



Increased insertion number leads to increased sperm transfer and fertilization success in a nursery web spider



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Across animals, a male's fitness is largely dictated by his ability to fertilize eggs; and there exists a plethora of male adaptations associated with increasing fertilization success. In the nursery web spider, *Pisaurina mira*, males restrain females prior to and during copulation by wrapping them with silk. Previous research demonstrates that copulatory silk wrapping reduces a male's chance of being sexually cannibalized and increases the number of sperm transfer opportunities (termed insertions) that a male can achieve within a mating. While avoiding cannibalism provides an obvious survival benefit to males, the impact of insertion number on male fitness remains unknown. This study tested the hypothesis that increased insertion number realized through copulatory silk wrapping increases (1) the quantity of sperm transferred and (2) fertilization success. To accomplish this, we directly quantified the amount of sperm in male pedipalps (i.e. the male sperm storage organ) before mating and after obtaining one or two insertions. We also, indirectly quantified fertilization success by measuring the number of hatched offspring when males were capable of achieving one versus two insertions within a mating. In support of our hypotheses, we found that males transfer roughly twice the amount of sperm when achieving two insertions compared to one. We additionally found that the amount of sperm transferred is negatively related to female size. In terms of offspring number, females obtaining two insertions had more offspring compared to females obtaining only one insertion. These results show that males achieve a fertilization benefit from increased insertion number, which is obtained through the male behaviour of copulatory silk wrapping.

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Differences observed in male and female reproductive strategies have been proposed to explain the evolution of sex-specific traits. For example, female fitness often depends on maximizing resource investment to developing offspring (Andersson, 1994; Bateman, 1948; Trivers, 1972) while male fitness is hypothesized to be dependent on maximizing the number of eggs fertilized (Andersson, 1994; Bateman, 1948; Parker, & Swanson, 1995). Positive selection is thus predicted, and often observed, on traits that allow males to secure matings and/or increase fertilization success.

In addition to facilitating copulations, many male-specific mating strategies or morphological traits may function to increase copulation duration, which can have important implications for sperm transfer and thus, sperm competition. Male traits such as 'grasping traits' (e.g. Sakaluk, Bangert, Eggert, Gack, & Swanson, 1995), larger nuptial gifts (Svensson, Petersson, & Frisk, 1990) and barbed or spiny

male genitalia (Edvardsson & Canal, 2006; Hotzy & Arnqvist, 2009) have all been documented to extend copulation duration. Male sagebrush crickets, *Cyphoderris strepitans*, for example, have a grasping device called a gin trap, which secures females to males during copulation, prolongs the duration of copulation and increases the chance of complete transfer of the spermatophore to the female (Sakaluk, et al., 1995). As previously suggested, benefits of longer copulations include increased male fertilization success due to increased sperm transfer (e.g. Campbell & Fairbairn, 2001; Engqvist & Sauer, 2003; Pilastro, Mandelli, Gasparini, Dadda, & Bisazza, 2007; Schneider, Gilberg, Fromhage, & Uhl, 2006).

In some cases, the traits (including behaviours) that benefit male reproductive success can appear potentially harmful to female mating partners. For example, males of many species use 'harassment' or 'coercive' mating strategies (reviewed in Arnqvist & Rowe, 2013; Clutton-Brock & Parker, 1995), or possess morphological traits (e.g. larger body size, structures for grasping/holding) that appear to function in restraining females. Many of these male strategies/traits are proposed to increase the females' mating rates or copulation duration past their phenotypic optima (Arnqvist &

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Rowe, 2013; Parker, 1979, 2006). For example, prolonged copulation in females can reduce foraging rates, increase risk of predation, injury, parasites, etc., and decrease control over fertilization and mate choice (reviewed in Arnqvist & Rowe, 2013). Indeed, cryptic female choice, the ability of females to exert choice on male sperm following copulation, is suggested to have evolved in response to primarily male-controlled copulations (Eberhard, 1996; Thornhill & Alcock, 1983). Additionally, across a diversity of animal groups, females are observed to engage in behaviours associated with seeming attempts to terminate copulations, such as kicking their male mating partner (Edvardsson & Canal, 2006), sexual cannibalism (Elgar, Schneider, & Herberstein, 2000; Herberstein et al., 2011) or simply attempting to remove the male (e.g. Mazzi et al., 2009). Ultimately, in many taxa, there appears to be a conflict between the sexes in terms of copulation duration (reviewed in Arnqvist & Rowe, 2013).

