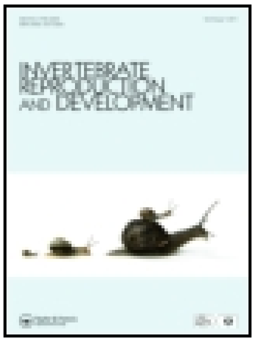

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Strong first sperm precedence in the freshwater hermaphroditic snail *Planorbella trivolvis*

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ABSTRACT
Post-copulatory sexual selection, through either sperm competition or cryptic female choice, is an important aspect of sexual selection in hermaphrodites. We investigated sperm precedence, non-random fertilization of eggs by multiple partners, to determine the potential for sperm competition in the hermaphroditic freshwater snail *Planorbella trivolvis*. We provided snails with two sequential partners, and assessed paternity using albinism as a genetic marker. We mated albino snails first to a pigmented partner then an albino (PA; n = 26) or first to an albino then a pigmented individual (AP; n = 27) and calculated the proportion of eggs fertilized by the second partner (P2) during the 1st, 3rd, and 5th week post-mating. We collected all egg masses laid each week (~300) and examined the embryos to determine paternity by the presence or absence of eye pigmentation. P2 values were quite low (0.18 ± 0.04) indicating strong first sperm precedence and thus little opportunity for sperm competition. We also found that mating order influenced precedence; when the first mate was an albino P2 values were higher than when the first mate was pigmented, suggesting a difference in sperm transfer or viability or a preference for pigmented partners.

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Gastropoda; simultaneous hermaphrodite; sperm precedence; post-copulatory sexual selection

Introduction

Sperm competition, first defined by Parker (1970) as competition among the sperm from multiple males to fertilize the eggs of a female, is recognized as an important aspect of sexual selection (Parker 1998 and other references in this volume). Males have evolved a variety of strategies to increase post-copulatory reproductive success, including complex reproductive structures and behaviours that increase the likelihood of fertilization or prevent re-mating (Birkhead and Pizzari 2002; Wada et al. 2010; Cordero-Rivera 2016), modifications to sperm transfer with increased competition risk (Gage 1991; Gage and Barnard 1996; Loose and Koene 2008; Kimura and Chiba 2013), and accessory gland proteins transferred during copulation that interfere with female reproductive function (Chapman et al. 1995; Koene et al. 2010). The theory of sperm competition was first developed in gonochorists (species with male and female reproductive organs in different individuals) and most empirical examples come from them. However, the importance of this phenomenon in hermaphrodites (individuals with both male and female reproductive structures), first theorized by Charnov (1996), is gaining attention (Michiels 1998; Scharer et al. 2014 and references therein). In fact, because hermaphrodites by definition do not exhibit sexual dimorphism, it has been suggested that post-copulatory sexual selection may be more important than pre-copulatory mate choice or competition in these organisms (Scharer et al. 2014). To fully understand reproductive strategies in hermaphrodites, we need to expand studies of mate assessment, mate choice, and post-copulatory activities to more model systems (Anthes 2010).

Although hermaphrodite species occur across animal taxa (they are present in 70% of animal phyla; Jarne and Auld 2006), we focus here on gastropods, which are becoming model systems for studying reproductive strategies in hermaphrodites (Nakadera and Koene 2013). Many hermaphroditic gastropods exhibit reproductive attributes expected to lead to sperm competition (Michiels 1998; Baur 1998). Internal fertilization is common and individuals typically mate with and store sperm from multiple partners (Mulvey and Vrijenhoek 1981; Vianey-Liaud et al. 1987; Wethington and Dillon 1991; Bürkli and Jokela 2017). Sperm may be stored for long durations, ranging from 13–16 days in the opisthobranch *Alderia modesta* (Angeloni 2003) to several months in some pulmonates – 2 months in *Biomphalaria glabrata* (Vianey-Liaud 1995) and *Lymnaea stagnalis* (Nakadera et al. 2014), and more than a year in *Helix aspersa, Arianta arbustorum*, and *Crepidula coquibensis* (reviewed in Nakadera and Koene 2013). Furthermore, because many hermaphrodites can self-fertilize (Jarne et al. 1993), sperm from a mate (allosperm) may also compete with autosperm produced by the focal

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individual. As in gonochoristic species, the fate of sperm may influence sperm competition, but in hermaphrodites, it may also influence the gender choice of the donor and/or recipient and thus sex allocation, the relative amount of resources directed to male vs female function (Charnov 1996; Scharer et al. 2014). Thus to fully understand reproductive strategies in hermaphrodites, we need to know the fate of received sperm.

