using Newson's formula for weights (1966), which is based on litters of known gestational age. General linear models assuming normal distribution and a logit link function were used to test the effects of maternal testosterone and cortisol levels (separately), female weight, pregnancy age, and litter size, on offspring sex ratios. As female weight, pregnancy age, and litter size are intercorrelated, we verified that the variation inflation factor was low, which allowed us to retain all variables in the model (VIF between 0.8–1.9). Fetal weight and length from crown to rump in relation to sex were tested using linear regression, with maternal identity included as a random factor. In order to test whether sex was a significant predictor of the heaviest fetus in the litter, we used the likelihood ratio (deviation from 0.5 probability; chi-square).

The significance of sex in predicting extreme growth retardation in our sample was tested using a binomial probability test. Fetuses exhibiting extreme growth retardation were defined as weighing below 75% of the average fetal weight. These fetuses had also been included in the sex ratio analysis, since we had no way of accurately determining their viability before the culling and subsequent freezing. Model fitting and tests were all done in the JMP software (version 12, SAS Inc., Cary, NC, USA). Since no behavioral data were collected, no blinded methods were used.

**Data availability** All data analyzed during this study are included in the supplementary information files.

## Results

Using necropsies of 82 pregnant nutrias, we found that the most significant predictor of litter sex ratio is estimated pregnancy age (Table 1). As pregnancy progressed, the initially high male biases in sex ratios decrease, as male fetuses are potentially terminated (Fig. 1b; Table 1). We also found that while female weight was not associated with testosterone ($F_{3,78} = 0.9$, $P = 0.45$), both affected sex ratios (Table 1). Litter size was not associated with sex ratios (Table 1). Surprisingly, females that had higher testosterone had female-biased sex ratios (Fig. 1a; Table 1). In addition, females that were heavier had male-biased sex ratios (Fig. 1c; Table 1). Overall, the model explained 16% of the variance in litter sex ratios ($R^2 = 0.16$, $F_{4,81} = 3.658$, $P = 0.0088$). No association was found between maternal testosterone and litter size ($R^2 = 0.0006$, $n = 82$, $P = 0.63$), estimated pregnancy age ($R^2 = 0.007$, $n = 82$, $P = 0.45$), nor seasonality ($F_{3,81} = 0.34$, $P = 0.8$).

Maternal cortisol, the dominant nutria glucocorticoid (Wilson et al. 1964; Callard and Leathem 1969), was not related to offspring sex ratio ($F_{1,81} = 0.0075$, $P = 0.93$). There was also no association between maternal cortisol and litter size ($R^2 = 0.006$, $n = 82$, $P = 0.48$) nor maternal testosterone levels ($R^2 = 0.0005$, $n = 82$, $P = 0.85$). We found a positive relationship between maternal cortisol and estimated pregnancy age ($R^2 = 0.05$, $n = 82$, $P = 0.038$).

In 68% of litters (mean = 5.63 offspring/litter), the heaviest fetus was a male ($\chi^2_1 = 7.99$; $P = 0.0027$). When the entire litter was considered, male fetuses at the final stages of the pregnancy (estimated pregnancy age of 121–138 days; $n = 47$) were significantly heavier than female fetuses ($F_{1,37} = 4.67$, $P = 0.037$) and significantly longer from crown to rump ($F_{1,29} = 10.88$, $P = 0.0026$), even after controlling for maternal effects (mother ID). In addition, some fetuses exhibited extreme growth retardation, weighing between 12 and 75% of the average fetal weight. Growth-retarded males were more common than growth-retarded females (nine males vs. two females, $P = 0.0327$, binomial probability of 9/11).

## Discussion

Contrary to our predictions, elevated maternal testosterone was related to female-biased litters. This result was surprising since we hypothesized that good maternal condition would be related with higher testosterone, and male-biased litters, as seen in several mammalian studies (Grant 2007; Grant and Irwin 2005; Shargal et al. 2008; Helle and Laaksonen 2008; Grant et al. 2011). In other systems, maternal testosterone has also been shown to affect litter size (Rutkowska et al. 2005; Ryan et al. 2014), female reproduction (Christiansen 2001), and sex differences in the immune system (Libert et al. 2010). Since testosterone levels may also reflect maternal social rank (Grant et al. 2011), and increase in response to chronic stress (Grant et al. 2011), it may serve as a mediator between the maternal environment and internal prenatal sex ratios. Thus, if the ultimate mechanism maintaining biased sex ratios in nutria is sexual differences in variation and fitness return for the two sexes (Trivers and Willard 1973), then high testosterone may indicate maternal stress or poor body condition driving the production of female-biased litters. Alternatively, if the adaptive value of male-biased litters lies in reducing local competitions with related daughters (LRC hypothesis, Clark 1978), then low female testosterone may

