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Male body size and condition affects sperm number and production rates in mosquitofish, *Gambusia holbrooki*

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Abstract

Sperm number is an important predictor of paternity when there is sperm competition. Sperm number is often measured as maximum sperm reserves, but in species where mating is frequent, males will often be replenishing their reserves. Thus, variation in how quickly males can produce sperm is likely to be important in determining male success in sperm competition. Despite this, little is known about how male size, body condition or diet affects sperm production rates. We counted sperm number in large and small *Gambusia holbrooki* (eastern mosquitofish) after 3 weeks on either a high or low food diet. Sperm number was significantly higher in both larger males and in well-fed males. We then stripped ejaculates again either 1, 2, 3, 4 or 5 days later to investigate subsequent sperm production. The rate of sperm replenishment was influenced by an interaction between size and diet. Large, well-fed males had consistently high levels of sperm available over the 5 days (i.e. rapid replenishment), whereas small poorly fed males showed consistently low levels of sperm availability over the 5 days (i.e. slow replenishment). In contrast, large, poorly fed and small, well-fed males increased their sperm numbers over the first 3 days (i.e. intermediate replenishment). Our study highlights that when mating is frequent and sperm competition is high, size and condition dependence of maximal sperm number and of sperm production rate might both contribute to variation in male reproductive success.

Introduction

Sperm competition is an important selective pressure that influences the evolution of ejaculate traits (Parker, 1970; Hosken & Ward, 2001; Parker & Pizzari, 2010). When ejaculates from multiple males overlap at the site of fertilization, such as when females mate multiply in internally fertilizing species, males with more competitive ejaculates will receive a greater share of paternity (Andersson & Simmons, 2006). Sperm number is an important component of ejaculates that is often a good predictor of male fertilization success when males face sperm competition (Parker, 1990; Simmons, 2001). This is the so-called raffle principle – males with more tickets have a better chance of collecting the prize.

In species where mating is frequent the number of sperm a male has available to allocate towards a mating is dependent not only on his maximal sperm capacity, but also on how fast he is able to produce sperm and replenish reserves. Sperm depletion has been experimentally demonstrated in a wide range of taxa, including butterflies (Svärd & Wiklund, 1989; Bissoondath & Wiklund, 1996a,b), birds (Westneat *et al.*, 1998) and crabs (Sato *et al.*, 2006). This research suggests that males may rarely find themselves in a position to mate with fully replenished sperm reserves. As such, sperm production rates may be equally, if not more, important than maximal sperm capacity in determining male reproductive success when there is sperm competition. Males with slower rates of sperm production are at a competitive disadvantage as they effectively have a longer 'time-out' after mating than males that are able

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to replenish sperm reserves quickly (Clutton-Brock & Parker, 1992; Lemaitre *et al.*, 2009).

There is an evidence from butterflies that species with higher levels of polyandry have a higher capacity for sperm production than monandrous species (Svärd & Wiklund, 1989; Bissoondath & Wiklund, 1996b). Within species, however, little is known about the factors that affect individual males' rates of sperm production despite the potentially important consequences for male reproductive success. Two measures of male quality – size and body condition – are often associated with the expression of sexually selected traits in general (Johnstone, 1995; Cotton *et al.*, 2004) and have also been shown to influence sperm number. For instance, larger males tend to have more sperm available when their sperm reserves are fully replenished, as testes size increases with body size (Simmons, 2012; Lüpold *et al.*, 2014). Likewise, males that are in good condition have been shown to have more sperm (Vermeulen *et al.*, 2008; Rahman *et al.*, 2013), although a meta-analysis did not find a strong correlation between male ornamentation and sperm traits (Mautz *et al.*, 2013). However, studies measuring males' current sperm reserves are usually carried out on males that have not mated recently and have thus fully replenished their supply and attained their maximum sperm number. It does not necessarily follow that males that are larger or in better condition have faster rates of sperm production – they could merely accumulate their sperm reserves over a longer time period. To our knowledge, the simultaneous effects of size and condition on sperm production rates, hence the speed of replenishment, have not yet been tested using an experimental approach in wild animals.

Here, we measure sperm number in males that had time to fully replenish their sperm reserves. We then measured their subsequent sperm production by stripping and counting the sperm of males again either 1, 2, 3, 4 or 5 days after their initial counts. We studied *Gambusia holbrooki* (eastern mosquitofish), which is a poeciliid fish with a coercive mating system, frequent mating attempts and intense sexual selection (Wilson, 2005; Callander *et al.*, 2012), making it an ideal system to study sperm production rates. We tested for an effect of body size, diet and their interaction on maximum sperm reserve size and sperm production.

