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Mating Dynamics of the Calanoid Copepods Acartia tonsa and Acartia hudsonica

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Copepods are the most abundant metazoans in the ocean, link the microbial plankton to upper trophic levels, and affect ecosystem function through their role in biogeochemical cycles. This thesis focuses on an understudied area in copepods-- factors that determine mating success. First, I tested the hypothesis that female-biased adult sex ratios of *Acartia tonsa* may be explained by ratios already skewed at birth. Offspring of field-caught females were raised to adulthood and the corresponding adult sex ratios were used as proxies for birth ratios. Almost one-fifth of mothers produced significantly female-biased sex ratios, suggesting that secondary processes commonly used to explain sex ratio biases may be less important than hypothesized.

Second, I measured lifetime spermatophore-production rates for males of *Acartia tonsa* and *Acartia hudsonica* under high, low, and no food. Spermatophore-production rates and lifetime totals for males of *A. tonsa* were not affected by food abundance; in *A. hudsonica,* rates were significantly higher under high food. For both species, there was also a significant decrease in spermatophore-production rates and numbers with age.

Third, I tested whether female mating status affected mate choice in males of *Acartia tonsa* and *Acartia hudsonica*. The costs and benefits associated with mating twice versus once for females were also tested. In both species, males mated more frequently with virgin females compared with females that had already mated, suggesting that female reproductive status is important for mating. Frequencies of females carrying double spermatophores in the field were

consistently low, implying that mate choice is also present in natural populations. There were no particular costs or benefits to mating a second time.

Fourth, I measured whether variation in encounter time, previous social experience with the same or opposite sex, and food availability influenced the strength of mate choice in *Acartia tonsa* and *Acartia hudsonica*. The strength of mate choice between species and among the sexes varied significantly.

Finally, the strength of mate choice was measured over two seasons in field populations of *Acartia hudsonica*. Mate choice was present in the field, but its strength varied within and between seasons and correlated with a number of ecological factors.

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Mating Dynamics of the Calanoid Copepods *Acartia tonsa* and *Acartia hudsonica*

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Table of Contents

Chapter 1:

Introduction

Background:

Copepods are the most abundant metazoans in the ocean and are important food sources for many larval fish species; hence, they link primary producers to upper trophic levels. Copepods are also important in control of the microbial food web and biogeochemical cycles in the ocean. Population maintenance and growth depend on the ability of a species to survive and reproduce. Oceanographers usually see reproduction as a function of temperature and food availability (Williamson and Butler, 1987). Little attention has been paid to the ability to mate successfully. Information on the factors influencing mating-- adult sex ratios, male mating capacities, and sexual selection through mate choice-- are unknown for most species of copepods. Even such basic information as the frequencies of mated individuals in field populations or the factors that may influence a copepod's decision to accept or reject a mating advance, is unknown for most pelagic copepods. As a result, many hypotheses dealing with mating in copepods have been constructed based on limited data. For example, a common, but unproven, assumption about copepod mating is that female copepods experience fertilization-limitation as a result of too few males in the population (Hopkins, 1982; Kiørboe, 2006; Kiørboe, 2007). But in fact, the seasonal proportions of fertilized females in the field are known for only one species, *Oithona davisae* (Uye and Sano, 1995, 1998). Even less is known about male mating abilities or how changes in environmental parameters (food availability, temperature), population parameters (density and sex ratios of adults), and mate preferences (for size and reproductive state) might impact reproductive success and the strength of mate choice in copepod populations. This dissertation is the first to show that female-skewed adult sex ratios may be already biased at birth in a calanoid copepod, to measure male mating capacity under

different food regimes, and to quantify changes in mate choice of *Acartia tonsa* and *Acartia hudsonica* as ecological parameters vary in both field and laboratory populations.

Population success depends largely on the fitness of its members; i.e. the number of offspring that survive to adulthood produced by individuals. In some copepods, mating with a large mate can increase the size and number of offspring, thereby increasing individual fitness (Weatherhead and Robertson, 1979; Kiørboe and Hirst, 2008; Ceballos and Kiørboe, 2010; Sichlau and Kiørboe, 2011). It is not surprising, then, that mate choice for body size is prevalent in the few species of copepods that have been studied, with both sexes preferring to mate with large mates (Ceballos and Kiørboe, 2010). Through mate choice, sexual selection may occur in copepod populations, especially since copepod mating needs are diverse (Ceballos and Kiørboe, 2010). Differences between species in morphology, behavior, and population biology may be explained by the strength of sexual selection (Trivers, 1972). Similarly, information on mate choice and mating dynamics in copepods may help to explain the evolution of sexual size dimorphism (females are generally larger than males). Stronger preferences (mate choice) for large females among males may explain the observation that sex size-ratios in calanoid copepods are relatively constant and female-biased (Clutton-Brock and Harvey, 1977; Maly, 1984). Finally, interest in optimizing copepod yields from cultures for use in ecotoxicological tests and as feed in larval fish aquaculture provides an economic reason for studying copepod mating dynamics. Thus, understanding how mate choice impacts mating and reproductive success as a function of culture conditions (food abundance, density and sex ratio) will help improve culturing techniques.

Acartia tonsa and *Acartia hudsonica* are dominant copepod species present in Long Island Sound during the summer/fall and the winter/spring months, respectively. Although they

belong to one of the most commonly studied copepod genera, there are large gaps in our knowledge of their mating dynamics. Males in both species are smaller than females (*A. tonsa:* Parrish and Wilson, 1978; *A. hudsonica*: personal observation), on average by 0.15 mm and 0.1 mm in body length, respectively (personal data). In the laboratory, males have shorter lifespans than females (Parrish and Wilson, 1978) and as a result also have a shorter breeding period (~20 days for females, ~10 days for males of *A. tonsa*)(Ceballos and Kiørboe, 2010). Both species locate mates through hydromechanical signals, and in *A. tonsa*, mates can be detected up to 7 mm away (Bagøien and Kiørboe, 2005). After a potential mate is encountered, it is assessed through 7-8 synchronized hops, at which point a male will leap towards and catch the female (Bagøien and Kiørboe, 2005). The male then holds the female with its specialized fifth leg and attaches a spermatophore (sperm packet) to the female's gonopore (Bagøien and Kiørboe, 2005). Mating lasts for only a few seconds. Because females lack a spermathecum to store sperm (Ohtsuka and Huys, 2001), they need to mate multiple times over their lifespan. In the lab, spermatophores remain attached for an average of 10 days in *A. tonsa* (Ceballos and Kiørboe, 2010) and an unknown amount of time for *A. hudsonica*.

Research Objectives:

This dissertation aimed to provide information on factors influencing copepod mating success that have previously been underrepresented in the literature. Specifically, adult sex ratios, male mating capacities, and sexual selection through mate choice. Goals for each chapter were:

- **Chapter 2: First evidence of biased sex ratios at birth in a calanoid copepod**

To determine whether female-biased adult sex ratios could be the result of ratios already skewed at birth in *Acartia tonsa.*

- **Chapter 3: Male reproduction as a function of food abundance and age in two calanoid copepods, *Acartia tonsa* and *Acartia hudsonica***

To measure lifetime spermatophore production as a function of food abundance in *Acartia tonsa* and *Acartia hudsonica* to understand male energetics and mating abilities.

- **Chapter 4: Female mating status affects mating and male-mate choice in the copepod genus *Acartia***

To determine if male-mate choice in *Acartia tonsa* and *Acartia hudsonica* is influenced by female reproductive status (virgin vs. mated) in both laboratory and field populations, and whether there is a fitness penalty for females that mate multiple times.

- **Chapter 5: Variation in mate-choice strength in laboratory experiments of two calanoid copepods, *Acartia tonsa* and *Acartia hudsonica***

To determine if mate choice is constant or variable in the two species of copepods. I measured variation in mate-choice strength in both sexes as a function of four variables (encounter time, previous exposure to the opposite sex, previous exposure to the same sex, and food availability).

- **Chapter 6: Variation in the strength of mate choice for body size in field populations of the calanoid copepod *Acartia hudsonica***

To corroborate the finding of the previous chapter by determining if mate choice for body size is present in field populations, and whether it varies as ecological parameters fluctuate over two seasons.

Chapter 7: Summary

To summarize the main findings of chapters 2-6 are explore their implications.

Chapter 2*:

First evidence of biased sex ratios at birth in a calanoid copepod

* This chapter was submitted as a manuscript to Limnology and Oceanography.

Abstract:

To test the hypothesis that the sex ratio of the copepod *Acartia tonsa* is biased at birth, adult sex ratios of the offspring of families (21 families in 2011 and 72 in 2013) produced by field-caught females were determined in the laboratory under controlled conditions. Sex ratios at birth were estimated from the adult sex ratio, and after applying a correction in which all dead/missing individuals were counted as the rarer sex (males). Before correction, the overall population sex ratio was female biased during both years, with over 42% of mothers producing clutches that were significantly different from unity. After correction, 25% (2013) to 33% (2011) of mothers still produced significantly biased sex ratios. The ratio of deaths of males to females had to be 1.75 to 1 (2011) and 7.25 to 1 (2013) for sex ratios not to be biased at birth. There was no evidence of sex reversal in any individual from juvenile (C4 stage) to adult, implying that juvenile sex ratios were also significantly biased. Hence, neither sex reversal nor differential mortality of the sexes is a likely explanation for the observed skewed sex ratios. The sex ratios at birth varied widely from the expectation from Mendelian inheritance of sex chromosomes, suggesting that another mechanism is responsible for skewed sex ratios at birth. Since the majority of families with biased sex ratios at birth were female dominated as adults, biased sex ratio at birth may contribute to the skewed sex ratio of adults observed in the field.

Introduction:

Sex determination is one of the least understood aspects of copepod biology even though the mode of sex determination can have a substantial influence on the population size and adult sex ratio of a species (Voordouw and Anholt 2002). An individual's sex can be influenced by a number of genetic as well as environmental factors, including elements that may not necessarily be adaptive (Hickey 1982; review by Godfray and Werren 1996). For instance, under Mendelian inheritance of sex chromosomes, sex ratios are generally 1:1with little potential for variation among families (Falconer 1954; Edwards 1970; Hohenboken et al. 1988). Conversely, under environmental sex determination and environmental sex reversal, large variations in birth ratios are expected both between families and also between generations as the environment changes (Bull 1983; Korpelainen 1990). Hence, the sex ratio at birth can provide valuable information about the potential mechanism of sex determination in a species.

Sex ratios at birth have been measured in only a handful of harpacticoid copepod species, and show that individual families can produce offspring that is heavily male-skewed, which in turn reflects the male-skewed adult sex ratios in the field (Battaglia 1958; Igarashi 1964; Voordouw et al. 2005). There are no data on birth ratios in calanoid copepods, but adult populations in both the field and laboratory tend to be female-dominated (Kiørboe 2006; Gusmão and McKinnon 2009; Hirst et al. 2010). Whether this skew is due to biased sex ratios at birth is not known.

In calanoid copepods of the genus *Acartia*, the evidence suggests that sex is determined genetically and not altered during the developmental process. Sex is determined by chromosomes; males are heterogametic (XO) and females are homogametic (XX) (Goswami and Goswami 1974; Lécher et al. 1995). Hence, one would expect that under Mendelian inheritance

of sex chromosomes, the sex ratio at birth, and subsequently in adulthood, would be unity. However, if sex at birth is determined via some other mechanism (parental control, cytoplasmic inheritance, etc.), or if sex reverses during late juvenile stages, then adult sex ratios may be biased both within families and in the offspring population as a whole.

There is no information on juvenile sex ratios for *Acartia tonsa*, but sex ratios of adults are often strongly female-biased in laboratory cultures as well as in the field (Heinle 1966; Lee and McAlice 1979; Burris 2014). In many animals, the sex-determining mechanism can have a significant impact on adult sex ratios. In calanoid copepods, if biased sex ratios at birth translate to biases in sex ratios of adults, then population growth rates may be negatively affected. In those species that require re-mating to continue producing offspring as *Acartia tonsa* does (Wilson and Parrish 1971; Barthelemy et al. 1998; Barthelemy 1999), female-biased sex ratios can reduce mate encounter rates and limit fertilization (Hopkins 1982; Williamson and Butler 1987; Kiørboe 2006). Knowing the degree and variation of sex ratio skew at birth in copepod families can provide information about the mechanism(s) important in determining sex in copepods, and ultimately, population success.

Since there is no way yet to determine the sex of a copepod embryo the sex ratio at birth is often assumed to be unity (Kiørboe and Sabatini 1994; Dam and Tang 2001). Evidently, adult sex ratios may not necessarily reflect those sex ratios at birth. Skewed sex ratio of adults may instead be the result of high mortality rates of males throughout their lives (Maly 1970; Hairston et al. 1983; Hirst et al. 2010). In addition, switching sex from male to female as late stage juveniles in response to changes in temperature or food abundance may also bias sex ratios towards females in adult copepods (Fleminger 1985; Voordouw and Anholt 2002; Gusmão and McKinnon 2009). Thus, in order to get a better understanding of the mechanism(s) behind sex

determination in calanoid copepods, the above factors need to be accounted for during any study on birth ratios. We estimated sex ratios at birth of offspring from individual families reared to adulthood in the laboratory under constant environmental conditions. We kept track of egg and juvenile mortality as well as any instances of sex reversal from C4 copepodid stages to adulthood. Using this information, we applied a correction to sex ratios (males to females) of families assuming all dead or missing individuals were males, the rarer sex. Because this approach yields an estimate of sex ratio in the direction of males, it provides the highest possible male-to-female ratio at birth. Hence, any deviations from unity in corrected sex ratios must reflect a biased sex ratio at birth. For example, assume that out of 100 eggs, 80% of adults are females, and mortality from egg to adult is 20%. We can estimate the maximum number of eggs that are born males by assuming all mortality was due to the rarer sex (males in this case): 16 adult males + 20 dead males = 36 males at birth. Because the mortality of the dominant sex is assumed to be zero, the sex of females at birth remains the same as adults, 64 in this case. Hence, the sex ratio (36 males to 64 females) had to be female-biased at birth. Using this approach in a laboratory study, we tested if sex ratios at birth in families of the calanoid copepod *Acartia tonsa* differed from unity and varied among families.

Methods:

Sex ratios of *Acartia tonsa* were obtained from triplicate, weekly plankton tows from early June to late December 2013. Horizontal tows were done on incoming tides off of the Groton Long Point bridge (41.3144° N, 72.0078° W) using a 202 µm mesh plankton net. Contents of the tows were immediately preserved with 10% buffered formalin. The sex ratio was determined by counting adult males per 35 females. The proportion of males in the population was calculated as an average of the triplicate tows.

Fertilized female *Acartia tonsa*, as evidenced by the presence of an attached spermatophore, were collected in mid-October of 2011 and 2013, also at Groton Long Point, with a 90-µm mesh plankton net and brought immediately to the laboratory. Thirty adult females (2011), and 110 adult females (2013) of similar size were randomly chosen and transferred by pipet to individual Petri dishes containing 700 µg C/L total of a mixture of the diatom *Thalassiosira weissfloggi* and the green flagellate *Tetraselmis sp.* (50% of each by carbon) in 70 mL filtered seawater. This concentration represents saturated food conditions for *Acartia tonsa* (Houde and Roman 1987; Besiktepe and Dam 2002). Dishes were placed in a refrigerator at 15˚C (LIS water temperature during collection) and exposed to a 12 hour light/dark cycle. Females were fed at the above food concentrations for 3 days to standardize their diet and to ensure eggs from were well provisioned (Wilson and Parrish 1971; Tester and Turner 1990). Thus, maternal-diet effects were minimized.

Females of *Acartia tonsa* remain fertilized for much longer than three days (Ceballos and Kiørboe 2010; Burris and Dam 2014) and so females did not need to be re-mated in the laboratory to produce viable offspring. On the third day, females were transferred to new food solutions and all eggs produced over the following 24 hours were collected for the study. Clutch size was recorded and eggs from the same family were placed together in 100mL Petri dishes with food solutions, as described above. Dishes were checked daily for egg hatching, and only families with 100% hatching success and at least 20 individuals were followed to adulthood. Families with fewer than 20 individuals were not included because of low statistical power (<0.5).