Sperm precedence, nonrandom differences in fertilization success after copulation with multiple sperm donors (Parker 1970), is one way to assess the influence of mating order on paternity. It is typically measured by providing individuals with two consecutive mates and determining P2, the proportion of eggs fertilized by the second mate (Boorman and Parker 1976). Sperm precedence is thus a measure of both the potential for and outcome of sperm competition. If the values are not 0 (sperm from the first mate fertilize all the eggs) or 1 (sperm from the second mate are exclusively used to fertilize eggs), the situation is ripe for sperm competition. Pulmonate gastropods have P2 values ranging from 0.23–0.60, with substantial variation among individuals (reviewed in Nakadera and Koene 2013). In many species studied thus far, P2 values do not significantly differ from 0.5, indicating that eggs are equally likely to be fertilized by the first and second sperm donors, and for those species in which P2 significantly differs from random fertilization, P2 values are generally lower than 0.3, indicating first sperm precedence. (One exception here is the ppisthobranch *Aplysia californica* with an average P2 value of 0.73, indicating that sperm from the second donor is more successful than that from the first).

While the above data are informative, most studies measure sperm precedence in only the first clutch of eggs, and represent the only handful of studies addressing this topic in hermaphrodites. Our lab is developing *Planorbella trivolvis* (Say 1817, Hygrophila: Planorbidae, also known as *Helisoma trivolvis*), a hermaphroditic freshwater pulmonate snail widely distributed across the US and Canada (Johnson et al. 2013), as a model hermaphrodite system. Several unique features of *P. trivolvis* make it a good candidate for investigating these questions. As simultaneous hermaphrodites, individual snails can act as male or female during mating, but also have the ability to mate reciprocally (Abdel-Malek 1952; Norton et al. 2008). Unlike most pulmonate snails, self-fertilization is almost non-existent (Escobar et al. 2011; Norton and Newman 2016; Norton et al. 2018a) which removes the complication of eggs fertilized by autosperm. Snails store sperm for approximately 16 weeks post-mating (Norton and Newman 2016) and produce numerous egg masses with embryos easily observed during early development. Albinism, often used as a genetic marker of paternity, is inherited as a single locus recessive allele (Norton et al. 2018a), and populations of both albino and pigmented individuals with similar genetic background are kept in our lab.

Our goal was to determine whether the potential for sperm competition occurs in *P. trivolvis* and to assess the nature of sperm precedence to understand how mating history may influence gender roles, adding to the growing body of knowledge about reproductive strategies in these often neglected hermaphrodites. By mating snails first to one partner and then a second, using albinism as a genetic marker to determine paternity, we estimated P2 values as measures of sperm precedence. Based on data from other pulmonates, we hypothesized that precedence would vary among individuals, but average close to 0.5, setting up a situation for competition.

Materials and methods

Study population

Pigmented snails were sampled from a population of *Planorbella (Helisoma) trivolvis* collected from the wild and maintained in the lab for four years. Albino snails were sampled from another lab population that originated from the F_3 progeny from crosses between the above pigmented snails and an albino laboratory population two years before the experiment began. Pigmented snails have a dark mantle color and black eyes, whereas albinos are pigment-free both on the mantle and eyes, so appear red due to hemoglobin in their blood.

Although they are hermaphrodites, *P. trivolvis* reproduce almost exclusively by outcrossing, so eggs are fertilized by allosperm produced and transferred from the mate, not autosperm produced by the mother (Norton and Newman 2016; Norton et al. 2018a). Additionally, mating is almost always reciprocal, with each snail simultaneously acting in both male and female roles, thus transferring and receiving sperm from their partner (Norton, unpublished data). Sperm produced in the ovotestis of the donor pass through the hermaphroditic duct into the sperm duct, are mixed with seminal fluid from the prostate, pass through the vas deferens and exit through the male genital opening during muscular contractions of the verge (Abdel-Malek 1954). During mating, ejaculate enters the seminal receptacle duct of the partner through the vagina. Sperm may then move up the vagina, likely by the action of cilia, or may be stored in the seminal receptacle sac. Fertilization is presumed to take place in the fertilization sac near the oviduct or in the hermaphroditic duct itself. Fertilized eggs receive albumen and other secretions as they pass through the female reproductive tract, are packaged into a capsule (egg mass) in the oothecal gland and move out of the body through the muscular contraction of the vagina.

