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UNIVERSITY OF CALIFORNIA  
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The Effect of Sex Ratio on Copulation, Sperm Transfer, and Sperm Reserves in the  
Waterstrider, *Aquarius remigis*

A Thesis submitted in partial satisfaction  
of the requirements for the degree of

Master of Science

in

Evolution, Ecology, and Organismal Biology

by

Clayton James Houck

December 2011

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The Thesis of Clayton James Houck is approved:

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**Chapter 1: The Effect of Sex Ratio on Copulation, Sperm Transfer, and Sperm Reserves in the Waterstrider, *Aquarius remigis***

**Abstract:**

Although male reproductive success is expected to increase with additional copulations and the transfer of numerous or large sperm to females, these may reduce male sperm reserves and affect future reproductive effort. The waterstrider *Aquarius remigis* Say (Gerridae, Heteroptera) has large sperm (5mm length), copulates frequently (averages one per day) and has a polygynandrous mating system. We test the hypothesis that sperm reserves will decrease as mating opportunities increase by comparing the behavior and sperm reserves of male *A. remigis* after nine days under three sex ratios (3 males:9 females, 6 males:6 females, 6 males:0 females) and for single copulation trials with two sex ratios (1 male:3 females, 2 males:2 females). We show that males with additional mating opportunities copulate more often and for shorter durations but do not become sperm depleted and frequently have copulations of insufficient duration to result in sperm transfer. The number of sperm transferred was not related to copulation duration, sex ratio, or number of eggs in females. This suggests lack of strategic sperm allocation by males. Multiple mating did not increase female fertility and the number of sperm in female reproductive tracts. Thus, non-virgin females appear to gain no direct benefit from additional sperm transfer, although past research shows females *in copula* receive less harassment from males. Our results indicate that variation in the success and duration of copulations is best explained by female influence over copulation rather than by male reproductive tactics.

Key words: Reproductive tactics, Sperm, Mating, *Aquarius remigis*

## **Introduction:**

Comparisons between the sexes indicate that male animals are generally more willing to mate than females (Darwin 1871; Trivers 1972; Dawkins 1976; Andersson 1994; Thornhill and Alcock 2001; Simmons 2001; Arnqvist and Rowe 2005). The pervasive eagerness of males "to pair with any female" (Darwin 1871) has often been interpreted as evidence that males can easily and quickly refill their sperm reserves (Trivers 1972; Dewsbury 1982). Individual sperm are small compared to eggs and have historically been considered inexpensive to produce (Parker et. al. 1972; Nakatsuru and Kramer 1982; Dewsbury 1982; Arnold and Duvall 1994; Simmons 2001). However, evidence suggests that male sperm reserves can be low in many species (e.g. Almquist and Hale 1956; Rutowski et. al. 1987; Birkhead 1991; Cook & Wedell 1996; Bissoondath and Wiklund 1996; Preston et al. 2001; Simmons 2001; Velando et al. 2008) and that sperm depletion may limit male paternity success (Parker 1998; Preston et. al. 2001; Smith et. al. 2009; Hettyey 2009; Perez-Lachaud 2010). Males are most likely to face depletion of sperm reserves if they copulate repeatedly and transfer numerous sperm. By transferring large numbers of sperm, males increase the probability that at least some sperm will survive in the female reproductive tract (Birkhead et. al. 1993). More significantly, if females are polyandrous and store sperm, transferring large numbers of sperm gives males an advantage in competition with the sperm of rival males (Parker 1998; Simmons 2001). Some species produce large sperm and evidence suggests that large sperm size could be an adaptation for sperm competition (Gomendio and Roldan 1991; Briskie et. al. 1997; LaMunyon and Ward 1998, Gomendio and Roldan 2008;

Lupold et. al. 2009). Species with very large sperm tend to have low numbers of sperm, which suggests a trade-off between sperm size and number (Pitnick and Markow 1994; Pitnick et. al. 1995; Parker 1998), and the presence of such a trade-off itself suggests some limitation on male sperm reserves. Replenishing sperm reserves involves testes that must be developed and maintained at some cost to the male (Dewsbury 1982; Pitnick and Markow 1994; Pitnick 1996). Thus, when faced with opportunities to mate repeatedly, males are expected to try to achieve numerous copulations involving the transfer of large numbers of viable sperm but must also deal with limitations on their sperm reserves.

It is difficult to predict the effect of additional mating opportunities and potential depletion of sperm reserves on male behavior and sperm reserves (Lemaitre et. al. 2009). Males may maintain sperm reserves through tactics such as reduced frequency of copulation (Thornhill and Alcock 2001), increased rate of sperm production, or strategic allocation of sperm to females based on sperm competition risks, number of mating opportunities and female characteristics related to fecundity (Gage 1991; Evans and Magurran 2000; Pilastro et al. 2002; Pizzari et. al. 2003; Harris and Moore 2005; delBarco-Trillo and Ferkin 2006; Smith et. al 2009). If males do not alter their sperm production or mating frequency (Preston et. al. 2001; Lemaitre et. al. 2009), they are likely to face a reduction in reproductive success due to lack of sperm in future copulations (Preston et. al. 2001).

We report a series of experiments designed to determine if males in a polygynandrous species (i.e., where both sexes mate with multiple partners): (1) risk

depletion of their sperm reserves when faced with multiple mating opportunities, and (2) adjust their sperm production, mating frequency or number of sperm transferred per ejaculate to reduce the risk of sperm depletion. Our study species, the waterstrider, *Aquarius remigis* (Heteroptera, Gerridae) has a number of traits that make it particularly suitable for investigating the influence of additional mating opportunities on depletion of male sperm reserves and strategic sperm allocation. Males and females typically mate daily over a reproductive lifespan lasting a month or more under natural conditions and each sex mates with multiple partners (Preziosi and Fairbairn 2000; Ferguson and Fairbairn 2000). Mating attempts typically begin with a vigorous and energetically costly struggle and the majority of attempts end in failure (Weigensberg and Fairbairn 1994; Watson et. al. 1998; Vermette and Fairbairn 2002). If the male is successful in overcoming female resistance, the resulting copulation is likely to be long (avg. 2-3 hrs., max.>12 hrs.; Fairbairn 1988; Weigensberg and Fairbairn 1994) and males do not eat while *in copula*. Thus mating is costly for males in terms of both energy expended and lost opportunities for additional matings (Vermette and Fairbairn 2002). Sperm transfer requires a minimum initial lag of 15 - 20 minutes and always occurs at the end of copulation, even when copulations last many hours (Rubenstein 1989; Campbell and Fairbairn 2001). The polygynadrous mating system and female sperm storage creates an opportunity for sperm competition (Fairbairn 1988; Campbell and Fairbairn 2001, Vermette and Fairbairn 2002), and males produce large sperm (5mm,  $\geq 1/3$  body length; Miyata et al 2011). Further, past experiments suggest relationships between sperm use and reproductive behavior. An experiment with irradiated males in co-sex pools

(Vermette and Fairbairn 2002) indicated that quantity of sperm transferred influences reproductive success in males of *Aquarius remigis*. The same experiment demonstrated that under natural conditions of multiple mating by both sexes, male paternity success is positively correlated with number of matings while mating duration had a negative effect. This negative effect was due to long copulations limiting males from additional copulations. Therefore, quantity of sperm transferred and number of matings are expected to be important for male paternity success in *Aquarius remigis* though both may deplete male sperm reserves.

In experiment 1, we test the hypothesis that male *A. remigis* experience short term reductions in their sperm reserves when the local sex ratio is biased toward females and hence mating opportunities are abundant. We predict that under these conditions, males will have more copulations and less sperm in their seminal vesicles than males kept under an even sex ratio. Similarly, we predict that males kept with females will have less sperm in their seminal vesicles than males kept without access to females. We also predict that males will increase sperm production to replenish their sperm reserves and hence the number of sperm developing in the testes should be highest when the sex ratio is female biased and lowest when males are kept without mating opportunities.

In experiment 2, we test the hypothesis that males adjust the number of sperm transferred per mating in accordance with their perception of additional mating opportunities and the risk of sperm competition. *A. remigis* has many characteristics that make male sperm allocation particularly likely; males experience significant sperm competition, have opportunities to mate multiple times in natural conditions and have



sperm that are likely costly to produce. Therefore, males would be expected to use strategic sperm allocation to increase their chances of fertilizing eggs over rival males and to conserve sperm for future copulations. We compare the number of sperm transferred on first copulation by two males given access to two mature virgin females to the number of sperm transferred on first copulation by one male given access to three mature virgin females. We predict that males will transfer less sperm in the absence of a competitor than when a competitor is present. The rationale for this prediction is two-fold. First, males perceiving three potential mates and no competition may transfer less sperm to their first mate in order to conserve sperm for additional copulation. Second, males facing a competitor will transfer more sperm to better compete with the rival male for fertilizations (Parker 1998). Thus both the perceived number of mating opportunities and the risk of competition should favor reduced sperm per ejaculate in the absence of the rival male. The combination of both effects should increase our probability of detecting strategic sperm allocation in our *A. remigis* males.

## **Methods:**

### **Experimental animals**

All experiments used first generation lab-reared animals derived from eggs laid by adults collected on Rattlesnake Creek (a stream in Santa Barbara, California) on July 3rd, 2010. The rearing and experimental trials were done at room temperature (mean  $\pm$  SD, 22.4 C  $\pm$  .51, range 21.3 - 24.0 C) and 14hL. The stream tanks used for rearing animals and used for experiment 1 were 135 x 42 cm, with water to a depth of approximately 7 cm. All tanks used a water pump to mimic water currents found in natural conditions. Food (frozen *Acheta domestica* and *Drosophila melanogaster*) in excess of daily requirements for maximum fecundity (Blanckenhorn et al. 1995) was added to stream tanks and old food was removed daily between 9 am and 10am. Upon eclosion males and females were separated into different tanks. All individuals were virgins at the beginning of experiments. Experiments were conducted in the fall of 2010. All statistical analyses were performed using SPSS 17.0 © 2008 and the statistical program R version 2.9.2 © 2009.

Males and females in both experiments were photographed in ventral aspect and body components were later measured using these images. Pictures were taken using the computer program Spot Basic 3.4 © 1997-2001 and a Leica Wild M3C microscope hooked up to a computer. Body components were photographed at 40x and genital and pre-genital components at 125x magnification.

Measurements were taken using the computer program Sigma Scan Pro 5.0 © 1987-1999. We measured the lengths of the head, thorax, genitals, abdomen, total soma,

and total body (including genitals). We also measured segment 8. The length of the head, thorax, abdomen, and genitals were used in a principal component analysis with a correlation matrix. The first principal component (PC1) was taken from this analysis (see table A4.1 and table A4.2 for factor loadings of body components and eigenvalues of the first 4 principal components). PC1, the length of the soma, and the total length, were used as measures of general body size. The first principal component (PC1) accounted for 56.0% of the variance of males and 80.4% of the variance of females. These body measurements were used due to their relationships to mating success. Large male mating advantage has been documented in this species (Fairbairn 1988; Krupa and Sih 1993; Fairbairn and Preziosi 1994; Preziosi and Fairbairn 1996, 2000; Weigensberg and Fairbairn 1996; Sih et. al. 2002) though this is due to selection acting on male genital length (Preziosi and Fairbairn 1996, 2000; Sih et. al. 2002; Bertin and Fairbairn 2005). Length of segment 8 (the first genital component) is likely the primary target of this selection (Bertin and Fairbairn 2005). Males with a smaller soma length have been indicated as being favored for mating (Fairbairn and Preziosi 1996; Preziosi and Fairbairn 1996, 2000; Ferguson and Fairbairn 2000; Sih et. al. 2002; Bertin and Fairbairn 2005). All of the measurements will be referred to collectively as body components for sake of brevity.

### **Experiment 1: Nine-day assays examining sperm depletion**

To determine if males with ready access to mating opportunities become sperm depleted, we conducted two replicates of three experimental treatments. Treatments

consisted of 1 stream tank with 6 males without females (males only), 1 stream tank of 6 males and 6 females (even sex ratio), and 2 stream tanks of 3 males and 9 females (female-biased sex ratio). Males and females were 6 - 8 weeks post eclosion for replicate 1, and 8 - 12 weeks post eclosion for replicate 2. Animals were identified by individual numbers painted on their dorsal cuticle. Daily inspections verified that no individuals died or were lost from stream tanks prior to or during the experiments.

We assayed copulation frequency and duration by scanning each tank every 10 minutes for one minute and recording the mating status of each individual. Copulations were identified by visually verifying intromission. Scans were conducted between 10am and 8pm (the most active hours of *A. remigis*; Gentile 1998) on the 1st, 2nd, 6th, 8th, and 9th day after initiation of the treatments. All animals were photographed immediately after the last scan on the 9th day for later measurement of body components (as described above). All animals were then immediately placed in a container containing chloroform and were then dissected to make estimates of sperm abundance in reproductive organs.

Copulations lasting less than 20 minutes rarely result in sperm transfer (Rubenstein 1989; Campbell and Fairbairn 2001). Under laboratory conditions, copulations typically average 1.5h – 3h but can last more than 12h (Fairbairn 1988, 1990; Weigensberg and Fairbairn 1994, Campbell and Fairbairn 2001). We used 2h (120 minutes) to demarcate relatively short and relatively long copulations and categorized copulations as < 20 min (probably unsuccessful), 20 min to <120 min (successful short), or  $\geq$  120 min (successful long).

To determine the number of sperm remaining in male sperm reserves, we removed the seminal vesicles and placed them in a 1.5 ml, low retention centrifuge tube containing 1 ml of 1% phosphate buffer saline (PBS) solution. The tube was placed on ice and sonicated 3 times for 10 seconds, with 1 minute breaks between rounds of sonication, to break apart sperm clumps and cells. After sonication, the tube was centrifuged for 5 minutes at 10,000 rpm to concentrate cell components in a pellet. The excess fluid was removed and 5  $\mu$ L of a solution of 3.5 micro-grams of 4',6-diamidino-2-phenylindole (DAPI) per mL of 1% PBS was added to the container to stain the cell nuclei. Another round of sonication was done for 10 sec and this was repeated at one-minute intervals until the pellet was fully broken apart. The tube was then placed in an Eppendorf mixer for 10 minutes to create an even distribution of cells in the fluid. The resulting solution was spread on a transparent grid cut from the bottom of an Ibidi brand gridded micro-dish and observed using a Zeiss Axiovert 10 fluorescence microscope at a magnification of 630x total magnification. Sperm nuclei could easily be identified by their elongated shape. To estimate the number of sperm nuclei in each sample, we counted all sperm nuclei within seven standard quadrates evenly spaced on the slide. The sampled area comprised 23.16 % of the grid, so total numbers were estimated as the count/0.2316 (additional details about dissections and the sperm counting methodology can be found in Appendix 1- 3).

To estimate of abundance of sperm developing in the testes, the testes were removed and placed in a modified Carnoy fixative (3 absolute ethanol : 1 glacial acetic acid). Dr. Olga Kiseliova then stained the testes in aceto-orcein for 3-4 days and squashed

the testes on a slide, following the protocols from Kiseliova et. al. (1998). The relative numbers of sperm in different stages of development were estimated under 1600x magnification on an Olympus™ BX51 (DIC) microscope for one testis from each male. This allowed for a close inspection of a thin layer of the squashed testes. Mature sperm were defined as sperm with elongated, fully developed nuclei and immature sperm were defined as sperm with round nuclei or not fully developed nuclei. The abundances of immature and mature sperm were ranked on a scale of 0 (no sperm at that stage of development), 1 (approximately < 20% on average of the fields of view of the testes contained sperm), 2 (approximately 20 - 40% on average of the fields of view of the testes contained sperm), and 3 (approximately >40% on average of the fields of view of the testes contained sperm).

