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Virility does not imply immensity: Testis size, accessory gland size and ejaculate depletion pattern do not evolve in response to experimental manipulation of sex ratio in *Drosophila melanogaster*

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ABSTRACT

Sperm competition theory predicts that with increase in sperm competition, males either invest more in reproductive organ(s) and/or improve ejaculate investment. We test this idea using experimental evolution in *Drosophila melanogaster*. We maintained replicate populations of *Drosophila melanogaster* under male (M) and female (F) biased sex ratio regimes for more than a hundred generations with the result that males from the M regime evolved higher sperm competitive abilities relative to males from the F regime. In the present study, we measured the testes and the accessory gland size of virgin and singly mated males from the M and F regimes. The M and F males do not differ in either testis or accessory gland size. Additionally, ejaculate investment is not different in the M and F males, as measured by reduction in testis and accessory gland sizes. Thus, contrary to theoretical prediction and evidence from other species, we found that evolved differences in sperm competitive ability are not necessarily due to evolution of testis/accessory gland size or strategic ejaculate investment in these populations.

1. Introduction

Females from promiscuous species store sperm from more than one male, leading to post-copulatory sexual selection. Sperm from different males compete inside the female reproductive tract for fertilization success- a phenomenon known as sperm competition (Parker, 1970; Wedell et al., 2002). According to sperm competition theory, reproductive investment depends on intensity as well as risk of sperm competition. Theoretical models predict that with increase in sperm competition risk reproductive investment increases (Ball and Parker, 1998; Birkhead and Møller, 1998; Parker, 1998; Parker and Pizzari, 2010) but it decreases with increased sperm competition intensity (Parker et al., 1996). In either case, sperm competition and the resulting post-copulatory sexual selection can significantly alter male reproductive behavior (Bretman et al., 2009, 2010; Cook and Wedell, 1996; Dickinson, 1986; Gage and Barnard, 1996; Nandy and Prasad, 2011; Price et al., 2012; Wedell and Cook, 1999a, 1999b; Simmons et al., 1993) and physiology (Wolfner, 1997). Since a large number of sexual species are promiscuous, sperm competition is expected to be a widespread phenomenon influencing the evolution of male anatomy, physiology and behavior. There are at least two ways in which males can increase their sperm competitive ability: by increasing ejaculate quantity and/or by improving ejaculate efficacy.

Under competitive conditions a male can, in principle, increase his progeny number, if he increases the number of sperms transferred during mating (Eady, 1995; Garbaczewska et al., 2013). Increase in sperm number is likely to be a result of increased testes size. Stockley et al., (1997) found an increase in gonad size and sperm number with increase in intensity of sperm competition across several species of fish. Risk of sperm competition leads to an increase in gonad size (Gage, 1994; Hosken and Ward, 2001; Stockley, 1997) and sperm number (Gage and Morrow, 2003) in other taxa like butterflies and yellow dung flies.

In species like *Drosophila melanogaster*, in addition to sperm, accessory gland proteins (ACPs) are also known to play an important role in sperm competition (Gillott, 2003). Along with facilitating sperm transfer, ACPs exert wide-ranging and long term effects on female reproductive behavior, thus improve the males' chances of siring a significant proportion of offspring (Aigaki et al., 1991; Chapman, 2001; Avila, 2012; Apger-McGlaughon and Wolfner,

Abbreviations: M, male-biased population with adult ratio 3male: 1females; F, female-biased population with adult ratio 3female: 1males; ACPs, accessory gland proteins.

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2013; Wigby et al., 2009; Wolfner, 2009). Thus, increased levels of post-copulatory sexual selection can lead to increased production of one or more components of accessory gland proteins, which might reflect in an increase in accessory gland size. For example, selection for increased accessory gland size may result in increased production of some ACPs (e.g., sex peptide) but not of others (e.g., ovulin) (Wigby et al., 2009).

Although increased investment in the reproductive organs involved in ejaculate production is an indicator of increased sperm competitive ability, the amount of ejaculate transferred during each mating is arguably the most important determinant of a male's sperm competitive success (Moller, 1987). However, ejaculate production is a costly affair (Dewsbury, 1982) which can result in males showing adaptive plasticity in response to intensity and/or risk of sperm competition. Bretman et al., (2009) have shown that males alter their ejaculate investment (measured as mating duration) according to the level of sperm competition. Nandy and Prasad (2011) have shown that *Drosophila melanogaster* males, based on their perception of sperm competition risk, can plastically vary copulation duration which is positively correlated with sperm defense ability.

A study by Engqvist and Reinhold (2005) discusses the pitfalls in testing sperm competition theories with adult sex-ratio manipulation studies. With increased male-male competition in the male-biased sex-ratio regime, the males might be selected for increased investment in pre-copulatory traits which might trade-off with investment in post-copulatory traits. Thus, males under male-biased sex ratio might not be selected for increased investment in post copulatory traits. Therefore, while testing sperm competition theories using populations maintained at different sex-ratios, it is important to first establish that the populations differ in their sperm competitive ability (or in their post copulatory sexual selection).

