BIOLOGY LETTERS

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Cite this article: Firman RC, Tedeschi JN, Garcia-Gonzalez F. 2020 Sperm sex ratio adjustment in a mammal: perceived male competition leads to elevated proportions of female-producing sperm. *Biol. Lett.* **16**: 20190929.
http://dx.doi.org/10.1098/rsbl.2019.0929

Received: 18 December 2019
Accepted: 27 April 2020

Subject Areas:
evolution

Keywords:
male-driven sex allocation, sexual selection, offspring sex ratios, local mate competition, social environment, mice

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Electronic supplementary material is available online at https://doi.org/10.6084/m9.figshare.c.4983296.

THE ROYAL SOCIETY PUBLISHING

Evolutionary biology

Sperm sex ratio adjustment in a mammal: perceived male competition leads to elevated proportions of female-producing sperm

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Mammal sex allocation research has focused almost exclusively on maternal traits, but it is now apparent that fathers can also influence offspring sex ratios. Parents that produce female offspring under conditions of intense male–male competition can benefit with greater assurance of maximized grand-parentage. Adaptive adjustment in the sperm sex ratio, for example with an increase in the production of X-chromosome bearing sperm (CBS), is one potential paternal mechanism for achieving female-biased sex ratios. Here, we tested this mechanistic hypothesis by varying the risk of male–male competition that male house mice perceived during development, and quantifying sperm sex ratios at sexual maturity. Our analyses revealed that males exposed to a competitive 'risk' produced lower proportions of Y-CBS compared to males that matured under 'no risk' of competition. We also explored whether testosterone production was linked to sperm sex ratio variation, but found no evidence to support this. We discuss our findings in relation to the adaptive value of sperm sex ratio adjustments and the role of steroid hormones in socially induced sex allocation.

1. Introduction

Strategies of male–male competition take on many different forms, including elaborate displays, female mate guarding and physical combat [1]. Male–male competition also extends to the post-copulatory arena when females mate multiply and the sperm of rival males vie for fertilizations [2]. Therefore, be it energy spent on defending a territory against a rival or the requirement to produce more sperm to gain a fertilization advantage over a rival, competition is inherently costly to males [3,4]. Because of this cost, males are expected to be prudent when it comes to the development of competitive traits (e.g. ornaments, weaponry, sperm) [5]. This is especially true in light of the potential for trade-offs with critical processes (e.g. immune function; [6]). Males may acquire important information on the value of investing in a competitive phenotype at sexual maturity via environmental cues perceived during early-life. Correspondingly, exposure to different social conditions during development can lead to adaptive male responses. For example, it has been shown that males reared in the presence of (more) rivals or their cues will adapt to competition with the development of larger body [7–9] and testes [7,9–13] size, as well as elevated sperm production rates [10,14–19]. However, the social conditions that males experience during development may also be reflective of the environment that their offspring will face. Indeed, in order to maximize their inclusive fitness, both males and females have a vested interest in ensuring