To determine the number of sperm transferred to females, we extracted the entire gynatrial complex (gynatrial sac plus spermathecal tube) after removing the dorsal abdominal cuticle and removing the gut. The gynatrial complex was placed in a 1.5 mL low retention tube with 5 µL of a solution containing 3.5 µL DAPI stain per 1 ml of 1% PBS solution. The low retention tube was placed on ice, sonicated once for 10 sec. and repeated as necessary, and then processed following the protocol for counting sperm in the seminal vesicles (above). The number of eggs within females was also recorded during dissection. All eggs were viewed with a 4x dissecting microscope with a 10x ocular lens (40 x total magnification) and were counted.

Two weeks after the completion of each replicate, 100 eggs were collected from each tank in the experiment to estimate the percent of eggs successfully fertilized. Twelve

to 13 eggs were haphazardly selected on each of the 8 resting cups in each tank and examined under a dissecting microscope at 40x power. Eggs with eyespots or developing embryos, and eggs that had been split open (indicating hatched eggs) were counted as successfully fertilized eggs.

### **Experiment 2: Single copulation trials**

To determine if males alter their pattern of sperm allocation in response to the availability of mating opportunities, we conducted single-copulation mating trials with 2 males with 2 virgin females (even-sex ratio; 7 trials) or 1 male with 3 virgin females (female-biased sex ratio; 9 trials). Males and females were 11 - 15 weeks post-eclosion and had been maintained in large single-sex stream tanks (135 cm by 42 cm, with a water pump to mimic water currents found in natural conditions) prior to the experiment. Prior to the experiments, numbers were painted on all the experimental animals to allow individual identification and images were taken of body components using the method described above. Each mating group was placed in a separate container (28 cm by 13 cm) with water to a depth of 5 cm, an air bubbler to create surface water movement, and resting sites made from foam. Food (frozen *Acheta domestica* and *Drosophila melanogaster*) in excess of daily requirements for maximum fecundity (Blanckenhorn et al. 1995) was added to containers immediately before the waterstriders were placed in the containers at 10am.

All containers were scanned continuously until termination of the first copulation. Trials in each container were terminated after a single copulation regardless of duration

of copulation. In one even sex ratio trial, the two males copulated at the same time and both copulations were allowed to terminate naturally. The significance of results did not change when including or excluding the second copulation from this trial and thus, this copulation was included in analysis. Copulation durations were determined by recording the start and end times for all copulations. Upon termination of trials, animals were placed in a container containing chloroform until insects were killed. We then counted the number of eggs carried by all females and we immediately made slides in order to count the number of sperm in the female's gynatrial complex and male seminal vesicles for animals that had copulated, using the methods described above. Within 3 days of preparing slides, sperm numbers were estimated from slides using the methods described above.



## **Results:**

### **Experiment 1: Nine-day behavioral assays**

Male body components (PC 1, genital length, soma length, and length of segment 8) were not related to the frequency of copulation or sperm abundance in the reproductive organs (Appendix 4, tables A4.1 – A4.5). Therefore male body components were not included in further analyses.

Shapiro-Wilk normality tests were used on the dependent variables of interest (copulation frequency and sperm in reproductive organs). When data were not normally distributed, we transformed the data to best approximate normality and then conducted the analyses using untransformed, transformed, and ranked data. In no case did transformation or ranking influence our statistical conclusions. Nevertheless, where transformation was necessary to approximate normality, we present the analyses for the transformed data only.

We initially tested our hypotheses using nested ANOVA with tank nested in treatment and replicate. This design acknowledges that within each treatment, individual waterstriders were grouped within tanks. The nested component was never significant and removal of the nested component did not change the significance level of treatment effect. We therefore simplified the analyses by removing tank as a nested component.

#### *Frequency and duration of copulations for males:*

Males experiencing the female-biased sex ratio averaged significantly more copulations than males in the even-sex ratio but the number of copulations  $\geq 20$  min did

not differ between treatments (table 1; figure 1a). This discrepancy can be understood by comparing the relative frequency of long and short copulations in the two treatments (figure 2). The difference between treatments is highly significant ( $\chi^2 = 76.3$ ,  $df = 2$ ,  $p < 0.001$ ) with the proportion of copulations lasting  $\geq 20$  min being much lower in the female-biased sex ratio than in the even sex ratio (37% versus 94%;  $\chi^2 = 42.112$ ,  $df = 1$ ,  $p < 0.001$ ). The even sex ratio also had a higher proportion of very long copulations lasting  $\geq 120$  min when including or excluding copulations  $< 20$  min. ( $\chi^2 = 74.8$ ,  $df=1$ ,  $p < 0.001$  and  $\chi^2 = 29.979$ ,  $df = 1$ ,  $p < 0.001$ , respectively). Thus males with access to more females copulated more but did not gain more copulations of sufficient duration to permit sperm transfer.

Females were all virgins at the beginning of the experiment. The change in female mating status from virgin to mated to multiply-mated may have influenced the tendency of males to transfer sperm or of females to accept sperm over the course of the experiment. To determine if this occurred we asked if day of the experiment affected the probability of sperm transfer as determined by the proportion of copulations  $\geq 20$  min. A covariance analysis indicated a significant effect of treatment ( $F_{1,20} = 73.914$ ,  $p < 0.001$ ) and day ( $F_{1,20} = 6.752$ ,  $p = 0.009$ ) but not of replicate ( $F_{1,20} = 4.698$ ,  $p = 0.058$ ) or the interaction of day and treatment ( $F_{1,20} = 2.502$ ,  $p = 0.117$ ) on the proportion of copulations  $\geq 20$  min. We therefore combined replicates and regressed the proportion of copulations  $\geq 20$  min. on day of the experiment for the two treatments (figure 3). The ANCOVA of the data replicates combined indicated a significant effect of day ( $F_{1,6} = 10.695$ ,  $p = 0.017$ ) but not of treatment ( $F_{1,6} = 2.139$ ,  $p = 0.194$ ), or the interaction of

treatment and day ( $F_{1,6} = 5.724$ ,  $p = 0.054$ ) on the proportion of copulations  $\geq 20$  min. Because the interaction term was so close to significance, we conducted separate regressions for the two treatments. The average proportion of copulations lasting  $\geq 20$  min. did not change significantly over time in the even sex ratio ( $F_{1,3} = 0.579$ ,  $p = 0.502$ ,  $R^2 = 0.162$ ) but did decline significantly in the female-biased sex ratio ( $F_{1,3} = 12.009$ ,  $p = 0.040$ ,  $R^2 = 0.800$ ). Thus, the willingness of either females or males to engage in copulations long enough to allow insemination decreased over time in the treatment where females are abundant, but not when the sex ratio is even.

*Sperm in seminal vesicles and testes:*

Treatment significantly influenced the number of sperm in the seminal vesicles and the ranked abundance of immature sperm in the testes (table 1). This effect was entirely due to the all-male treatment in which males had significantly more sperm in their seminal vesicles and fewer immature sperm in the testes (table 1). The two co-sex treatments did not differ from each other for either variable, but both differed from the all-male treatment. The ranked score of mature sperm in the testes was not affected by treatment (table 1). Although access to females clearly reduced the number of sperm in male seminal vesicles, males in both co-sex treatments still had enough sperm to inseminate females (see below). Further, the similarity of sperm numbers in the two co-sex treatments is consistent with the above observation of similar numbers of copulations of sufficient duration to result in sperm transfer.

*Sperm transferred to females and female frequency of copulation:*

The average number of copulations per female was not affected by sex ratio but females in the female-biased sex ratio averaged fewer copulations  $\geq 20$  min. (table 2; figure 1b). Sex ratio did not affect the number of sperm females had in their gynatrial complexes (table 2) or the proportion of fertilized eggs laid:  $87.5\% \pm 3.5\%$  (mean  $\pm$  SE) of the eggs were fertilized in the even sex ratio treatment compared to  $84.8\% \pm 3.2\%$  of the eggs in the female-biased treatment ( $t_{(4)} = -0.523$   $p = 0.628$ ). Therefore, there was no evidence that the reduced number of copulations lasting  $\geq 20$  min in the female-biased sex ratio treatment reduced female fertility or the number of sperm in gynatrial complexes.

Figure 1a: Mean number of copulations per male in the even sex ratio (6 males : 6 females) and female-biased sex ratio treatments (3 males : 9 females). Data are given for all copulations and for copulations lasting  $\geq 20$  min. Blue = even sex ratio; red = female-biased sex ratio.

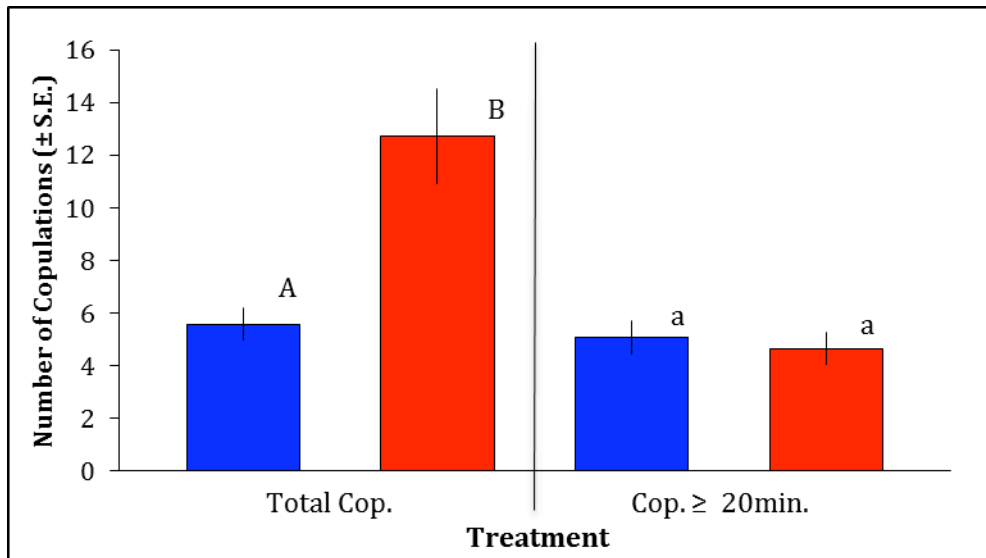


Figure 1b: Mean number of copulations per female in the even sex ratio (6 males : 6 females) and female-biased sex ratio treatments (3 males : 9 females). Data are given for all copulations and for copulations lasting  $\geq 20$  min. Blue = even sex ratio; red = female-biased sex ratio.

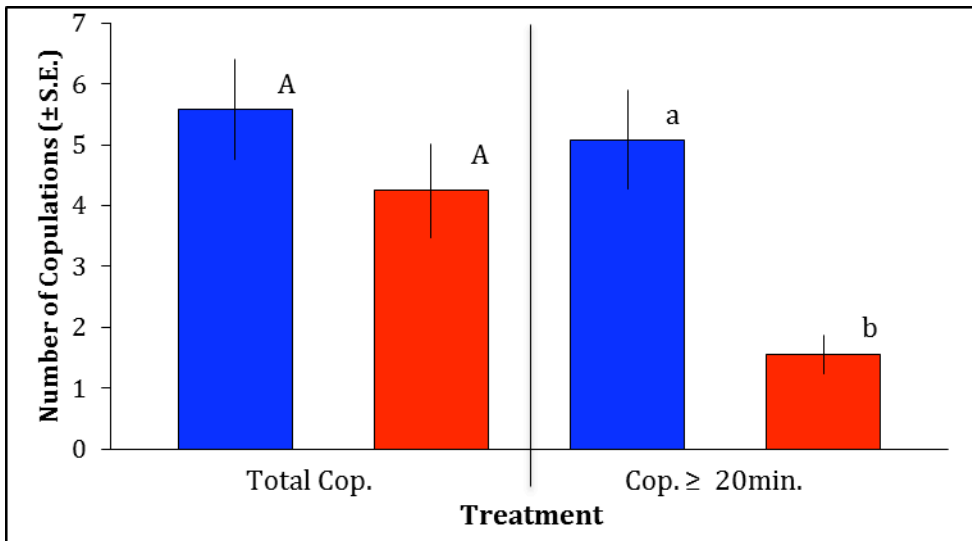


Figure 2: Distribution of number of copulations < 20 min, between 20 and 120 min, and  $\geq$  120 min in the even and female-biased sex ratio treatments of experiment 1. Data from both replicates are combined as replicates did not differ statistically. Blue = even sex ratio; red = female-biased sex ratio.

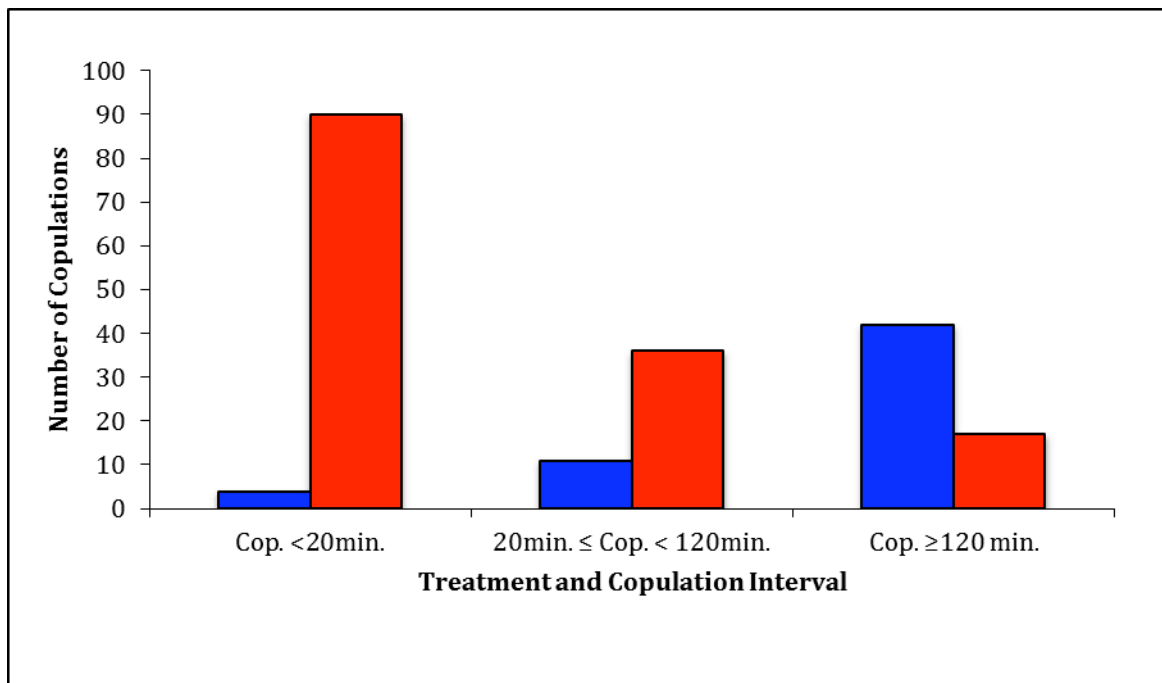


Figure 3: Average proportion of copulations  $\geq 20$  min by day for each treatment. Blue triangles = even-sex ratio; red circles = female-biased sex ratio. Lines are univariate regressions (see text).

