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Do male secondary sexual characters correlate with testis size and sperm length in the small hairy maggot blowfly?

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ABSTRACT

The phenotype-linked fertility hypothesis proposes that secondary sexual characters (SSCs) advertise a male's fertility to prospective mates. However, findings from empirical studies attempting to test this hypothesis are often ambivalent or even contradictory, and few studies have simultaneously evaluated how both morphological and behavioural SSCs relate to ejaculate characteristics. Males of the small hairy maggot blowfly, *Chrysomya varipes*, possess conspicuous foreleg ornaments and display highly stereotyped courtship behaviour. These traits are favoured by females during pre-copulatory mate choice, but it remains unknown whether they correlate with post-copulatory traits expected to influence male fertility. The aim of this study was to investigate whether male courtship and ornamentation correlate with testis size and sperm length in *C. varipes*. We found that males investing more in courtship had bigger testes, and males with more extensive foreleg ornamentation released sperm with longer tails. Based on the assumption that larger testes enable males to produce more sperm, and that sperm with longer tails have greater propulsive force, our findings suggest that more vigorous and more ornamented males may be more fertile. These findings lend support to the phenotype-linked fertility hypothesis. However, a complete test of this hypothesis will require evaluating whether testis size and sperm length influence male fertilisation ability, as well as female fecundity and/or fertility.

1. Introduction

The phenotype-linked fertility hypothesis predicts positive relationships between traits shaped by pre- and post-copulatory selection (Sheldon, 1994). Theoretically, a male's quality is condition-dependent and reflected by his ability to invest both in secondary sexual characters (SSCs) (such as ornaments and courtship display) and ejaculate traits (such as sperm number and sperm size) (Pizzari et al., 2004). In turn, SSCs provide a reliable signal of ejaculate traits that affect fertilisation efficiency (Sheldon, 1994). Males of high quality are therefore expected to simultaneously produce more elaborate SSCs and more functional post-copulatory traits (such as high numbers of good-quality sperm). However, empirical tests of the phenotype-linked fertility hypothesis have yielded ambiguous and often conflicting results. For example, in support of this hypothesis, several empirical studies have demonstrated concordant positive relationships between pre- and post-copulatory traits (e.g. Locatello et al., 2006; Pitcher et al., 2007; Rogers et al., 2008; Navara et al., 2012; Mehlis et al., 2013; Kekäläinen et al., 2014). By contrast, several studies have either found negative relationships, indicating trade-offs between pre- and post-copulatory traits (e.g. Pitcher et al., 2009; Evans, 2010; Rowe and Pruett-Jones, 2011; Simmons et al., 2011; Galeotti et al., 2012), or failed to find any relationship between male SSCs and ejaculate traits (e.g. Birkhead and Fletcher, 1995; Hettyey et al., 2009; Doyle, 2011).

In an effort to understand the general relevance of the phenotype-linked fertility hypothesis, Mautz et al. (2013) conducted a meta-analysis investigating the average relationship between male SSCs and four measures of ejaculate quality: sperm number, viability, swimming speed and size. The study calculated effect sizes from 38 studies of 21 species representing five animal classes (Mammalia, Aves, Amphibia, Pisces and Insecta) and was based on the implicit assumption that each of the sperm traits examined reliably predicts female fertility (i.e. the proportion of eggs fertilised) (Møller and Jennions, 2001). Overall, the study found that male SSCs were significantly positively correlated (albeit weakly) with sperm viability, and that mean relationships with sperm number, swimming speed and size were consistently positive. Critically, however, the study lacked the statistical power required to test for taxonomic differences (empirical studies were heavily biased towards birds and fish), as well as variation in

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effect sizes for different types of SSCs (i.e. morphological versus behavioural traits). Consequently, Mautz et al. (2013) concluded that many more empirical studies (spanning a diversity of taxonomic groups) are required to elucidate sources of variation in the relationship between male SSCs and ejaculate traits.

Arguably, future studies aiming to explore relationships between male SSCs and reproductive traits expected to influence fertility should focus on investigating links between multiple pre- and post-copulatory traits in the same species. To our knowledge, only one study has simultaneously considered both ornamental and behavioural elements when evaluating the phenotype-linked fertility hypothesis. Birkhead and Fletcher (1995) quantified male song rate and fluctuating asymmetry of the chest-band plumage in zebra finches and found no evidence that variation in either of these traits co-varied with five measures of ejaculate quality: number of sperm, absolute number of sperm, percentage of live sperm, sperm length or sperm swimming velocity. Nevertheless, a strong argument can be made for the existence of links between multiple pre-copulatory male traits and fertility in other species (Trivers, 1972; Candolin, 2003).