Materials and methods

Origin and maintenance

We collected male *G. holbrooki* from two ponds in Canberra, Australia (35°18′27″ S 149°07′27.9″ E) in February–March 2014. Males were initially kept in single-sex tanks (60 L), with a 14:10 h photoperiod at 28 °C for approximately 1 month before experiments began. They were fed *ad libitum* on a diet of *Artemia* nauplii and commercial flakes.

Experimental design

To investigate how male size, diet and their interaction affect sperm number and sperm production, we used a 2 × 2 factorial design. We manipulated the diet of large and small males. To generate our size classes, we weighed 490 wild-caught males and chose those in the top (> 190 mg) and bottom (< 150 mg) thirds (large: 246.9 ± 3.2 mg; small 129.0 ± 1.0 mg). (Note: all descriptive statistics are presented as mean ± SE). Standard length and weight were highly correlated ($r = 0.96$, $n = 294$, $P < 0.001$). Males were then placed in individual aquaria (1 L) and randomly assigned to one of two diets. Males on the high food diet were fed *ad libitum* twice daily with *Artemia* nauplii, whereas those on the low food diet were fed approximately 2% of their body mass (2.01 ± 0.02%) (following methods for guppies; Rahman *et al.*, 2013).

Effect of diet on body condition

The change in mass of males was significantly affected by both their initial size class ($F_{1,292} = 63.520$, $P < 0.001$) and diet ($F_{1,292} = 912.357$, $P < 0.001$) (two-way ANOVA). Males on the high food diet gained mass (+10.13 ± 1.17 mg), whereas males on the low food diet lost mass (−31.06 ± 0.97 mg). There was a significant interaction between male size and diet ($F_{1,292} = 7.680$, $P = 0.006$): large under-fed males lost more mass, and large well-fed males gained less mass, than their smaller counterparts.

Collecting and counting sperm

Following 3 weeks of high or low food diets, we stripped sperm from each male to estimate his maximum sperm number (males had not mated for at least 6 weeks). This ensured that all males were fully sperm depleted so that we could then estimate net sperm production either 1, 2, 3, 4 or 5 days afterwards. For each treatment by day combination (4 treatments × 5 days), we had 14–16 males (total = 300 males).

To strip ejaculates, we anaesthetized males in ice-cold water, patted them dry with paper towel and placed them on a glass slide under a dissecting microscope with their gonopodium (intromittent organ) swung forward. We then gently applied pressure to the base of their gonopodium to eject all recoverable sperm. We assumed any error is random with respect to treatment type. All sperm strips were carried out by the same person for consistency. Using a micropipette, we transferred the stripped ejaculate to an Eppendorf tube containing 100 μL of 0.9% saline solution. We then vortexed the solution for 20 s, to break up sperm bundles and to ensure an even distribution of sperm in the sample.

We counted sperm following standard methods (Evans, 2009). Briefly, we averaged sperm counts from five squares of an improved Naubauer hemocytometer under ×400 magnification (microscope specifications: Kyowa Medilux-12; Kyowa Optical Co., Ltd., Sagamihara, Japan). Counts across the five squares were highly repeatable ($r = 0.89$, $n = 600$). After initially being stripped of sperm (day 0), we allowed males to recover in a tank under aeration for 20 min, returned them to their own tank and maintained them on their previous high or low food diet until they were stripped again either 1, 2, 3, 4 or 5 days later. Each male was only stripped of sperm once after day 0.

Statistical analyses

To test for an effect of diet and male size on maximum sperm count, we ran a two-way ANOVA with day 0 sperm count as the response variable and male size category, diet and their interaction as fixed factors. We excluded three males (1%) that had no sperm on day 0. To look at sperm replenishment, we ran a general liner model (GLM) with sperm counts across the 5 days as the response variable, male size and diet as fixed factors and day (1–5) as a covariate. We included all two-way and three-way interactions in this model.

To help interpret the significant three-way interaction found during this analysis, we ran seperate one-way ANOVAS for the four treatment groups, with sperm counts across the 5 days as the response variable and day (1–5) as a fixed factor.

We transformed data, using the power transform function in the *car* package of *R*, to ensure all model residuals were normally distributed. We ran all models using SPSS v. 22.0 (Armonk, NY, USA).

Results

Maximum sperm number

Larger males ($F_{1,292} = 36.370$, $P < 0.001$) and males on a high food diet ($F_{1,292} = 4.014$, $P = 0.046$) had significantly larger sperm reserves on day 0 (see Fig. 1). There was no effect of the interaction between male size and diet ($F_{1,292} = 0.912$, $P = 0.340$).

