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Resolving mechanisms of short-term competitive fertilization success in the red flour beetle

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ABSTRACT

Postcopulatory sexual selection occurs when sperm from multiple males occupy a female's reproductive tract at the same time and is expected to generate strong selection pressures on traits related to competitive fertilization success. However, knowledge of competitive fertilization success mechanisms and characters targeted by resulting selection is limited, partially due to the difficulty of discriminating among sperm from different males within the female reproductive tract. Here, we resolved mechanisms of competitive fertilization success in the promiscuous flour beetle *Tribolium castaneum*. Through creation of transgenic lines with fluorescent-tagged sperm heads, we followed the fate of focal male sperm in female reproductive tracts while tracking paternity across numerous rematings. Our results indicate that a given male's sperm persist and fertilize eggs through at least seven rematings. Additionally, the proportion of a male's sperm in the bursa (the site of spermatophore deposition), which is influenced by both timing of female's ejecting excess sperm and male size, significantly predicted paternity share in the 24 h following a mating. Contrary to expectation, proportional representation of sperm within the female's specialized sperm-storage organ did not significantly predict paternity, though spermathecal sperm may play a role in fertilization when females do not have access to mates for longer time periods. We address the adaptive significance of the identified reproductive mechanisms in the context of *T. castaneum's* unique mating system and ecology.

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1. Introduction

Postcopulatory sexual selection, which includes sperm competition to fertilize eggs (Parker, 1970) and cryptic female choice among competing ejaculates (Eberhard, 1996), is credited with generating rapidly evolving (reviewed in Swanson and Vacquier, 2002) and highly divergent traits (reviewed in Pitnick et al., 2009a). Such selection consequently impacts variation in reproductive success within populations (Pizzari and Parker, 2009) and reproductive isolation between populations and species (e.g., through conspecific sperm precedence; Howard et al., 2009; Manier et al., 2013a). Resolving the processes underlying variation in competitive fertilization success, along with the sex-specific targets of accompanying selection, is thus important for our understanding of biodiversity. Yet, such knowledge is presently limited.

Mating system differences among species, including female remating frequency and mate number, determine sperm competition intensity for males and the potential for choice among ejaculates for females. These mating system characteristics in turn influence the evolution of traits that determine variation in competitive fertilization success, including sperm and other ejaculate traits (Pizzari and Parker, 2009; Snook, 2005) and female reproductive physiology, morphology and behavior (Eberhard, 1996; Lüpold et al., 2013). Both sperm quantity (Parker and Pizzari, 2010) and sperm quality (e.g., Malo et al., 2006; Pattarini et al., 2006; Lüpold et al., 2012), as well as morphological relationships between sperm and female sperm-storage organs (García-González and Simmons, 2007; Miller and Pitnick, 2002), have been found to influence competitive fertilization success in different systems. Fertilization success can also be influenced by female behaviors, such as the timing or quantity of sperm ejected after copulations (e.g., Pizzari and Birkhead, 2000; Bussière et al., 2006; Lüpold et al., 2013), differential storage of preferred sperm (e.g., Pilastro et al., 2004; Bretman et al., 2009), or altering oviposition behavior (Bretman et al., 2006). Ultimately, it is a species mating system that will determine the relative importance of these or other reproductive adaptations to postcopulatory sexual selection.

When discerning mechanisms underlying patterns of competitive fertilization success, it is critical to identify the subset of sperm

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that are used to fertilize eggs (i.e., the "fertilization set" *sensu* Parker et al., 1990). Not all locations that sperm can occupy within the female reproductive tract have an equal probability of supplying sperm for fertilization (Manier et al., 2013c; Pitnick et al., 2009b). Few studies have identified the fertilization set as well as the more general spatiotemporal dynamics of sperm storage and use following competitive matings (but see LaMunyon and Ward, 1998; Bussière et al., 2010; Manier et al., 2010, 2013b; Holman et al., 2011; Lüpold et al., 2012, 2013) due to the difficulty of distinguishing sperm from different males within the female reproductive tract (but see for unique methodological solutions: Otronen and Siva-Jothy, 1991; Otronen et al., 1997; Schärer et al., 2007; Bussière et al., 2010; Manier et al., 2010).

Finally, whereas the majority of investigations of characteristics that influence paternity outcomes have employed a standard experimental design using virgin females that are then mated with two males, paternity estimates from field collected specimens suggest that mating with more than two males is common in many species (e.g., Zeh et al., 1997; Bretman and Tregenza, 2005; Simmons et al., 2007; Demont et al., 2011). Relatively few studies have investigated the dynamics of postcopulatory success when three or more males compete for fertilization (but see Radwan, 1991, 1997; Zeh and Zeh, 1994; Cooper et al., 1996; Eady and Tubman, 1996; Lewis and Jutkiewicz, 1998; Arnaud et al., 2001; Drnevich, 2003; Lewis et al., 2005; Bjork et al., 2007). Because mating system, including remating frequency, is expected to influence mechanisms of postcopulatory sexual selection, it is critical to understand the persistence of a focal male's sperm in the female reproductive tract throughout multiple rematings.