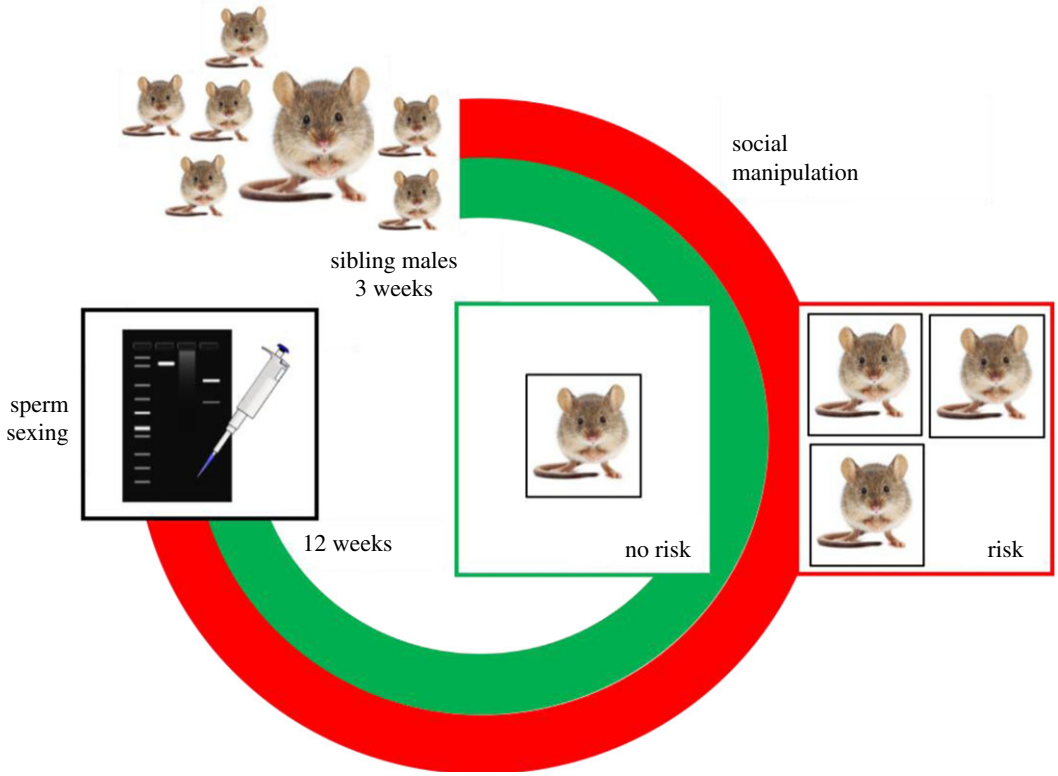

Figure 1. The experimental design. Wild-sourced male house mice were reared under standard conditions from birth to weaning, and then exposed to one of two social environments that reflected either a 'risk' or 'no risk' of male–male competition. Brothers were used across treatments. Serum testosterone concentration and sperm sex ratios were measured at approximately 90 days of age.

that offspring phenotype aligns adaptively with the social conditions that prevail in their local neighbourhood.

Offspring sex is a phenotypic trait that is likely to have a significant impact on an individual's inclusive fitness in the context of variation in the social environment. Theory predicts that the production of female offspring will provide parents with the most beneficial outcome under conditions of an intense male–male competition (*sensu* the local mate competition hypothesis) [20]. The underlying rationale is intuitive: in a male-dominated environment the production of female offspring—who are guaranteed high mate availability—eliminates the exposure of male offspring to a highly competitive scenario [20]. In species where females have direct control over offspring sex, mothers have been shown to maximize their fitness by mediating offspring sex ratios based on local social conditions (e.g. haplodiploid invertebrates [21] and birds [22–24]). Social instability has been linked to the production of female offspring in mammals [25–28], which is often attributable to elevated maternal stress [29,30]. However, both female *and* male mammals contribute to offspring sex: fertilization and offspring development occur inside the mother, but fathers provide the sex-determining gamete. Males that produce ejaculates containing high numbers of good quality sperm may benefit by skewing offspring sex ratios toward sons who will inherit their father's superior fertility (*sensu* the male fertility hypothesis; [31]). Indeed, recent evidence has indicated that mammal sex allocation is not at the exclusive liberty of mothers [31–34]. To this end, adaptive paternal control over offspring sex ratios has emerged as an important area of research [35,36].

Our recent investigation revealed that the sperm sex ratio ($n_{\text{Y-CBS}}/n_{\text{total sperm}}$) is a variable trait that is sensitive to the social environment that an individual experiences during sexual development [9]. Specifically, we found that, relative to high-female density conditions, exposure to high-male density social conditions resulted in both an increase in testes size (a proxy for high fertility) and an elevation in the production of Y-CBS [9]—results that align with the male fertility hypothesis [31]. We speculated that variation in testosterone production may be the underlying mechanism accounting for these differences [9]. Here, we explicitly test this hypothesis by applying an experimental design proven to elicit adaptive responses among males exposed to reproductive competition during sexual development [8,14,37]. Thus, we quantified variation in testosterone concentration and plasticity in sperm sex ratios in relation to the perceived risk of male–male competition in an established mammalian model, the house mouse (*Mus musculus domesticus*). We hypothesized that a competitive social environment would lead to elevated testosterone levels, which in turn would favour the production of Y-CBS.

2. Material and methods

(a) Social manipulation

In this experiment, we used third generation, laboratory-born wild house mice (*Mus musculus domesticus*), the ancestors of which were originally sourced from Rat Island (28°42′ S, 113°47′ E; Western Australia) (see [8] for more details). Following protocols routinely performed in our laboratory, we manipulated the social experience of males during their sexual development to create variation in the perception of male–male competition risk via differential exposure to rival males and their scents (figure 1). Brothers from a total of 24 families were used across treatments. Males were exposed to

different social environments that reflected either a 'risk' (two rivals) or 'no risk' (no rivals) of competition from weaning until sexual maturity (approx. 90 days old) (figure 1). The social manipulation methodology can be found in the electronic supplementary material, as well as in our previously published work [8,14,37]. The experimental subjects were initially used in an investigation that tested whether different social conditions shaped plasticity in testes tissue architecture (i.e. corresponding to previous findings on sperm production rates, [14,16]; see [8]). To test the hypothesis outlined here, we extracted and stored sperm and blood serum from the males as detailed below.