Inseminated snails lay eggs approximately daily in masses containing 13–33 eggs ($\bar{x} = 22.9 \pm 0.6$; Norton and Newman 2016). Embryos develop in the egg capsule and hatch about 7–10 days after laying. The difference between the two morphs can be detected by examining eye pigmentation while embryos are still in the egg case. Pigmented offspring have small black eye spots at the base of their antennae and albinos lack this pigment.

We sampled 30 pigmented and 30 albino snails from the above populations and allowed them to lay eggs. We collected egg masses from 26 pigmented and 29 albino snails and reared them until the offspring were approximately 70 days old (prior to sexual maturity). At this time we isolated three offspring from each of the 26 pigmented adults and five from each of the 29 albinos to serve as our experimental individuals. These snails were transferred to individual 300 ml plastic cups filled with tap water treated to remove chlorine and fed small squares of boiled organic Romaine lettuce two times per week. Snails were kept in the lab at approximately 25ºC with natural lighting.

Matings

When snails were approximately 95 days old, we assessed their body size by measuring shell diameter (a reliable and repeatable indicator of size – Norton et al. 2008). We chose 25 albino snails (one from each original parent) as our focal individuals. We mated the focal snails with a similarly sized mate to ensure size disparities would not interfere with mating. ‘Mating’ consisted of placing both snails in a plastic cup with fresh water. Twelve of these were mated to another albino snail from the same source (designated AP) and 13 were mated to a pigmented snail (again one from each of the original adults, designated PA). We marked snails with a small dot of nail polish to aid in individual identification. We also matched snails in the two experimental groups for size. Twenty-four hours later we separated the pair and placed the focal snail with a mate of the opposite type – thus the designations: AP snails were mated first to an albino and then to a pigmented partner, and PA snails were mated first to a pigmented snail then to an albino. After another 24-hour period, the focal snails were placed in individual cups and the mates were discarded. One week later we repeated the experiment with another 28 albino snails; 15 were mated first to an albino partner and then a pigmented partner (AP) and 13 were mated to a pigmented and then albino partner (PA). Overall there were 27 AP snails and 26 PA.

Determining paternity

We collected all egg masses laid by focal snails during the first, third and fifth week post-mating to assess sperm precedence and determine whether it might change as sperm stores became depleted. We removed snails and transferred them to fresh cups, and using scissors cut the plastic around each egg mass from the cup and placed each in the well of a 6-well tissue culture plate filled with dechlorinated tap water. We observed each egg mass under an inverted microscope with total magnification of 40x and counted the number of albino and pigmented embryos in each egg mass. We collected between 2 and 15 egg masses from each snail per collection, observing a total of 910 egg masses (about 300 each of the three weeks of measurement) with an average 16.7 eggs per mass for a total of 15,262 embryos. Embryos were initially kept in the lab until an air conditioning unit broke when we transferred them to environmental chambers kept at approximately 22–24ºC.

Statistical analysis

To determine sperm precedence we divided the number of embryos resulting from fertilization by the second mate (pigmented offspring for the AP group and albino for the PA group) by the total number of embryos produced for each snail – the P2 value for each individual snail. We then calculated the average P2 value for each treatment group (AP or PA) for each of the two trials. We did this for each sampling week (1, 3, and 5) and also for the total of all embryos collected. Because there were no differences in P2 values between trials for the individual weeks (Week 1 ANOVA: $F_{[1,50]} = 0.748$, $p = 0.39$; Week 3 ANOVA: $F_{[1,49]} = 0.745$, $p = 0.39$; Week 5 ANOVA: $F_{[1,42]} = 0.036$, $p = 0.85$) or the total (ANOVA: $F_{[1,51]} = 0.803$, $p = 0.37$), we combined trials for further analysis.

To determine whether there was any change in sperm precedence over time, we carried out a repeated measures ANOVA (IBM SPSS Statistics v. 22) for P2 values in weeks 1, 3, and 5. To determine whether there was an effect of the order of mating (albino first and then pigmented or pigmented first and then albino) we carried out ANOVAs comparing P2 values between AP and PA groups during weeks 1, 3, 5 as well as for the total sampling period. Finally, to determine whether P2 distributions were significantly different from those expected if there is no sperm precedence (a normal distribution with an average P2 of 0.5) we carried out Kolmogorov-Smirnov goodness of fit tests for each mating order type.