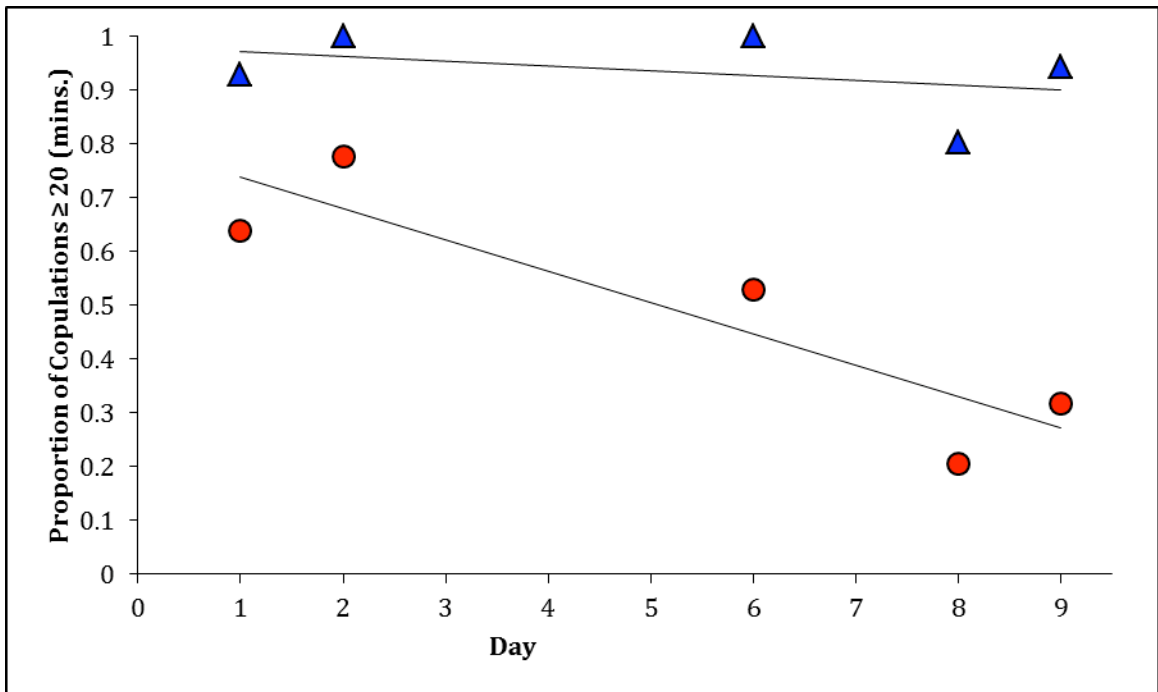




Table 1: Results of ANOVA comparing the males in experiment 1. Dependent variables are total number of copulations, total number of copulations lasting  $\geq 20$  min, total number of sperm in the seminal vesicles, and the ranked scores for numbers of mature and immature sperm in the testes for each male. Untransformed means and standard errors for each treatment are shown for the replicates combined because replicates did not differ. Superscripts indicate the results of post-hoc Tukey HSD tests. Where the ANOVA are based on transformed data, this is indicated below the variable name.

Dependent variable	Treatment mean $\pm$ SE			Treatment	Replicate	Treatment x Replicate
	6m:0f	6m:6f	3m:9f			
Number of copulations (log)	NA	5.58 $\pm$ 0.61	12.75 $\pm$ 1.81	$F_{(1,20)} = 19.823$ $p < 0.001$	$F_{(1,20)} = 3.489$ $p = 0.077$	$F_{(1,20)} = 0.003$ $p = 0.957$
Number of copulations $\geq 20$ min	NA	5.08 $\pm$ 0.62	4.67 $\pm$ 0.59	$F_{(1,20)} = 0.247$ $p = 0.625$	$F_{(1,20)} = 0.245$ $p = 0.626$	$F_{(1,20)} = 2.850$ $p = 0.107$
Number of sperm in seminal vesicles (square root)	14693 $\pm$ 1604 <sup>a</sup>	6821 $\pm$ 1031 <sup>b</sup>	7111 $\pm$ 1850 <sup>b</sup>	$F_{(2,30)} = 9.454$ $p = 0.0007$	$F_{(1,30)} = 0.0007$ $p = 0.980$	$F_{(2,30)} = 2.921$ $p = 0.069$
Immature sperm developing in the testes	1.08 $\pm$ 0.36 <sup>a</sup>	2.17 $\pm$ 0.27 <sup>b</sup>	2.17 $\pm$ 0.32 <sup>b</sup>	$F_{(2,30)} = 4.246$ $p = 0.024$	$F_{(1,30)} = 1.231$ $p = 0.276$	$F_{(2,30)} = 2.588$ $p = 0.092$
Mature sperm developing in the testes	0.75 $\pm$ 0.35	1.33 $\pm$ 0.31	1.67 $\pm$ 0.40	$F_{(2,30)} = 1.716$ $p = 0.197$	$F_{(1,30)} = 0.461$ $p = 0.502$	$F_{(2,30)} = 1.236$ $p = 0.305$

Table 2: Results of ANOVA comparing the females in experiment 1. Dependent variables are total number of copulations, total number of copulations lasting  $\geq 20$  min, and total number of sperm in the gynatrial complex for each female. Untransformed means and standard errors for each treatment are shown for the replicates combined. Where the ANOVA are based on transformed data, this is indicated below the variable name.

Dependent variable	Treatment mean $\pm$ SE		Treatment	Replicate	Treatment x Replicate
	6m:6f	3m:9f			
Number of copulations (square root)	5.58 $\pm$ 0.83	4.25 $\pm$ 0.77	$F_{(1,44)} = 2.080$ $p = 0.156$	$F_{(1,44)} = 1.803$ $p = 0.186$	$F_{(1,44)} = 0.029$ $p = 0.866$
Number of copulations $\geq 20$ min (square root)	5.08 $\pm$ 0.81	1.56 $\pm$ 0.31	$F_{(1,44)} = 17.462$ $p = 0.0001$	$F_{(1,44)} = 0.142$ $p = 0.708$	$F_{(1,44)} = 1.391$ $p = 0.245$
Number of sperm in gynatrial complex (square root)	833 $\pm$ 180	687 $\pm$ 93	$F_{(1,44)} = 0.916$ $p = 0.344$	$F_{(1,44)} = 1.873$ $p = 0.178$	$F_{(1,44)} = 0.321$ $p = 0.574$

## **Experiment 2: Single mating trials**

### *Sperm transferred to females and sperm in male seminal vesicles:*

The distributions of sperm in the seminal vesicles and sperm transferred to females did not depart significantly from normality. Analyses were conducted on all copulations and on just copulations resulting in sperm transfer. If the two analyses produced the same statistical conclusion, we present only the analysis for all copulations.

None of the male or female body or genital measures significantly influenced the number of sperm transferred to females and including these in the overall regression models did not alter the statistical conclusions concerning treatment effects (Appendix 4: Table A4.7 for males, table A4.8 for females). Male genital length and segment 8 length did influence the number of sperm in male seminal vesicles following copulations, but including these in the regression models did not alter the conclusions concerning treatment effects (Appendix 4: Table A4.6). Therefore we do not include size variables in the results reported below. Similarly, full analyses indicated that the number of eggs carried by females did not significantly influence the number of sperm transferred or the effects of treatment on number of sperm transferred (Appendix 4 table A4.8). We therefore did not include number of eggs in the analyses of treatment effects described below.

The number of sperm transferred to females in a single copulation was not influenced by sex ratio treatment ( $t_{15} = -0.101$ ,  $p = 0.921$ ; figure 4). As expected, sperm transfer did not occur until coupling had lasted approximately 20 minutes, though, overall, duration of copulation was not related to the number of sperm transferred (linear

regression:  $\beta = -0.003$   $F_{1,16} < 0.001$ ,  $p = 0.999$ ,  $R^2 < 0.001$ ; figure 5). The three copulations that did not result in sperm transfer lasted 21, 18, and 10 minutes respectively, and the only copulation that was  $< 20$  minutes and resulted in sperm transfer lasted 19 minutes. The few short copulations not resulting in sperm transfer did not cause an overall positive relationship between copulation duration and number of sperm transferred and excluding these did not affect the conclusions. The trend for copulations resulting in sperm transfer is still not significant and, if anything, is slightly negative rather than positive (linear regression:  $\beta = -1.149$ ,  $F_{1,13} = 0.669$ ,  $p = 0.999$ ,  $R^2 = 0.053$ ). After an initial latency of approximately 20 minutes, there is clearly no relationship between the number of sperm transferred by males and the duration of their copulations.

The sum of the numbers of sperm transferred (ST; mean  $\pm$  SE,  $1,396 \pm 272$ ) and the numbers of sperm remaining in the seminal vesicles after copulation (SRV; mean  $\pm$  SE,  $16,660 \pm 1,619$ ) provides an estimate of the number of sperm in the seminal vesicles before copulation for each male that copulated. The ratio  $ST/(ST + SRV)$  then estimates the proportion of sperm transferred in one copulation. For all copulations, the average percentage of sperm transferred was only 9.5%. Excluding copulations not resulting in sperm transfer raised this percentage only to 11.5% (SRV: mean  $\pm$  SE,  $16,306 \pm 1,534$ ; ST: mean  $\pm$  SE,  $1,695 \pm 267$ ). The number of sperm transferred was not related to the number in their seminal vesicles before copulation for all copulations ( $F_{(1,16)} = 0.106$ ,  $p = 0.750$ ,  $R^2 = 0.007$ ) or for only copulations resulting in sperm transfer ( $F_{(1,13)} = 0.005$ ,  $p = 0.942$ ,  $R^2 < 0.001$ ). The regression of ST to SRV further indicates a lack of relationship

between these variables (all copulations:  $F_{(1,16)} = 0.954$ ,  $p = 0.344$ ,  $R^2 = 0.060$ ; only copulations resulting in sperm transfer:  $F_{(1,13)} = 0.457$ ,  $p = 0.512$ ,  $R^2 = 0.037$ ).

Unexpectedly, after one copulation females held larger quantities of sperm than females at the conclusion of 9 days in experiment 1 (experiment 1: mean  $\pm$  SE,  $724 \pm 82.4$ ; experiment 2: mean  $\pm$  SE,  $1396 \pm 272$ ; t-test,  $t_{(19)}=2.369$ ,  $p=0.029$ ). Thus, sperm is not accumulating in females with multiple copulations.

Figure 4: Average number of sperm in the female's gynatrial complex after a single copulation in experiment 2.

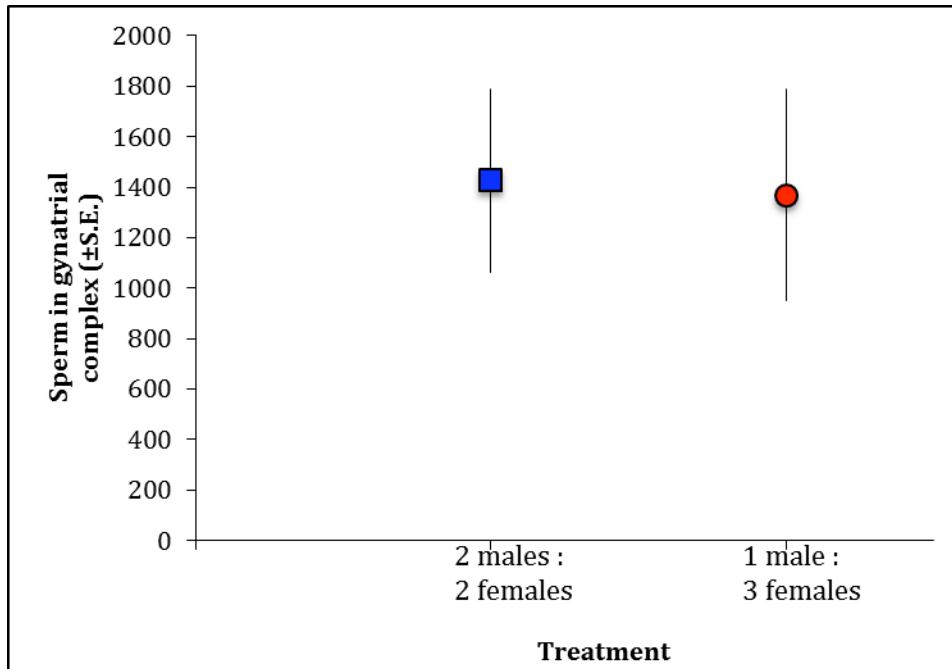
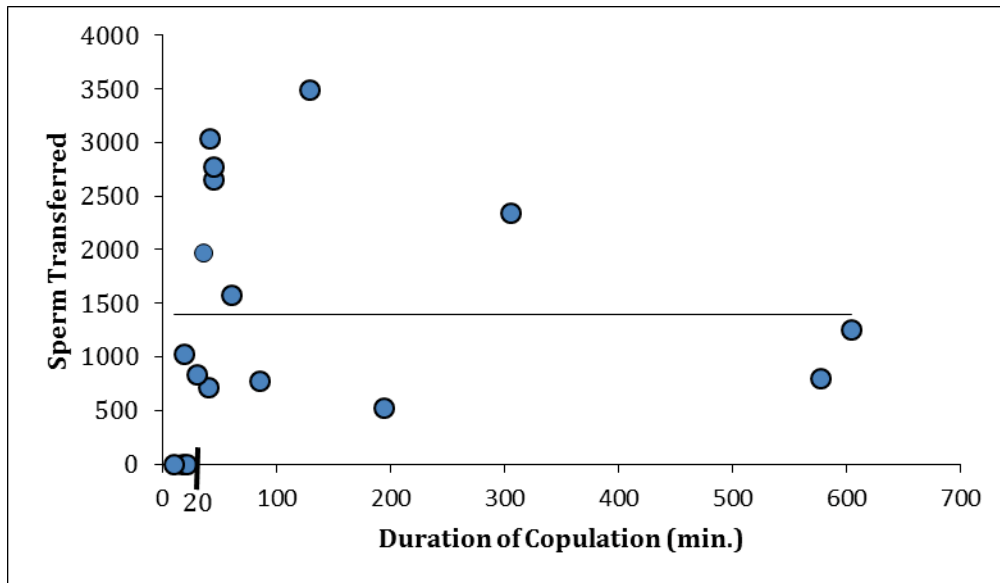


Figure 5: Number of sperm in the female's gynatrial complex as a function of the duration of copulation in the single mating trials of experiment 2. The vertical line marks 20 minutes, the threshold previously used to delineate copulations sufficiently long to allow insemination. The horizontal line is the best fit linear regression (see text).



## **Discussion:**

### **Experiment 1: Male sperm reserves**

Experiment 1 tested the hypothesis that male *A. remigis* experience short term reductions in their sperm reserves when the sex ratio is biased toward females and hence when mating opportunities are abundant. We predicted that males experiencing a female-biased sex ratio would have more copulations and less sperm in their seminal vesicles than males in an even sex ratio. Our results are clearly contrary to these predictions. Although males in a female-biased sex ratio did copulate more often than males in an even sex ratio, they did not have more successful copulations (copulations  $\geq 20$  min. that likely result in sperm transfer) or less sperm in their seminal vesicles.

As predicted, males kept with females had less sperm in their seminal vesicles than males kept without access to females. Males in the all-male treatment had more sperm in their seminal vesicles than males in either co-sex treatment. Therefore, either sperm numbers in seminal vesicles decline when males are allowed to copulate, sperm numbers in seminal vesicles increase when males are withheld from copulating, or both.