(b) Sperm sexing

We sampled epididymal sperm [8,14,38] and extracted genomic DNA from these samples [9,39] according to methodology that is routinely performed in our laboratory (see the electronic supplementary material). We measured the proportion of Y-CBS using an absolute quantification qPCR protocol that we had previously optimized and validated for mouse sperm samples [9]. Briefly, we amplified a standard concentration of [100 ng µl^{-1}] of DNA for the *G6pd2* and *Sry* genes in triplicate 10 µl singleplex reactions (see [9] for reaction ingredients and cycling conditions). The fluorescent signals captured at the end of each amplification cycle produced the threshold cycle (Ct). The mean of the replicate *G6pd2* gene and *Sry* gene Ct values for each sperm sample was used for calculating the proportion of X- and Y-CBS [40]. The proportion of Y-CBS was calculated from the ratio between the quantities of X- and Y-CBS using the following equation [40,41]:

$$\text{proportion Y} - \text{CBS} = \frac{n}{n+1},$$

where $n = \text{Ct}_{\text{Y-CBS}}/\text{Ct}_{\text{X-CBS}}$.

Thus, the number of X- and Y-CBS were calculated for each sample using the (i) proportion measured in the qPCR assay, (ii) required volume of sperm suspension used in the assay and (iii) overall sperm concentration that was measured at the time of sperm isolation.

(c) Testosterone assays

Males were sacrificed between 9:00 and 10:00 am and blood was immediately collected via cardiac puncture, refrigerated overnight (4°C) and then centrifuged (20 min; 3250 r.p.m.). The serum was removed, aliquoted and stored (−80°C). Duplicate testosterone ELISAs were performed according to the manufacturer's instructions.

(d) Statistical analyses

All statistical analyses were conducted in R v. 3.5.1 [42]. Implemented within the package *lme4* [43], a model on testosterone concentration was initially fitted using the function *lmer* (linear mixed model; LMM). The model on sperm sex ratio was fitted using *glmer* (generalized linear mixed model; GLMM) with a binomial error distribution and the command '*cbind*' so that the response variable contained information about the numbers of X- and Y-CBS leading to a ratio for each sample. To account for issues of data dispersion in our GLMM we applied quasi-likelihood by correcting the standard errors of the estimates by the dispersion factor and then recomputing *Z*- and *p*-values accordingly. Family ID and tub ID were included as random factors in the models (see the electronic supplementary material). Interactions between the fixed factors were included in the models. To control for differences owing to body size, we included body mass (mean-centred) as a covariate in our LMM testing testosterone responses. Testes mass and testosterone concentration (mean-centred) were included as covariates in our GLMM to test their mechanistic implication on sperm sex ratio variation. Significance of the fixed effects in the LMMs was calculated using maximum likelihood and Wald tests, using the function Anova (*car* package), while parameter estimates were calculated using restricted maximum likelihood (REML) as recommended [44]. Diagnostic plots were visually checked to validate the models. The interaction terms in each model were non-significant and therefore removed. 'No risk' treatment level was the reference level for the treatment factor. One 'risk' male was excluded from our analyses owing to abnormally low testis mass (2.1 mg).

Table 1. Linear mixed model testing the effect of the social environment on serum testosterone concentration in male house mice. *p*-values in bold are significant at <0.05.

fixed effects	estimate	± s.e.	type II, Wald χ^2	d.f.	*p*-value
intercept	1.7858	0.2183			
treatment	0.6625	0.3310	4.280	1	**0.039**
body mass	−0.0799	0.0847	0.952	1	0.329

3. Results

Our LMM revealed that there was a significant social environment treatment effect on testosterone concentration (table 1). Males exposed to a perceived 'risk' of male–male competition during development had, on average, higher testosterone levels (mean ± s.e.: 15.7 ± 2.2 ng ml^{-1}) compared to males that developed under 'no risk' of competition (mean ± s.e.: 8.7 ± 1.2 ng ml^{-1}). However, an inspection of the data revealed that this difference was driven by six 'risk' males having comparatively very high testosterone levels (i.e. greater than 30 ng ml^{-1}; figure 2*a*).