Three-quarters of the food solution was removed every third day by pipette; the remaining water and copepods were gently transferred to a clean dish and new food solution was

added. The sex of each individual was recorded at stage C4 (as per Conway 2006) and after metamorphosis to adult. The number of individuals from each family that survived to adulthood was recorded and only those families (21 in 2011, and 72 in 2013) that had 60% or higher survival were used in the analysis. This survival value was used to minimize the influence of mortality by individuals of unknown sex on our results and to maximize the number of families that could be included in the analysis. What is more, the statistical tests of the family clutch sizes obtained would have had very low power (<0.5), and thus unreliable results. In addition, using the mortality correction on these low survival families would have heavily skewed the distribution of sex ratio among families, which could unduly suggest non-Mendelian inheritance of sex chromosomes (see below).

Z-tests using Yates' correction (to prevent the overestimation of statistical significance as a result of small sample sizes) were performed on each family to determine if sex ratios of the offspring differed from unity. To determine if the population sex ratio was at unity, all offspring from the 72 families were pooled and a two-tailed z-test was performed on the combined sample size and proportion, using software from SigmaPlot 11.0.

A linear regression was performed to determine if brood size (number of offspring) was associated with the proportion of males produced. If more males are produced in smaller clutches, then our results may be biased in the direction of females by not including families that had fewer than 20 embryos. Similarly, for both years, a linear regression was performed to determine if family survival was associated with the proportion of males produced. If the families that had higher proportions of male offspring also had lower survivorship, then our results could also be biased towards females by not including families that had <60% survival.

To ensure that the observed skewed sex ratios were not the result of differential mortality of the rarer sex (males), all family and population-level sex ratios were recalculated after assigning all dead/missing individuals as males. This was done for all families, regardless of whether or not their sex ratios differed from unity. One-tailed z-tests were then rerun on these new proportions. As pointed out, earlier this procedure yields the highest possible male-to-female sex ratios; thus, any deviation from unity indicates a biased sex ratio.

To determine if sex ratios of families followed a binomial distribution as expected through Mendelian segregation of chromosomes, a chi-square test was performed on the distribution of male offspring produced by family against a binomial distribution, for both uncorrected and corrected sex ratios. Sex ratios were pooled at each extreme (<0.2 and >0.8) for the analysis to prevent problems due to low expected values and high Type I error (following Voordouw et al. 2005).

Results

From June to December 2013, field-populations of *Acartia tonsa* were consistently female-biased (Fig. 1). The proportion of adult males in the population ranged from as low as 10% (± 0.02 S.E.) during the second week in November to as high as 42.7% (± 0.02 S.E.) in mid-July.

In the laboratory study, there was no evidence of sex reversal from stage C4 to adults. Of the 30 families used for experiments in 2011, nine were excluded from the analysis because they either produced few viable eggs (<20), or had high mortality rates (>40%). Similarly, in 2013, 38 of 110 families were not included in the analysis. In 2011 on average, females produced about 44 embryos and about 77% (±0.02 S.E.) of offspring survived for each family, with a

range of 59 to 88% (Fig. 2A). On average for the 72 families included in 2013, 29 embryos were produced per female (ranging from 22 to 45 embryos per female), for a total of 2074 embryos. Survival to adulthood for individual families ranged from 61% to 97% (Fig. 2B), with an average of 78.3 % survival (±0.01 S.E.), and a total of 1637 individuals. Of the 21 families in 2011, 38% (8 of 21) produced sex ratios that did not differ from unity, while the remaining 13 families produced significantly biased sex ratios (Fig 3A). Of these, four families were male biased whereas nine were female-skewed (Fig. 3A). Between 69 and 80% of the offspring in male-dominated families were males, while 72 to 100% of offspring in female-dominated families were females. The four male-skewed families accounted for 45% of the males in the offspring, whereas the nine female-dominated families produced over 50% of the female offspring. Pooling all offspring produced by the 21 families, the population was significantly female-biased ($z = 3.8$; $p<0.001$). There was no correlation between the number of offspring produced and the proportion of males in each family ($r = 0.2$, $p>0.05$) or between survivorship and male proportion ($r = 0.27$, $p>0.05$).

Out of the 72 families included in the analysis for 2013, 42 (58.3%) produced sex ratios that were not statistically different from unity (Fig. 3B). The percentage of males produced in these families ranged from as low as 16.7% to as high as 83% (Fig. 3B), with an average of 44% (±0.03 S.E.). The remaining 30 families had statistically significant skewed sex ratios (Fig. 3B). More than one-third of all families (26 families; 36.1%) produced female-biased sex ratios, whereas only four families produced male-skewed sex ratios (Fig. 3B). Between 86 and 100% of offspring in these clutches were males (average 91%, ±0.03 S.E.). These four male-biased families (5.6% of all families) accounted for 15.6% of the total males produced in the population. The female-skewed families produced on average only 8.8% males (± 0.01 S.E.), with five

families producing solely females. The 26 families that had female-skewed sex ratios accounted for 48.4% of all females produced in the population. Upon pooling all offspring produced, the population was significantly female-biased and composed of only 35.9% males (z= 8.1; p<.001). There was no correlation between the number of offspring in each family and the proportion of males produced (r = 0.18; p >0.05). There was a weak positive relationship between family survivorship and the proportion of males produced (r = 0.27; p =0.03).

For all families, we recalculated the sex ratio at birth by assigning all dead/missing individuals as males (the rarer sex). Recall that this approach assumes no mortality for females; hence, it yields the maximum male-to-female sex ratio. After applying this approach, 33.3% of families in 2011 (7of 21) and 25% of families in 2013 (18 of 72) still produced statistically skewed sex ratios at birth (Fig. 3). Five families produced male-biased clutches whereas two produced significantly female-biased offspring in 2011 (Fig. 3A). In 2013, nine families produced male-skewed sex ratios (average: 85% male, ± 2.9 S.E.) and nine others produced female-biased sex ratios (average: 22.2% male, ± 1.1 S.E.) (Fig.3B). Population sex ratios moved to unity in both 2011and 2013 with the correction (2011 families: 53.6% male, z = 1.549, p = 0.12; 2013 families: 49.5% male, z = 0.291, p = 0.77). However, this approach unrealistically assumes no mortality by females. Thus, we also calculated the proportion of males to females that would have to die in order for the observed female-skewed population sex ratio to be unity. In 2013, a mortality proportion of 7.25 males to every one female (of 447 dead in this study: 370 males and 51 females) is the lowest value needed to explain the observed female-skewed sex ratios. In 2011, when the total number of individuals in the population was smaller, the mortality proportion was 1.7 males for every 1 female (of 208 dead: 131 males and 77 females).

For both 2011 and 2013 families, the distributions of uncorrected and corrected family sex ratios were statistically different from what would be expected under Mendelian segregation of chromosomes (2011 families: uncorrected: $\chi^2 = 69.7$, $p<0.0001$; corrected: $\chi^2 = 18$, $p<0.01$; 2013 families: uncorrected: $\chi^2 = 392$, $p<0.0001$; corrected: $\chi^2 = 37$, $p<0.001$) (Fig. 4).

Discussion:

We present the first evidence of biased sex ratios at birth in a calanoid copepod, *Acartia tonsa.* Because there is yet no way to determine the sex of an embryo in copepods, sex ratios at birth were inferred from adult sex ratios of families reared from egg to adulthood in the laboratory under controlled conditions (high food concentration, constant salinity and temperature, absence of predators) to minimize confounding variables that may affect adult sex ratios. By applying a correction for mortality that assumed all missing/dead individuals were males (the rarer sex), we obtained the highest possible male-to-female sex ratios at birth for 21 families in 2011 and 72 families in 2013. Deviation from unity in these corrected ratios indicates a biased sex ratio at birth. We highlight three main findings of our study and discuss their implications for field populations. First, the offspring of families in both years were significantly female biased, and were due to a preponderance of families producing female-biased sex ratios (Fig. 3). Second, after the correction for mortality, over 25% of families in both years still had sex ratios at birth that differed significantly from unity. In 2013, when 72 families were tracked, a male-to-female mortality of 7:1 would have been required to keep the sex ratio at unity. Third, the variation in sex ratios produced by families was not explained by Mendelian inheritance of sex chromosomes (Fig. 4), implying that some other mechanism is responsible for the observed skewed sex ratio at birth.

In populations that are very female biased, mate choice by males may evolve. In addition, a female-biased population would select for females to mate so that they maximize the proportion of sons they produce (Fisher, 1930). Thus, female preferences for male traits may also change as a result of skewed sex ratios. Females collected from the field during both years produced significantly female-biased offspring. This was due to most families producing more female than male offspring, even if sex ratios of individual families were not statistically different from unity (Fig. 3). Since there are differences in maturation time and longevity between the sexes (males are thought to mature earlier and die sooner than females) (Landry 1975; Hirst and Kiørboe 2002; Hirst et al. 2010), then the sex ratio at birth will not necessarily reflect the sex ratio of adult populations. However, if females are constantly producing more female offspring than male offspring, there should always be more adult females entering the population, on average, than males, as long as juvenile mortality is equal in the sexes. There is little information on field mortality, stage-duration times, and sex ratios of juvenile copepods (but see Hirst et al. 2010), and almost nothing known for individual species, specifically *Acartia tonsa*. Thus, it is unknown if female-biased sex ratios at birth, as determined here, remain that way during juvenile stages in field populations. More information is needed about the processes that occur during the juvenile stages before sex ratios at birth can be directly linked to adult sex ratios in the field. However, since predation is the main mode of adult mortality in field populations (Hirst et al. 2010), the fact that there is no significant difference in predation rates between the sexes of *Acartia* (Landry 1978; Hirst and Kiørboe 2002; Hirst et al. 2010) suggests that the biased sex ratios of adult *Acartia tonsa* may reflect the sex ratio at birth.

Differential mortality of the sexes or change in sex of juveniles are offered as explanations for the observed female-skewed sex ratio in laboratory experiments, even when

neither of these two processes were measured (Peterson 1986; Irigoien et al. 2000). We applied a mortality correction assuming that 100% of dead or missing individuals were males. Over 25% of sex ratios of families were still biased after this correction. In addition, while the sex ratio of families was no longer significantly different from unity, this correction unrealistically assumed no deaths by females. Therefore, we calculated the lowest proportion of male to female mortality necessary for the observed population sex ratios to become unity. In 2013, the proportion of male to female deaths required would have to be extremely high: 7.25 males to1 female. While the proportion during 2011 is more reasonable (1.7 males to 1 female), these copepods were raised in ideal laboratory conditions with no predators, constant temperature, and high food. Male copepods, in general, are considered the weaker sex; they have shorter lifespans (Finiguerra et al. 2013), higher susceptibility to starvation (Finiguerra et al. 2013), and often higher predation rates than females (Hirst and Kiørboe 2002; Hirst et al. 2010). However, none of these variables were a factor in our laboratory study. Thus, differential mortality of the sexes is unlikely to be the cause of the biased sex ratios measured here, at least during 2013. Additionally, animals were sexed both as juveniles and after metamorphosis to adults. There was no evidence of sex change during these stages (i.e. 100% of identified C4 males became adult males), implying that the observed adult sex ratios were not explained by juvenile sex-reversal, but rather by a bias present at birth.

Our study did not include data from families that had low survival (<60%) or small clutch sizes (<20 eggs) because their corresponding sex ratios would not necessarily be accurate. It is possible, but improbable, that these families produced mostly male offspring, thus biasing our results towards families that produced females because we found no correlation between the size of the clutch and the number of male offspring produced. While this correlation is limited by not

including those very small broods, we did have a broad range of brood sizes (from 20 to 62 egg clutches) and thus should have been able to detect at least a weak correlation. In fact, no pattern was found: the second largest family (61 embryos) and the smallest family (20 embryos) were male-biased. Families with low survival (<60%) that were also not included in the analysis may have been male-dominated as well. Yet, this is also unlikely because there was no correlation (2011) or a weakly positive correlation (2013) between offspring survivorship and the proportion of males produced. Rather, this suggests that by excluding families with low survival, we may have excluded female-dominated families and skewed our results towards males.

In species with chromosomal sex segregation, there is often little potential for variation in the sex ratio at birth because Mendelian transmission is very stable (Falconer 1954; Edwards 1970; Foster and McSherry 1980). This tendency to produce equal sex ratios, and many deviations from it, can often be explained through adaptive sex ratio theory (Fisher 1930), wherein there is selection for the sex ratio that maximizes the transmission of autosomal alleles to future generations (Fisher 1930; Trivers and Willard 1973; Chamov 1982). Thus, we would expect little variation in the sex ratios of *Acartia tonsa* if sex chromosomes were randomly inherited. The large observed variation in the sex ratios among families (Fig. 4), however, suggests that some mechanism other than Mendelian inheritance of sex chromosomes determines sex at birth. Genetic (chromosomal and polygenic) and environmental modes of sex determination (ESD) have been hypothesized for many animals, including copepods (Fleminger 1985; Miller et al. 2005; Gusmão and McKinnon 2009), and are not necessarily mutually exclusive. In species with true environmental sex determination, such as marine worms and mollusks, fish, and many reptiles, sex is not determined at conception, but later by some

environmental factor acting on genes (Bull 1983; Korpelainen 1990). These species do not have sex-determining chromosomes (Bull 1980; Lécher et al. 1995), and as is hypothesized for a number of harpacticoid copepods, changes in temperature or food abundance determine the sex of an individual (Fleminger 1985; Voordouw and Anholt 2002; Gusmão and McKinnon 2009). *Acartia,* however, bears sex chromosomes (Goswami and Goswami 1974; Lécher et al. 1995), and so environmental sex determination should not occur. However, the environment can still influence sex determination in many animals that have sex chromosomes, through a process known as environmental sex reversal (ESR) (Bull 1983; Valenzuela et al. 2003; Baroiller et al. 2009). Under ESR, an individual's genetic sex (determined by sex chromosomes or sex genes) can be overridden by environmental factors (pH, temperature, food abundance, crowding, pathogens, etc.) during development and the sex phenotype is reversed (Bull 1983; Valenzuela et al. 2003; Baroiller et al. 2009). In copepods, genetic males are thought to switch sex to phenotypic females as late stage juveniles rather than after they become adults (Svensen and Tande 1999; Voordouw and Anholt 2002; Gusmão and McKinnon 2009). Since copepods do not molt as adults, they would have no way to phenotypically change sex after maturation (Gusmão and McKinnon 2009). If copepods reversed sex early in life, there would be a greater chance that environmental conditions would be different by the time they became adults. Thus, by changing sex as late in the juvenile stage as possible (C5), individuals can become the sex that benefits most from the current environment (Charnov and Bull 1989; Bull 1983). The sex ratios of species that exhibit ESR or ESD are often very variable in nature (Charnov 1982); this strategy provides high reproductive output and ensures maintenance of populations that live in very variable environments (Fleminger 1985; Svensen and Tande 1999; Miller et al. 2005). In the present study, however, there was no evidence of male sex reversal from C4 to adult,

suggesting that, at least under constant laboratory conditions, sex reversal was not the cause of the observed female-biased families. In summary, the wide variation in sex ratio among families and the lack of evidence for sex reversal suggest that sex ratio at birth is not determined by Mendelian inheritance of sex chromosomes in *Acartia tonsa*.

Non-Mendelian inheritance of sex chromosomes has been shown in three harpacticoid copepod species, *Tigriopis californicus*, *T. japonicus* and *Tisbe gracilis,* all of which produce significantly skewed sex ratios at birth (Battaglia 1958; Igarashi 1964; Voordouw et al. 2005). In *T. californicus* and *T. gracilis,* the sex-determining mechanism is thought to be polygenic, the sum of many genes having minor additive effects determines the final sex (Vacquier and Belser 1965; Egloff 1966; Voordouw et al. 2005). In *Tigriopus japonicus*, however, a cytoplasmic mode of sex determination is hypothesized because the offspring sex ratio is exclusively determined by the mother's genotype (Igarashi 1964). In cytoplasmic inheritance, mitochondria, plastids, and cytoplasmic microorganisms are passed through the egg cytoplasm by the mother, and skew sex ratios strongly towards females since they are the sex that transmits them to future generations (Sager 1965; Godfray and Werren 1996). Therefore, maternally inherited microorganisms are not adaptive for individuals and can eventually lead to extinction of males (Hamilton 1967; Hatcher 2000). Thus, cytoplasmic inheritance may explain the female-biased sex ratios observed in *Acartia tonsa*. However, the biased offspring sex ratios may also have been caused by the unequal transmission of the sex chromosomes from the male parent, a process known as meiotic drive (Lyttle 1991; Jaenike 2001). Since this mechanism also commonly results in excess production of female offspring (Jaenike 2001), it is also a potential mode of sex determination in *A. tonsa*.