**Table 1** Model effects test and parameter estimates for predicting litter sex ratio. Italics denotes significant effects ($n = 82$)

| Parameter | Estimates | Std error | Sum of squares | *F* ratio | Prob > *P* |
|---|---|---|---|---|---|
| Estimated pregnancy age | − 0.0047 | 0.0016 | 0.463 | 9.14 | *0.0034* |
| Maternal testosterone | − 0.0413 | 0.018 | 0.268 | 5.29 | *0.0241* |
| Maternal weight | 0.0662 | 0.0298 | 0.249 | 4.92 | *0.0295* |
| Litter size | − 0.0105 | 0.0175 | 0.018 | 0.36 | 0.5499 |

indicate a kin-dense local environment, triggering the production of more sons, the dispersing sex in nutria.

Our morphological finding that females that weighed more tended to produce male-biased litters supports parental investment theory, where mothers in better condition should invest more in male offspring, as male offspring size and/or condition may produce greater fitness returns (Trivers and Willard 1973). This result is in line with previous studies, which found that nutria mothers in better condition produce more sons (Gosling et al. 1984; Gosling 1986). Overall, males in polygamous species are expected to show higher variation in fitness returns. Nutrias are polygynous, with extensive variation in male reproductive success (Gosling and Baker 1989; Guichón et al. 2003). Large males control large territories and have greater reproductive success (Gosling and Baker 1989; Guichón et al. 2003; Túnez et al. 2009). Although all of these morphological findings are consistent with TW, they may not constitute conclusive evidence supporting this hypothesis (e.g., Hewison and Gaillard 1999; Brown 2001; Schindler et al. 2015). Poor nutrient supplies may be the cause of abortion at various stages of gestation in the nutria (Gosling et al. 1984). In other mammalian species, maternal weight, diet, fat reserves, and nutritional status were associated with sex ratio biases, where better nutritional condition was linked with a male-biased sex ratio (e.g., Rivers and Crawford 1974; Ryan et al. 2011). However, this relationship may not be universal (Javad Zamiri 1978).

Poor condition, whether resulting from density, social, or nutritional environments, may lead to maternal stress. While elevated cortisol might imply maternal stress (reviewed in Navara 2010), we did not find an association between maternal cortisol and sex ratios. We found only a weak positive relationship between maternal cortisol and estimated pregnancy age ($R^2 = 0.05$, $n = 82$, $P = 0.038$), which can be accounted for by the rise in maternal glucocorticoids with the progression of pregnancy, observed in many species (Nathanielsz et al. 1975; Kitterman et al. 1981; Chan et al. 1993; Patel et al. 1996). However, stressful conditions may be associated with elevated testosterone in females (Powell et al. 2002; Roos et