The observed mating strategy of male nursery web spiders, *Pisaurina mira*, suggests that a conflict might exist between females and males regarding copulation duration or the number of successful sperm transfer events achieved by males (i.e. male insertion number). In this cannibalistic species, males always constrain the female's movement by wrapping her legs with silk prior to and during copulation (Supplementary Fig. S1). Most virgin females are receptive to mating and appear to allow males to initially mount and silk-wrap them prior to transferring sperm (A. G. Anderson, personal observation). Sperm transfer relies upon independent paired external organs called pedipalps (Foelix, 2011), which function in sperm storage as well as transmission. In many spider species, including *P. mira*, males insert and transfer sperm using one pedipalp at a time (termed an insertion). In *P. mira*, females begin to appear aggressive towards their male mating partner immediately following the first insertion. Females begin to struggle in a seeming attempt to get out of the silk wrapping, suggesting an effort to shorten copulations. In response, males typically attempt to restrain and rewrap the females to achieve one more insertion (for a total of two), after which males quickly flee. Prior work has manipulated a male's ability to engage in copulatory silk wrapping and found that the silk wrapping reduces rates of postcopulatory sexual cannibalism and increases the likelihood of a male achieving two pedipalp insertions versus only one (Anderson & Hebets, 2016). Similarly, binding females in silk during mating has been linked to reduced rates of sexual cannibalism in two other spider species (*Caerostris darwini*: Gregorić, Suen, Cheng, Kralj-Fišer, & Kuntner, 2016; *Nephila pilipes*: Zhang, Kuntner, & Li, 2011), as well as increased copulation duration in one species (Zhang et al., 2011). We hypothesize that silk wrapping in *P. mira* provides males increased fitness benefits by increasing copulation duration.

Although copulation duration is often correlated with increased fertilization success (i.e. increased offspring production given the number of eggs available) due to an increase in the quantity of sperm transferred (e.g. Arnqvist & Danielsson, 1999; Schneider et al., 2006; Svensson et al., 1990), this need not always be the case (Bukowski, Linn, & Christenson, 2001; Linn, Molina, Difatta, & Christenson, 2007; Schneider & Elgar, 2001; Snow & Andrade, 2004). For example, in the orb-weaving spider *Nephila clavipes*, increased copulation duration was not associated with the amount of sperm transferred, but was instead found to decrease female receptivity to future matings. Additionally, increased sperm transfer need not reflect the amount of sperm stored and available for fertilization as females of some species are known to manipulate sperm storage (e.g. Eberhard, 1996; Herberstein et al., 2011). Indeed, several studies have failed to find a relationship between copulation duration and fertilization success (Assis & Foellmer, 2016; Gilchrist & Partridge, 2000; Mazzi et al., 2009). Thus, in attempting to understand male–female mating dynamics and

potential costs and benefits of copulation duration, it is important to directly quantify the relationships between copulation duration, sperm transfer and fertilization success, as well as their relationship to female and male size.

This study uses the nursery web spider *P. mira* to test the hypothesis that increased insertion number, facilitated by copulatory silk wrapping, increases male fitness. Specifically, we predicted that two insertions (versus one) would increase (1) the quantity of sperm that males transferred to females and (2) the number of offspring that females produced.

METHODS

Species Collection and Maintenance

We collected immature female and male *Pisaurina mira* at night from Wilderness Park, Lancaster County, Lincoln, Nebraska, U.S.A. during 29 March–28 April 2015 and 4 April–26 April 2016. Collected individuals were transported to the University of Nebraska–Lincoln where they were individually housed in 87.3 × 87.3 × 112.7 mm clear plastic containers (763C, AMAC Plastics, Petaluma, CA, U.S.A.). We covered the outside of each container with opaque tape to maintain visual isolation between individuals. Spiders were maintained under a 12:12 h light:dark cycle, fed four crickets approximately 0.64 cm in length per week (Ghann's cricket farm, GA, U.S.A.) and provided water ad libitum. Spiders were checked each day for the presence of a moult and to determine the date of sexual maturity. Our research adhered to the ASAB/ABS guidelines for the treatment of animals in research, the legal requirements of the U.S.A., and all guidelines of the University of Nebraska – Lincoln. All animals were handled and maintained within the laboratory under proper conditions.

Experiment 1: Insertion Number and Sperm Quantity

To determine the relationship between insertion number and the amount of sperm transferred, we staged mating trials between 6 May and 21 May 2016 in which age-matched (15 days ± 2 days postmaturation) females and males were randomly paired. Mating arenas and procedures were similar to those carried out in a previous study (Anderson & Hebets, 2016). We separated males from females at three time points: immediately after (1) the silk wrapping was laid (i.e. no sperm transferred/zero insertions), (2) the first insertion (one insertion), or (3) the second insertion (two insertions). From each of these three treatment groups, we took 15 males and quantified the amount of sperm remaining in each of their pedipalps. Sperm quantification took place immediately after the male was separated from the female.

To quantify sperm, we used methods adapted from Snow and Andrade (2005), which were originally adapted from Bukowski and Christenson (1997) and Bukowski et al. (2001). The modifications of methods were reported to improve a uniform distribution of sperm and reduce sperm clumping. Briefly, we removed each male's left and right pedipalps using soft forceps and dissecting scissors and then placed them into a 0.5 ml Eppendorf tube containing 75 µl of spider sperm counting solution. The counting solution consisted of 150 µl of a solution containing 10 ml of saline and 10 µl of Triton X detergent, which was then mixed with 10 ml of spider saline (Juusola & French, 1998). Within the Eppendorf tubes, each pedipalp was crushed using disposable pellet pestles (Fisher Scientific, Waltham, MA, U.S.A.). We vortexed each sample for 30 s and subsequently centrifuged each sample at 4000 revolutions/min for 10 min. We repeated the vortexing and centrifuging steps two additional times. Following sample preparation, we pipetted 10 µl of each sample into an improved Nebauer double-chamber

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