The red flour beetle, *Tribolium castaneum,* has unusually high mating rates as compared to other internally fertilizing model systems commonly used to study postcopulatory sexual selection (e.g. fruit flies, dung flies, bruchid beetles, crickets, and birds), with male and female flour beetles observed to mate multiple times an hour (Fedina and Lewis, 2008). Due to the expected influence of mating system on traits important to competitive fertilization success, unique mechanisms are predicted to underlie variance in postcopulatory success in *T. castaneum* than have been identified in other systems (e.g. proportion of focal male sperm in specialized storage organs in *Drosophila* (Lüpold et al., 2012; Manier et al., 2013c, 2010) and *Gryllus* crickets (Bretman et al., 2009)).

Here, we used transgenic lines of the red flour beetle, *Tribolium castaneum*, featuring males that produce sperm that have heads tagged with green (GFP) or red fluorescent proteins (RFP) to address the persistence of focal male sperm through multiple rematings and the sperm's continued relevance to fertilization. These fluorescently labeled lines enable the tracking of a focal male's ejaculate through multiple matings as well as connecting patterns of sperm storage to patterns of paternity. A greater understanding of postcopulatory sexual selection is particularly desirable in this species due to extreme female promiscuity, a general absence of precopulatory mate choice (Sokoloff, 1974) and substantial, yet largely unexplained (but see Edvardsson and Arnqvist, 2000) variation in competitive fertilization success (Lewis and Austad, 1990).

2. Materials and methods

2.1. Experimental system and culturing

During mating, male *T. castaneum* transfer a spermatophore to the female that rapidly everts to release sperm into the bursa copulatrix (henceforth "bursa"). Following spermatophore eversion, sperm move to storage in the spermatheca, are retained in the bursa (Fig. 1), or are ejected from the female reproductive tract along with remnants of the spermatophore (Fedina, 2007). Sperm remain viable for fertilization for many months after mating (Bloch Qazi et al., 1996). The female's last mate generally sires a majority of offspring (i.e., displays "last male sperm precedence"; but see (Edvardsson and Arnqvist, 2000; Fedina and Lewis, 2004)).

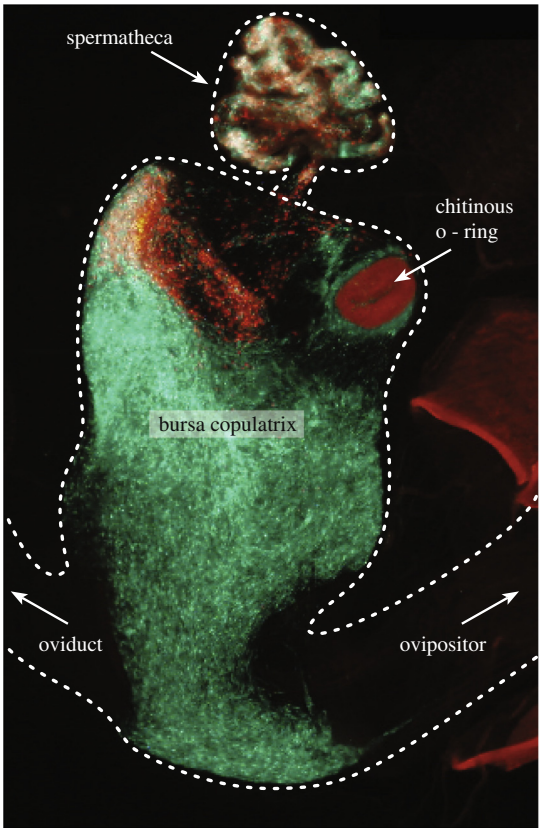

Fig. 1. *Tribolium castaneum* female reproductive tract under fluorescent illumination after mating with an RFP male followed by GFP males. Fluorescently labeled sperm are visible in the main and specialized sperm storage organs (i.e. bursa copulatrix and spermatheca, respectively). Major organs are outlined and labeled. Scale bar represents 200 μm.

All experimental females are from the WLIN (West Lafayette, Indiana) population that has been maintained at large population sizes since their collection in 2008 (see Drury et al., 2009 for collection details). Unless otherwise noted, experimental males are from transgenic lines bearing sperm marked with GFP or RFP tagged protamines, a protein specific to DNA packaging in sperm heads, enabling identification of individual male's sperm after transfer to the female reproductive tract. The transgenic protamine lines are referred to as GFP and RFP hereafter. Preliminary experiments also used Blk males, which carry a homozygous, naturally arising, semi-dominant mutation causing black body color. The WLIN and Blk lines were generously provided by Dr. Mike Wade (University of Indiana, Bloomington IN), whereas the GFP and RFP lines were created by the authors.

Beetle stocks were cultured in quart jars filled with standard yeast-enriched flour medium of 95% whole wheat flour, 5% yeast by weight, supplemented with 0.0003% Fumagillin to prevent microbial infection in a dark and humid growth chamber. All lines were maintained with overlapping generations since their arrival to the Pitnick lab. Populations of beetles were moved to fresh media every two months with initial population densities of approximately 1 beetle/1 g medium. Experimental beetles were sexed as pupae and maintained separately by sex to ensure virginity. Beetles were 4–14 days old at the time of the experiments.

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