Several studies in the past have used experimental evolution to examine the effects of post-copulatory sexual selection on the evolution of testis and accessory gland size and their depletion patterns. Wigby and Chapman (2004) found no difference in testes size and accessory gland size in populations of *Drosophila melanogaster* evolved under altered sex-ratio. McNamara et al., (2016) found no difference in testes and accessory gland size in male seed beetle, *Callosobruchus maculatus*, maintained under experimentally enforced polyandry and monandry. Linklater et al., (2007) studied the gonad size and ejaculate depletion patterns in *Drosophila melanogaster* males from populations evolving under male-biased (MB) and female-biased (FB) sex ratio. Virgin males from the two regimes showed no difference in either testis or accessory gland size. However, when allowed to mate successively with five virgin females, males from the MB regime depleted their ACPs at a much faster rate than males from the FB regime (as measured by the reduction in accessory gland size upon mating). However, there is no evidence that MB males have higher sperm competitive ability than FB males. Therefore, the biological significance of increased ejaculate depletion pattern in the MB males found in this study is not clear, highlighting the concerns raised by Engqvist and Reinhold (2005). The discrepancies in the results of previous studies make it all the more important to conduct the study described below.

In the present study we used populations of *Drosophila melanogaster* evolved under male-biased (M), female-biased (F) operational sex ratios. An earlier study using these laboratory populations by Nandy et al. (2013a) had found that males from M regime had evolved higher sperm defense (P1) and sperm offense (P2) ability compared to males from F regime, when competing against males from a common population. Typically, in sperm competition assays, each female is mated successively with multiple males, one of which is the focal male. In such a case, P(n) (n = 1,2,. . .) is defined as the proportion of the progeny sired by the focal male when it is the n^{th} male to mate with the female (Morrow et al., 2005). In both the P1 and P2 experiments, males from M regime sire higher proportion of progeny, as compared to males from F regime. We have clear evidence of evolved sperm competitive ability in males from M regime over males from F regime, thus establishing the fact that there exists differential post-copulatory sexual selection in these two regimes.

In the present study, we propose the hypothesis that the differential sexual selection is correlated with differential investment in reproductive tissues. In order to test this hypothesis, we asked the following questions:

a) Are the testes and/or accessory gland sizes different between M and F regime males?
b) Is the ejaculate investment pattern different between M and F regime males? Following Linklater et al., (2007) we used the change in the testes/accessory gland area post mating as a measure of ejaculate investment.

2. Materials and methods

2.1. Maintenance of population

In the present study we used populations of *Drosophila melanogaster* belonging to two regimes- the male-biased sex ratio regime 'M' and female-biased sex ratio regime 'F' as described in Nandy et al. (2013b). The M regime had an adult sex-ratio of three males to one female while the F regime had an adult sex-ratio of one male to three females. The selected populations were derived from a long-term laboratory adapted population of *Drosophila melanogaster*, LH_{st} (Prasad et al., 2007). LH_{st} is a derivative of LH base-line population (see Chippindale and Rice, 2001 for details of the maintenance regime) having a recessive autosomal "scarlet eye" marker. Each regime had three replicate populations, called "blocks" ($M_{1-3}$and F_{1-3}) (see Nandy et al. (2013b) for details of selection history). The populations were maintained on a 14-day discrete generation cycle, at 25 °C, 60% relative humidity (RH) and 12 h: 12 h light/dark cycle. They were fed on standard cornmeal–molasses–yeast food in glass vials (90-mm length × 30-mm diameter). Flies were grown in controlled larval density of 140–160 per vial. We collected virgin flies each generation and held them in single sex vials (eight individuals per vial) till the 12th day after egg collection. They were then combined according the respective sex ratio regimes in food vials provisioned with ~8 mL food supplemented with 0.47 mg of live yeast per female. Two days later, the flies were transferred to vials containing fresh food for oviposition. The eggs laid during the window of 18hrs were controlled for density (140–160 eggs per vial) and used to start the next generation.

2.2. Generation of experimental flies

Males from M (male-biased) and F (female-biased) selection regimes were used for the experiment. For the mating trials, common females from LH_{st} (base-line population) were used. We generated all the experimental flies under controlled larval density (150 per vial) and standard culture conditions (25 °C, 60–80% RH, 12 h–12 h light/dark cycle). 150 eggs were cultured in 8–10 mL of cornmeal-molasses food per vial, for each of the populations (2 selection regimes × 3 blocks, LH_{st}). On the 10th day after egg collection we collected M and F males as virgins by isolating them within 6 h of eclosion under light CO_2 anesthesia. The males were held at a density of 8 per food vial for 2 days. Virgin LH_{st} females were collected and maintained in a similar manner as described above.