Our GLMM showed that 'risk' males produced lower sperm sex ratios, corresponding to greater proportions of X-CBS, compared to 'no risk' males (table 2 and figure 2*b*). Testosterone concentration and testes mass (in absolute terms as well as relative to body mass) did not account for variation in sperm sex ratios (table 2). The six 'risk' males with the highest testosterone concentration produced sperm sex ratios that fell close to or below the median treatment value, ranging from 0.486 to 0.509 (figure 2*b*).

4. Discussion

Recent research has indicated that the sperm sex ratio is a plastic trait that responds to prevailing social conditions [9], which highlights the potential that these adjustments function as a mechanism of male-driven sex allocation [31–34]. In a previous experiment on house mice, we found that exposure to high-male density conditions during sexual development (3–12 weeks of age) resulted in the production of higher proportions of Y-CBS and larger testes [9], which taken together support the male fertility hypothesis [31]. Despite there being strong evidence that low testosterone levels lead to the production of female offspring [45], the precise mechanism by which testosterone influences sperm sex ratios is currently unknown. In the current investigation, we tested whether sperm sex ratio adjustments are linked to variation in testosterone production. Contrary to our expectation,

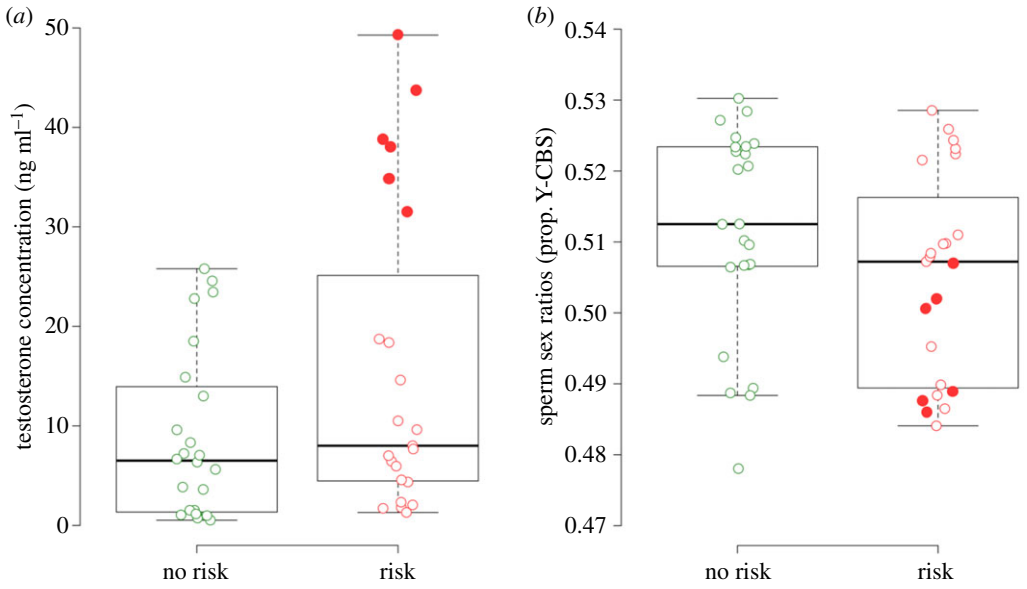

Figure 2. Serum testosterone concentration (*a*) and sperm sex ratios (*b*) for male house mice reared under a perceived 'risk' or 'no risk' of male–male competition. Solid circles indicate samples with testosterone concentrations greater than 30 ng ml^{-1}. The median (box midline), third (upper box line) and first (lower box line) quartiles, and range (whiskers) are presented.

Table 2. Generalized mixed models testing the effect of the social environment on sperm sex ratios in male house mice, including testosterone and testes mass (*a*) or the residuals of testes mass regressed on body mass (*b*) as covariates. *p*-values in bold are significant at <0.05.

fixed effects	estimate	±s.e.	*Z*	d.f.	*p*-value
(*a*) with testes mass and testosterone					
intercept	0.0466	0.0057			
treatment	−0.0267	0.0111	−2.491	1	**0.013**
testes mass	−0.0001	0.0001	−0.961	1	0.336
testosterone	−0.0042	0.0026	−1.601	1	0.109
(*b*) with residuals of testes mass and testosterone					
intercept	0.0471	0.0058			
treatment	−0.0284	0.0107	−2.642	1	**0.008**
testes mass residuals	−0.0001	0.0003	−0.346	1	0.729
testosterone	−0.0036	0.0026	−1.409	1	0.159