Sperm production

There was a marginally significant three-way interaction between male size, diet and the day sperm were stripped ($F_{1,274} = 3.914$, $P = 0.049$). There were also strong main effects (size: $F_{1,274} = 40.239$, $P < 0.001$; food: $F_{1,274} = 18.928$, $P < 0.001$; day: $F_{1,274} = 8.488$, $P = 0.004$), which remained if the interaction was dropped from the model. Large males under the high food diet had a high number of sperm from the first day onwards (i.e. replenishment occurred more rapidly)

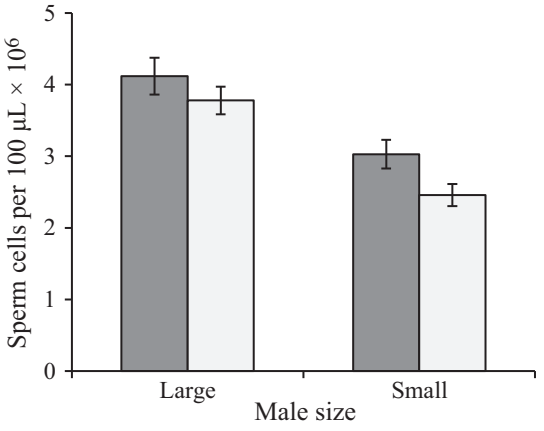

Fig. 1 Mean ± SE sperm counts of large and small males on day zero after 3 weeks in high food (grey bars) or low food (white bars) diets. Sample sizes are displayed above the error bars.

(day: $F_{4,67} = 0.472$, $P = 0.756$). Small males under the low food diet had low sperm numbers across all 5 days (i.e. replenishment was slow) (day: $F_{4,63} = 0.280$, $P = 0.890$). For both large males on a low food diet and small males on a high food diet sperm numbers changed over time (day: Large/Low: $F_{4,68} = 3.822$, $P = 0.007$; Small/High: $F_{4,64} = 2.438$, $P = 0.027$): both had low sperm numbers on days 1 and 2 and higher levels of sperm thereafter. In the full model, there were no significant two-way interactions (size × food: $F_{1,274} = 2.701$, $P = 0.101$; size × day: $F_{1,274} = 0.105$, $P = 0.746$; food × day: $F_{1,274} = 0.057$, $P = 0.811$).

Finally, the daily mean sperm numbers were all lower than the mean on day 0 for all four treatment groups. This suggests that sperm replenishment was incomplete. It should be noted, however, that none of the sperm counts on day 5 were significantly lower than that for day 0 (i.e. all < 2 SE from the mean) (see Fig. 2).

Discussion

The maximum number of sperm males had available when fully replenished (i.e. sperm reserves on day 0) was clearly affected by both their body size and recent diet. The strongest effect was for body size: larger males had far greater maximum sperm reserves than smaller males. This is expected based on results from other fish species where testes size increases with body size in an allometric fashion (Comparative analysis: Stockley *et al.*, 1997) and also supports a previous study on *G. holbrooki* that showed larger males have more sperm (Locatello *et al.*, 2008). The effect of diet, from which we infer the effect of body condition, was smaller but still clear: males in better condition had more sperm on day 0. This provides evidence of condition dependence in maximum

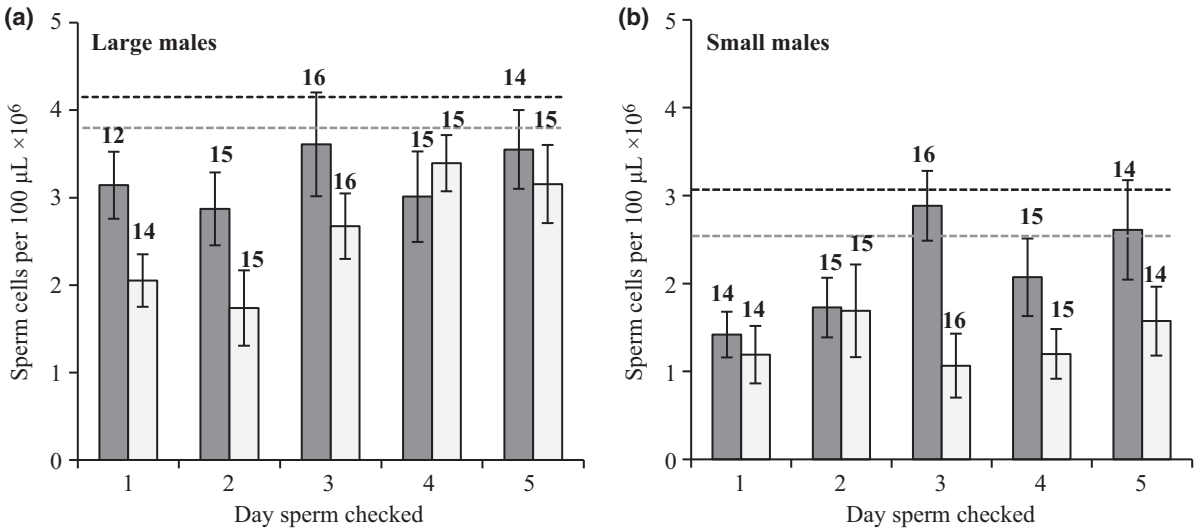

Fig. 2 Mean ± SE sperm counts of (a) Large males and (b) Small males, either 1, 2, 3, 4 or 5 days after being stripped of all recoverable sperm. Grey = high food diet, White = low food diet. Sample sizes are displayed above the error bars. Horizontal dashed lines depict average sperm counts on day zero: Black = high food diet, Grey = low food diet.

