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Effects of mating status on copulatory and postcopulatory behaviour in a simultaneous hermaphrodite

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Mating status is one of the most important predictors of the mating propensity of an individual. This is because mating lowers the amount of sperm cells and seminal fluids available to donate for males and increases the amount of ejaculate received by females, which may both have an effect on the mating propensity. In simultaneous hermaphrodites with reciprocal copulation, the mating status is expected to affect the mating propensity in both the male and the female sex function within a single individual, but empirical evidence is scarce. We experimentally tested the effect of the mating status of an individual and its partner on copulatory and postcopulatory behaviour in the free-living flatworm *Macrostomum lignano*, an outcrossing simultaneous hermaphrodite. These worms have frequent reciprocal copulations and often display a postcopulatory suck behaviour, potentially involved in removing ejaculate components from their own sperm-receiving organ. Virgin pairs copulated more, earlier and for longer than sexually experienced pairs. Moreover, we observed fewer sucks in virgin than sexually experienced pairs, all consistent with a higher willingness both to donate and to receive sperm in virgins. We investigated whether the lower suck frequency in virgin pairs depends on the mating status of the focal individual or on that of its partner. Surprisingly, the results suggested that the suck frequency depends on the mating status of the partner. We discuss these results in the context of potential sexual conflicts over the performance of the suck behaviour.

In copulating animals, matings are crucial events in which males and females are expected to allocate their reproductive resources strategically over multiple matings and partners to maximize their own fitness (Jennions & Petrie 2000; Wedell et al. 2002; Kokko & Mappes 2005; Parker & Pizzari 2010; Edward & Chapman 2011). The mating propensity of an individual is expected to depend on the costs and benefits of copulating, which may vary between the sexes and also across different mating opportunities, for example, because of varying amounts of available gametes and varying attractiveness of the available partners.

During copulation, males donate an ejaculate, which is usually composed of both sperm cells and seminal fluids. An important determinant of male reproductive success is the amount of transferred sperm cells, since males transferring more sperm cells have been shown to outcompete the sperm cells of competing males (e.g. Gage & Morrow 2003; but see Snook 2005). In addition, seminal fluids may interact with sperm, and thereby also influence male reproductive success (reviewed in Chapman 2001; Arnqvist & Rowe 2005), notably by manipulating female physiology and behaviour (e.g. Chen et al. 1988; Heifetz et al. 2000). Although males are expected to gain fitness benefits from inseminating numerous females with large ejaculates, the ejaculate also represents a costly investment, which requires time and energy to produce and to replenish (e.g. Nakatsuru & Kramer 1982; Royer & McNeil 1993; Schärer & Vizoso 2007). Hence, given that male reproductive success depends on the amount of ejaculate transferred (e.g. Gage & Morrow 2003; Wigby et al. 2009) and that the amount of available ejaculate is influenced by previous mating events (e.g. Brauer et al. 2007; Hettyey et al. 2009), sexually deprived males are expected to have a higher mating propensity than recently mated males.

During copulation, females receive an ejaculate, which is often stored and provides the sperm required for the fertilization of the eggs (reviewed in Orr & Zuk 2012). On the one hand, female reproductive success might be limited by the amount of sperm available to fertilize the eggs, for example because of difficulties in obtaining sufficient sperm or in finding mates (Wedell et al. 2002; Kokko & Mappes 2005), and females may benefit from multiple matings (Jennions & Petrie 2000). On the other hand, the receipt of ejaculate may also have detrimental effects on female reproductive success, which are likely to increase with repeated copulations, for

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example because of the risk of polyspermy (reviewed in Birkhead et al. 1993) or seminal fluid-mediated costs (reviewed in Chapman 2001; Arnqvist & Rowe 2005). Therefore, female mating propensity is expected to vary according to the amount of sperm stored to optimize the eggs' fertilization and the female's reproductive success. In addition, female mating propensity may also be manipulated by previous mating partners (Johnstone & Keller 2000), notably through the seminal fluid transferred during copulation (e.g. Chen et al. 1988). Consequently, female mating propensity can also be expected to depend on the amount and the composition of the ejaculates received from previous mating partners.

In addition to its mating status, an individual's mating propensity may also vary according to the attractiveness of the partner. When mates vary in their reproductive quality, both sexes are expected to be choosy about their mating partners, and thus display higher mating propensity with partners that are expected to provide higher fitness benefits (reviewed in Dewsbury 1982; Jennions & Petrie 2000; Edward & Chapman 2011). For instance, it has been shown that males mate preferentially with more fecund and/or virgin females (e.g. Johnson & Hubbell 1984; Schneider et al. 2011) and/or tailor the ejaculate size to the level of sperm competition (e.g. Wedell 1992; Gage & Barnard 1996; reviewed in Parker 1998). Similarly, females may preferentially mate with males providing material and/or genetic benefits (Jennions & Petrie 2000; Møller & Jennions 2001).

Consequently, since the costs and benefits of copulating can depend on the previous mating events of both mating partners, the mating status of both is expected to contribute significantly to mating propensity in both males and females. The effect of mating status on mating propensity has mainly been studied in species with separate sexes (Kokko & Mappes 2005; Edward & Chapman 2011), whereas fewer studies have focused on species with different sexual systems.