we found that males reared under a risk of competition produced lower sperm sex ratios (i.e. more X-CBS biased) compared with males that matured in the absence of rivals. It is interesting that males exposed to the competitive environment in the current experiment (risk) produced more X-CBS biased sperm ratios than males exposed to the non-competitive environment (no risk), while the opposite result was observed in our previous study (competitive environment = 'high-male density'; non-competitive = 'high-female density') [9]. While these results are seemingly contradictory, differences in experimental design are likely to account for the different responses. The degree of perceived male–male competition was comparatively less intense in our previous experiment (i.e. males maturing within the same room as other males; [9]) than what was applied in the current experiment (i.e. rival males maturing within close proximity to one another within the same experimental tub), which highlights the intriguing possibility that variation in the *intensity* of competition (and not just presence/absence) leads to different sperm sex ratio responses.

Theory predicts that it would be maladaptive for parents to produce male offspring in a mate competitive environment because they would be forced to compete for access to females and/or be subjected to sperm competition [20]. Conversely, with guaranteed mate availability, high-male density conditions will be evolutionarily favourable for females. Thus, the production of daughters under these conditions is expected to be advantageous to both mothers and fathers [20]. Adaptive maternal sex allocation in relation to male density within the local neighbourhood has been demonstrated in diverse species, including spider mites [21] and house mice [29]. Here, we used house mice sourced from an island population where dispersal capacity is severely restricted and consequently parents and offspring often experience the same local social conditions (see the electronic supplementary material for more information). As a consequence, it is likely that males are forced to compete with both related (*sensu* local mate competition; [20]) and unrelated males for access to females. We demonstrated that male house mice reared under conditions of intense male–male competition produced higher proportions of X-CBS relative to males not subjected to competition—an outcome that has the potential to have adaptive paternal consequences. Certainly, if increased numbers of female-producing sperm translate to more female offspring, sperm sex ratio adjustments could potentially be an effective strategy for males to enhance their grand-parentage under competitive conditions. We plan to explore this currently untested hypothesis in our future research.

The precise mechanism(s) controlling sex allocation in mammals is currently not well understood. In terms of paternally driven proximate mechanisms, recent research has linked variation in sperm sex ratios [34] and differential X- and Y-CBS motility to offspring sex ratios [46,47]. Further

to this, there is evidence to suggest that the ultimate cause of socially induced sex ratio biases involves physiological responses via endocrine signalling [30,48]. Here, we found that the social environment influenced testosterone concentration, but only as a consequence of elevated levels in a subset of 'risk' males. Although these individuals produced proportions of Y-CBS at the lower end of the scale, our statistical analyses provided no evidence that sperm sex ratio plasticity is driven by variation in testosterone production. The division in testosterone levels in the 'risk' treatment (i.e. less than 20 ng ml^{-1} and greater than 30 ng ml^{-1}) may be indicative of hormone profiles linked to social status. The default assumption is that social hierarchies are associated with differential testosterone levels, but, in fact, more often than not there is no predictive pattern (e.g. see [49] and references therein). For example, it is only the most aggressive dominant male mice that display elevated testosterone levels (relative to less aggressive dominant males and subordinate males) [49], which likely explains the pattern we have observed in our 'risk' treatment. Stress hormones, such as corticosterone, are more commonly associated with social status in male mice, although the direction of the effect has been inconsistent [49]. Offspring sex ratio biases have been linked to maternal stress in a number of mammals [29,30], yet the role that paternal stress plays in sex allocation remains an open question. To address this gap in knowledge, our future research will focus on the relationship between socially induced paternal stress and variation in the sperm sex ratio.

Ethics. The work reported in this article followed the guidelines for the ethical treatment of animals in research under UWA Ethics Committee approval (03/100/1456).

Data accessibility. The data file is provided in the online electronic supplementary material.

Authors' contributions. R.C.F. conceived the study, performed the sperm extraction and ELISA assays, and drafted the manuscript. J.N.T. optimized the qPCR assay. F.G.-G. analysed the data. All authors engaged in valuable discussion and provided edits on the drafts of the manuscript.

Competing interests. We declare we have no competing interests.

Funding. This research was funded by the Australian Research Council (grant no. FT180100625 to R.C.F.), the Spanish Ministry of Economy (grant nos. CGL2012-34685 and CGL2016-76173-P to F.G.-G.) and UWA (Research Collaboration Award to R.C.F. and F.G.-G.).

Acknowledgements. We thank Gonçalo Igreja André who performed the social manipulation and Misha Lavoie who was employed to run the qPCR assays.

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