Results

Planorbella trivolvis exhibited primarily first sperm precedence, with no significant change over time (Figure 1; Repeated measures ANOVA: $F_{[2,38]} = 0.189$, $p = 0.83$; note that any snail which did not produce eggs in one

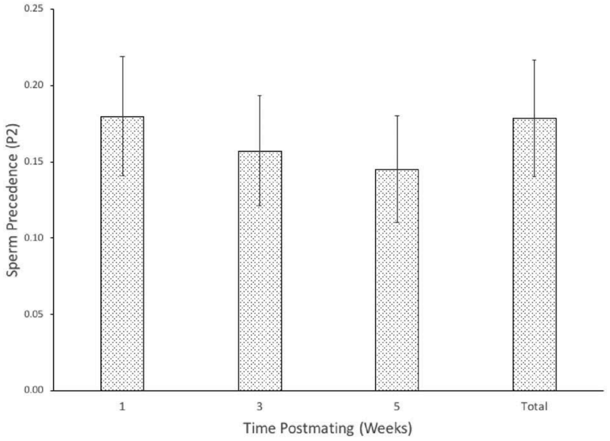

Figure 1. Sperm precedence in *Planorbella trivolvis* measured at 1, 3, and 5 weeks post mating. Albino snails were paired with an albino partner then 24 hours later with a pigmented snail (AP; n = 22) or the reverse (PA; n = 21). Columns represent the average P2 (proportion of eggs fertilized by the second partner) during the first, third, and fifth week post-mating for the 43 individuals, regardless of mating order, that laid eggs during all 3 periods. Total P2 (over all three measurement periods) is also reported. Error bars represent ± 1 standard error.

of the three sampling periods was omitted from the analysis, so total sample size for this analysis was 43: 22 AP and 21 PA). P2 values were low for each of the one-week periods after mating, with an average overall P2 of 0.18 ± 0.04 across all individuals. There was, however, a significant influence of mate order on precedence; P2 values were significantly higher when the first mate was albino ($\bar{x}$ = 0.28 ± 0.07) than when the first mate was pigmented ($\bar{x}$ = 0.07 ± 0.02) (Figure 2; ANOVA for total P2: $F_{[1,51]}$ = 8.87, p = 0.004). Individual P2 values also varied more when the first mate was albino (Figure 3). The variance in P2 value of the AP group was 0.122 whereas that of the PA group was 0.009, almost ten-fold less. Additionally, when the first mate was albino, in 4/27 cases no sperm came from this partner – all of the progeny were pigmented (P2 = 1), but when the first mate was pigmented in no case did progeny result from only the second mate. In both cases the distributions were significantly different than those predicted if fertilization is random with regard to the two sperm donors (Figure 3; Kolmogorov-Smirnov = 0.214, p = 0.003 for group AP and K-S = 0.272, $p < 0.001$ for group PA).

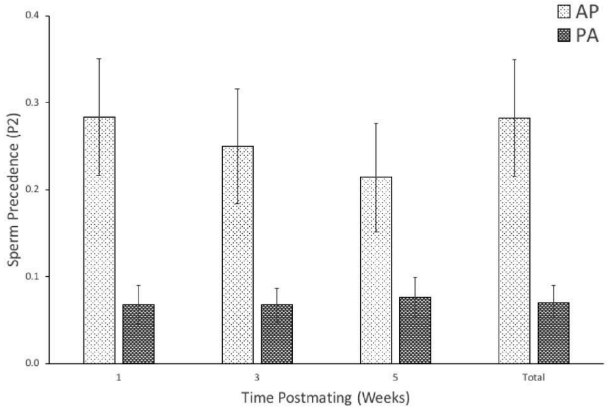

Figure 2. Sperm precedence in groups of *P. trivolvis* presented with mates in different order. Individuals were presented with either an albino partner followed by a pigmented partner (AP; n = 27) or a pigmented partner followed by an albino (PA; n = 26). Columns represent the average P2 (proportion of eggs fertilized by the second partner) during weeks 1, 3, or 5 as well as the total overall P2 for all collection periods for snails in each mating order group (AP or PA). Error bars represent ± 1 standard error.

Discussion

These data indicate strong first sperm precedence in *P. trivolvis*. The average P2 value we report (Figure 1; $\bar{x}$ = 0.18 ± 0.04) is lower than those reported for other pulmonate gastropods (0.23–0.60; Nakadera and Koene 2013). There was also substantial variation among individuals in the proportion of eggs fertilized by the second mate (Figure 3). These individual differences are not unusual and have also been reported in other species including the freshwater snail *Lymnaea stagnalis* (Koene et al. 2009) and land snails *Ariana arbustorum* (Baur 1994) and *Helix aspersa* (Evanno et al. 2005).