Another way that parents may influence offspring sex is through differential investment in the sexes (Komdeur et al. 1997). If the fitness benefits of offspring of each sex differ according to the current ecological or social environments, then parents should change the proportion of each sex produced to maximize fitness (Trivers and Willard 1973; Werren and Charnov 1978; Charnov 1982). Species can actively modify sex ratios to avoid fitness loss. By allocating more resources to offspring of one sex (males, for example) parental phenotypes can alter selection acting upon zygotic sex determiners. While this most commonly occurs after birth in animals that have parental care (i.e. by providing more food to one sex over the other), it can also occur during egg formation (Mead et al. 1987; Anderson et al. 1997; Cordero et al. 2001). For instance, females may provision male and female eggs differently by making eggs of one sex larger than the other (Nilsson and Svensson 1993; Smith and Bruun 1998; Styrsky et al. 1999). While not affecting the sex ratio at birth, this would skew the adult sex ratio if one sex was more likely to survive than the other sex. Thus, egg sexual dimorphism may be adaptive if the smaller, disadvantaged sex (copepod males, for instance) had better provisioned eggs and thus lower mortality (Anderson et al. 1997), or if it favored the offspring with higher reproductive return (Cordero et al. 2001). Egg size is a widely used predictor of reproductive investment in animals (Winkler and Wallin 1987; Sinervo and Licht 1991; Bernardo 1996), but there is little information on embryonic provisioning in copepods, and none on whether parents invest differently in male versus female offspring. However, some copepods are known to increase the size of their embryos at the expense of the number of embryos produced (Guisande et al. 1996), suggesting that provisioning differences may exist. This could explain our female-biased results if large eggs, which are theoretically more costly to produce, became male offspring, and smaller, less costly, eggs became female offspring. While egg size was not measured in the

present study, there was no relationship between the size of the brood and the proportion of males produced. This suggests that there were no size differences in eggs between sexes. In addition, in copepods, size differences between the sexes do not become evident until stage C4, when juveniles start developing secondary sexual characteristics, implying that males are not better provisioned than females.

In addition to possible size differences in male and female eggs, specific products placed in the egg by the mother can also be important during early development since the zygotic genotype is not expressed during early mitotic division (Werren and Hatcher 2000). Thus, any gene products (proteins, lipids, or mRNA) placed in the developing egg could have significant effects on sex determination (Werren and Beukeboom 1998). For instance, in reptiles, the mother's seasonal allocation of yolk hormones (Bowden et al. 2000) or her condition (Robert and Thompson 2001) can influence the amount of these substances placed on developing embryos. In the copepod *Cyclops kolensis*, old females produce smaller eggs than young females, possibly because of a reduction in their lipid reserves or other products (Jamieson and Santer 2003). Thus, investment in embryos decreases as females age, suggesting that perhaps the more expensive sex (males) is produced early in a female's life and the cheaper sex (females) is produced later in life. Since we used field-caught mothers to produce families, differences in age and condition could explain the observed variation in the family sex ratios as well as the seasonal variation in adult sex ratios measured in the field (Fig. 1). It is possible that younger females produce male-dominated clutches and older females produce female-dominated clutches. Thus, differential investment in the sexes is another possible mechanism for our female-biased sex ratios.

Sex determination is one of the least understood aspects of copepod biology. The mode of sex determination can have important consequences for population size and sex ratio (Voordouw and Anholt 2002), both of which can impact the reproductive success and genetic properties of a species (Bulmer and Bull 1982; Bull and Charnov 1988). While the sex-determining mechanism is still unknown in *Acartia tonsa*, our results show that a genetic mechanism with non-Mendelian inheritance of sex chromosomes is likely responsible for the significant variation in the female-biased sex ratios produced at birth. Once the mechanism is understood, the adaptive (or possibly non-adaptive) significance of producing female-biased clutches may be explained.

Figure legend:

Figure 1: Sex ratios (mean ± standard error, $n = 3$) in field populations of *Acartia tonsa* during 2013. The proportion of males in the population ranged from as low as 10% (± 0.02 S.E.) during the second week in November to as high as 42.7% (± 0.02 S.E.) in mid-July.

Figure 2: Survivorship (egg to adult) for 21 families in 2011 (A) and 72 families in 2013 (B) of *Acartia tonsa* in laboratory experiments for determination of adult sex ratio. On average (solid line), 0.77 (±0.02 S.E.) of offspring survived in 2011; the proportion survived ranged from 0.59 to 0.88. In 2013, the proportion of offspring surviving to adulthood ranged from 0.61 to 0.97 across families, with 0.78 surviving on average (±0.01 S.E.) as shown by the solid line.

Figure 3: Proportion of male offspring produced by families during 2011 (A) and 2013 (B). The solid line shows equal male:female production of offspring. In 2011 (A), 38% of families (eight out of 21) had sex ratios not statistically different from unity (unfilled circles), while 13 families (~62%) had statistically skewed sex ratios (gray and black circles). Of those, four families produced predominantly male offspring, while nine produced predominantly female offspring. After recalculating sex ratios by assuming all deaths were due to males (see methods), seven families still had skewed sex ratios in 2011; five male-biased and two female-biased (black circles). In 2013 (B), almost 60% of families (42 out of 72) had sex ratios that did not statistically differ from unity (unfilled circles). Thirty families produced significantly skewed sex ratios (gray and black circles); 26 families produced female-dominated clutches and four families producing male-dominated clutches. After correction for mortality of males, 18 families

had biased sex ratios (black circles), with nine female-biased families and nine male-biased families.

Figure 4: Expected and observed distributions of sex ratios (proportion male) assuming Mendelian segregation of chromosomes for 2011 (A) and 2013 (B) families. Uncorrected (white bars) and corrected (grey bars) distributions of sex ratios were significantly different from the expected (black bars) binomial distribution in both years (2011 families: uncorrected: $\chi^2 = 69.7$, $p<0.0001$; corrected: $\chi^2 = 18$, $p<0.01$; 2013 families: uncorrected: $\chi^2 = 392$, $p<0.0001$; corrected: $\chi^2 = 37$, $p<0.001$). Extreme proportions (≥ 0.8 and ≤ 0.2) were combined (see text).

Figures:

Figure 1:

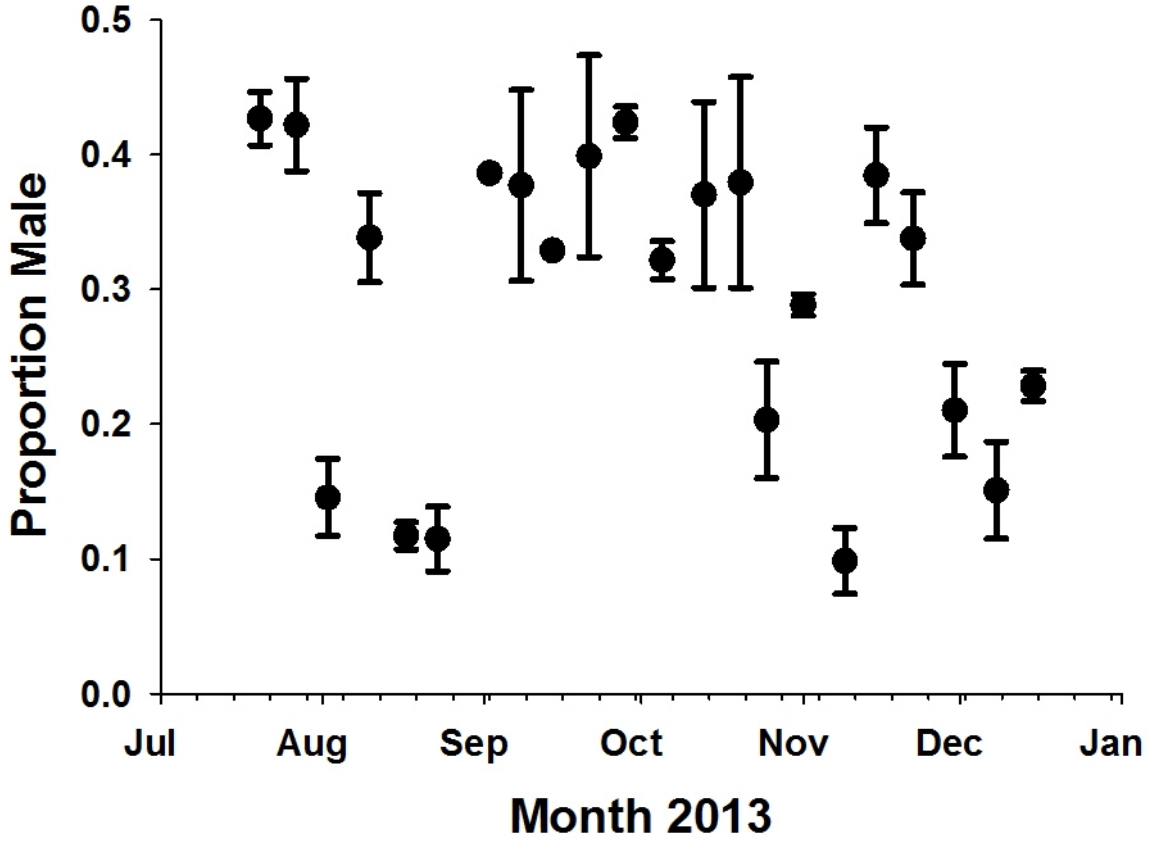


Figure 2:

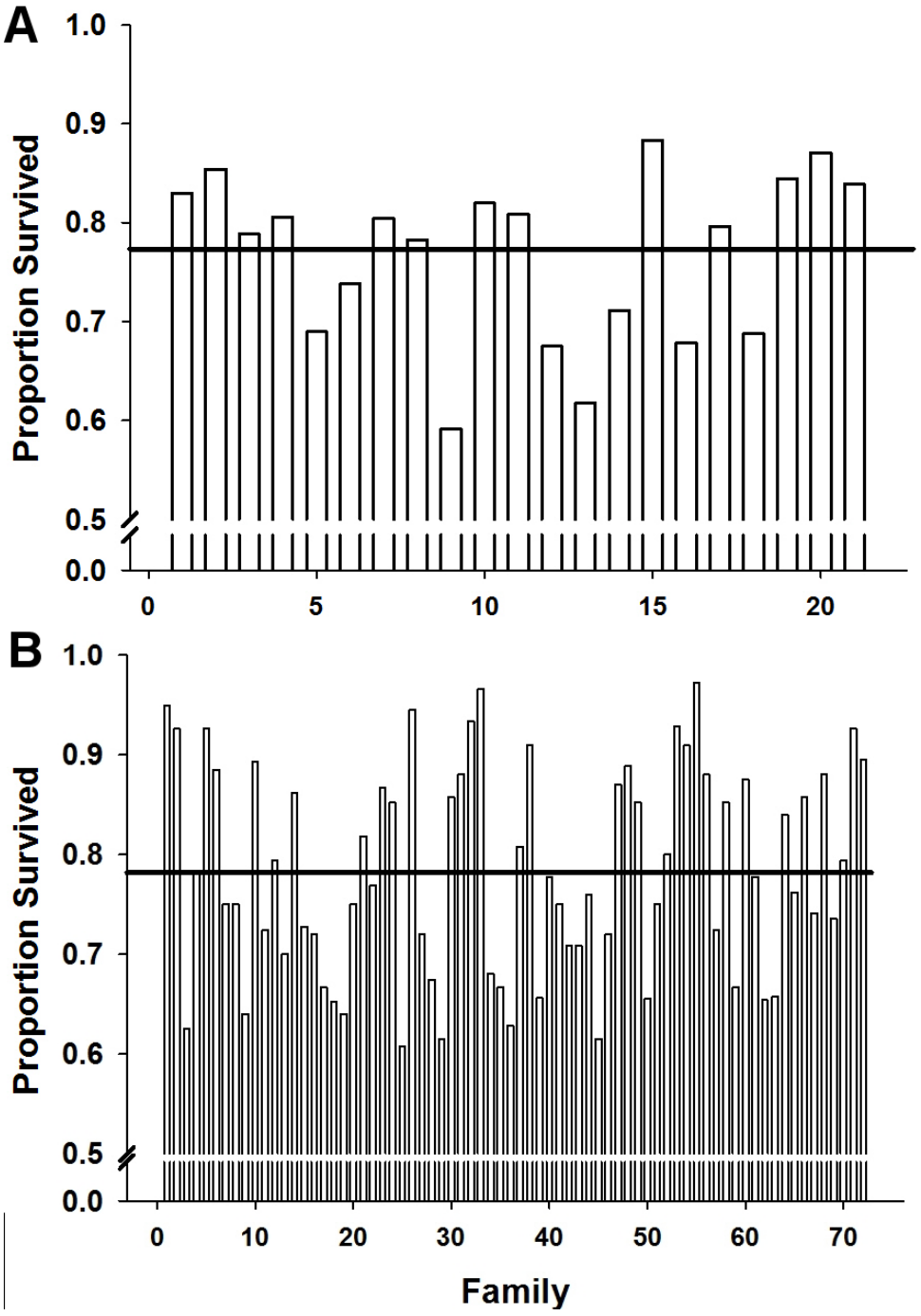

A
1.0
0.9
0.8
0.7
0.6
0.5
0.0
Proportion Survived
0
5
10
15
20
B
1.0
0.9
0.8
0.7
0.6
0.5
0.0
Proportion Survived
0
10
20
30
40
50
60
70
Family

Figure 3:

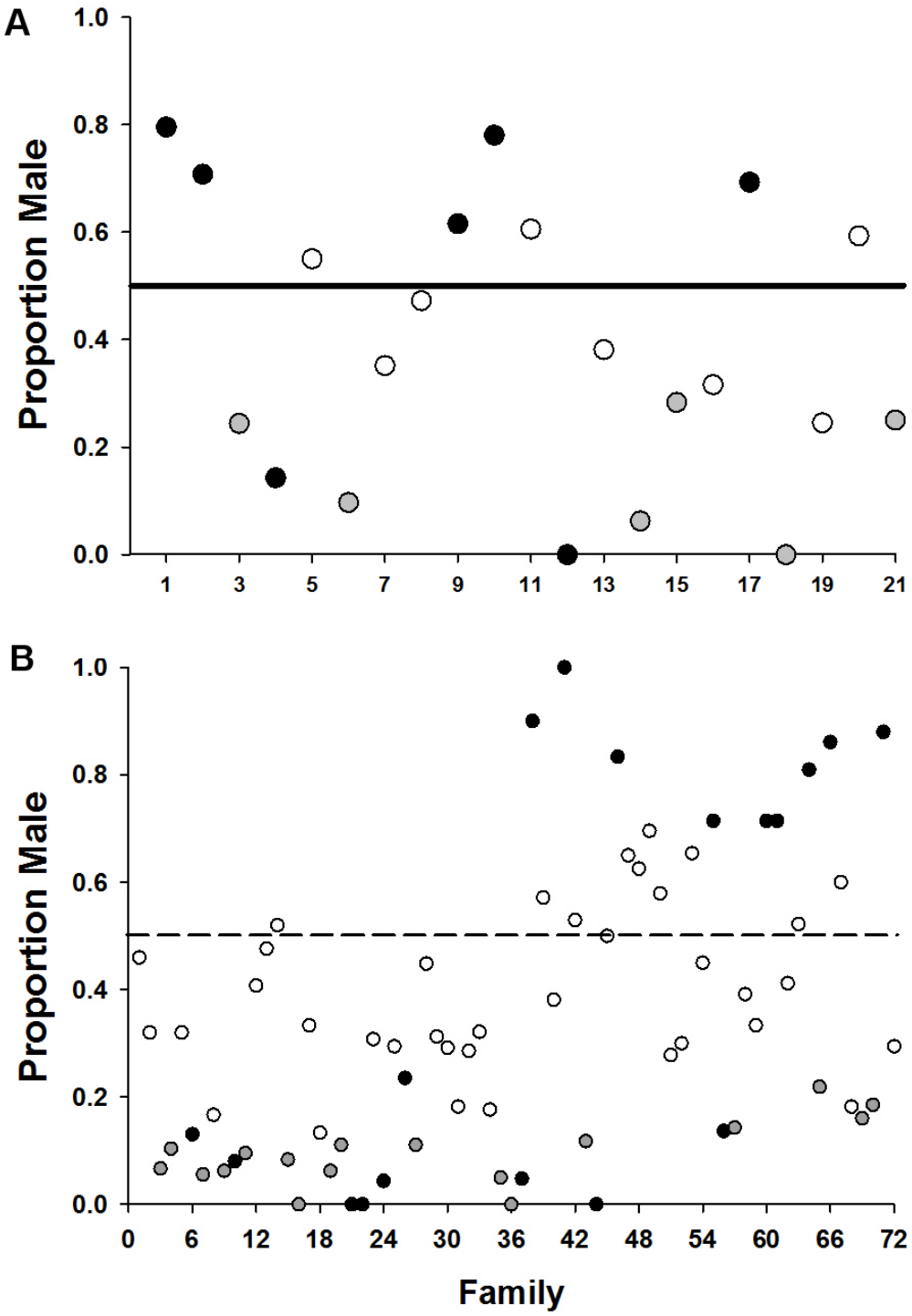
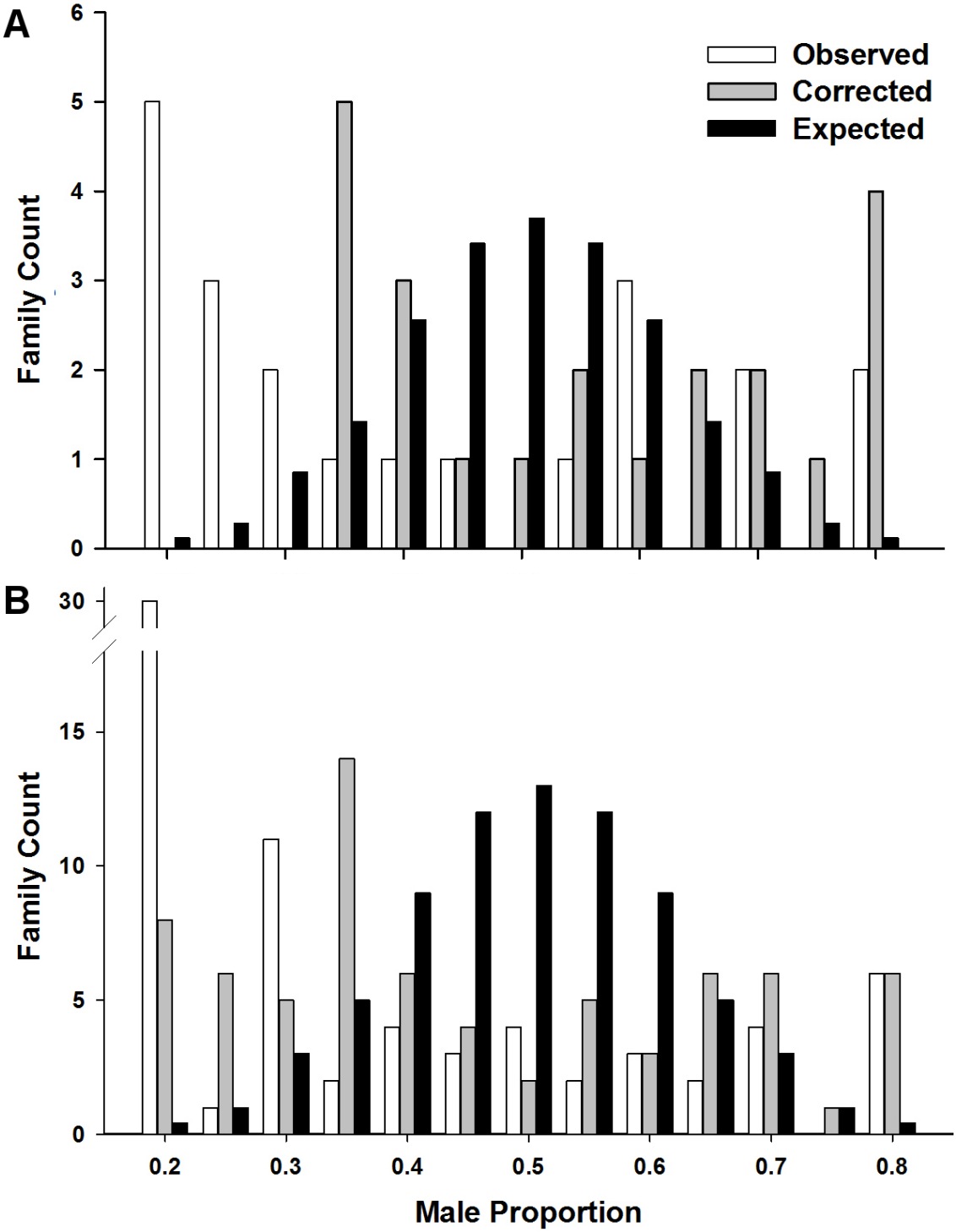

A
Proportion Male
1.0
0.8
0.6
0.4
0.2
0.0
1
3
5
7
9
11
13
15
17
19
21
B
Proportion Male
1.0
0.8
0.6
0.4
0.2
0.0
0
6
12
18
24
30
36
42
48
54
60
66
72
Family

Figure 4:


A
B
Observed
Corrected
Expected
Family Count
Male Proportion
0
1
2
3
4
5
6
10
15
30
0.2
0.3
0.4
0.5
0.6
0.7
0.8

Chapter 3:

Male reproduction as a function of food abundance and age in the calanoid copepods, *Acartia tonsa* and *Acartia hudsonica*

Abstract:

Lifetime-spermatophore (sperm packet) production, which can provide valuable information about male energetics and mating, is not known for any species of copepod. Spermatophore production could limit female fertilization in species in which females require multiple matings to remain fertilized, and in populations that often have adult sex ratios highly skewed toward females. Spermatophore-production rates were measured in the laboratory for two species of calanoid copepods, *Acartia tonsa* and *Acartia hudsonica,* from time of maturation until death, and under three food regimes (high, low, and no food). Spermatophore-production rates of *A. tonsa* were independent of food treatment, but significantly different among treatments in *A. hudsonica*-- with those in high food having the highest peak rates. For both species, there was a significant decrease in production rates and numbers of spermatophores with age, regardless of food treatment. The majority of males ceased spermatophore production with around 50% of their lifespan remaining. However, *A. hudsonica* males in the low-food treatment did not show a significant reduction of spermatophore production with age, and made up for their early low-production rates by continuing to reproduce into old age. Therefore, there was no difference in the average lifetime number of spermatophores produced in males of *A. hudsonica* fed at high or low-food conditions. Males with no food, however, produced significantly fewer spermatophores than fed males. In *A. tonsa*, there was no difference in the average number of spermatophores produced over a male's lifespan among food treatments. Finally, males of *A. tonsa* held with females had significantly shorter lifespans than males held alone, supplying further evidence that sexual activity and spermatophore production may result in a tradeoff in longevity. Both species had similarly low rates of spermatophore production compared to other

values reported in the literature, suggesting that even under high-food conditions, female fertilization rates may be limited.

Introduction:

Copepods are the most abundant metazoans in the oceans and are important links to upper trophic levels. Because copepods reproduce sexually, successful mating is a requirement to sustain or grow the population. Reproduction in pelagic copepods is not solely dependent on female fecundity, but may be constrained by the mating capacity of males (Kiørboe, 2006). For instance, regardless of food quantity and quality, significant numbers of adult females in nature have been reported to produce either non-hatching eggs (Irigoien et al., 2002; Maps et al., 2005) or none at all (Williamson and Butler, 1987; Uye and Sano, 1995). This has been hypothesized to be due to fertilization-limitation (Parrish and Wilson, 1978; Hopkins, 1982; Williamson and Butler, 1987; Ceballos et al., 2014), as a result of fewer males than females in the population coupled with low-spermatophore (sperm packet) production rates (Kiørboe, 2007; Ceballos et al., 2014). Despite its potentially important implications for population dynamics, our knowledge of male reproductive investment (spermatophore-production rates) is limited (but see Ianora and Poulet, 1993; Ianora et al., 1995, 1996, 1999). The lack of information on spermatophore production is in stark contrast to female egg production rate, which has been extensively studied both in field and laboratory populations (Marshall and Orr, 1972; review: Kiørboe and Sabatini, 1994). During mating in calanoid copepods, males attach a single spermatophore near the female's gonopore (Hopkins and Machin, 1977; Ferrari and Dojiri, 1987; Bagøien and Kiørboe, 2005). Thus, low rates of spermatophore production can limit the number of matings that a male can perform (Hopkins, 1982; Kiørboe, 2007). The few measurements of spermatophore-production rates suggest that production may be so low (0.1 to 3 day^{-1}) that even at low population densities they may limit male reproduction more than encounter rates with females

(Hopkins, 1982; Ianora and Poulet, 1993; Ianora et al., 1995, 1996, 1999; Kiørboe and Bagøien, 2005; Kiørboe, 2007).

While reproductive theory suggests that producing sperm is energetically less expensive than producing eggs (Bateman, 1948; Trivers, 1972; Charnov, 1982), energy for sperm production and sperm transfer may be limiting for many animals (Dewsbury, 1982; Birkhead and Møller, 1998). In copepods, however, energetic investment in spermatophore production may be equally expensive as in egg production (Mauchline, 1998). Male copepods do not emit sperm gamete by gamete. Instead, in order to increase the chance of fertilization, they place hundreds to thousands of spermatozoa in spermatophore bundles (Mauchline 1998, Sichlau and Kiørboe, 2011). Thus, female fertilization rates are actually limited by the number of spermatophores, not spermatozoa, that a male can produce (Dewsbury, 1982). The costs associated with producing spermatophores are likely considerable since males may place additional, costly substances (proteins, lipids, carbohydrates, mineral, hormones, etc.) in their spermatophores as 'nuptial gifts' for the females (Defaye et al., 2000). This is a common, and costly, phenomenon among insects and other animals (Vahed, 1998; Arnqvist and Nilsson, 2000). Therefore, the cost of spermatophore production may not be trivial for male copepods.

Information on spermatophore production is rare; to date there are published rates for only six species (see Table 1). Most of these rates have been measured over only a single day or in the presence of a single potential mate. However, one may assume that spermatophore-production rate varies with age of males and may also depend on mate choice. We know that female egg production is not constant and in fact decreases with age (Rodríguez-Graña et al., 2010; Sichlau and Kiørboe, 2011), and there is some evidence that male spermatophore-production rates are age-dependent and cease entirely in old age (Ceballos and Kiørboe, 2010;

Sichlau and Kiørboe, 2011). Similarly, using a single potential mate to obtain spermatophore-production rates may underestimate the actual male production rate. Recent work suggests that many copepod species (and often both sexes) may exhibit mate choice (Burton, 1985; Lazzaretto et al., 1990; Anstensrud, 1990, 1992; Heuch and Schram, 1996; Ali et al., 2009; Ceballos and Kiørboe, 2010, 2011; Sichlau and Kiørboe, 2011, Heuschele and Kiørboe, 2012; Burris and Dam, in press). By providing a single mate, the observed rates may reflect a male's choice to refuse a mate rather than his actual ability to mate. Therefore, any attempt to measure actual spermatophore production requires following individual males over their lifetimes in the presence of abundant female mates.

The calanoid copepods *Acartia tonsa* and *Acartia hudsonica* are the dominant species present in Long Island Sound during the fall/winter and the spring/summer months, respectively (Peterson, 1986). Females in both species lack a spermathecum and as a result cannot store sperm (Hammer, 1978; Ohtsuka and Huys, 2001), requiring that they re-mate to ensure continued fertilization. Males transfer a single spermatophore per mating (verified by checking females immediately after mating) and so the rate of mating is assumed to be equivalent to the rate of spermatophore production. Males continue to feed after maturation but have minimal fat and lipid reserves (Lee et al, 2006). This might suggest that spermatophore production may be tied to food intake (Defaye et al., 2000), since lipids and other nutritional substances are often placed in insect spermatophores (reviewed by Boggs, 1990, 1995; Gwynne 1997, 2008). Copepods likely experience instances of food limitation over their lives, since factors affecting algal biomass (quantity) and elemental composition (quality) are constantly changing (Durbin et al., 1992; Bergreen et al., 1988; Muller-Navarra and Lampert, 1996; Gulati and Demott, 1997; Sterner and Schulz, 1998). Similarly, adult males have been shown to age more rapidly than

females (Rodríguez-Graña et al., 2010), implying that reproductive investment may decrease or even cease much earlier in males than in females. In the present study we tested the hypotheses that male spermatophore-production rates were dependent on food abundance as well as age in both species of copepods. In addition, we were interested in a number of other aspects related to male mating biology: 1) are some males much more (or less) successful in producing spermatophores than others? 2) what is the average number of spermatophores produced and mates mated with over a male's life? 3) do males produce multiple spermatophores on the same day? 4) are those multiple spermatophores placed on the same or different females? 5) do males held in the presence of females have shorter lifespans than those held alone? Information on the energetics and basic biology of spermatophore production in copepods may provide answers to the question of whether males limit female fertilization under field conditions.

Methods:

Copepods used in experiments were from laboratory cultures of *Acartia tonsa* and *Acartia hudsonica* initiated with animals collected from Groton Long Point, Long Island Sound, USA (Latitude: 41.3271 N, Longitude: 72.00150 W) within the past year. Cultures were kept in 34 L buckets in an environmental chamber (15°C, 12:12 light:dark cycle) and fed *ad libitum* on a standard diet of the diatom *Thalassiosira weissflogii* and the green flagellate *Tetraselmis sp.* Algae were grown at 18°C as semi-continuous cultures on F/2 medium (Guillard, 1975).

Male copepods were separated as stage C4 juveniles and held in an 8L container together and fed as above. Males were checked daily for maturation. Once mature, males were randomly placed into individual 60 x 30 mm Petri dishes filled with ~30 mL of >700 (food-replete) or ~200 μg C L^{-1} (food-limited) food solutions (Besiktepe and Dam, 2002), or filtered sea water (no-food treatment). The food solutions consisted of 0.2-μm filtered sea water to which

Thalassiosira weissflogii and *Tetraselmis sp.* (50% of each by carbon) were added. Sample sizes for each treatment ranged from 18-31 males.

Five unmated females were then placed with each individual male. These females were picked from cultures every few days as either C4 juveniles or as unmated adults (no spermatophore present). Females were checked for mating after 24 hours (indicated by an attached spermatophore on the gonopore) and, regardless of whether or not they mated, were replaced by five new females. The number of spermatophores produced and the number of females mated with were recorded daily for each male until death (4- 35 days depending on treatment, see Fig. 6). Water and food solutions were changed daily. In both species, male survivorship, number of mating events per day over their lifetime, number of females mated with, average rate of mating, and mating rate per day were calculated as an average of all males for each food treatment.

To test whether spermatophore size depends on food availability, images of the spermatophores deposited by male *Acartia hudsonica* were recorded using an inverted microscope attached to a camera. Length and width of each spermatophore were measured to one thousandth of a millimeter using the program ImageJ. Spermatophore volume was calculated as an ellipsoid shape (Ceballos and Kiørboe, 2010). In addition, mated females were then placed into separate dishes and checked daily for the length of time that spermatophores remained attached (an indication of the length of time that females remain fertilized).

An additional study was performed one month later to test the effect of female presence on male longevity. Recently matured *Acartia tonsa* males were placed in one of the same three food treatments as above and either placed with 5 females or held alone. Food solutions were changed daily and males were followed until death. For longevity analysis, survival (l_x) was

calculated as the ratio of individuals alive at time x, n_x, and at the start of the experiment, n_0: $l_x = n_x/n_0$.