We also predicted that males would increase sperm production to replenish their sperm reserves and hence the number of sperm developing in the testes would be highest when the sex ratio is female biased and lowest when males are kept without mating opportunities. There was no indication of differences in sperm production in the testes between co-sex treatments but there were fewer immature sperm in the testes of males in the all-male treatment than males in either co-sex treatment. The treatment effect on sperm production in the testes probably reflects a feedback between number of sperm in



the seminal vesicles and sperm production. In the absence of females, males may have reduced their sperm production when their seminal vesicles became fully distended. By the same argument, males in the co-sex treatments may have increased their sperm production in response to reductions in the number of sperm stored in their seminal vesicles. Our data do not permit a distinction between these alternatives, and of course, both processes could be occurring.

The results presented here are surprising because males in the female-biased sex ratio had an increased frequency of copulation in comparison to males in the even sex ratio but had similar numbers of sperm in their seminal vesicles. We found no evidence that this was caused by changes in sperm production or male strategic sperm allocation (see experiment 2). Instead, our results indicate that males had similar numbers of sperm in their seminal vesicles in the two treatment the frequencies of successful copulations (copulations  $\geq 20$  min. that likely result in sperm transfer) did not differ.

### **Experiment 1: Female influence over copulation duration and sperm transfer**

Though frequency of successful copulation may explain the similarity in sperm reserves for males in co-sex treatments, it raises the question, why did the female-biased sex ratio have a high proportion of copulations that were unsuccessful. We argue that female reproductive tactics, as opposed to male reproductive tactics, best explain these unsuccessful copulations. We base this assertion on the costs and benefits of unsuccessful copulation for both sexes and the accordancy of this reasoning with the pattern of change in proportion of successful copulations by day in each treatment.

Male tactics to limit sperm depletion are unlikely to be the cause of unsuccessful copulations because unsuccessful copulations carry significant costs for males. Males have to find, lunge at, and struggle with a female to initiate copulation. Hence they entail most of the energetic costs and risks of predation or injury that successful copulations do (Watson et al 1998), but produce no fitness gains. Further, under natural mating conditions males typically have to engage in many attempted copulations before being able to achieve an intromission (Weigensberg and Fairbairn 1994; Eldakar 2010a). Given these time and energy costs, as well as the low probability of achieving an intromission with another female, it seems unlikely that a male would forgo insemination after achieving intromission.

Although the declining proportion of successful copulations over time in the female-biased sex ratio could reflect a shift in male mating tactics if males tended to refrain from ejaculating with non-virgin females (females were virgins at the start of the experiment), this is unlikely in *A. remigis*. Such a tactic would be unlikely to evolve in this species because males rarely come in contact with virgin females in natural populations. Both sexes mate daily over a prolonged mating season and so the vast majority of mating encounters are between non-virgin males and females. Further, transferring sperm to non-virgin females still significantly increases male paternity success (Rubenstein 1989; Vermette and Fairbairn 2002), so having gone through the time and energetic expense to secure a copulation, it is unlikely that a male would benefit by withdrawing without ejaculation. We do not know if male *A. remigis* can determine female mating status but evidence from another species of waterstrider (*Gerris lateralis*)

indicates that males do not alter number of sperm transferred based on female mating status (Arnqvist and Danielsson 1999a). Additional evidence against the hypothesis that the decline in proportion of successful copulations is a male mating tactic to preserve sperm comes from comparing the even-sex ratio and female-biased treatments. The proportion of successful copulations did not decline in the even-sex ratio treatment which suggests that the tactic is only manifest in highly female-biased sex ratios. Sex ratios average unity in the wild on the population level but range from male-biased to female-biased on scales of meters to tens of meters. Highly female-biased sex ratios such as we used are extremely unlikely in natural populations except as transient groupings on small spatial scales (Eldakar et. al., 2010b). The decline in proportion of successful copulations that we observed in our female-biased sex ratio treatment is not likely to be an expression of a male tactic for conserving sperm.

Alternatively female influence over copulation may explain unsuccessful copulations. We found that increasing the number of successful copulations does not translate into acquisition of more sperm or increased fertility for females. Even though females in the female-biased sex ratio had fewer successful copulations per female than those in the even sex ratio, they did not have a lower percentage of fertilized eggs or average less sperm in the gynatrial complex. Similarly, females had more sperm immediately after a single copulation (experiment 2) then after 9 days in co-sex pools (experiment 1). Rubenstein (1989) also found no difference in the number of fertilized eggs laid by females that copulated with one versus two males. In another species of waterstrider (*Gerris odontogaster*), females received no major genetic benefit from re-

mating (Arnqvist 1989) but we do not have similar data for *A. remigis*. These results indicate that the relationship between female fitness and number of successful copulations is likely to plateau at relatively low mating frequencies. The lack of correlation between number of copulations and fertility or number of sperm retained is not explained by females quickly depleting their sperm reserves as they fertilize their eggs. Our observations indicate that females maintain full fertility and fecundity for at least 15 days after isolation from co-sex pools and Rubenstein (1989) recorded females laying fertilized eggs for 30 days after double matings. It is also unlikely that sperm disappear because females are digesting ejaculates for nutrients as no evidence of this has been found in other species of waterstriders (Andersen 1982, Kaitala 1987, Arnqvist 1989). However, in another species of waterstrider (*Gerris lateralis*), female sperm dumping was suspected from behavioral observations and analyses to determine sources of variance for male postcopulatory reproductive success (Arnqvist and Danielsson 1999b). Thus, female *A. remigis* may also be dumping excess sperm between matings. Alternatively, limitations on number of sperm held in the spermathacea could result in excess sperm remaining in the gynatrial sac where it would be destroyed by subsequent copulations (Fairbairn et al 2003). The later of these may explain why females in experiment 1 (9-day trials) had fewer sperm in their gynatrial complex than females in experiment 2 (single copulation trials). Whatever the mechanism, it is clear that additional copulations do not significantly increase the number of sperm in the gynatrial complex or increase female fertility in *A. remigis*.

Given that females do not increase their fertility by engaging in more, long copulations, it is likely that the long copulations typical of *A. remigis* reflect other benefits of being *in copula*. Females *in copula* have increased energetic demands and predation risks (Fairbairn 1993, Watson et al 1998). However, they also experience less harassment from other males and so are able to increase their foraging efficiency (Wilcox 1984; Blanckenhorn et al 1995). Therefore, long copulations are beneficial to females when facing significant male harassment but are costly to females when facing little male harassment. Each female in the female-biased sex ratio received potentially low levels of harassment because there were three females present for every male and this would be expected to tip the balance in favor of fewer, shorter copulations. This assessment explains previous results of changes in copulation when waterstriders are placed in different social conditions. Clark (1987, 1988) found that copulations in *A. remigis* were significantly longer in duration in a male biased-sex ratio as opposed to a female-biased sex ratio. Krupa and Sih (1993) found that females were more likely to mate when in a male-biased sex ratio. Further, in the water strider, *Gerris odontogaster*, females were less reluctant to mate in male-biased sex ratio and this was consistent with a sexual conflict model whereby female behavior is explained by reluctance to mate based on level of harassment (Arnqvist, 1992).

The same arguments can explain why the proportion of successful copulations declined over time in the female-biased treatment but not in the even sex ratio treatment. At the beginning of the experiment all females were virgins that had been deprived of males. As expected, both co-sex treatments had a high proportion of copulations likely

resulting in sperm transfer early in the experiment. The treatment effect on proportion of successful copulations appears only later in the experiment when all of the females have mated sufficiently to acquire enough sperm for maximum fitness.

We have argued that variation in the proportion of copulations that are too short to permit sperm transfer is best explained by female influence over copulation duration. However, variation between treatments in duration of copulation for successful copulations is consistent with benefits to males. Successful copulations were shorter in the female-biased sex ratio than in the even sex ratio treatment. Vermette and Fairbairn (2002) showed that long copulations reduce male fitness (paternity success) because of a negative correlation between copulation duration and number of matings over a 4-day period. Shorter copulations when females are common and males are rare thus benefits males by permitting them to mate with more females. From the female perspective, copulations should be shorter in the female-biased sex ratio because harassment rates are lower and shortening duration reduces the energetic costs and predation risks of copulation. In the even-sex ratio, females benefit from longer copulations due to limiting harassment from males and increasing foraging efficiency. Our results on short successful copulations are thus consistent with adaptive plasticity in mating behavior of both sexes. However, longer copulations in the even-sex ratio and frequent unsuccessful copulations in the female-biased sex ratio seem to be best explained by adaptive plasticity in females.

The results of this experiment also suggest that unsuccessful copulations in *A. remigis* are due to sexual conflict rather than sexual selection for both the initiation and

duration of copulation. The initiation of copulation has been hypothesized in waterstriders to be due to males utilizing forced copulation or convenience polyandry (Arnqvist and Rowe, 2005). That is, females may allow males to initiate copulation because the costs of continuing to fight off copulation attempts are greater than the costs for being *in copula*. Thus, copulations could be initiated even when females receive few reproductive benefits from the copulation. Alternatively, it has been proposed that female waterstriders are evaluating males during mating struggles and that females sexually select males by allowing for the initiation of copulation (Eberhard, 1996). Our results cannot rule out sexual selection as indirect benefits have not been studied in *A. remigis*. However sexual selection theory would predict that females would become less choosy when males are rare and our results found the opposite; that is, copulations were less likely to result in sperm transfer when males were rare. Our results also indicate that females copulate even when they receive no sperm and this is best explained by male force, convenience polyandry, or female strategies to limit harassment. Male force, convenience polyandry, and female strategies to limit harassment may also explain why the minority of females that were not holding eggs and were unlikely to be laying eggs, still copulated even though they likely received no reproductive benefit (see Appendix 5). Throughout this discussion we have argued that females strongly influence duration of copulation and that this influence is highly tied to social conditions. We have also argued that additional sperm acquisition is rarely beneficial to non-virgin females and that females may shorten copulation duration to an extent such that sperm transfer does not occur. Therefore, males may initiate copulation counter to the female's best interests

while females may influence copulation duration counter to the male's best interests and the result of this is a high frequency of unsuccessful copulations.

### **Experiment 2: Number of sperm transferred**

Experiment 2 tested the hypothesis that males alter the number of sperm transferred to females based on sex ratio. We predicted that males would transfer less sperm to females in a female-biased sex ratio than in an even sex ratio. This was predicted based on (1) males preserving sperm for future copulations when in the female-biased sex ratio and hence having additional mating opportunities, and (2) males transferring more sperm in the even sex ratio to better compete with the additional males to fertilize eggs (additional sperm competition). This experiment also allowed us to determine if male strategic sperm allocation could explain the number of sperm in seminal vesicles in experiment 1. To test the hypothesis that males adjust the number of sperm transferred per mating in accordance with their perception of additional mating opportunities and the risk of sperm competition, we compared the number of sperm transferred on first copulation by males given access to three mature virgin females (female-biased sex ratio) to the number of sperm transferred by males in the presence of two mature virgin females and one additional male (even sex ratio with sperm competition). The relationship between number of sperm transferred and copulation duration was also studied because this could be a component of male strategic sperm allocation (i.e. males could have longer copulations in order to transfer additional sperm).



Contrary to our predictions, we found no evidence of male strategic sperm allocation based on sex-ratio, level of sperm competition or copulation duration.

This experiment did, however, confirm a delay of approximately 20 minutes between intromission and sperm transfer, a threshold previously observed by Rubenstein (1989) and Campbell and Fairbairn (2001). A demarcation of approximately 20 minutes is therefore a good indicator of successful versus unsuccessful copulations. Similarly, short copulations (less than 15 minutes) in *Gerris lateralis* are less likely to result in sperm transfer through copulations of shorter duration can result in sperm transfer in this species (Arnqvist and Danielsson, 1999a). In *A. remigis* the evolutionary reason for this delay until sperm transfer is not known and warrants further research. It has been commonly proposed in other species that delay until sperm transfer is a method of male or female choice of mates. For instance, Vahed et. al. (2011) found in a meta-analysis of 54 species of bushcricket that the latency to transfer sperm was related to the size of the spermatophore transferred and that this delay may allow males to assess mates before transferring a costly spermatophylx. Alternatively, a delay between intromission and sperm transfer could be due to a female imposed barrier whereby females are assessing males during copulation by such means as internal courtship (Eberhard, 1996). Another possibility is that latency until sperm transfer could be due to the time needed for males to prepare for sperm transfer and in some species this could be related to male condition (diet, size, Etc.; Gabor and Halliday 1997; Taylor and Yuval, 1999).

Male genital length and segment 8 length were related to the number of sperm in male seminal vesicles. Male genital length (Preziosi and Fairbairn 1996, 2000; Sih et. al.

2002; Bertin and Fairbairn 2005) and segment 8 length (Bertin and Fairbairn 2005) have previously been indicated to positively influence male mating success. Therefore, males with a larger genital length and segment 8 length tend to have more copulations. There may be developmental relationships between the amount of sperm held in the seminal vesicles and male genital length and segment 8 length. In *Drosophila* a genital disc gives rise to the genital ducts, oviducts, vas deferens, and other accessory components of internal and external genitalia (Bodenstein, 1994). Heteroptera have a genital disc. However, the developmental relationship between the genital disc and genital structures has not been determined in *A. remigis*. Further, the relationship between male genital length and segment 8 length to sperm in the seminal vesicles was not found in experiment 1.

Interestingly, males in experiment 2 only transferred on average 1,695 sperm or 11.5% of the total number of sperm in their seminal vesicles per copulation. Previous evidence that male *A. remigis* transfer only a small portion of their sperm reserves comes from mating trials using irradiated males. Vermette and Fairbairn (2002) found that under semi-natural conditions of multiple mating by both sexes and even sex ratios, irradiated males only begin losing their share of paternity after approximately 4 days and an average of four successful copulations. Thus, in that experiment, males carried enough stored sperm to fully support at least four inseminations.

Based on the average number of sperm transferred to females from a single copulation (experiment 2), unsuccessful copulations in experiment 1 were unlikely to be due to sperm depletion in males. Males in co-sex treatments averaged 6,967 sperm in the seminal vesicles at the conclusion of experiment 1 and this was more than enough sperm

to engage in additional successful copulations. The high proportion of unsuccessful copulations in the female-biased sex ratio and the large number of sperm in male seminal vesicles after 9-days in experiment 1 indicate that other factors besides male sperm depletion cause unsuccessful copulations to occur.

Transferring only a small proportion of sperm reserves may well be a male tactic of *A. remigis* to prepare for additional copulations and this could be due to the potential cost for producing the large sperm (5mm in length with 2.5 mm head; Miyata et al 2011) found in this species. In many *Drosophila* species with large sperm, males only transfer a small portion of their entire sperm supply to females and this could be due to males preparing for additional copulations (Pitnick and Markow 1994; Mery and Joly, 2002). Males of the tropical tephritid fruit fly (*Anastrepha oblique*) also transfer only a small portion of their entire sperm supply to females and this has been hypothesized to have evolved to limit depletion of male sperm reserves during this species short mating season when copulations are frequent (Perez-Staples and Aluja, 2006). Thus, species that have evolved in conditions with frequent mating opportunities and that have short mating seasons or sperm that are costly to produce, may use this tactic to maximize fitness across multiple copulations. This is in comparison to species that are not noted for having sperm that are costly to produce or having frequent copulations during a short mating season. In these species, number of sperm in ejaculates tends to significantly decrease in successive copulations (Jones, 1973; Sims, 1979; Synnott et. al., 1981; Leegwater-van der Linden and Tiggelman, 1984; Dewsbury and Sawrey, 1984; Rubolini, 2007).