Although the biology of sperm storage in *P. trivolvis* is not well understood, the mechanism behind first sperm precedence may simply be filling of the sperm storage organ, so that additional allosperm from a subsequent mating is either unable to be transferred or quickly digested. Or as in the land snail *Cornu aspersum*, sperm from the first mating may preferentially attach to the reproductive tract or cluster, preventing sperm from the second mate from persisting (described in Garefalaki et al. 2010). Very little is known about the location or physiological mechanisms of sperm storage in *P. trivolvis* (Abdel-Malek 1954). Because genital anatomy and sperm storage may be important influences on overall fertilization success as well as sperm precedence, it will be important to expand our knowledge of these factors. Investigating how a longer delay in re-mating influences sperm precedence should be informative here. If precedence is influenced by first sperm taking up space or initially preventing additional sperm from entering the female reproductive structure, longer delays should deplete allosperm stores so the frequency of second mate fertilization would be expected to be higher. Our finding of first sperm precedence is markedly different from the case of *Lymnaea stagnalis* (with P2 values closer to 0.5) where, with only 24 hours between matings, sperm are hypothesized to be mixed in storage and used

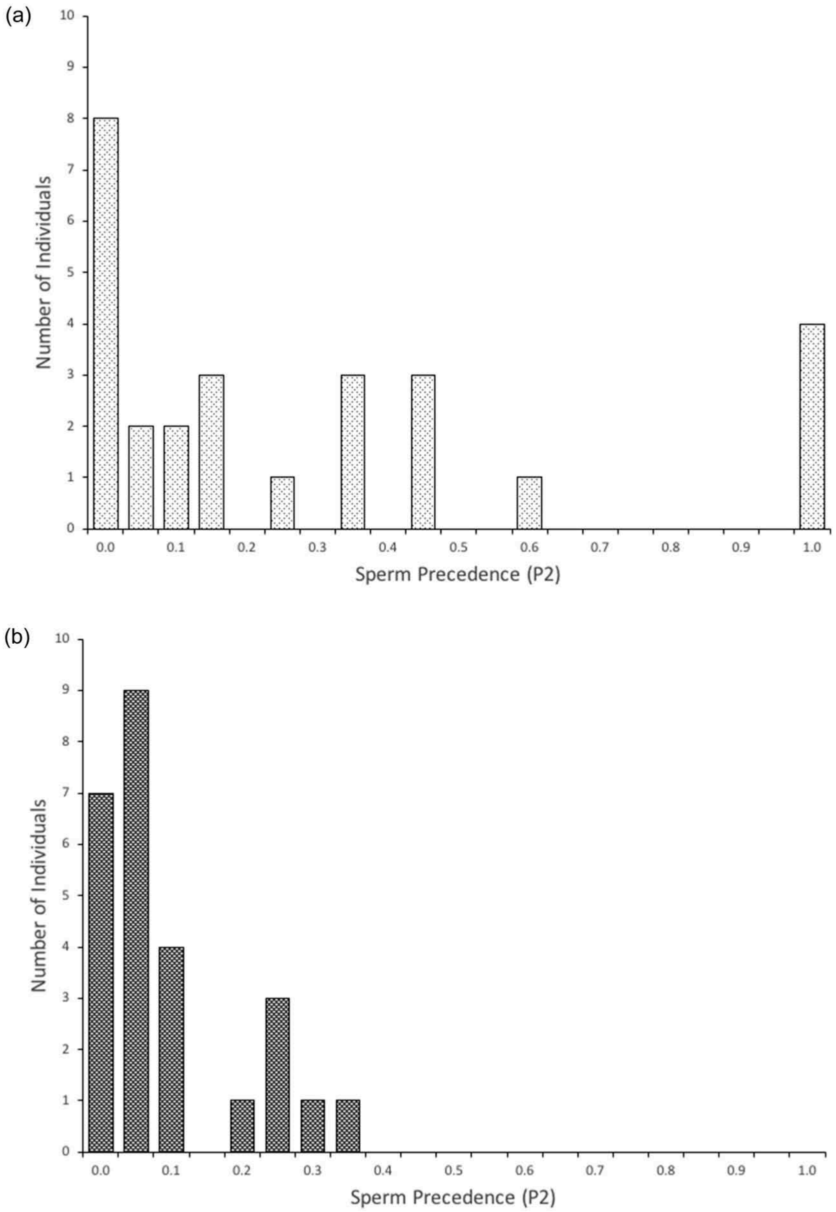

Figure 3. Variation in sperm precedence in *P. trivolvis*. Frequency distribution of P2 values during the first 5 weeks post-mating. (a). Individuals presented first with an albino partner and then a pigmented partner (AP; n = 27). (b). Individuals presented first with a pigmented partner and then an albino partner (PA; n = 26).

randomly to fertilize eggs (Koene et al. 2009). Differences in the location and/or mechanism of sperm storage may help to explain this variation between species.