sperm number, supporting previous work on guppies (another poeciliid fish) (Gasparini *et al.*, 2013; Rahman *et al.*, 2013) (but see (Devigili *et al.*, 2013) for a nonsignificant effect of diet on sperm number) as well as work on insects [e.g. red flour beetle: (Perry & Rowe, 2010); *Drosophila*: (McGraw *et al.*, 2007)]. Still, there are far fewer experiments testing condition dependence of postcopulatory traits in wild animals compared to the extensive literature on condition dependence of precopulatory traits such as male weapons and ornaments (Johnstone, 1995; Cotton *et al.*, 2004).

We also found that in *G. holbrooki* the rate of sperm production depended on both male body size and body condition (which we assume is elevated for males on a high food diet). In general, large males replenished their sperm reserves faster than small males. This finding differs from that in butterflies, which have shown that male size does not affect the size of ejaculates across successive matings (Bissoondath & Wiklund, 1996a; Hughes *et al.*, 2000). Furthermore, well-fed males replenished their sperm reserves faster than low-fed males, although an interaction (albeit weak) with male size means that this effect leads to different patterns of condition dependence for large and small males. For small males, those on a low food diet had low levels of sperm production over the 5 days, whereas those on a high food diet recovered their sperm reserves within 5 days. In contrast, for large males those on the low food diet recovered their sperm reserves within 5 days, but less quickly than those that were well fed. We noted that during the 5 days after fully stripping sperm the mean sperm numbers in all four treatment groups were consistently lower than the maximum counts obtained on day zero, although not significantly so. This could partly be due to a slowing down in sperm production as males approach their maximal reserve capacity (i.e. males accumulate small additions of sperm over an extended period of time). Males measured at day 0 had been deprived of females for the preceding 6 weeks. In addition, males at day 0 had also been kept in male stock tanks with access to fish flakes prior to placement in their 3-week diet treatment. It is therefore also possible that social environment or dietary differences had a small effect on sperm production.

The number of sperm males transfer to females during mating should be a good predictor of male fertilization success when there is sperm competition due to female multiple mating (Parker, 1970, 1982; Simmons & Fitzpatrick, 2012). This has, for example, been experimentally demonstrated in poeciliid fish (guppies: Boschetto *et al.*, 2011). Both a male's sperm capacity and his sperm production rate influence the number of sperm he has available to transfer during mating. Consequently, body size and/or condition are likely to have important effects on male reproductive success in species where females mate multiply. We predict that the rate of sperm production will be particularly important for male success in sperm competition in these species because males tend to mate often. This is likely to hold even if males do not allocate all of their sperm to a single mating (e.g. strategic ejaculation; Kelly & Jennions, 2011). Having a lower total sperm number would not affect a males' competitiveness in a single mating if he

can still allocate the same amount of sperm as other males but, across successive matings, he will eventually be at a distinct disadvantage if he becomes fully sperm depleted sooner than other males (assuming equal production rates). In *G. holbrooki*, a previous study found large and small males transferred similar numbers of sperm per mating but that small males have higher success rates than large males (Pilastro *et al.*, 1997). Therefore, while the small males appear to be advantaged, our results suggest that their insemination success should diminish faster than that of large males. Furthermore, variation in sperm production rates might directly influence male sperm allocation decisions. For instance, males that are able to produce and replace sperm quickly will have a shorter timeout after mating and so can potentially afford to be less choosy about who they allocate their sperm to (Kokko *et al.*, 2012).

Our results demonstrate that it is important to consider sperm production rates in addition to sperm number when looking at variation among males in their sperm competition performance. Most studies on ejaculate traits measure sperm reserves after males have been allowed to fully replenish sperm (i.e. males are deprived of females for days/weeks). This is a perfectly sensible approach if the aim is to standardize measurements and estimate maximal sperm reserves. In species where multiple mating rates are high, however, it is also prudent to consider the speed of sperm production. In these wild populations few males are likely to have maximum sperm reserves because of the shorter interval between successive matings. The time since last mating and, crucially, the rate of sperm production are more likely to predict a male's current ejaculate size and, by extension, his reproductive output under sperm competition. Having more data on sperm production rates could allow a comparative analysis to explore the effect of female polyandry on male sperm production rates.

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