Though males in this experiment transferred only a small portion of the sperm in their seminal vesicles, it is not known if male *A. remigis* in the wild carry less sperm and transfer a higher portion of sperm in their seminal vesicles per copulation. Males in this experiment were reared in laboratory conditions without predators and with an abundant food supply and they were separated from females for a significant length of time after eclosion. Thus, our males had time to produce many sperm and had few energetic demands. Nevertheless, it is interesting that males with many sperm in their seminal vesicles are either not able to or elect not to transfer a large portion of sperm from their seminal vesicles to females.

The main results of this experiment are that no relationships were found in *A. remigis* between number of sperm transferred and sex ratio, sperm competition risks or copulation duration. These results allowed us to rule out strategic sperm allocation as an explanation for sperm numbers in seminal vesicles in experiment 1.

The results of experiment 1 and 2 indicate that similar numbers of copulations resulting in sperm transfer best explain similar numbers of sperm in the seminal vesicles of males in the female-biased sex ratio and the even sex ratio in experiment 1. This was even though males in the female-biased sex ratio had more copulations; though many of these were < 20min. and unlikely to result in sperm transfer. There was no indication of strategic sperm allocation based on sex ratio. Based on cost-benefit analyses for each sex, the frequency of copulations unlikely to result in sperm transfer in the female-biased sex ratio is best explained by female influence over copulation duration and subsequently

sperm transfer. Therefore, female influence over copulation duration and sperm transfer in *A. remigis* needs to be considered in future studies on male sperm reserves.

**Future research:**

Future research on reproductive behavior in waterstriders should continue to examine the evolutionary significance of male harassment for the successful transfer of sperm. For instance, in the wild males are often found in "hot spots" (locations where large congregations of males are found; Krupa and Sih, 1993). Past research suggests that "hot spots" may increase female willingness to copulate due to increased male harassment and Krupa and Sih (1993) found a higher than average female mating frequency in these locations. The research presented here suggests that not only will "hot spots" encourage females to copulate but they may also increase the duration of copulation and the likelihood of sperm transfer. However, females may avoid or move from locations where they experience significant male aggression and this results in localized female-biased sex ratios (Eldakar et. al., 2010b). Eldakar et. al. (2010b) interrupted this result to explain how less aggressive males, in certain situations, could get more copulations than hyper-aggressive males. Evidence presented here indicates that males in a female-biased sex ratio may have more copulations as stated by Eldakar et. al. (2010b) but males in these sex ratios are also more likely to have copulations that do not result in sperm transfer. Understanding that changes to the likelihood of sperm transfer is part of the trade-offs for males in "hot-spots" and males in female-biased sex ratios is important for considering the evolution of social and spatial variation in this species.

The large size of the sperm in *A. remigis* could be related to the lack of sperm accumulation in females. Parker (1998) suggested that large sperm would only evolve when the transfer of additional sperm provided little reproductive advantages to males and when large sperm gave males significantly higher chances of fertilizing eggs. Across taxa, sperm length has been correlated with the size of the female sperm storage organ (Pitnick et. al., 2009) and one interpretation of this is that large sperm evolve to limit displacement (Briskie et. al., 1997). In *A. remigis*, females are either dumping a significant number of sperm or sperm is being damaged by males in future copulations. This may limit the advantages to male for transferring additional sperm to females and increase the benefits of transferring sperm that are difficult to displace (i.e. large sperm).

The results of these experiments suggest that female reproductive tactics can affect sperm transfer and that these reproductive tactics may evolve in response to costs and benefits of copulation other than those relating directly to transfer of sperm. This could be important in many species. Females often do not increase fertility with additional copulations (Bateman 1948; Andersson 1994; Arnqvist and Rowe 2005) and females copulate for many reasons unrelated to acquiring sperm. These reasons may include gaining food resources (Hunter et. al. 1993; Vahed and Gilbert 1996), gaining protection (Lovell-Mansbridge and Birkhead 1998), forced copulation (Gowaty and Buschhaus 1998; Arnqvist and Rowe 2005) or in rare cases, acquiring non-food resources (Hunter and Davis, 1998). Females also have costs of copulation that include energetic demands, time constants, and predation risks (Andersson 1994; Arnqvist and Rowe 2005). Future researchers studying male sperm reserves and reproductive tactics need to

be aware of changes to female costs and benefits of copulation with differing treatment regimes. As our experiments illustrate, understanding how female costs and benefits affect copulation behavior is important for understanding sperm transfer during copulation and ultimately the number of sperm in male sperm reserves.

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## **Appendix 1: Technique for extracting and counting sperm in the seminal vesicles**

### **General methodology:**

The sperm of *A. remigis* are extremely long (approximately 5 mm) and about half of that length is a long rigid head (approximately 2.5 mm; Miyata et al. 2010). The heads are thin and difficult to distinguish from the tails using standard light microscopy (Figure A1.1).

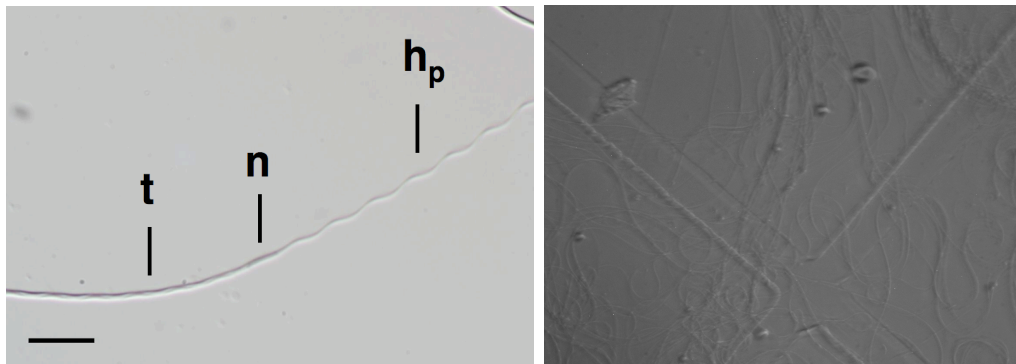


Figure A1.1 Top left: The components of the 500 micrometer long sperm. t=tail, n=nucleus, hp=helical portion. [courtesy of Haruhiko Miyata] Top right: Sperm viewed on a slide with a gridded cover slip under 200x magnification. Note that the long thread-like sperm overlap each other and extend out of the field of view. Image taken with the computer program Simple PCI 6.

These giant, elongated sperm are formed and ejaculated intertwined in bundles (Figure A1.2). Techniques designed to disperse the sperm for counting tend to break the sperm themselves, leading to overestimates of sperm numbers as pieces of sperm can be easily mistaken for whole sperm. These difficulties necessitated developing a method for counting sperm that would disperse the sperm and still allow individual sperm to be distinguished.



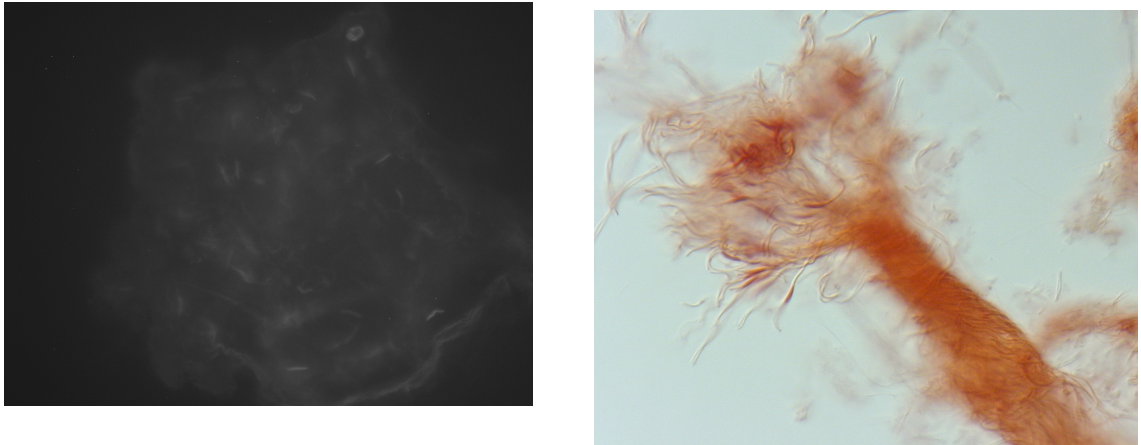


Figure A1.2. *Aquarius remigis* sperm clumping. Top left: A clump of sperm extracted from the seminal vesicles and after using a homogenizer. Individual sperm can be identified by their bright, elongated nuclei which have been stained with the fluorescence dye DAPI. Image taken with the computer program Simple PCI 6 at 630x magnification. Top right: A bundle of sperm extracted from the testes placed in a fixative of a modified Carnoy solution (3 absolute ethanol : 1 glacial acetic acid), and stained in aceto-orcein for 3-4 days. Image taken at 1600x magnification. [courtesy of Olga Kiseliova]

Hemocytometers allow for an even distribution of cells on a grid by using capillary action. However, this method proved unsatisfactory for *A. remigis* sperm because the large rigid heads and significant clumping prevented the sperm from being pulled by capillary action and evenly spread through a hemocytometer. It also proved impossible to count the sperm by homogenizing the seminal vesicle tissue and spreading the resulting suspension of cells evenly over a gridded slide. This method failed to fully disentangle the bundles of sperm, resulting in a clumped distribution on the slide. This

method could be used to count all the sperm on the slide but the significant clumping made it unsuitable for sub-sampling grid squares to estimate total sperm numbers. The same problem precluded subsampling by diluting of the sample and only counting sperm from subsamples of the total volume.

To resolve the problem of clumping, we used sonication rather than a homogenization to break apart the sperm bundles. Sonication breaks apart both the head and tail regions of sperm while not damaging the nucleus and thus, allows for sperm clumps to be broken apart. However this exacerbates the problem of distinguishing individual sperm from broken pieces of heads and tails in the resulting suspension. To ensure that individual sperm were reliably distinguished and counted only once, we counted sperm nuclei rather than whole sperm by visualizing them using fluorescence microscopy. This is a common methodology for estimating total sperm numbers (Locher and Baur 1997; Vermeulen et. al. 2008) and has been used in other species having sperm with long tails to avoid confusion when counting the sperm (Kullmann and Sauer 2009).

#### **Detailed protocol:**

To dissect out the seminal vesicles, males were pinned ventral side up through the upper thorax to a wax dissection plate. A lateral incision was made across the mid-thorax. From the midpoint of this incision a second incision was made along the ventral midline of the abdomen to the genitalia. The left and right ventral components of the exoskeleton were then folded back and pinned to the wax plate. This revealed the internal organs and loosened the exoskeleton around the seminal vesicles. The seminal vesicles were then

carefully pulled caudally out of the body by delicately pulling on the external genitalia (Figure A1.3). The external genitalia were then separated from the seminal vesicles with tweezers and a scalpel.



Figure A1.3. Top left: Male external genitalia before dissection at 125x magnification. These genitalia are partially inflated; the internal genitalia (vesica and dorsal plate) can be seen emerging from between the pygophore and proctiger (segments 9 and 10). Top right: Male seminal vesicles with attached external genitalia at 125x magnification after removal from the male's abdomen. Image captured using the program SPOTBasic and a Leica microscope hooked up to a computer.

Immediately upon dissection, the seminal vesicles were placed in a 1.5ml low retention micro-tube containing 1 ml of isotonic phosphate buffer saline (PBS; 2mM  $\text{NaH}_2\text{PO}_4$ , 10mM  $\text{Na}_2\text{HPO}_4$ , 135mM  $\text{NaCl}$ ). (Low retention tubes were used to prevent cells from sticking to the sides of the tube during centrifugation.) The tube was then placed in a beaker of ice to prevent overheating and sonicated with a Branson Sonifer 250. We sonicated each sample a minimum of three times at a power output of 2 for 10

seconds with one-minute breaks between rounds of sonication. If the seminal vesicles were not clearly broken apart (i.e. clumps of tissue could be viewed floating in the fluid) then additional rounds of sonication were applied. After sonication, tubes were allowed to sit in ice for 1 minute to cool and then centrifuged for 5 minutes at 10,000 rpm to concentrate the cells into a pellet. Using a pipette, the supernatant was transferred to another container. Initially we examined the extraneous fluid from five male dissections under a microscope to ensure that it contained no cells. For the experiments reported in this thesis, we checked the extraneous fluid from one out of every five males dissected. Cells were never found in the supernatant.

After removal of the supernatant, we added five micro-liters of a solution containing 3.5 micro-grams of 4',6-diamidino-2-phenylindole (DAPI) per ml of PBS to pellet in the tube and allowed this to sit for 15 minutes to dye the cell nuclei (Figure A1.2 and A1.4). After waiting 15 minutes, the pellet was then broken apart in the solution containing DAPI stain by sonicating at an output power of 1 for 10 seconds. Additional rounds of sonication (10 seconds in duration with one minute breaks between rounds of sonication) were applied if the pellet was not fully broken apart. The container was then placed into an Eppendorf Mixer 5432 and mixed for 10 minutes to aid in the even dispersal of sperm in the tube.

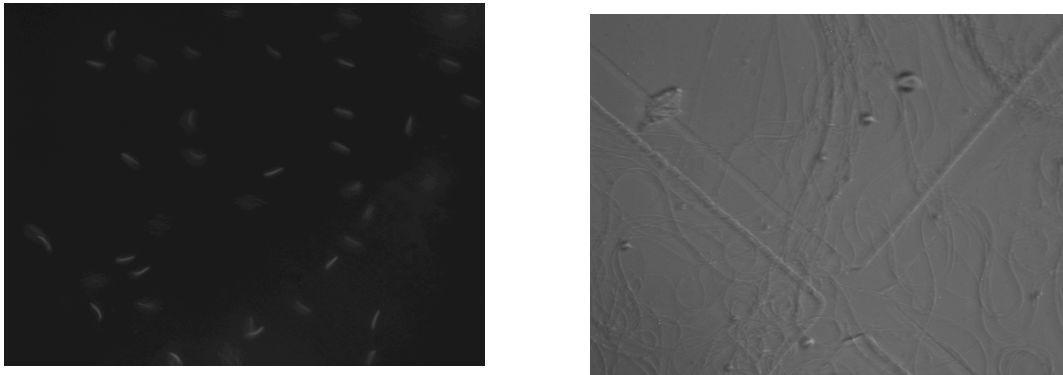


Figure A1.4. Top left: Dispersed sperm nuclei dyed with the florescence dye DAPI and viewed under 630x magnification. Notice the elongated shape of the sperm nuclei. Image taken with the computer program Simple PCI 6. Top right: The components of the 500 micrometer long sperm. t=tail, n=nucleus, hp=helical portion. [courtesy of Haruhiko Miyata] Bottom right: Sperm viewed on a slide with a gridded cover slip under 200x magnification. Note that the long thread-like sperm overlap each other and extend out of the field of view. Image taken with the computer program Simple PCI 6.