A two year field study for *Acartia hudsonica* (1 year for *A. tonsa*) was conducted during 2013 and 2014 in Long Island Sound to measure the extent of fertilization limitation in field populations. Plankton tows were conducted on a weekly basis (see chapter 7 for methods). The frequency of mated females (indicated by spermatophore presence) for each date was recorded.

Differences among food treatments were tested with a one-way ANOVA. When the assumptions for the parametric ANOVA were not met, a Kruskal-Wallis ANOVA on ranks was used. When results were significant, ($P<0.05$), post hoc comparisons were performed using the Tukey HSD test (for ANOVA) or Dunn's test (for ANOVA on ranks). Multiple-comparison Chi-square tests were used to examine differences among proportions. The significance of the slope of a linear regression, for each food treatment, was used to test whether spermatophore-production rate depended on male age. Differences in survival among *A. hudsonica* in different food treatments were tested using the Gehan–Breslow test (Lee and Wang, 2003). Differences in survival among *A. tonsa* in different food treatments and in the presence/absence of females were tested using a two-way ANOVA.

Results:

Proportion of mated males

In both *Acartia tonsa* and *Acartia hudsonica*, more than half of males mated at least once over their lifetime (Fig. 1). A greater proportion of *Acartia hudsonica* males (range 0.57-0.78) mated compared with *Acartia tonsa* males (range 0.67-0.91). There was no obvious pattern between food treatment and proportion of mated males in *A. tonsa* (Fig. 1A), but the proportion

of mated males appeared to decrease as food concentration decreased in *A. hudsonica* (Fig. 1B). However, statistically, the proportion of mated males was independent of food treatment for both species (*A. tonsa* proportion mated: High: 0.63, n=27, Low: 0.57, n=30, No Food: 0.78, n=31, chi-square=3.1, p=0.22, Fig. 1A; *A. hudsonica* proportion mated: High: 0.91, n=22, Low: 0.84, n=25, No Food: 0.67, n=18, chi-square=4.0, p=0.13, Fig. 1B).

Total lifetime number of spermatophores produced

Acartia tonsa males produced a maximum of nine spermatophores over their lifetime under all food conditions, while the maximum numbers produced by *Acartia hudsonica* were17 under high food, 12 under low food, and four under no food.

For *A. tonsa* there was no significant difference in the average number of spermatophores produced between food treatments (Kruskal-Wallis ANOVA on ranks; all males: H=3.1, p=0.21; mated males: H=0.5, p = 0.79) (Fig. 2A). When all males (unmated and mated) were included in the averages, those in the high treatment produced an average of 1.8 spermatophores over their lifetimes (±0.4 S.E.), compared with 1.9 (±0.5) for those in low food, and 2.5 (±0.4) for males in no food (Fig. 2A, black bars). When only males that mated at least once in their life were included, those fed at high food produced an average of 2.8 spermatophores (±0.5 S.E., n=17), while those in low food averaged 3.3 (±0.6, n=17) and those in no food produced 3.2 (±0.5, n=24) (Fig. 2A, grey bars).

Conversely, for *Acartia hudsonica* males, the average lifetime spermatophore production was dependent on food treatment, with males in no food producing the fewest spermatophores (all males: H= 18.3, p <0.001; mated males: H=17.7, p<0.001)(Fig. 2B). On average for all individuals (Fig. 2B, black bars), males produced a total of 7.2 (±1 S.E.) spermatophore in high

food, 5.3 (±0.7 S.E.) in low food, and 1.7 (±0.4 S.E.) under no food. Using only males that mated, those with no food still produced significantly fewer spermatophores (2.6 ±0.3 S.E.) than high (8.0±1.0) and low (6.3±0.7) food treatments (Fig. 2B, grey bars). In both cases, there were no significant differences in the averages between high and low-food treatments, but the no-food treatment produced statistically fewer spermatophores than the other two treatments.

Spermatophore-production rate

Acartia tonsa produced, in all food treatments, the majority of spermatophores during the first four days, and spermatophore production ceased entirely after about 12 days (Fig. 3A). In all treatments, the rate of spermatophore production decreased significantly with age (slopes of linear regression were all significantly different from 0: high food: F=22, low food: F=16.7, no food: F=22, P<0.01 for all). During peak production (first four days), there was no significant difference among treatments in the average number or rate of spermatophores produced (all: H = 5.409; p = 0.067; mated: H=2.9, P=0.23). Averaged for all males over their lifetime, males in high food produced spermatophores at a rate of 0.2 day $^{-1}$ (±0.04S.E.) (mated males: 0.3±0.05 day $^{-1}$), in low food 0.24 day $^{-1}$ (±0.07) (mated: 0.42±0.1), and with no food 0.31 day $^{-1}$ (±0.05) (mated: 0.4±0.05) (Fig. 4A). Again, there was no significant difference in average-lifetime rates among food treatments (all males: H= 5.1; mated: H= 3.6, p> 0.05 for both) (Fig. 4A). Neither was there a significant difference among food treatments in the average last day that *Acartia tonsa* males produced a spermatophore (H= 1.95, p=0.38). Males in the high-food treatment had 45% (±6.6) of their lifespan left after producing their last spermatophore, in low food 42.9% (±4.4) and with no food 44.8% (±6.2).

Acartia hudsonica males in the high-food treatment had their highest spermatophore-production rate during the first four days of their lives, and stopped producing spermatophores after about 12 days. Males in the low-food treatment experienced a significant drop in spermatophore-production rate after eight days, but continued to produce spermatophores until death (Fig. 3B). Males in the no-food treatment experienced a decrease in spermatophore-production rate after about seven days but, as with high food, stopped producing spermatophores well before death. In summary, a significant decrease in spermatophore production with increasing age was observed for the high-food and no-food treatments (slope of regressions all significantly different from 0: high: $F=63$, Starved, $F=4.8$, all $p<0.05$), but not in the low-food treatment (slope of regression not different from 0: $F=1.2$, $p=0.28$). A significant difference among treatments in the last day that *A. hudsonica* males that had mated produced a spermatophore was also observed. Males in the low-food treatment produced spermatophores later into their lives than those in the high-, or no-food treatments (low vs. starved: $t=4.6$; low vs. high: $t=3.8$; $P< 0.001$ for both; high vs. starved: $t=1.4$, $p> 0.05$). Males in the high-, and no-food treatments had about half of their lives remaining when they produced their last spermatophore (high: 44.2 ±4.5% S.E., no food: 53.3±5.2%), twice as long as males in the low-food treatment (22.4% ±3.6). Spermatophore-production rates during the first four days were significantly greater in the high-food treatment than the other two treatments ($H=14.2$, $P <0.001$). Spermatophore-production rate averaged for all males over their lifetime was 0.38 day $^{-1}$ (±0.06 S.E.) (mated males: 0.42 ±0.06 day $^{-1}$) for the high-food treatment, 0.25 day $^{-1}$ (±0.03) (mated: 0.3±0.03) for the low food, and 0.13 day $^{-1}$ (±0.03) (mated: 0.2±0.03) for the no-food treatment (Fig. 4B). The average spermatophore production over a lifetime was only statistically different

between the high- and no-food treatments (all: H=11.4, p = 0.003; mated :H=8.3, p = 0.016) (Fig. 4B).

Multiple matings

Interestingly, males of both species often produced more than one spermatophore a day (up to four for *Acartia tonsa* and up to five for *Acartia hudsonica*). When this occurred, males almost always mated with multiple females (over 97% of the time). The number of days in which a single male *A. tonsa* produced multiple spermatophores was independent of food treatment: 39.4% (high food), 40% (low food), and 38% (no food) (chi-square=3.01, p>0.05). Thus, on days when mating occurred males produced 1.46 spermatophores (±0.15 S.E.) in high food, 1.42 (±0.12) in low food, and 1.5 (±0.12) in no food (no significant difference among treatments: H=0.37, p=0.83).

The number of days in which a single *Acartia hudsonica* male produced multiple spermatophores was independent of food treatment: 26.6% (high food), 19.1% (low food), and 11.1% (no food) (chi-square=4.0, p>0.05). Similar to *Acartia tonsa*, the number of spermatophores produced on days when mating occurred was also independent of food treatment (H=5.6, p = 0.06): 1.84 (±0.4) for high food, 1.2 (±0.05) for low food, and 1.25 (±0.13) for no food.

Spermatophore volume and time attached

Neither the size (volume) of spermatophores nor the length of time they remained attached was affected by food abundance in *Acartia hudsonica* males. Average spermatophore volume was 0.12 ±.01S.E. $x10^{-2}mm^3$ (n=22) in the high-food treatment, 0.13 ±.01 (n=21) in low

food, and 0.12±.01 (n=11) in no food (F=0.546, p=0.58) (Fig. 5A). Spermatophores remained attached to females for an average of 4 ±0.4 days (high food), 5.4 ±0.5 (low food), and 4.4 ±0.8 (no food) (H=5.7, p= 0.059) (Fig. 5B).

Longevity

The longevity of *Acartia tonsa* males depended significantly on whether they were with females, and on food treatment (Fig. 6A). Under high food, males held with females had shorter lifespans on average (16.2 ±1.2 days S.E.; n=45) than males held alone (20.8±1.3 days; n=40). Under low food, males held with females also had shorter lifespans (7.7 ±0.6 days; n=41) than those held alone (11.5 ±0.8 days; n=38). Under no food, longevity was independent of the presence of females (with females: 6.4 ±0.5 days; n=45; alone: 5.6 ±0.3 days; n=38). When only longevities of males held alone were compared, males had longer average lifespans as food concentration increased. Lifespan of males held with females was also longer for the high-food treatment relative to low- and no-food treatments. There was a significant interaction between the presence of females and the food treatment (F=6.0, p=0.003). The negative effect of female presence on male longevity increased as food levels increased.

For *Acartia hudsonica*, longevity was measured only for males held with females (Fig. 6B). There was no difference in the average lifespan of males in high- (19.5±1.2 days S.E.; n=22) and low-food treatments (21.8±1.3 days S.E.; n=25) (Gehan-Breslow test=1.1, p = 0.31), but males in the no-food treatment had significantly shorter lifespans (14.9±1.0 days S.E.; n=18) (High vs. No food: stat=7.9, Low vs. No food: stat=13.9, $p < 0.05$ for both).

Field-fertilization frequencies

During 2013 and 2014, not once were 100% of females in the population mated for either species (Fig. 7). On average over the 32 weeks for *Acartia tonsa*, 80.3% of females in the population had mated (±1.3%) (range: 68% to 96.7%) (Fig.7A). Averaged over the season for *Acartia hudsonica*, 77% (±1.9%) of females were mated in 2013 and 75% (±2%) in 2014. However, values ranged from 40% to 88.4% in 2013 (from 50% to 92.7% in 2014) (Fig. 7B).

Discussion:

The present study shows that spermatophore production in male copepods may be limited by both food abundance and male age. In addition, this is the first study to measure spermatophore-production rates for individual male copepods over their lifetimes, and as a function of food abundance. Both *Acartia tonsa* and *Acartia hudsonica* showed a significant decrease in spermatophore production with age. In *A. hudsonica*, spermatophore-production rate was also food dependent. Most importantly, even during the peak time for production (early in the male's life) the spermatophore-production rate for both species was very low, ~ 0.6 spermatophores male^{-1} day^{-1} for *A. tonsa* and ~1 male^{-1} day^{-1} for *A. hudsonica.* The effect of these low spermatophore-production rates on fertilization was evident in the field during 2013; at no point in time were all females fertilized.

The low spermatophore-production rates measured in this study are consistent with the few other reports in the literature of <1 and up to 3 spermatophores male^{-1} day^{-1} (Ianora and Poulet, 1993; Ianora et al., 1995, 1996, 1999; Miralto et al., 1995; Turner et al., 2001; Kiørboe, 2007; Ceballos and Kiørboe, 2010, 2011; Sichlau and Kiørboe, 2011). Ceballos and Kiørboe (2010) measured a rate of production over 24 hours of 1 spermatophore male^{-1} for *A. tonsa*, similar to the rate measured in this study under high food. The combination of low

spermatophore-production rates and their decrease with male age suggests that males may be responsible for limiting female fertilization rates in the field. In these two copepod species, in which adult sex ratios are often highly skewed toward females (Lee and McAlice, 1979; Conover, 1956; Deevy, 1960; chapter 6), and females must re-mate multiple times to stay fertilized (Hammer, 1978; Ohtsuka and Huys, 2001), low spermatophore-production rates may severely limit population growth. The field data (Fig. 7) collected on a weekly basis over 2013 (for both species) and 2014 (for *A. hudsonica*) show that on no occasion were all females in the population mated. In actuality, between 68-97% of *A. tonsa* and between 40-93% of *A. hudsonica* females were mated (i.e. been fertilized). Thus, the low spermatophore-production rates measured under ideal conditions in our laboratory study coupled with female-biased sex-ratios (Chapters 1 and 6) suggest that males are indeed limiting fertilization in field populations.

Food limitation did not have the same effect on spermatophore production in *Acartia tonsa* and *Acartia hudsonica*. In *A. tonsa* there was no significant effect of food abundance on either the total number of spermatophores produced or the rate at which they were produced. Conversely, *A. hudsonica* males produced significantly fewer spermatophores, and at a lower rate in the no-food treatment than in the other two food treatments (Figs 2-4). While there was no difference in the total number of spermatophores produced over a male's lifetime in the high- and low-food treatments, the majority of spermatophores were produced at a much earlier age in the high-food than in the low-food treatment. The significant reduction in spermatophore production as food becomes limiting suggests an energetic cost to spermatophore production in *A. hudsonica*. Conversely, no such cost was apparent for *A. tonsa*. It is surprising nonetheless, that spermatophore production in the no-food treatment was not lower. Some species of

copepods in which males do not feed after maturation are able to produce a limited number of spermatophores (*Euchaeta norvegica* produces up to 3 over their short lifespan: Hopkins, 1978).

Another possibility is that spermatophore production in *A. tonsa* is dependent more on reserves acquired as juveniles rather than food consumed as adults. This is unlikely, though, since this species is not known to have lipid reserves (Lee et al., 2006; Finiguerra et al., 2013). A more plausible explanation, however, is that, instead of the number of spermatophores decreasing, some other aspect of reproduction is affected. For instance, the volume of the spermatophores, the quantity or quality of spermatozoa, or the addition of other substances in the spermatophore may decrease under food limitation, as is the case in many insects (Arnqvist and Nilsson, 2000; Cardoso and Gilbert, 2007). While little is known about the actual contents of spermatophores in copepods, two types of materials of different densities are present (Defaye et al., 2000). This implies that males may provide (possibly costly) materials other than sperm to their mates. Sperm quality, but not necessarily spermatophore production, decreased when males of *Temora stylifera* were fed poor diets (Ianora et al., 1999). In addition, there is evidence to suggest that sperm quality also decreases with age in some copepods (Rodríguez-Graña et al., 2010; Ceballos and Kiørboe, 2011; Sichlau and Kiørboe, 2011). Thus, in *A. tonsa,* it would not be surprising if sperm quality is affected by food abundance even while spermatophore production is not. We did measure spermatophore volume and attachment length for males of *A. hudsonica*, but found no differences by food treatments. Since large spermatophores contain more spermatozoa than small ones (Sichlau and Kiørboe, 2011), it appears that *A. hudsonica* copes with food limitation by reducing the total number of spermatophores produced rather than the amount of sperm they contain. It is also possible that, because the spermatophores of *A. tonsa* are larger than those of *A. hudsonica* (unpubl. data), that there are differences between the

species in the amount of glue needed to cement spermatophores onto females. If *A. tonsa* requires more glue, and if that glue is expensive to make, then spermatophores produced by males in the absence of food may not remain attached for as long as those produced by males fed high food. Future work is needed to determine if other aspects of male reproduction are affected by food limitation.