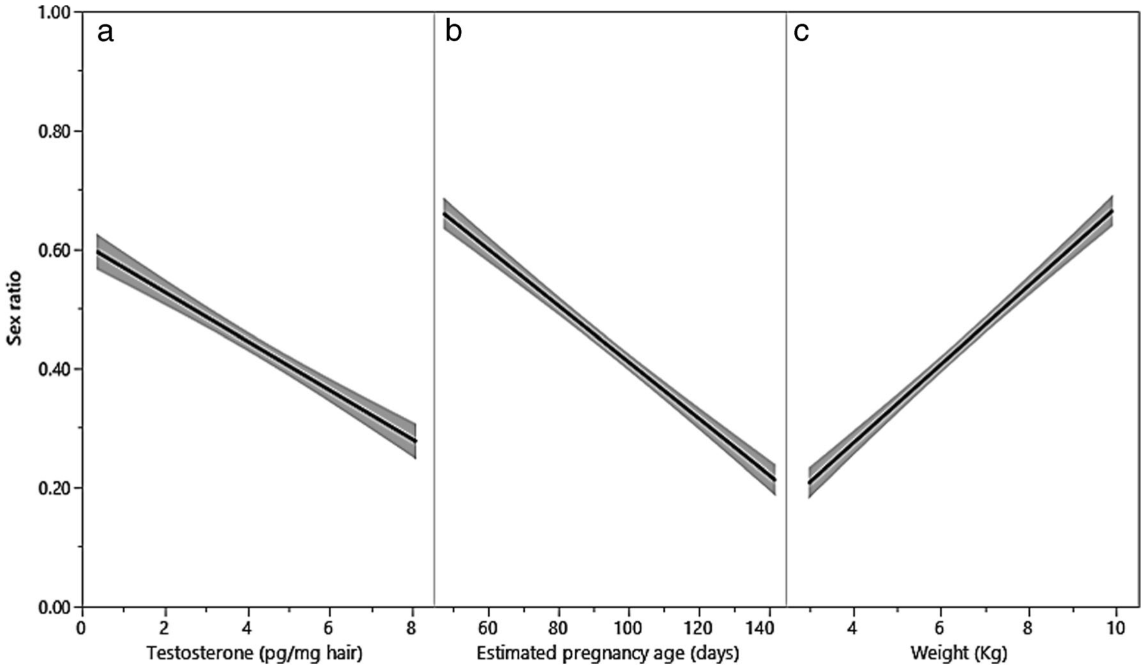

**Fig. 1** Estimated model parameter slopes and offspring sex ratios. Sex ratios are the ratio of males in a litter, where 1 indicates all male litters and 0 indicates all female litters. Shaded area indicates confidence intervals. **a** Higher maternal testosterone is associated with female-biased sex ratios. Maternal testosterone is expressed in picograms per milligram of hair. **b** As pregnancy progresses, sex ratios decline. Pregnancy age is estimated in days according to Newson (1966). **c** Higher maternal weight is associated with male-biased sex ratios

al. 2011; Christiansen 2001; King et al. 2005; Grant et al. 2011; Bryan et al. 2015; but also see Ward and Weisz 1984; Kunstmann and Christiansen 2004; Schöpper et al. 2012), possibly linking environmental stressors to reproductive suboptimality and sex allocation (Grant et al. 2011; James 2015). High levels of testosterone may have adverse effects on female reproduction, such as an increased risk of infertility and cycle disturbance, fetal loss, preterm birth, and low birth weight (Balen et al. 1995; James 2015). Testosterone may be linked to nutrias' adjustment to changes in the environment by fetal absorptions and abortions (~50–60% of embryos not reaching full term; Newson 1966; Willner et al. 1979). In human females, for example, high levels of circulating testosterone are associated with a high incidence of ovarian dysfunction (Smith et al. 1979; Steinberger et al. 1981), and in rabbits (*Oryctolagus cuniculus*), administration of testosterone during pregnancy resulted in increased rates of abortion and resorption (Fuller et al. 1970). Stress had been shown to effect sex ratio, reduce litter size, and cause differential male mortality pre-and post-conception (e.g., Pratt and Lisk 1989; Bacon and McClintock 1999). Males seem to be especially vulnerable to stressful environments, from conception to infancy (e.g., Kruger and Nesse 2004; Catalano and Bruckner 2006; Baxter et al. 2012). This appears to be reflected in our study, since we found that sex ratios decreased as pregnancies progressed. We also found significantly more growth retardation and fetal absorption in male than female fetuses (9 vs. 2, respectively), suggesting post-implantation sex ratio adjustments (Gosling 1986), independently, or in addition to pre-implantation sex allocation. In the last stage of pregnancy, male weight and length were higher than females', suggesting higher in utero growth rate and greater demands for maternal resources (Gosling et al. 1984).

Trade-offs between litter size and sex ratios have been attributed to both testosterone and cortisol, suggesting an indirect effect of the hypothalamic–pituitary–adrenal axis activation on sex ratio via stimulation of adrenal testosterone (Ryan et al. 2014). Though we did not find a relationship between cortisol and testosterone in our study, we cannot exclude the possibility that stress affected adrenal or ovarian androgen production. In addition, hair testing is not the most suitable matrix to detect short-term associations between steroid levels under acute stress. Contrary to blood, the steroid levels that we measured in hair probably represent average circulating levels over the early weeks of pregnancy. Stress may mediate the hypothalamic–pituitary–gonadal axis in females via activation of a sympathetic neural pathway originating in the hypothalamus and releasing norepinephrine into the ovary (Toufexis et al. 2014). Chronic or repeated sympathetic nerve activation, caused by stress, can also stimulate androgen secretion from the ovary, affecting reproductive function (Lara et al. 2002; Greiner et al. 2006; Bernuci et al. 2008; Toufexis et al. 2014). At this point, the mechanisms behind our results remain a mystery. However, we suggest that maternal testosterone may be an important mediator of litter sex ratio adjustment through its reflection of environmental conditions and its role in reproduction.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** The nutria was introduced into the wild in Israel during the 1950s and is currently considered a pest. Culling efforts are managed by the KKL-JNF, the managing organization of the Agmon Hula Park. We collected nutria that were culled during 2013–2015 and did not initiate culling. No permits are needed for collecting nutrias since they are not protected by the Israeli law.

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