Using a low retention pipette tip, all the liquid in the tube (5 microliters) was transferred onto a gridded coverslip (described below), spread with a blunt metallic probe and allowed to dry. Low retention pipette tips were used as a precaution though there was no evidence that sperm were sticking to the sides of the pipette tip. Transferring the sperm directly onto the gridded coverslip rather than into the slide allowed for better images of the sperm and grid when focusing the microscope. Spreading sperm is commonly used in methods for estimating sperm numbers as it helps create an even distribution of sperm (e.g. Gage and Barnard 1996). Air-drying liquid on slides is commonly used for estimating sperm numbers (e.g. Arnqvist and Danielsson, 1999) as it

prevents cells from moving and thus allows for easier counting of cells. The cover slips were made from Ibidi brand sterile and untreated gridded micro-dishes marked with 932 complete 500 x 500  $\mu\text{m}$  squares. The Ibidi dish is thin (180 micrometers thick) allowing for high resolution when using high magnification, emits extremely low autofluorescence, and has a high optical quality (Ibidi micro-dish instruction manual; 2010). Care was taken to transfer fluid containing sperm to the side of the coverslip that normally comes into contact with cells (when the micro-dishes are used as dishes). This side of the coverslip was pushed onto the slide with the dried fluid between the coverslip and slide. The coverslip was firmly attached to the slide using transparent adhesive tape. Gridded slides are commonly used to estimate sperm numbers (Garcia-Gonzalez and Gomendio 2004; Perez-Staples and Aluja 2006). However, the squares within the micro-dish grid were smaller than many commercially available gridded cover slips and the small size of the grid aided in counting sperm numbers under high magnification. The slide was viewed under an inverted fluorescence microscope and the sperm nuclei were counted using 630x (Figure A1.4). Lower magnifications (200x and 400x) were also used in conjunction with the computer program Simple PCI 6 to view sperm cells.

Sperm nuclei of *A. remigis* can be distinguished from other cell nuclei because they are distinctly elongated whereas other cell nuclei are circular (Figures A1.4 and A1.5). This was verified by placing a male's seminal vesicles on a slide, using a solution containing the fluorescence dye DAPI to dye cell nuclei, and then activating cells by adding approximately 10 micro-liters of a mixture containing 20 micrograms of Trypsin per 1 ml of PBS. Trypsin is known to activate sperm cells in many species and has been

found to activate sperm cells of *A. remigis* (H. Miyata, personal communication 2010). Cells were then viewed under the fluorescence microscope. Only cells with elongated nuclei were activated by Trypsin. Thus, cells with rounded nuclei were either not sperm cells or not fully mature sperm cells ready for transfer to females.

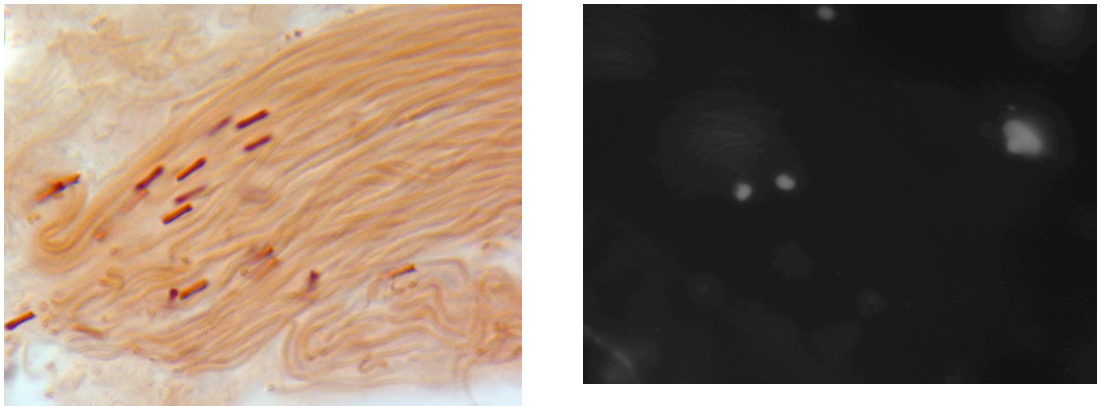


Figure A1.5. Top left: *Aquarius remigis* sperm showing elongated sperm nuclei, courtesy of Olga Kiseliova. The long, thickened strands are sperm heads anterior to the nuclei. Sperm cells were placed in a fixative of a modified Carnoy solution (3 absolute ethanol : 1 glacial acetic acid), and stained in aceto-orcein for 3-4 days. Image taken at 1600x magnification. Top right: Non-sperm cell or undeveloped sperm cell nuclei dyed with DAPI and under 630x magnification. These cells have round nuclei (compare with Figure 2.4). Image taken with the computer program Simple PCI 6.

The number of sperm nuclei found in each quadrat (row) of the cover-slip grid was recorded on a printed diagram of the grid (Figure A1.6). This was initially used to

find the total number of sperm for the entire slide and thus, the total number of sperm in the male's seminal vesicles. Complete counts of sperm in quadrates were later used to establish a sub-sampling technique to estimate total sperm numbers (see Appendix 3).

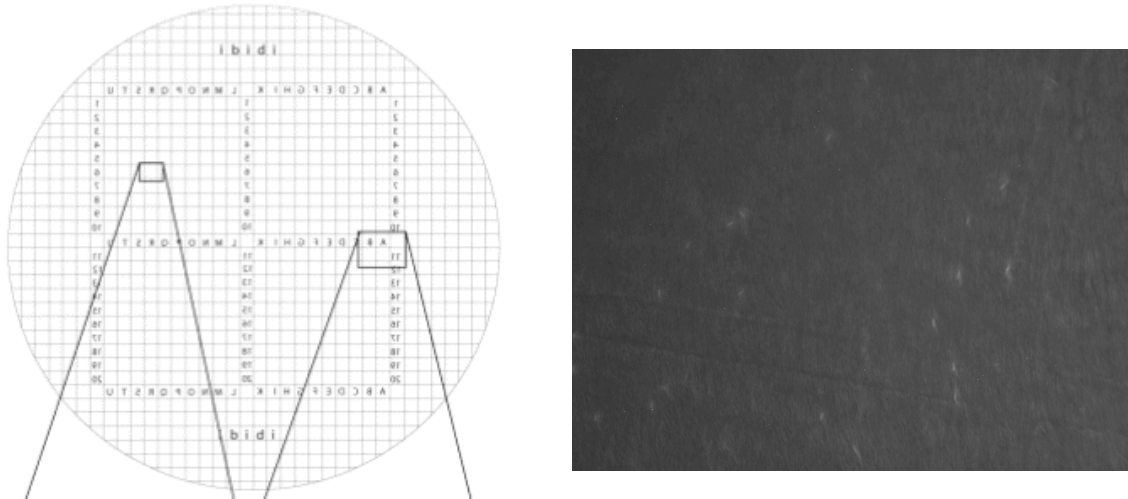


Figure A1.6. Top left: Diagram of the gridded micro-dish with 960, 500 micrometer x 500  $\mu$ m squares. Top right: The sperm nuclei and grid viewed at 200x magnification. The nuclei of cells were dyed with fluorescence dye DAPI.



## **Appendix 2: Technique for extracting and counting sperm in the gynatrial complex**

### **General methodology:**

The methodology employed was similar to that of the seminal vesicles of males. The gynatrial complex was removed and sonicated to break apart clumps of sperm. DAPI was used to stain the nuclei of sperm cells and the sperm cells were smeared on a gridded cover-slip for counting. The gynatrial complex broke apart easily with sonication and thus fewer rounds of sonication were required than necessary for the seminal vesicles of males (see below for more details).

### **Detailed description of protocol:**

Females were pinned dorsal side up on a wax dissection plate. Incisions were made across the top of the abdomen and down the sides of the abdomen toward the posterior end. The internal organs of the female were exposed by gently removing the dorsal exoskeleton of the abdomen, The gut was then removed revealing the gynatrial complex (Figure A2.1, left panel). The surrounding tissue was separated from the gynatrial complex using tweezers and a scalpel. Using tweezers, the gynatrial complex was removed and placed in a low retention tube with 5 micro-liters from a stock solution containing 3.5 micro-grams DAPI per ml of PBS (Figure A2.1, right panel).

Damage to the gynatrial sac and spermatheca could cause sperm to be lost from these organs (typical protocols for sperm counting in other species involve splitting open these organs and spilling the sperm held within these organs onto a slide; e.g. Arnqvist

and Danielsson 1999). To verify that this was not occurring, we removed the gynatrial complexes of 11 females and placed them on individual slides.

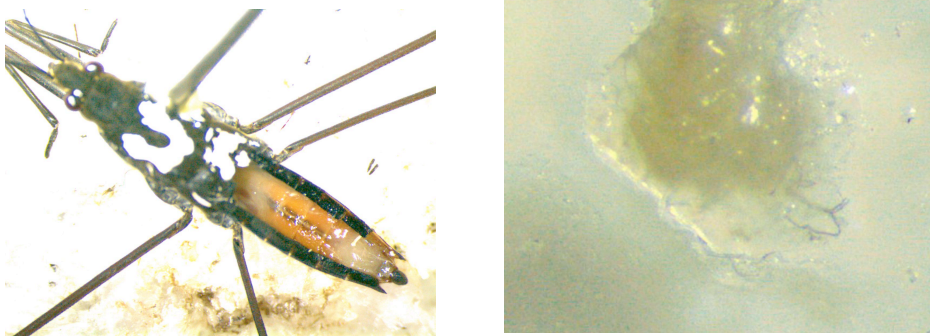


Figure A2.1. Top left: the female with the intestines removed and the gynatrial sac and spermatheca remaining. Image taken at 50x magnification. Top right: The gynatrial sac and spermatheca viewed at 125x magnification after removal from the female)

A coverslip was then delicately squashed down over the gynatrial complex. The slide was placed under a light microscope to examine damage to the fecundation canal, fecundation pump, spermatheca, and the gynatrial sac as a whole (Figure A2.2). Ten samples were undamaged and one had slight damage to the spermatheca but was otherwise intact. Therefore, the dissection technique was not damaging the gynatrial complex and was appropriate for the experiment.

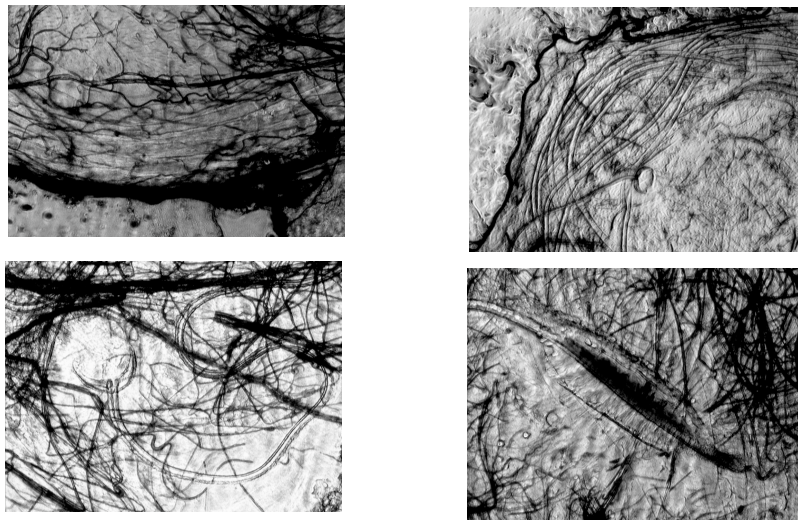


Figure A2.2: Examples of images of the gynatrial sac and spermatheca used to verify that dissections were not damaging the gynatrial sac and spermatheca. All images taken with the computer program Scion Image. Top left: the spermatheca viewed under 100x magnification. Top right: the spermatheca and fecundation canal viewed under 40x magnification. Bottom left: the fecundation canal viewed under 100x magnification. Bottom right: the fecundation pump viewed under 100x magnification.

After removal, the gynatrial complex was placed in a tube containing 5  $\mu$ L of 3.5 micro-grams of DAPI per ml of PBS. The tube was put in a container of ice and sonicated for 10 seconds at power output 1. Sonication was repeated until the gynatrial complex was fully broken apart (i.e., no clumps of tissue could be seen with the naked eye). Unlike the male seminal vesicles, the gynatrial sac and spermatheca did not scatter over the tube but instead quickly broke down and mixed with the solution. Further, the gynatrial complex contains relatively few sperm. Because of this, only one sonication was generally needed for tissue and sperm clumps to be broken apart. The tube was then placed in an Eppendorf Mixer 5432 for 10 minutes to ensure that the solution was

adequately mixed. It was then transferred to an Ibidi brand gridded cover-slip made from an Ibidi micro-dish, using a pipette with a low retention pipette tip. A blunt metal probe was used to spread the contents of the solution evenly over the cover-slip. The solution was then allowed to air dry, and the grid was firmly adhered to a slide using transparent adhesive tape. Sperm numbers were then estimated by a sub-sampling technique (see Appendix 3).

### **Appendix 3: Technique for estimating sperm numbers from a sub-sample**

#### **Sub-sampling technique:**

To establish a protocol for estimating sperm numbers by sub-sampling, four males and three females were dissected. All individuals used were wild caught individuals that had been separated by sex for eight weeks. To get a protocol accurate for sub-sampling a range of sperm numbers, we dissected two males shortly after they had copulated and two males that had not been allowed to copulate after being captured. The three females were dissected within 20 minutes after copulation.

We counted all sperm within each male and female and recorded the totals for each row (quadrate) of their respective gridded cover-slips. Sperm nuclei that were on a line between rows of the grid were always counted for the row above the line to limit edge effects (the over counting of specimens that reside in multiple quadrates). After examining prepared slides, it became clear that males only transferred a small portion of the sperm in their seminal vesicles to females and this was further examined in experiment 2. The two males that had copulated had 13,438 and 27,357 sperm in their seminal vesicles. Yet, the females they copulated with had only 1,262 and 1,335 sperm in their gynatrial complexes. The other female (who had copulated with a male that was not dissected) had 2,077 sperm. The two males that had not copulated had 24,643 and 20,471 sperm in their seminal vesicles.

After counting all of the sperm from these males and females and examining the distribution of sperm on each row of the grid, a protocol was established for estimating sperm numbers from a subsample of rows on the grid. Each row, consisting of a linear

series of 500  $\mu\text{m}$  by 500  $\mu\text{m}$  squares, was considered to be a separate quadrat, and there were 34 rows (quadrats) on each coverslip. Each square could easily be viewed with the 20x magnification and a 10x ocular lens (200x total magnification) and thus, locations on the grid could be easily found. Further, it was easy to scan squares within this grid with a 63x magnification with a 10x ocular lens (630x total magnification). This higher magnification allowed for a closer examination of cells when needed (i.e. when determining if a cell nucleus was elongated or round). Additionally, these quadrats extended from the left to right side of the circular cover slip and moving the slide from left to right could easily be done. Finally, Krebs (1999) recommends using elongated quadrats as they capture potential heterogeneity of samples better than circular or square quadrats on a per area basis.