Theoretically, there are three ways in which multiple ornaments that signal direct benefits (e.g. fertility benefits) might evolve. First, past selection episodes might result in males possessing old ornaments that are no longer favoured by females, but are nonetheless retained alongside new desirable ornaments so as to meet a mating threshold (Holland and Rice, 1998). Second, females might evolve a preference for one primary ornament, and males might then evolve secondary ornaments to exploit the existing preference (Endler and Basolo, 1998). Third, females might possess multiple preferences that each evolved independently, and multiple ornaments advertising male quality might then coevolve with these preferences via a handicap process (Brooks and Couldridge, 1999). Under this final scenario, multiple ornaments might indicate: (i) different aspects of male quality (the multiple messages hypothesis); or (ii) the same aspect of male quality (the back-up signal hypothesis) (Møller and Pomiankowski, 1993; Johnstone, 1996). Crucially, if multiple ornaments influence female mate choice, disentangling the relationship between pre- and post-copulatory traits may not be as simple as quantifying investment in one pre-copulatory trait alone. As such, there is a current need for studies that evaluate various pre- and post-copulatory male traits simultaneously. These types of inclusive analyses are likely to significantly advance our understanding of the general relationship between male phenotype and ejaculate characteristics.

The small hairy maggot blowfly, *Chrysomya varipes*, provides an ideal opportunity to investigate links between multiple pre- and post-copulatory traits in the same species. Female *C. varipes* assess multiple males before mating, and males possess striking ornamentation in the form of white hairs on their forelegs, which are used as part of their highly stereotyped courtship display. Males do not force mating, and only provide mates with ejaculates, so any benefits of mate choice must come through either indirect genetic mechanisms, or direct benefits associated with the assimilation of seminal fluid products or the improvement of fertility (Wagner and Harper, 2003). It has previously been demonstrated in *C. varipes* that males that invest more in courtship are more attractive to females. Furthermore, there is evidence that more ornamented males have a higher probability of mating (Jones et al., 2014). Such male mating advantages are likely to have arisen from female preferences for males that invest more in SSCs, which may provide direct benefits to females if SSCs advertise male fertility. However, post-copulatory traits have never been examined in this species, and whether male foreleg ornamentation and/or courtship behaviour correlate with ejaculate traits remains unknown.

The general aim of our study was to test for a relationship between the expression of pre- and post-copulatory traits in *C. varipes*. In order to address this aim we undertook two laboratory experiments. In the first experiment, we aimed to determine if male ornamentation and courtship investment were related to testis size. In the second experiment, we aimed to determine if male ornamentation was related to sperm size. Based on the widely held assumption that larger testes enable males to release more sperm per ejaculate, and that more sperm and longer sperm improve a male's probability of fertilisation success (Mautz et al., 2013), we predicted that males investing more in courtship and ornamentation would have bigger testes and/or longer sperm. Significant positive relationships between pre- and post-copulatory traits would provide partial support for the phenotype-linked fertility hypothesis and justify further research in *C. varipes* aimed at testing for links between male post-copulatory traits and male fertilisation ability, female fecundity and female fertility.

2. Materials and methods

2.1. Fly collection and culturing

Flies were trapped on the 7th and 9th of January 2013, at the University of Wollongong, Australia. Following capture, flies were kept at a temperature of 23 °C (± 1 °C), with a 12:12 h light/dark cycle, in the University's Ecological Research Centre. Adult flies were provided with a constant supply of raw sugar and water, and were maintained in plastic cages (300 mm × 500 mm and 250 mm high) with a fly screen lid. Additionally, they were provided with kangaroo mince (ca. 100 g) (Luddenham Pet Meats, Sydney, Australia), for use as a food source and an oviposition medium. The meat was removed after eggs were laid, and the eggs were isolated in a plastic rearing container (130 mm × 190 mm and 70 mm high) with a fine mesh top. Upon hatching, extra kangaroo mince (ca. 200 g) was given to the maggots to ensure that food was not limiting, and the bottom of the plastic rearing container was covered with wheaten chaff as a pupation substrate. After pupation, the rearing container holding the pupae was relocated into a larger cage, permitting the free movement of adult flies following their eclosion. The flies were sexed within 24 h of emergence, and males and females were kept in separate cages (300 mm × 500 mm and 250 mm high) to ensure that all flies used in experiments were virgins.

2.2. Experiment 1: the relationship between testis size, courtship and ornamentation

To quantify courtship behaviour, male and female virgin flies were randomly selected and placed in mixed-sex pairs (one male and one female) into a sealed transparent petri dish (85 mm × 15 mm) containing approximately 0.5 g of kangaroo meat positioned centrally. The behaviour of flies was recorded with a CCTV recording system and eight Samsung SCB-2000P digital cameras under standard laboratory light and temperature conditions (23 ± 1 °C). Behavioural trials ran for 20 min, or until copulation occurred. Eight behavioural trials ran concurrently. The experimental arenas were surrounded with cardboard barriers to ensure that the flies in each trial could not see each other. All flies used in mating experiments were of the F1 generation, and all flies were aged 12 (± 2) days to ensure sexual maturity (Wallman, unpublished data). All trials took place between 1000 and 1700 h.

Video footage of behaviours was analysed using the behavioural analysis software package EthoLog 2.2 (Ottoni, 2000), and only trials in which courtship behaviour occurred were used for analysis. For each experimental replicate, the behaviour of the male was quantified from the time of courtship initiation until copulation (or until 20 min, whichever occurred first). Male courtship included the following seven behaviours: orienting towards a female, following