In simultaneously hermaphroditic animals (hereafter called hermaphrodites), individuals produce ejaculates and eggs at the same time and so both partners can donate and receive ejaculates. Hermaphrodites are therefore expected to allocate their ejaculate strategically over multiple matings and partners, while simultaneously aiming to ensure an optimal supply of sperm to fertilize their own eggs. Hence, mating propensity may depend on both the amount of sperm (hereafter called autosperm) and seminal fluids available to inseminate a partner and on the amount of received sperm available to fertilize the eggs (hereafter called allosperm; Anthes et al. 2006), which are both likely to vary according to the previous mating activity and social context (Schärer & Ladurner 2003).

To date, effects of mating status on mating propensity have been mainly studied in hermaphrodites with unilateral copulation, especially snails (reviewed in Anthes et al. 2006) while, to our knowledge, there are currently no experimental studies in reciprocally mating species (but see Tomiyama 1996 and Kupfernagel & Baur 2011 for correlational studies). For instance, sexual isolation has been shown to increase both female (Facon et al. 2007) and male mating propensity (Koene & Ter Maat 2005; Dillen et al. 2008). It has been argued that in some snail species male mating propensity may be regulated by the filling status of glands producing the seminal fluids, which appears to increase the fertilization success of a given amount of donated sperm (e.g. Koene & Chase 1998; Koene et al. 2005; Chase & Blanchard 2006).

In hermaphrodites with reciprocal copulation, mating events are expected simultaneously to replenish the amount of allosperm stored and to deplete the amount of autosperm and seminal fluids. Therefore, mating status is expected to have multiple effects on mating behaviour for hermaphrodites with reciprocal copulations, namely sexually isolated individuals are expected to display higher mating propensity to gain both male and female reproductive success than already mated individuals.

In this study, we tested experimentally the effect of mating status on both copulatory and postcopulatory behaviours in the free-living flatworm *Macrostomum lignano*. This species has reciprocal mating and performs a postcopulatory behaviour, the so-called suck behaviour, which is possibly involved in removing ejaculate components received during copulation (Schärer et al. 2004, 2011; Vizoso et al. 2010). In addition, it has recently been suggested that mating status affects mating propensity, since previously isolated worms that were offered two mating partners consecutively copulated more frequently with the first than with the second mate (Janicke et al. 2012).

We experimentally manipulated the mating status of worms, leading to virgin individuals and to individuals that were sexually experienced in both sex functions (i.e. in reciprocally mating species the mating status necessarily changes in both sex functions upon mating). In a first experiment, we observed pairs of virgin worms (called virgin pairs) and pairs of sexually experienced worms (called sexually experienced pairs) and compared their copulation frequency, the time to the first copulation, as well as the average copulation duration, and the suck frequency over the first five copulations. Since virgins have a lot of available ejaculate (see Appendix 1 for previously unpublished data on autosperm and seminal fluid of an experiment reported in Schärer & Janicke 2009) and lack allosperm (L. Marie-Orleach, personal observation), we expected that virgin individuals would show greater interest in both donating and receiving sperm and that they would therefore be likely to copulate more often and for longer. As we found that individuals within virgin pairs sucked less frequently than individuals within sexually experienced pairs, we performed additional experiments to test whether the suck frequency depends on the mating status of the focal worm or, alternatively, on the mating status of the partner. We expected the virgin individuals would show greater willingness to receive allosperm and so to suck less frequently than the sexually experienced individuals.

METHODS

Study Organism

Macrostomum lignano (Macrostomorpha, Platyhelminthes) is a free-living flatworm and a member of the meiofauna of the Northern Adriatic Sea (Ladurner et al. 2005b). Individuals used here stem from a genetically outbred laboratory mass culture (called LS1) descending from worms collected in 2003 in Lignano Sabbiadoro and Bibione, Italy (Ladurner et al. 2005b). Worms in mass cultures are kept at 20 °C in petri dishes in f/2 medium (Andersen et al. 2005) and fed ad libitum with the diatom *Nitzschia curvilineata*. Under these conditions body size reaches about 1.5 mm, generation time is about 18 days and worms have a median life span of about 200 days (Mouton et al. 2009). While young worms tend to be more male biased than older worms (i.e. worms are slightly protandrous, Vizoso & Schärer 2007), the worms we used in the experiments reported below were old enough to be mature in both sex functions. *Macrostomum lignano* is an outcrossing simultaneous hermaphrodite that copulates frequently (on average about 6 copulations/h, Schärer et al. 2004) and is highly promiscuous (Schärer & Ladurner 2003; Janicke & Schärer 2009). Copulation consists of reciprocal insertion of the male copulatory stylet into the female genital organ (the antrum) of the partner (Schärer et al. 2004), generally leading to the transfer of sperm and seminal fluid from a prostate-like accessory gland (Doe 1982; Ladurner et al. 2005a, b; Vizoso et al. 2010). The sperm reserves are