We used Weigert's method (Krebs 1999) to determine the minimum number of quadrats needed to maximize the benefit of sampling but also to limit the investment in time per sample. Weigert's method does this by finding values with the lowest or near lowest value from the following equation:

$$K = (\text{Relative cost}) \times (\text{relative variance})$$

According to Krebs (1999), relative time can be substituted for relative cost if time is the main issue of sampling. Thus the equation used for determining the number of quadrats to sample is as follows:

$$K = (\text{Relative time}) \times (\text{relative variance})$$

This equation can further be broken down. Relative time is equivalent to time to sample  $n$  quadrats ( $T_{\text{sample}}$ ) divided by the minimum time to sample 1 quadrat

( $T_{\text{minimum}}$ ). Relative variance is equivalent to variance found among estimates from sampling  $n$  quadrates ( $V_{\text{sample}}$ ) divided by the variance found among estimates from sampling 34 quadrates with replacement ( $V_{\text{minimum}}$ ). Therefore, the equation becomes the following:

$$K = (T_{\text{sample}} / T_{\text{minimum}}) \times (V_{\text{sample}} / V_{\text{minimum}})$$

As each additional quadrate takes an equal amount time to sample as past quadrates then the time to sample  $n$  quadrates ( $T_{\text{sample}}$ ) divided by the time to sample 1 quadrate ( $T_{\text{minimum}}$ ) is equivalent to number of quadrates sampled ( $N_{\text{quadrates}}$ ). Thus, the equation further breaks down into the following equation (for the purposes of this protocol):

$$K = (N_{\text{quadrates}}) \times (V_{\text{sample}} / V_{\text{minimum}})$$

For each sample, two to 34 quadrates were then randomly sampled, with replacement, and used to make an estimation of the total number of sperm on the slide. This was repeated 1,000 times with a bootstrapping method (i.e. 2 quadrates were randomly sampled 1,000 times and used to make estimates of total sperm numbers per sample, 3 quadrates were randomly sampled 1,000 times and used to make estimates of total sperm numbers per sample, etc.). Bootstrapping with replacement is typically used for estimating population parameters (Sokal and Rohlf 1995) - in this case the total number of sperm on the slide. When making estimates of the total number of sperm on the slide, each square was equivalent and, thus, a sampled quadrate with few squares was less influential for the estimate than a sampled quadrate with many squares. By bootstrapping, an average sampling variance and confidence interval was determined for

the estimates of total number of sperm on slides derived from samples of 2 to 34 quadrates.

The relative variance for sampling  $n$  quadrates was determined by the variance among the 1,000 estimates of total sperm numbers from sampling  $n$  quadrates divided by the variance among the 1,000 estimates of total sperm numbers from sampling 34 quadrates (which was typically the lowest variance found).

Weigert's value ( $K$ ) declined precipitously as  $n$  increased from 2 to 7 quadrats but then leveled off (figure A3.1). Although very modest gains were made for increasing  $n$  up to 20, the increased accuracy in the estimate would not have justified the additional time allocated per sample, given the number of samples to be processed. We therefore elected to use  $n = 7$ . However, to better account for heterogeneity of sperm density across the slide we spaced quadrates evenly across the coverslip (figure A3.1, left panel) rather than selecting them at random. These rows had an average number of squares of 30.857 and accounted for approximately 23.16% of the total area of the slide.



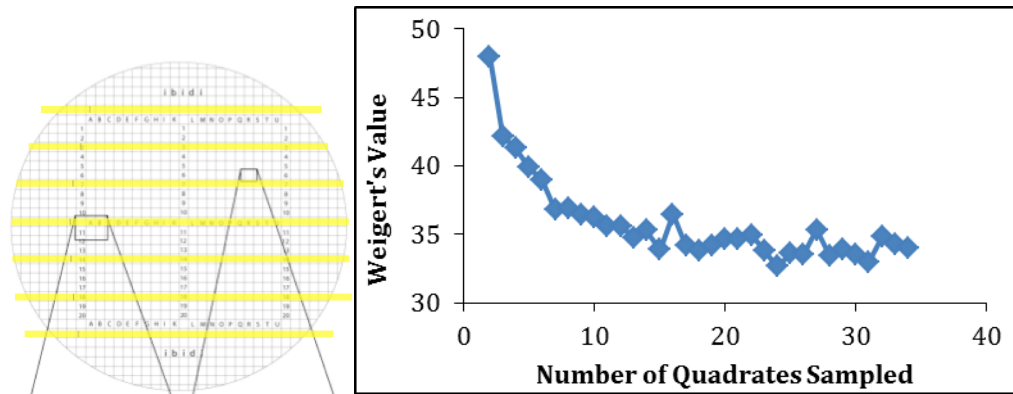


Figure A3.1. Top left: The 7 rows to be sampled. These rows were approximately equally spaced across the grid. Top right: The average Weigert's value of all 7 samples as a function of the number of rows sampled.

The bootstrap technique for sub-sampling 7 rows allowed for upper and lower confidence intervals to be calculated. Average confidence intervals were found by expressing the confidence interval as a proportion of the mean and then averaging the upper 95% confidence intervals of all 7 slides and by averaging the lower 95% confidence intervals of all 7 slides. From this, it was found that estimates of sperm numbers on slides have an upper 95% confidence interval of 14.8% above the mean and a lower 95% confidence interval of 11% below the mean.

To estimate the realized accuracy for sub-sampling seven evenly spaced quadrates, we first estimated the total number of sperm on each slide. We did this by dividing the total sperm count for all seven quadrates by the proportion of the total area of the slide that they included (0.2316). The realized estimates averaged 3.94% deviation from the actual number of sperm on the slides. The reason for the increased accuracy between the predicted sperm counts by randomly sampling seven rows using the

bootstrap method and the realized accuracy when sampling seven approximately evenly dispersed rows across the slide is likely due to evenly dispersed rows better accounting for heterogeneity of samples. This is particularly important when considering the occasional clumping of sperm even after sonication and other techniques to increase sample homogeneity.

**Concluding remarks on sperm counting methodology:**

This methodology established for counting sperm numbers in males and females of *A. remigis* provides a way to study sperm allocation and production in an organism with unusual and large sperm (5 mm in length, 2.5mm acrosome) and with sperm that becomes clumped. This method might be particularly useful for other species of *Gerris* where little is known about sperm production and transfer.

#### **Appendix 4: Relationships of body size to sperm abundance, frequency of copulation and number of sperm transferred to females**

A PCA was conducted to determine PC1. The length of the head, thorax, abdomen, and genitals were used in this PCA and the PCA used a correlation matrix. The factor loadings for these body components are listed in table A4.1. The eigenvalues of the first 4 principal components are listed in table A4.2.

To determine if male size influenced mating behavior or sperm abundance in experiment 1, we regressed estimates of male body size and the sizes of various body and genital components (PC1, total length, length of soma, length of genital segments, and length of segment 8) on total number of copulations per male, total number of copulations  $\geq 20$  min per male, and sperm abundance in the male seminal vesicles and testes. Initially a full linear regression model was run for each dependent variable with the independent variables body component, replicate, treatment, and their interactions. Neither 2- nor 3-way interactions were found to be significant, so they were removed from the models leaving only the additive main effects. The regression was then run a second time without the size variable. The two regression models were compared using the  $R^2$  values. This allowed us to determine the significance of the size variable on the dependent variable of interest. The regressions for sperm abundance include all three treatments, while those for frequency of copulation include only the two co-sex treatments. The results are given in tables A4.3– A4.7.

Table A4.1: Factor loading values for the body components used to determine PC1. A correlation matrix was used for the PCA analysis.

Factor loading for PC1 body components		
Body component/ Sex	Male	Female
LHead	0.859	0.848
LThorax	0.829	0.956
LAbd	0.900	0.889
Lgenitals	- 0.081	- 0.897

Table A4.2: The eigenvalues of the first 4 principal components as determined from a PCA analysis. PC1 was used for analysis.

Eigenvalues of principal components		
Principal component number / Sex	Male	Female
PC1	2.243	3.226
PC2	1.057	0.392
PC3	0.436	0.261
PC4	0.264	0.121

Table A4.3: Linear regressions of the effect of male body size variables, treatment and replicate on frequency of copulations for the males in experiment 1

Size variable	Significance of regression coefficients (probabilities)			Full model	Model excluding size variable	Significance of the size effect
	Size variable	Treatment	Replicate	F <sub>(df)</sub> , P, R <sup>2</sup>	F <sub>(df)</sub> , P, R <sup>2</sup>	F <sub>(df)</sub> , p
PC1	0.915	0.001	0.093	F <sub>(1,20)</sub> = 6.036 p = 0.004, R <sup>2</sup> = 0.477	F <sub>(1,21)</sub> = 9.495 p = 0.001 R <sup>2</sup> = 0.475	F <sub>(1,20)</sub> = 0.102 p = 0.999
LTotal	0.915	0.002	0.100	F <sub>(1,20)</sub> = 6.036, p = 0.004, R <sup>2</sup> = 0.476	F <sub>(1,21)</sub> = 9.495 p = 0.001 R <sup>2</sup> = 0.475	F <sub>(1,20)</sub> = 0.038 p = 0.847
LSoma	0.823	0.001	0.108	F <sub>(1,20)</sub> = 6.061 p = 0.004 R <sup>2</sup> = 0.476	F <sub>(1,21)</sub> = 9.495 p = 0.001 R <sup>2</sup> = 0.475	F <sub>(1,20)</sub> = 0.038 p = 0.847
LGenital*	0.895	0.005	0.008	F <sub>(1,11)</sub> = 8.873 p = 0.003 R <sup>2</sup> = 0.708	F <sub>(1,12)</sub> = 14.486 p = 0.001 R <sup>2</sup> = 0.707	F <sub>(1,11)</sub> = 0.034 p = 0.883
LSeg8	0.708	0.001	0.150	F <sub>(1,20)</sub> = 6.120, p = 0.004 R <sup>2</sup> = 0.479	F <sub>(1,21)</sub> = 9.495 p = 0.001 R <sup>2</sup> = 0.475	F <sub>(1,20)</sub> = 0.153 p = 0.700

\* Some values excluded due to genitals being extended and thus not providing an accurate representation of genital length.

Table A4.4: Linear regressions of the effect of male body size variables, treatment and replicate on frequency of copulations  $\geq 20$  min for the males in experiment 1.

Size variable	Significance of regression coefficients (probabilities)			Full model	Model excluding size variable	Significance of the size effect
	Size variable	Treatment	Replicate	$F_{(df), P, R^2}$	$F_{(df), P, R^2}$	$F_{(df), P}$
PC1	0.133	0.850	0.853	$F_{(1,20)} = 0.980$ , $p = 0.422$ $R^2 = 0.110$	$F_{(1,21)} = 0.227$ $P = 0.799$ $R^2 = 0.021$	$F_{(1,20)} = 2.000$ $p = 0.173$
LTotal	0.140	0.996	0.419	$F_{(1,20)} = 0.946$ $p = 0.437$ $R^2 = 0.124$	$F_{(1,21)} = 0.227$ $p = 0.799$ $R^2 = 0.021$	$F_{(1,20)} = 2.352$ $p = 0.141$
LSoma	0.071	0.944	0.349	$F_{(1,20)} = 1.383$ $p = 0.277$ , $R^2 = 0.172$	$F_{(1,21)} = 0.227$ $p = 0.799$ $R^2 = 0.021$	$F_{(1,20)} = 3.647$ $p = 0.071$
LGenital*	0.916	0.857	0.725	$F_{(1,11)} = 0.073$ $p = 0.973$ , $R^2 = 0.020$	$F_{(1,12)} = 0.113$ $P = 0.894$ $R^2 = 0.018$	$F_{(1,11)} = 0.019$ $p = 0.981$
LSeg8	0.717	0.631	0.782	$F_{(1,20)} = 0.190$ $p = 0.902$ , $R^2 = .028$	$F_{(1,21)} = 0.227$ $p = 0.799$ $R^2 = 0.021$	$F_{(1,20)} = 0.144$ $p = 0.708$

\* Some values excluded due to genitals being extended and thus not providing an accurate representation of genital length.

Table A4.5: Linear regressions of the effect of male body size variables, treatment and replicate on number of sperm in the seminal vesicles for the males in experiment 1.

Size variable	Significance of regression coefficients (probabilities)			Full model	Model excluding size variable	Significance of the size effect
	Size variable	Treatment	Replicate	$F_{(df), P, R^2}$	$F_{(df), P, R^2}$	$F_{(df), P}$
PC1	0.928	0.002, 0.001	0.983	$F_{(1,31)} = 3.980$ $P = 0.010$ $R^2 = 0.340$	$F_{(1,33)} = 5.474$ $p = 0.004$ $R^2 = 0.339$	$F_{(2, 31)} = 0.094$ $p = 0.999$
LTotal	0.975	0.002, 0.001	0.972	$F_{(1,31)} = 3.996$ , $p = 0.010$ $R^2 = 0.340$	$F_{(1,33)} = 5.474$ $p = 0.004$ $R^2 = 0.339$	$F_{(2, 31)} = 0.024$ $p = 0.976$
LSoma	0.830	0.002, 0.001	0.989	$F_{(1,31)} = 3.995$ , $p = 0.010$ $R^2 = 0.340$	$F_{(1,33)} = 5.474$ $p = 0.004$ $R^2 = 0.339$	$F_{(2, 31)} = 0.023$ $p = 0.977$
LGenital*	0.425	0.026, 0.003	0.470	$F_{(1,19)} = 3.138$ , $p = 0.040$ $R^2 = 0.411$	$F_{(1,21)} = 4.032$ $P = 0.022$ $R^2 = 0.389$	$F_{(2, 19)} = 0.355$ $p = 0.706$
LSeg8	0.590	0.002, 0.001	0.903	$F_{(1,31)} = 4.089$ , $p = 0.009$ $R^2 = 0.345$	$F_{(1,33)} = 5.474$ $P = 0.004$ $R^2 = 0.339$	$F_{(2, 31)} = 0.142$ $p = 0.868$

\* Some values excluded due to genitals being extended and thus not providing an accurate representation of genital length.



Table A4.6: Linear regressions of the effect of male body size variables, treatment and replicate on the ranked estimate of number of immature sperm in the testes for males in experiment 1.

Size variable	Significance of regression coefficients (probabilities)			Full model	Model excluding size variable	Significance of the size effect
	Size variable	Treatment	Replicate	F <sub>(df)</sub> , P, R <sup>2</sup>	F <sub>(df)</sub> , P, R <sup>2</sup>	F <sub>(df)</sub> , P
PC1	0.825	0.024, 0.026	0.371	F <sub>(1,31)</sub> = 3.980 p = 0.010 R <sup>2</sup> = 0.339	F <sub>(1,33)</sub> = 2.949 p = 0.048 R <sup>2</sup> = 0.217	F <sub>(2, 31)</sub> = 0.125 p = 0.9984
LTotal	0.894	0.024, 0.026	0.311	F <sub>(1,31)</sub> = 2.148, p = 0.098 R <sup>2</sup> = 0.218	F <sub>(1,33)</sub> = 2.949 P = 0.048 R <sup>2</sup> = 0.217	F <sub>(2, 31)</sub> = 0.001 p = 0.999
LSoma	0.881	0.024, 0.025	0.316	F <sub>(1,31)</sub> = 2.150, p = 0.098 R <sup>2</sup> = 0.218	F <sub>(1,33)</sub> = 2.949 p = 0.048 R <sup>2</sup> = 0.217	F <sub>(2, 31)</sub> = 0.001 p = 0.999
LGenital*	0.121	0.278, 0.597	0.911	F <sub>(1,19)</sub> = 1.070, p = 0.400 R <sup>2</sup> = 0.092	F <sub>(1,21)</sub> = 0.501 p = 0.686 R <sup>2</sup> = 0.073	F <sub>(2, 19)</sub> = 0.199 p = 0.821
LSeg8	0.569	0.025, 0.023	0.352	F <sub>(1,31)</sub> = 2.248, p = 0.087 R <sup>2</sup> = 0.225	F <sub>(1,33)</sub> = 2.949 p = 0.048 R <sup>2</sup> = 0.217	F <sub>(2, 31)</sub> = 0.16 p = 0.853

\* Some values excluded due to genitals being extended and thus not providing an accurate representation of genital length.

Table A4.7: Linear regressions of the effect of male body size variables, treatment and replicate on the ranked estimate of number of mature sperm in the testes for males in experiment 1.