Alternately, the precedence of sperm from the first mate may be explained by a shift in sex allocation or gender roles during the second mating. During the first mating, when both partners have stores of autosperm and have not previously mated, they would be expected to mate reciprocally and both transfer sperm. However, during the second mating period, the focal snail has now mated (presumable in both male and female roles). The second mate may act preferentially as female when paired with an already mated partner to avoid sperm competition, so only receives sperm from the focal individual and does not act in the male role or transfers less sperm (as predicted by Anthes et al. 2006). The focal individual may thus mate with the second partner, but solely or primarily in the male role, donating but not receiving sperm. Recent observations (Norton, unpublished) indicate that copulation is almost always reciprocal in *P. trivolvis* when both snails have not previously mated, but we have not assessed mating roles in recently mated individuals. More information about gender role preferences with previously mated vs. unmated partners would help to explain our current findings that in 15/53 cases only sperm from the first mate fertilized eggs – no sperm came from the second partner (P2 = 0). It is also possible that accessory proteins transferred along with sperm from the first mate may influence the likelihood of re-mating; although much is known about these allohormones in *Lymnaea* (Koene et al. 2010), they have not been investigated in *Planorbella* spp.

We also found that that sperm precedence depended on the pigmentation phenotypes of the first and second mate (Figure 2). When a pigmented individual was the first mate, sperm from the second (albino) partner fertilized less than 10% of the eggs ($\bar{x} = 0.07 \pm 0.02$), and in 7/26 cases fertilized none of them. Yet when the first mate was albino, a higher proportion of sperm from the second (pigmented) partner fertilized eggs ($\bar{x} = 0.28 \pm 0.07$) and in 4/27 cases fertilized all of the eggs laid by the focal individual. Differences in sperm precedence dependent on the order of mating could result from a difference in the amount of sperm transferred by the two types of snails. Because the albino snails have the genetic background of a long-time laboratory population, they may differ not only in colour from the pigmented population, but also in reproductive characteristics including sperm production or transfer. In crosses between individuals from the original albino and pigmented populations, snails mated to individuals from the wild derived (pigmented) population laid more eggs in each egg mass than those fertilized by sperm from the (albino) laboratory population, regardless of their own origin (Norton et al. 2018b). This was interpreted as a difference in the amount of sperm transferred since the number of eggs per mass was dependent on the partner acting in the male role. These differences could also result from differential viability or competitive ability of sperm produced by snails from the albino and pigmented populations. In crosses between albino and heterozygous pigmented *Biomphalaria* Vianey-Liaud (1995; 1996) observed an excess of sperm carrying pigmented alleles fertilizing eggs. They speculate that these differences may be related to differences in the size and potentially motility of sperm produced by homozygous pigmented and albino individuals (Vianey-Liaud et al. 1996; but see Minoretti and Baur 2006 who found that sperm length was not related to velocity or longevity of sperm in the land snail *Arianta arbustorum*). We have seen no such discrepancies, either in genetic crosses (Norton et al. 2018a) or in recent explorations of precedence within a single ejaculate for sperm carrying an albino or pigmented allele (Norton and Holm unpublished data).

None of the above explanations easily account for the complete absence of sperm from the first mate (P2 = 1) which occurred only when the first mate was albino and the second was pigmented. This outcome more likely reflects an absence of mating with the first albino individual during mating or the focal individual adopting a female gender role in those cases. No evidence to date suggests a preference for mating with a pigmented or albino mate, especially when the individuals were not presented with a choice.

Such strong first sperm precedence in *P. trivolvis* suggests that the first male-acting mate to copulate with a newly mature individual has a significant selective advantage over subsequent sperm donors, and predicts that prior experience should influence gender role choice. More empirical work on these hermaphrodites is needed to further understand the mechanisms of sperm competition and its consequences.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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