Size variable	Significance of regression coefficients (probabilities)			Full model	Model excluding size variable	Significance of the size effect
	Size variable	Treatment	Replicate	$F_{(df), P, R^2}$	$F_{(df), P, R^2}$	$F_{(df), P}$
PC1	0.331	0.085, 0.272	0.555	$F_{(1,31)} = 0.984$ , $p = 0.431$ , $R^2 = 0.113$	$F_{(1,33)} = 1.279$ $p = 0.298$ $R^2 = 0.107$	$F_{(2, 31)} = 0.09$ $p = 0.898$
LTotal	0.913	0.089, 0.262	0.508	$F_{(1,31)} = 0.933$ , $p = 0.458$ $R^2 = 0.108$	$F_{(1,33)} = 1.279$ $p = 0.298$ $R^2 = 0.107$	$F_{(2, 31)} = 0.001$ $p = 0.999$
LSoma	0.706	0.079, 0.276	0.540	$F_{(1,31)} = 0.970$ , $p = 0.438$ $R^2 = 0.111$	$F_{(1,33)} = 1.279$ $p = 0.298$ $R^2 = 0.107$	$F_{(2, 31)} = 0.070$ $p = 0.933$
LGenital*	0.495	0.175, 0.954	0.305	$F_{(1,19)} = 1.023$ , $p = 0.422$ $R^2 = 0.185$	$F_{(1,21)} = 1.236$ $p = 0.324$ $R^2 = 0.163$	$F_{(2, 19)} = 0.256$ $p = 0.777$
LSeg8	0.856	0.082, 0.264	0.499	$F_{(1,31)} = 0.939$ $p = 0.455$ $R^2 = 0.108$	$F_{(1,33)} = 1.279$ $p = 0.298$ $R^2 = 0.107$	$F_{(2, 31)} = 0.001$ $p = 0.999$

\* Some values excluded due to genitals being extended and thus not providing an accurate representation of genital length.

To determine if male size influenced the number of sperm transferred to females in a single copulation, we performed the same regression analyses for males in experiment 2, using the number of sperm transferred to females and the number of sperm remaining in the male seminal vesicles as dependent variables. These results are shown in tables A4.8 and A4.9.

Table A4.8: Linear regressions of the effect of male body size variables, treatment and replicate on the number of sperm in the male seminal vesicles following a single copulation in experiment 2. Results are given for all copulations and also for only copulations lasting  $\geq 20$  min.

Size variable	Significance of regression coefficients (probabilities)	Full model	Model excluding size variable	Significance of the size effect	
All copulations					
	Size variable	Treatment	F <sub>(df), P, R<sup>2</sup></sub>	F <sub>(df), P, R<sup>2</sup></sub>	F <sub>(df), P</sub>
PC1	0.488	0.752	F <sub>(1,14)</sub> = 0.292, p = 0.751 R <sup>2</sup> = 0.040	F <sub>(1,15)</sub> = 0.080, p = 0.781, R <sup>2</sup> = 0.005	F <sub>(1,14)</sub> = 0.690 p = 0.562
LTotal	0.744	0.759	F <sub>(1,14)</sub> = 0.093 p = 0.912 R <sup>2</sup> = 0.013	F <sub>(1,15)</sub> = 0.080 P = 0.781 R <sup>2</sup> = 0.005	F <sub>(1,14)</sub> = 0.113 p = 0.742
LSoma	0.407	0.692	F <sub>(1,14)</sub> = 0.405 P = 0.674 R <sup>2</sup> = 0.055	F <sub>(1,15)</sub> = 0.080 p = 0.781 R <sup>2</sup> = 0.005	F <sub>(1,14)</sub> = 0.748 p = 0.402
LGenital	0.009	0.382	F <sub>(1,14)</sub> = 4.658 p = 0.028 R <sup>2</sup> = 0.400	F <sub>(1,15)</sub> = 0.080, P = 0.781, R <sup>2</sup> =0.005	F <sub>(1,14)</sub> = 9.217 p = 0.009
LSeg8	0.003	0.548	F <sub>(1,14)</sub> = 6.223 p = 0.012 R <sup>2</sup> = 0.471	F <sub>(1,15)</sub> = 0.080 p = 0.781 R <sup>2</sup> = 0.005	F <sub>(1,14)</sub> = 12.333 p = 0.003
Copulations $\geq 20$ min					
PC 1	0.303	0.950	F <sub>(1,11)</sub> = 0.598, p = 0.567 R <sup>2</sup> = 0.098	F <sub>(1,12)</sub> = 0.026, p = 0.875 R <sup>2</sup> = 0.002	F <sub>(1,11)</sub> = 1.18 p = 0.301
LTotal	0.547	0.935	F <sub>(1,11)</sub> = 0.205, p = 0.818 R <sup>2</sup> = 0.036	F <sub>(1,12)</sub> = 0.026, p = 0.875 R <sup>2</sup> = 0.002	F <sub>(1,11)</sub> = 0.388 p = 0.546
LSoma	0.294	0.986	F <sub>(1,11)</sub> = 0.621 p = 0.555 R <sup>2</sup> = 0.102	F <sub>(1,12)</sub> = 0.026, P = 0.875 R <sup>2</sup> = 0.002	F <sub>(1,11)</sub> = 1.225 p = 0.292
LGenital	0.051	0.569	F <sub>(1,11)</sub> = 2.423 p = 0.134 R <sup>2</sup> = 0.306	F <sub>(1,12)</sub> = 0.026, P = 0.875 R <sup>2</sup> = 0.002	F <sub>(1,11)</sub> = 4.818 p = .051
LSeg8	0.027	0.518	F <sub>(1,11)</sub> = 3.277 p = 0.076 R <sup>2</sup> = 0.373	F <sub>(1,12)</sub> = 0.026 p = 0.875 R <sup>2</sup> = 0.002	F <sub>(1,11)</sub> = 6.509 p = 0.027

Table A4.9: Linear regressions of the effect of male body size variables, treatment and replicate on the number of sperm transferred to females in experiment 2. The dependent variable is the number of sperm in the female gynatrial complex after a single copulation. Results are given for all copulations and also for only copulations lasting  $\geq 20$  min.

Size variable	Significance of regression coefficients (probabilities)		Full model	Model excluding size variable	Significance of the size effect
All copulations					
	Size variable	Treatment	$F_{(df), P, R^2}$	$F_{(df), P, R^2}$	$F_{(df), P}$
PC1	0.438	0.883	$F_{(1,14)} = 0.323$ $p = 0.729$ $R^2 = 0.042$	$F_{(1,15)} = 0.010$ $p = 0.921$ $R^2 = 0.001$	$F_{(1,14)} = 0.600$ $p = 0.451$
LTotal	0.567	0.869	$F_{(1,14)} = .0177$ $p = 0.840$ $R^2 = 0.025$	$F_{(1,15)} = 0.010$ $p = 0.921$ $R^2 = 0.001$	$F_{(1,14)} = 0.345$ $p = 0.566$
LSoma	0.409	0.825	$F_{(1,14)} = 0.458$ $p = 0.699$ $R^2 = 0.050$	$F_{(1,15)} = 0.010$ $p = 0.921$ $R^2 = 0.001$	$F_{(1,14)} = 0.722$ $p = 0.410$
LGenital	0.274	0.762	$F_{(1,14)} = 0.654$ $p = 0.535$ $R^2 = 0.085$	$F_{(1,15)} = 0.010$ $p = 0.921$ $R^2 = 0.001$	$F_{(1,14)} = 1.285$ $p = 0.276$
LSeg8	0.817	0.978	$F_{(1,14)} = 0.032$ $p = 0.968$ $R^2 = 0.005$	$F_{(1,15)} = 0.010$ $p = 0.921$ $R^2 = 0.001$	$F_{(1,14)} = 0.056$ $p = 0.816$
Copulations $\geq 20$ min					
PC 1	0.412	0.879	$F_{(1,11)} = 0.391$ $p = 0.686$ $R^2 = 0.066$	$F_{(1,12)} = 0.026$ $p = 0.908$ $R^2 = 0.002$	$F_{(1,11)} = 0.733$ $p = 0.426$
LTotal	0.433	0.894	$F_{(1,11)} = 0.358$ $p = 0.707$ $R^2 = 0.061$	$F_{(1,12)} = 0.026$ $p = 0.908$ $R^2 = 0.002$	$F_{(1,11)} = 0.691$ $p = 0.424$
LSoma	0.362	0.939	$F_{(1,11)} = 0.480$ $P = 0.631$ $R^2 = 0.080$	$F_{(1,12)} = 0.026$ $p = 0.908$ $R^2 = 0.002$	$F_{(1,11)} = 0.933$ $p = 0.355$
LGenital	0.642	0.962	$F_{(1,11)} = 0.140$ $p = 0.871$ $R^2 = 0.025$	$F_{(1,12)} = 0.026$ , $P = 0.908$ , $R^2 = 0.002$	$F_{(1,11)} = 0.259$ $p = 0.621$
LSeg8	0.704	0.883	$F_{(1,11)} = 0.102$ $p = 0.904$ $R^2 = 0.018$	$F_{(1,12)} = 0.026$ , $p = 0.908$ $R^2 = 0.002$	$F_{(1,11)} = 0.179$ $p = 0.680$

To determine if the number of sperm transferred to females in a single copulation was influenced by female size or the number of eggs carried by females, we performed analogous regression analyses using estimates of female body size and the sizes of various body and genital components (PC 1, total length, length of soma, length of genital, and length of segment 8) and number of eggs dissected from the females after the experiment as independent variables, and the number of sperm transferred to females as dependent variables. The data are from experiment 2. These results are shown in table A4.10 .

Table A4.10: Linear regressions of the effect of female body size variables, egg number, treatment and replicate on number of sperm in the female's gynatrial complex after experiment 2. Results are shown for all copulations and for only copulations  $\geq 20$  min.

Size/egg variable	Significance of regression coefficients		Full model	Model excluding size variable	Significance of size effect
	Size/egg variable	Treatment	$F_{(df)}$ , P, $R^2$	$F_{(df)}$ , P, $R^2$	$F_{(df)}$ , p
All copulations					
PC 1	0.695	0.898	$F_{(1,14)} = 0.085$ p = 0.919 $R^2 = 0.012$	$F_{(1,15)} = 0.010$ p = 0.921 $R^2 = 0.001$	$F_{(1,14)} = 0.571$ p = 0.611
LTotal	0.802	0.921	$F_{(1,14)} = 0.037$ p = 0.963 $R^2 = 0.005$	$F_{(1,15)} = 0.010$ p = 0.921 $R^2 = 0.001$	$F_{(1,14)} = 0.056$ p = 0.8136
LSoma	0.764	0.910	$F_{(1,14)} = 0.051$ P = 0.950 $R^2 = 0.007$	$F_{(1,15)} = 0.010$ p = 0.921 $R^2 = 0.001$	$F_{(1,14)} = 0.085$ p = 0.775
LGenital	0.724	0.892	$F_{(1,14)} = 0.458$ p = 0.716 $R^2 = 0.096$	$F_{(1,15)} = 0.010$ p = 0.921 $R^2 = 0.001$	$F_{(1,14)} = 1.471$ p = 0.245
LSeg8	0.995	0.926	$F_{(1,14)} = 0.005$ p = 0.995 $R^2 = 0.0011$	$F_{(1,15)} = 0.010$ p = 0.921 $R^2 = 0.001$	$F_{(1,14)} = 0.001$ p = 0.999
Eggs in female	0.556	0.760	$F_{(1,14)} = 0.186$ p = 0.832 $R^2 = 0.026$	$F_{(1,15)} = 0.010$ p = 0.921 $R^2 = 0.001$	$F_{(1,14)} = 0.359$ p = 0.559
Copulations $\geq 20$ min					
PC 1	0.975	0.832	$F_{(1,11)} = 0.027$ p = 0.974 $R^2 = 0.005$	$F_{(1,12)} = 0.026$ p = 0.908 $R^2 = 0.002$	$F_{(1,11)} = 0.041$ p = 0.849
LTotal	0.745	0.767	$F_{(1,11)} = 0.082$ p = 0.922 $R^2 = 0.015$	$F_{(1,12)} = 0.026$ p = 0.908 $R^2 = 0.002$	$F_{(1,11)} = 0.145$ p = 0.711
LSoma	0.894	0.807	$F_{(1,11)} = 0.035$ p = 0.966 $R^2 = 0.006$	$F_{(1,12)} = 0.026$ p = 0.908 $R^2 = 0.002$	$F_{(1,11)} = 0.044$ p = 0.838
LGenital	0.582	0.838	F = 0.187 p = 0.832 $R^2 = 0.033$	$F_{(1,12)} = 0.026$ p = 0.908 $R^2 = 0.002$	$F_{(1,11)} = 0.352$ p = 0.565
LSeg8	0.565	0.752	$F_{(1,11)} = 0.203$ p = 0.819 $R^2 = 0.036$	$F_{(1,12)} = 0.026$ P = 0.908 $R^2 = 0.002$	$F_{(1,11)} = 0.388$ p = 0.546
Eggs in female	0.939	0.887	$F_{(1,11)} = 0.029$ p = 0.972 $R^2 = 0.005$	$F_{(1,12)} = 0.026$ P = 0.908 $R^2 = 0.002$	$F_{(1,11)} = 0.033$ p = 0.859

### **Appendix 5: Evidence for copulations by females that are not producing eggs**

In experiment 1, some females that had sperm in their gynatrial sac contained no eggs. In a concurrently running experiment designed to measure long term fecundity, males were found to copulate with females that did not or rarely laid eggs over the 21-day observation period (J. Arendt, University of California, Riverside, Personal communication, 2011). Both observations were unexpected, as females that are not producing eggs would not be expected to benefit from acquiring sperm. The following data extend these observations and connect the data from the two experiments by determining if low numbers of eggs laid by females in the daily fecundity experiment correlate with low numbers of eggs contained within the female.

On dissection, 11 of the 48 females in experiment 1 were found to contain no eggs. Of those 11, 8 were seen copulating. All of these females plus one female not observed copulating had sperm in the gynatrial complex. Thus at least 9 of the 11 females without eggs copulated and 9 were inseminated over the course of 9 days in experiment 1.

In order to determine if females that did not contain eggs on dissection were unlikely to have laid eggs earlier in the experiment, we dissected and counted the eggs in 8 females that had not laid eggs in the long term fecundity experiment, 6 that only initially laid one or two clutches of eggs and then did not lay eggs again, and 8 females that regularly laid eggs throughout the observation period. Only 2 of the 8 females that never laid eggs had eggs within them. Similarly, only 2 of 6 females that initially laid eggs and then stopped had eggs within them. Overall, only 4 of the 14 females not laying



eggs at the end of the experiment contained eggs (29%). In contrast, all of the 8 females that continually laid eggs had eggs in them upon dissection, a difference that is statistically significant (two-way chi-square,  $\chi^2= 10.476$ ,  $df=1$   $p=0.002$ ). The number of eggs in the females that continually laid eggs (median, mean SD, 12.5, 12.63, 3.38), laid one or two clutches of eggs (median, mean SD, 0, 4.83, 8.54) and females that never laid eggs (median, mean SD, 0, 2, 4.28) ,were statistically different as determined by an ANOVA ( $F_{(2,21)}= 7.935$   $p= 0.003$ ). A Tukey pairwise comparison of means indicated that the number of eggs in females that continually laid eggs was statistically different then the number of eggs in females that only laid one or two clutches of eggs ( $p = 0.042$ ) and females that never laid eggs ( $p = 0.003$ ). These results verify that fecundity assessed on dissection is a valid indicator of realized fecundity (oviposition). The females that did not contain eggs in experiment 1 were unlikely to have laid eggs during the experiment. This is clear evidence that female *A. remigis* do mate when they are not ready to oviposit.

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