In both *Acartia tonsa* and *Acartia hudsonica*, spermatophore production decreased significantly as males aged, and the duration of production was as short as half the lifespan. Decreases in reproduction with age are common in many animals (Clutton-Brock 1988; Saether 1990), and age effects in copepods can be manifested through reductions in sperm quantity, sperm quality, and mating success (Rodríguez-Grana, 2010; Ceballos and Kiørboe 2011, Sichlau and Kiørboe 2011). In the present study, the reproductive period under high food was short compared to the lifespan; most males remained fertile for only 55% of their life, which is consistent with the similarly short periods reported for the copepods *Temora longicornis* and *Oithona davisae* (Ceballos and Kiørboe, 2011; Sichlau and Kiørboe, 2011). Sichlau and Kiørboe (2011) suggest that the rather long "post-reproductive life" in these males is an adaptation to low-mate-encounter rates; males that have not mated should continue to be able to produce spermatophores into old age. In *O. davisae*, experiments in which males were not allowed to mate early in life suggest this may be the case (Ceballos and Kiørboe, 2011), and our results for *A. hudsonica* provide additional evidence. Males under low-food conditions produced spermatophores at a lower rate early in life than those in high food, but continued production to a much older age (78% of their lives reproductive compared with 55%). Therefore, by the end of their lives, males from both treatments had produced similar numbers of spermatophores. It is possible that, by delaying reproduction until a later date but not sacrificing the total number of

matings, males in field populations could increase their chance of finding and mating with high-quality mates (Ceballos and Kiørboe, 2011). Sexual selection theory predicts that females should be choosier than males in selecting a mate partly because investment in sperm, in contrast to eggs, is not costly (Bradbury and Andersson, 1987). If, however, investment in sperm production is costly, in terms of time or energy, then males should also be selective (Thornhill and Alcock, 1983). Because of the low rates of spermatophore production, which suggest a cost to males, as well as the short reproductive period that may limit the number of males available to females, the potential for strong male-mate choice is possible (Hirst and Kiørboe, 2002).

The results in the present study suggest that in both species of copepods, there is large variation among males in reproductive abilities, and that a small proportion of males may be responsible for much of the mating in field populations. Males of *A. hudsonica* produced up to seventeen spermatophores over their lifetimes, while *Acartia tonsa* males produced up to nine. The only other measured lifetime-spermatophore-production rates are for a copepod species that does not feed after maturation, *Euchaeta norvegica ,* which produces up to three spermatophores (Hopkins, 1978; Ohtsuka and Huys, 2001); much less than the two species in the present study. The difference in maximum numbers is, in all likelihood, due to the fact the males of *A. tonsa* and *A. hudsonica* continue to feed as adults, whereas production in *E. norvegica* depends on lipid reserves obtained as juveniles (Hopkins, 1978; Ohtsuka and Huys, 2001). Furthermore, for species in which females can store sperm and thus require only a single mating, lifetime-spermatophore-production rates may also be much lower than the ones observed here. For instance, in the small cyclopoid copepod *Oithona davisae*, females can fertilize all eggs with a single spermatophore (Uchima, 1985), and males are thought to mate only about five times over their lives (Ceballos and Kiørboe, 2011). Interestingly, in males of *A. tonsa*, even under high-

food conditions, a large percentage of males (between 22 and 43%) did not ever mate (Fig. 1). The percentage in *A. hudsonica* was smaller, but not trivial (between 9 and 33% depending on food treatment). Copepods in the present study were from laboratory cultures, which were supplemented with field-caught copepods every month; thus, it was unlikely that males were sterile because of inbreeding. Instead, it is likely that males that do not mate may be common in field populations; for example, a large proportion of males of *Temora longicornis* collected from field populations did not mate in experiments (Ceballos et al. 2014). The frequencies of sterile males present in field populations as well as the degree of variation in male-mating success remain to be measured.

In the present study, males often produced multiple spermatophores on the same day. When this happened, males mated with multiple females more than 97% of the time. *Acartia tonsa* under high-food conditions produced multiple spermatophores about 40% of the time, whereas males of *Acartia hudsonica* produced more than one almost 27% of the time. This suggests that field observations of females carrying multiple spermatophores (Burris and Dam, in press) are the result of multiple matings with different males, instead of males mating multiple times with the same female in order to prevent future matings with other males, as has been suggested for some parasitic copepods (Anstensrud, 1990; Mauchline, 1998).

Acartia tonsa males held with females had significantly shorter lifespans in high- and low-food treatments compared with males that were held alone, suggesting that the presence of females or of sexual activity may impose significant costs on males. It cannot be ruled out that these results are due to the presence of *any* individuals, not just the female, since a treatment of males held with other males was not run. Previous work on *Oithona davisae*, however, found no effect of other males' presence on individual male lifespan (Ceballos and Kiørboe, 2011).

Instead, male lifespan was reduced only in the presence of females, and results became more extreme as the number of female mates per male increased (Ceballos and Kiørboe, 2011). While lifespans in males held alone were not measured for *Acartia hudsonica*, lifespans of males that were held with females were no different between the high- and low-food treatments. In this case, food limitation cannot be the explanation for this observation. Instead, similar sexual activity between treatments is a possibility. Since there was no difference in the total number of matings between these two treatments, males in both groups may have experienced the same mortality costs and thus died at the same ages. In *A. tonsa*, there was no difference in longevity between males held with or without females in the absence of food, implying that costs to males associated with lack of food were greater than those from female presence. In many taxa, male mating has been shown to accelerate the process of ageing (Bonduriansky and Brassil, 2005; Rodríguez-Graña et al., 2010). There may be a significant cost to mating in terms of early mortality for male copepods as well.

Acartia species likely experience periods of food limitation over their lifetimes in the field (Durbin et al., 1992; Dam et al., 1994). Low- and high-food experiments in the present study were meant to be analogous to periods of food limitation and saturation. Since copepod lifespans are likely shorter in nature than in the laboratory (Peterson, 2001; Hirst and Kiørboe, 2002), it is possible that most males may never reach old age. If this is the case, then differences between high- and low-food treatments are likely more extreme in nature; that is, food-limited males would not have the time to make up for low initial spermatophore-production rates. What is more, other aspects of food limitation, such as slower swimming rates for males fed at low food compared to high, would likely affect the number of mate encounters and as a result, mating success.

The low spermatophore-production rates measured in our experiments, while limiting male mating capacity under ideal conditions, would certainly have a stronger effect on population growth when encounter rates with females are low; that is, under periods of low population density or female-skewed sex ratios. Taken together, low spermatophore-production rates, limited total spermatophores produced over a lifetime, and the short reproductive period of males may be responsible for the low frequencies of mated females in the field population and the occurrence of nonviable eggs.

Table and Figure Legend:

Table 1: Spermatophore-production rates in the literature for 6 species.

Figure 1: Proportion of males that mated at least once in their lifetime for *Acartia tonsa* (A) and *Acartia hudsonica* (B) under high, low and no-food treatments. Numbers within histograms are the proportion of mated males and the sample size for each treatment, respectively. There was no statistically significant effect of food treatment on the proportion of mated males in either species.

Figure 2: Average lifetime number of spermatophores produced per male at three food levels (high, low, and no food) for *Acartia tonsa* (A) and *Acartia hudsonica* (B). When all males were included in the averages (black bars) or when only the mated males were included (grey bars), there was no significant difference among food treatments for *A. tonsa*. For all males, those in high food produced 1.8 (2.8 for mated males) spermatophores over their lifetimes, compared with 1.9 (3.3) in low food and 2.5 (3.2) in no food. Conversely, average lifetime spermatophore production of *A. hudsonica* was dependent on food treatment: males with no food produced significantly fewer spermatophores than males in high- and low-food treatments. For all individuals, males averaged 7.2 (mated: 8.0) spermatophores in high food, 5.3 (6.3) in low food, and 1.7 (2.6) under no food. Bars are standard error.

Figure 3: Average spermatophore-production rates per male every two days until death for *Acartia tonsa* (A) and *Acartia hudsonica* (B) under high food (black circles), low food (white circles), or no food (grey triangles). For *A. tonsa,* at all food levels, the highest rate of

production occurred during the first four days of a male's life, and then decreased significantly with increasing age. For males of *A. hudsonica*, those in high food had a significant drop in production after 12 days, whereas those in no food had a significant decrease after 8 days. Both treatments had significant decreases in spermatophore production with age. Under low food, however, production did not decrease significantly with age, and most males produced spermatophores until death. Bars are standard error.

Figure 4: Average lifetime production rate (spermatophores male^{-1} day $^{-1}$) under high-, low-, and no-food treatments for *Acartia tonsa* (A) and *Acartia hudsonica* (B). When all *A. tonsa* males were included in the averages (black bars), spermatophore-production rates in high food were 0.2 day $^{-1}$ (mated males (grey bars): 0.3 day $^{-1}$), in low food 0.24 day $^{-1}$ (mated: 0.42), and in no food 0.31 day $^{-1}$ (mated: 0.4). There was no significant difference in average lifetime rates among food treatments for *A. tonsa.* For *A. hudsonica*, rates were statistically different between the high food and no-food treatments. Spermatophore-production rates in high food were 0.38 day $^{-1}$ (mated males: 0.42 day $^{-1}$), in low food 0.25 day $^{-1}$ (mated: 0.3), and in no food 0.13 day $^{-1}$ (mated: 0.2). Bars are standard error.

Figure 5: Average spermatophore volumes (A) and lengths of time they remained attached to females (B) for *Acartia hudsonica* under high-, low-, and no-food treatments. Neither spermatophore volume nor time attached was statistically dependent on food abundance. Under high food, spermatophores were 0.12 x10^{-2}mm^3, compared with 0.13 for low food, and 0.12 for no-food treatments. Spermatophores remained attached to females for an average of four days for males fed at high food, 5.4 in low food and 4.4 days for no food. Bars are standard error.

Figure 6: Survivorship curves for three food treatments for (A) *Acartia tonsa* males in the presence or absence of females and (B) *Acartia hudsonica* males in the presence of females. Under high food, *A. tonsa* males held with females had significantly shorter lifespans (ave.: 16.2 days) than males held alone (ave.: 20.8 days). Under low food, males held with females also had significantly shorter lifespans (7.7 days) than those held alone (11.5 days). The longevities of males without food were not affected by the presence of females (with females: 6.4 days; alone: 5.6 days). When just longevities of males held alone were compared, males had significantly longer average lifespans as food concentration increased. Longevities of males held with females showed significant differences between the high- and low-food treatments, and high- and no-food treatments. For *A. hudsonica*, there was no difference in the average lifespan of males held with females in high- (19.5days) and low-food treatments (21.8days), but males in the no-food treatment had significantly shorter lifespans (14.9days).

Figure 7: Female fertilization frequencies in field populations for *Acartia tonsa* (A) and *Acartia hudsonica* (B) in 2013 (black circles) or 2014 (white circles). Frequencies were obtained by counting the number of females with and without spermatophores present during weekly sampling (see methods). Frequencies never reached 100% for either species (*A. tonsa* range: 0.68-0.96; *A. hudsonica*: 0.4-0.88) suggesting that field populations may be fertilization limited due to low spermatophore-production rates.

Tables:

Table 1:

Species	**Spermatophore-production rate** (male^{-1} day^{-1} ±S.D.)	**Length of time measured**	**Age**	**Mates provided**	**Authors**
Acartia clausi	<1 for between 0-55 days	Between 0-55 days	Unknown (from field)	Couples; males replaced when dead	Ianora et al., 1996
Acartia tonsa	1 37% of the 4-day trial never mated. Only 10% mated every day.	24 hours to 4 days	Matured within 24 hours (lab cultures)	1 male:10 females (24 hrs.); 1 male:4 females (new females daily over 4 days)	Ceballos and Kiørboe, 2010
Centropages typicus	<1 over 12 days	12 days	Unknown (field)	Couples	Miralto et al., 1995
Oithona davisae	0.9	2-3 days	Matured within 24 hours (lab)	3-4 males with >30 virgin mates	Kiørboe, 2007
Oithona davisae	2.5 for first 4 days; decrease after that with age. 25% of males never mated.	4 days	Matured within 24 hours (lab)	1 male: 10 females for 4 days. 1 male: 5 females at 24 hrs., then 10 and 20 days old or at 24hrs, 5, 7, and 10 days.	Ceballos and Kiørboe 2011
Temora longicornis	Mated only during first 8 days; decline after 5 days.	10 days	Matured within 24 hours (lab)	1 male: 2 females (new females daily)	Sichlau and Kiørboe 2011
Temora longicornis	< 1.5 Between 28-57% of males did not mate.	24 hours	Unknown (field) Matured within 24 hours (lab)	1 male: 10 females from field Couples from lab cultures.	Ceballos et al., 2014
Temora stylifera	0.4±0.4 to 0.7±0.6	10-54 days; checked daily	Unknown (field)	Couples	Ianora and Poulet, 1993
Temora stylifera	0.09±0.2 to 0.7±0.6	Measured until female death: 9-30 days	Unknown (field)	Couples	Ianora et al., 1995
Temora stylifera	0.6 ±0.3to 1.4±0.2	15 days; checked daily	Unknown (field)	Couples	Ianora et al., 1999
Temora stylifera	0.14±0.08 to 0.61±0.23	15-20 days	Unknown (field)	3 males:3 females or Couples	Turner et al., 2001

Figures:

Figure 1:

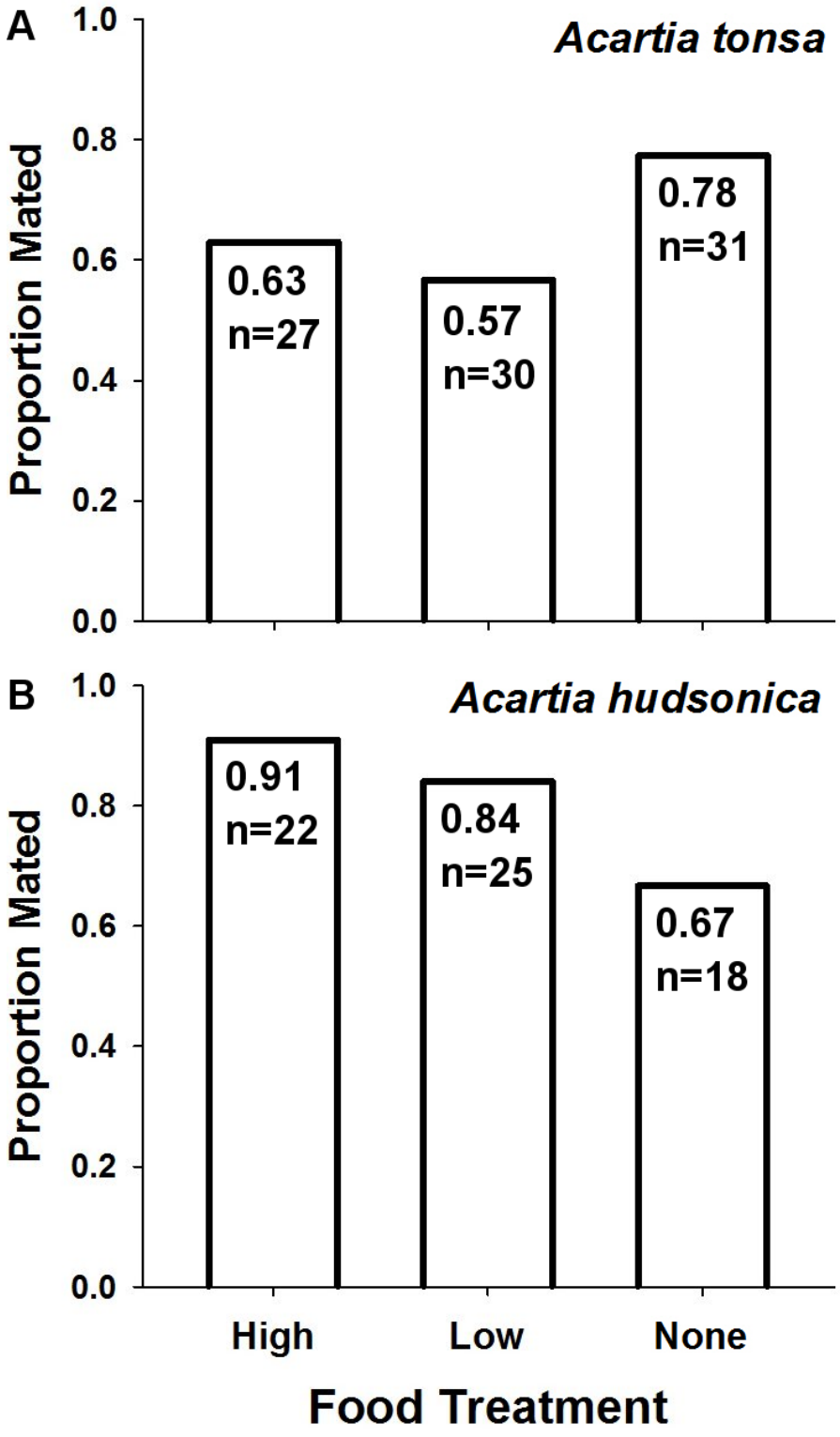


Figure 2:

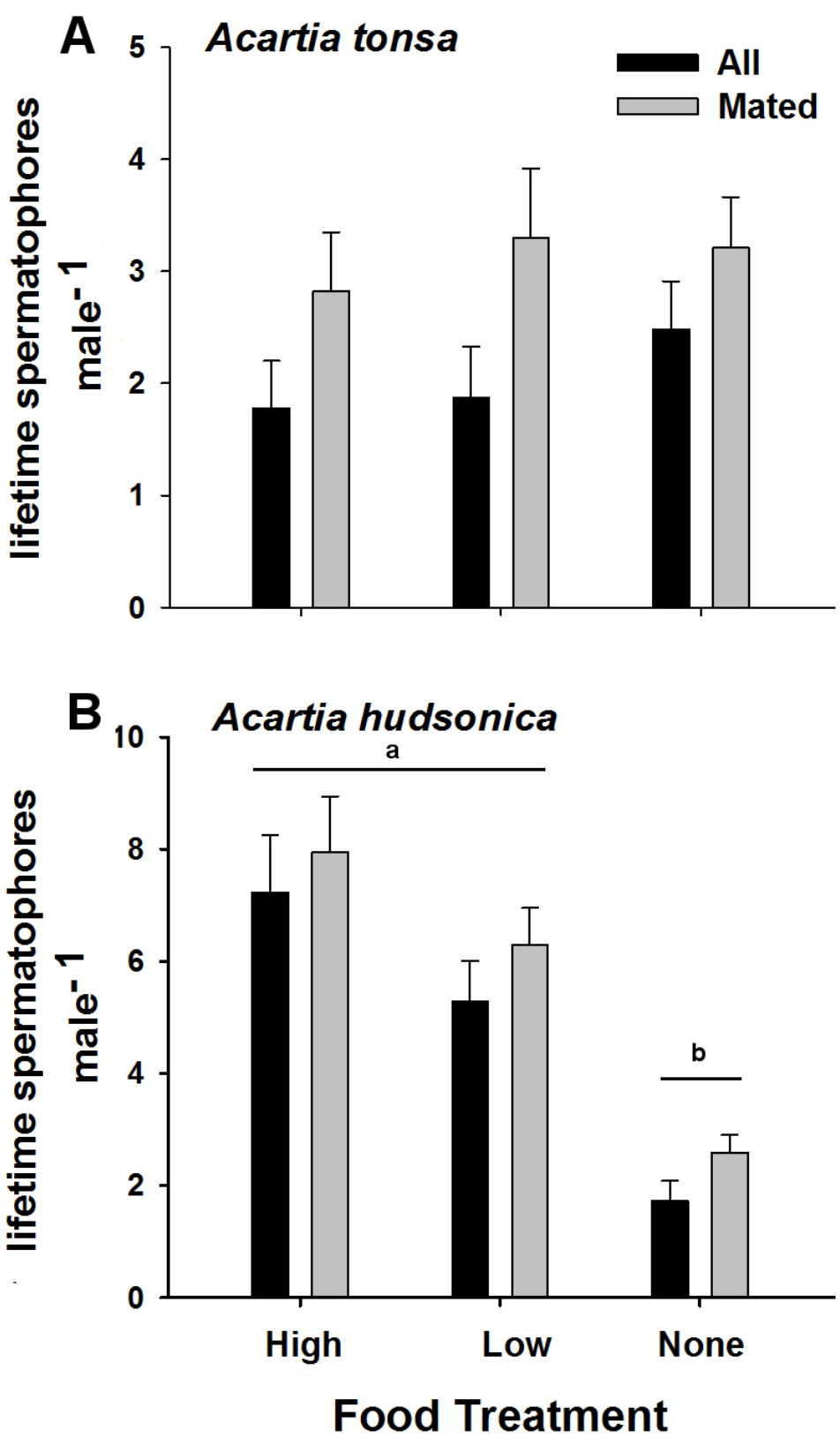

A
Acartia tonsa
All
Mated
lifetime spermatophores male-1
0
1
2
3
4
5
B
Acartia hudsonica
a
b
lifetime spermatophores male-1
0
2
4
6
8
10
High
Low
None
Food Treatment

Figure 3:

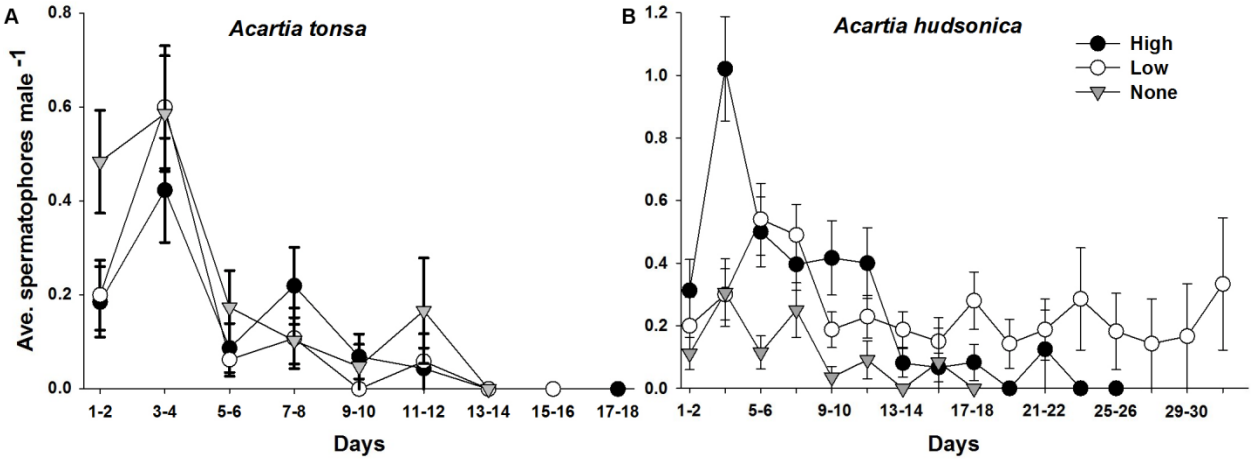

A
Acartia tonsa
Ave. spermatophores male -1
0.0
0.2
0.4
0.6
0.8
1-2
3-4
5-6
7-8
9-10
11-12
13-14
15-16
17-18
Days
B
Acartia hudsonica
High
Low
None
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1-2
5-6
9-10
13-14
17-18
21-22
25-26
29-30
Days

Figure 4:

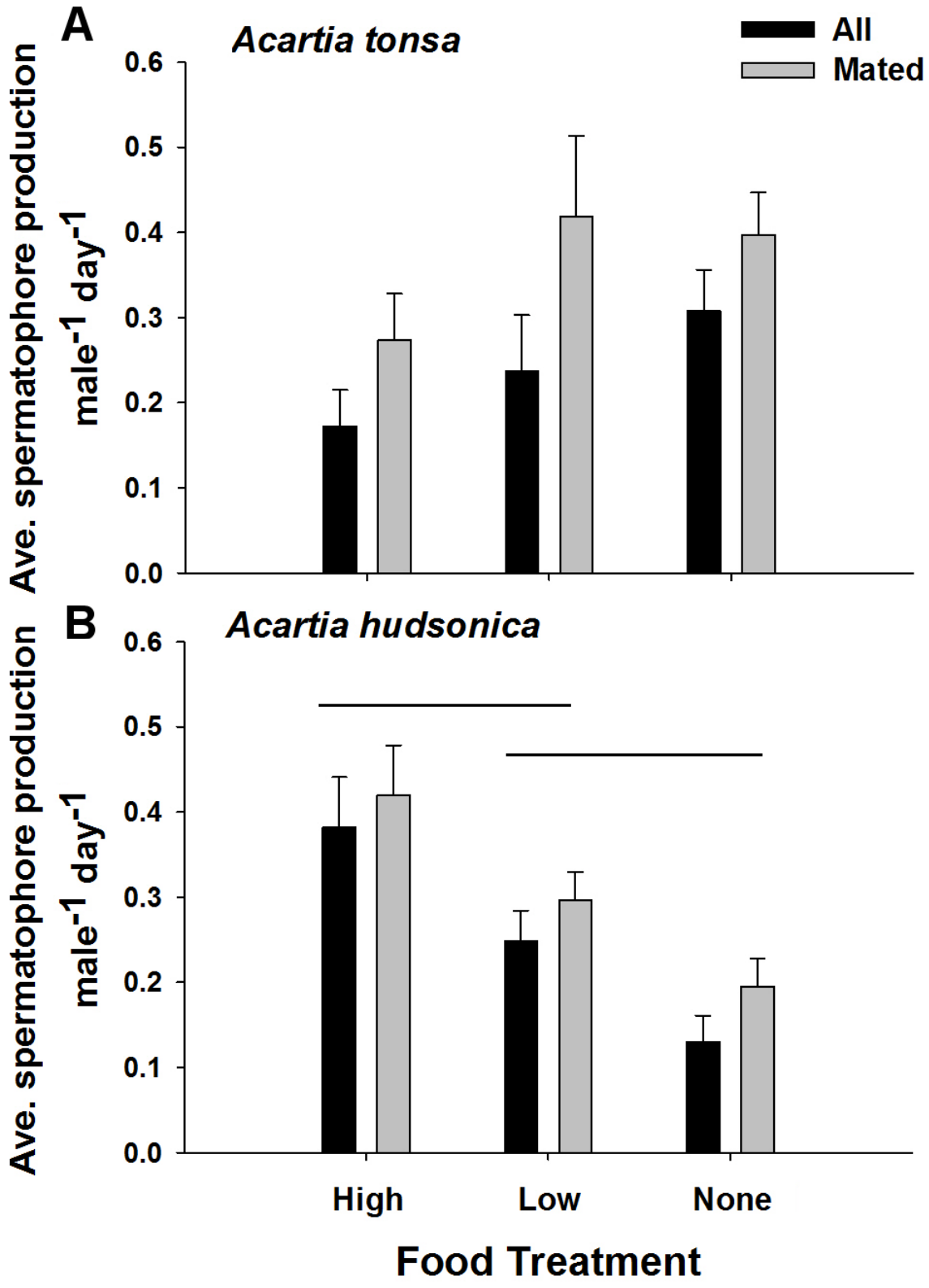

A
Acartia tonsa
All
Mated
Ave. spermatophore production
male-1 day-1
0.6
0.5
0.4
0.3
0.2
0.1
0.0
B
Acartia hudsonica
Ave. spermatophore production
male-1 day-1
0.6
0.5
0.4
0.3
0.2
0.1
0.0
High
Low
None
Food Treatment

Figure 5:

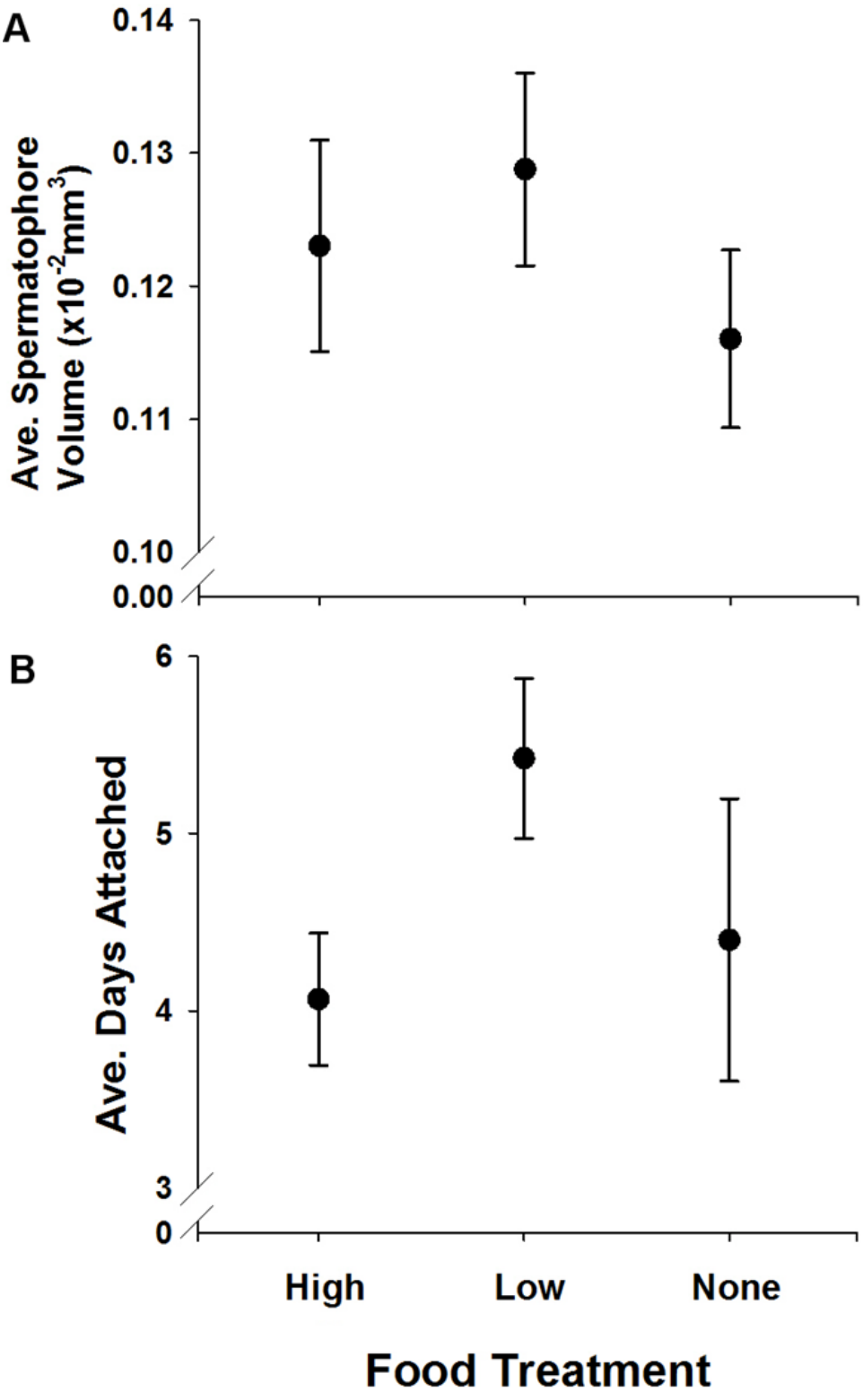

A
0.14
0.13
0.12
0.11
0.10
0.00
Ave. Spermatophore Volume (x10-2mm3)
B
6
5
4
3
0
Ave. Days Attached
High
Low
None
Food Treatment

Figure 6:

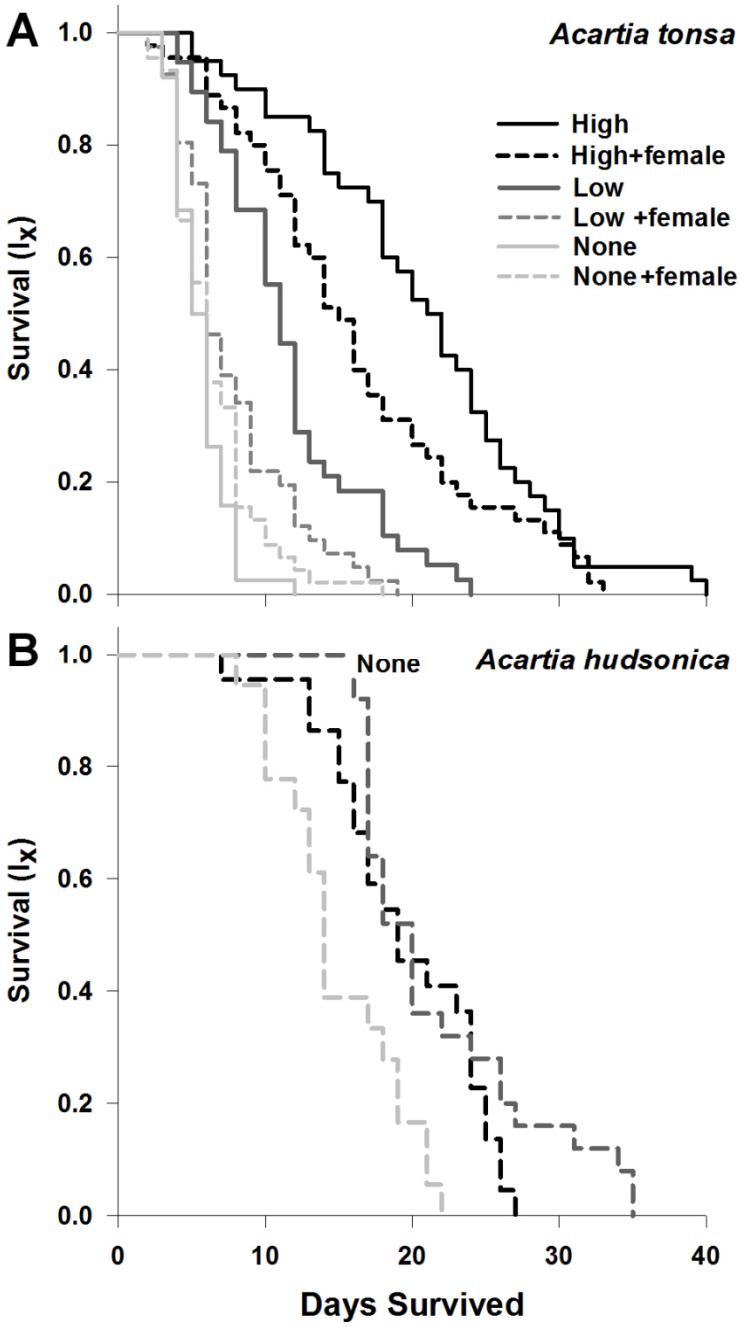
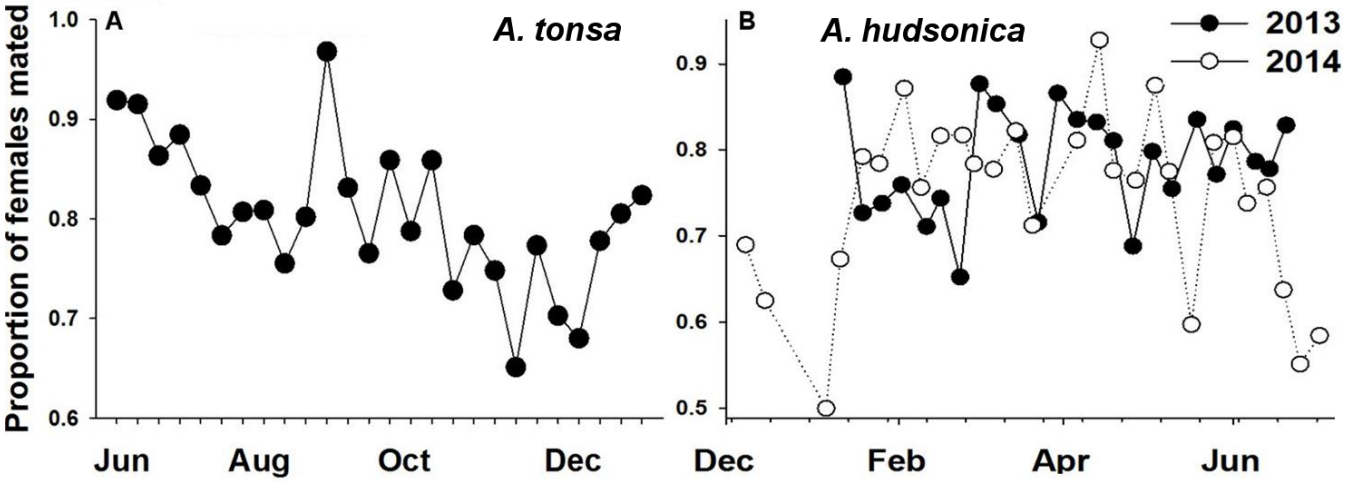

A
Acartia tonsa
High
High+female
Low
Low +female
None
None+female
Survival (lx)
1.0
0.8
0.6
0.4
0.2
0.0
B
None
Acartia hudsonica
Survival (lx)
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
40
Days Survived

Figure 7:


Proportion of females mated
1.0
0.9
0.8
0.7
0.6
A
A. tonsa
Jun
Aug
Oct
Dec
B
A. hudsonica
2013
2014
0.9
0.8
0.7
0.6
0.5
Dec
Feb
Apr
Jun

Chapter 4*:

Female mating status affects mating and male-mate choice in the copepod genus *Acartia*

*In press at Journal of Plankton Research

Abstract:

Mating in many copepod species is not random; moreover, individuals have preferences for certain qualities in their mates. This study tested whether female mating status (virgin vs. mated) affects mate choice in males in the copepod species *Acartia tonsa* and *A. hudsonica*, both in laboratory and field populations. In addition, the costs and benefits associated with mating twice versus once for females of both species were tested in laboratory studies. In both *A. tonsa* and *A. hudsonica*, males mated more frequently with virgin females compared with females that had already mated, suggesting that female reproductive status is important for mating and mate choice. Frequencies of females carrying double spermatophores in the field (at three sites and seasonally within a site) were consistently low, implying that mate choice for female reproductive status is present in natural populations as well. In *A. hudsonica*, the frequency of females carrying double spermatophores was positively related to spermatophore volume and male and female sizes, but negatively related to temperature. No such relationships were found for *A. tonsa*. However, the frequency of females carrying double spermatophores decreased as the season progressed. In both copepod species, there was no difference in the number of nauplii produced, length of fertilization, or lifespan between females that had mated twice compared with those that had mated only once. Hence, there was no particular cost or benefit to mating a second time. Our findings suggest that female reproductive status is important for mating success in these two coastal copepod species and that this is most likely due to male-mate choice.

Introduction:

Copepods are the most abundant metazoans in the ocean and are important food sources for many larval fish species. Hence, they are important links of primary producers to upper trophic levels. Copepods are also important in control of the microbial food web and biogeochemical cycles in the ocean and are used as standard test organisms in ecotoxicology. Thus, it is important to understand the factors that may impact their population maintenance and growth.

Through mate choice, sexual selection may occur in copepod populations. Since copepod mating needs are diverse (Kiørboe, 2006, 2007; Ceballos and Kiørboe, 2010), they are a potentially important group for studies of sexual selection and sexual size dimorphism (Clutton-Brock and Harvey, 1977; Maly, 1984). In addition, differences between copepod species in morphology, behavior, and population biology may be explained by differences in the strength of sexual selection (Trivers, 1972). Finally, it is important to understand the factors influencing mate choice in copepod species since it can have important consequences for population dynamics if not all adult individuals find a mate.

Until recently, little attention has been given to determining the factors important for successful mating in copepods, and even basic information on mating dynamics, such as the frequency of mated individuals over a reproductive season in field populations, is unknown for most pelagic copepods (but see Hopkins, 1982; Ferrari and Dojiri, 1987; Uye and Sano, 1995). Recent work has shown that for many species mating is not random, and that individual copepods have preferences for certain qualities in their mates. For instance, both sexes of the copepod species *Acartia tonsa* and *Temora longicornis* prefer larger mates, likely because larger individuals produce more and larger offspring (Weatherhead and Robertson, 1979; Kiørboe and

Hirst, 2008; Ceballos and Kiørboe, 2010; Sichlau and Kiørboe, 2011). In contrast, males of *Eudiaptomus graciloides* mate more frequently with small females, since large females are better at escaping mating attempts (Ali et al., 2009). Some species of copepods can distinguish between the ages of potential mates, with both males and females of *Temora longicornis* preferring young mates to old mates (Sichlau and Kiørboe, 2011). Since fertility and offspring quality decrease as copepods age, a copepod can maximize its fitness by mating with young mates (Uchima, 1985; Kelly et al., 1998; Choi and Kimmerer, 2008 and 2009; Ceballos and Kiørboe, 2011; Rodriguez-Grana et al., 2010; Sichlau and Kiørboe, 2011). In addition, the reproductive status of a female (virgin vs. mated) may be important in whether or not males decide to mate in several species of copepods. For instance, in the parasitic copepod *Lernaeocera branchialis*, male-mate choice depends on the age and the mating status of females. Males discriminate against mated and juvenile females in favor of virgin adult mates (Anstensrud, 1990, 1992; Heuch and Schram, 1996). Similarly, males of the cyclopoid copepod *Oithona davisae* can also distinguish between virgin females and those that have already mated (Heuschele and Kiørboe, 2012). In many harpacticoid copepods males also distinguish between female reproductive states by performing pre-copulatory mate guarding of juvenile females. Males grab and remain attached to a female until she matures, presumably to ensure offspring paternity (Ito, 1970; Burton 1985; Lazzaretto et al., 1990; Evstigneeva, 1993; Dahms and Schminke, 1993). Males will even switch females if an older juvenile stage female is encountered, likely to decrease the time spent guarding a mate (Lazzaretto et al., 1990; Burton, 1985).

In species in which females mate multiple times, sexual selection would likely favor males that can distinguish between female reproductive status because of the potential for sperm

competition (Ridley, 1983). Mating with a virgin female ensures no competition with another male's sperm, at least until the female re-mates (Parker, 1998). However, there have not been any studies on calanoid copepods. The calanoid copepods *Acartia tonsa* and *Acartia hudsonica* are the dominant species present in Long Island Sound during the fall/winter and the spring/summer months, respectively (Peterson, 1986). There are several reasons for having male-mate choice for female reproductive status in both copepod species. First, females require multiple matings to remain fertilized over their lifetime since they are not capable of storing sperm (Hammer, 1978; Ohtsuka and Huys, 2001). In the field, females are often found carrying multiple spermatophores, implying that multiple matings do occur and that females may be capable of using multiple spermatophores. Second, males have low rates of spermatophore (sperm packet) production (*A. tonsa:* 1 day^{-1} [Ceballos and Kiørboe, 2010]; *A. hudsonica:* 0.8 day^{-1} [unpublished data]), which limits copulation to about once per day. Because sex ratios in these species are often female-biased, males likely encounter more potential mates than can be mated with (Kiørboe and Bagøien, 2005; Kiørboe, 2006, 2007; Ceballos and Kiørboe, 2010).

The purpose of this study was three-fold: 1) To determine whether female mating status affects male-mate choice in *Acartia tonsa* and *Acartia hudsonica*. In particular, we expect that males will mate more frequently with virgin females compared with those that have already mated to ensure sperm usage. 2) To measure the frequency of multiple matings in field populations for both species of copepods to determine if male-mate choice occurs in the field, and if it correlates with other parameters (temperature, population density, sex ratio, adult size, etc.). If mate choice exists in field populations, then we would expect low frequencies of females carrying multiple spermatophores. 3) To determine the fitness benefits/costs of mating

multiple times for females of both species, in terms of total nauplii produced, length of time fertilized, and female longevity.

Methods:

Culturing of copepods

Live animals were collected by surface tow off of Groton Long Point, Long Island Sound, USA (Latitude: 41.3271 N, Longitude: 72.00150 W) using a conical plankton net with a 200-µm mesh and solid cod end. *Acartia hudsonica* females were collected in May and *Acartia tonsa* females were collected in October 2013. Mated females (indicated by the presence of an attached spermatophore) were sorted from the tows and kept at 15°C in an environmental chamber on a 12:12 light:dark cycle and fed a standard diet of the diatom *Thalassiosira weissflogii*, the green flagellate *Tetraselmis sp*., and the cryptophyte *Rhodomonas sp.* (equal parts by carbon). Algae were grown at 18°C as semi-continuous cultures on F/2 medium (Guillard, 1975). After one week, eggs were collected and raised to adulthood under the same conditions. These first generation individuals or subsequent generations were used in all experiments so that diet and age could be standardized.

Background on mating

Mating in both species lasts for only a few seconds and is concluded when the male attaches a spermatophore near the female's gonopores (Bagøien and Kiørboe, 2005). Females lack a spermathecum and as a result cannot store sperm (Hammer, 1978; Ohtsuka and Huys, 2001), requiring that they re-mate to ensure fertilization over their lifespan. In the lab, spermatophores remain attached for an average of 10 days in *Acartia tonsa* (Ceballos and

Kiørboe, 2010) and an unknown length of time for *Acartia hudsonica*. These copepods likely never encounter multiple potential mates at the same time, and thus have sequential mate choice: for each *individual* encounter the decision to mate with or reject the mate has to be made (Ceballos and Kiørboe, 2010). Thus, in all experiments, each individual will be paired with only one potential mate.

Male-mate choice for female reproductive status in laboratory populations

Male and female copepods of each species were sorted as juveniles from lab cultures and held with the same sex until maturation to ensure that individuals were virgins. Within 24 hours of maturation, 200 virgin females and 240 virgin males were collected and the prosome length (a proxy for body size, Mauchline, 1998) was photographed using a camera mounted on a microscope. Each image was then measured in ImageJ to 1/1000 of a millimeter. Copepods were then placed in individual 70mL petri dishes and fed at satiating food conditions (see above for diet information).

In order to get females of the same age but of different reproductive states (virgin vs. mated), two groups of females were assembled so that the average size of female prosomes was the same for each group. Females in the first group ("virgin treatment") were held alone for 48 hours (n= 49 for *Acartia tonsa,* n= 68 for *Acartia hudsonica*). During this time, females in the second group ("mated treatment") were each paired with two males and allowed to mate for 24 hours (n= 75 for *A. tonsa,* n= 160 for *A. hudsonica*). After this time, the females that had mated (as evidenced by the presence of an attached spermatophore) were then held alone for 24 hours to allow for recovery after mating. Females were then re-checked to ensure that their spermatophore was still attached. In *A. hudsonica*, almost half of the females had dropped their

spermatophore after 24 hours. Thus, for this species, an additional treatment was added to the experiment so that three reproductive states were tested: virgin females, females that mated and had a spermatophore attached, and females that mated but dropped their spermatophore. For *A. tonsa*, few females dropped their spermatophores, so only two treatments were compared ("virgin" and "mated with spermatophore attached").

After the different female reproductive states were obtained, the experiment started when a virgin male was paired with each individual female. The size differences between pairs were on average 0.093mm for *A. tonsa* and 0.097mm for *A. hudsonica*. After 24 hours, females were checked for mating. For each treatment, a mating frequency was calculated as the proportion of matings that occurred out of the total number of pairings. Differences in mating frequencies between the treatments were compared with a chi-square test (for 3 treatments, *A. hudsonica*) or Fisher's exact test (for 2 treatments, *A. tonsa*) using SigmaPlot 11.0 software.

Male-mate choice for female reproductive status in field populations

If male choice for female reproductive status occurs in the field, then we expect to find low frequencies of females carrying multiple spermatophores. If mate choice exists, it is also possible that it is constant (always occurring at the same level) or influenced by other parameters such as temperature, sex ratio, population density, etc. Therefore, to address both possibilities, a two-year field study was conducted for *Acartia hudsonica* (January 2013 to mid-July 2014), and a one-year study (January 2013 to December 2013) for *Acartia tonsa*. Three replicate horizontal plankton tows were taken at weekly intervals, using a conical plankton net with a 200-µm mesh and solid cod end, on incoming tides off of Groton-Long Point Bridge (site GLP), Long Island Sound, USA (Latitude: 41.3271 N, Longitude: 72.00150 W). The volume of water filtered was

calculated from a flow meter attached to the mouth of the net. Tows were preserved immediately in the field with 10% buffered phosphate formalin, for a final concentration between 3-5%. This preservative does not cause copepods to shrink or drop their spermatophores (unpublished data). It is conceivable that females drop spermatophores during the tow; yet, we think this is an unlikely possibility since spermatophores are attached by a cement-like substance (Hammer, 1978; Buskey, 1998). There is no evidence that females can remove spermatophores once attached, and thus attachment is most likely controlled by the quality and quantity of the male's glue rather than by the female. Water temperature and salinity were recorded with a YSI meter.

In addition, from January to December 2013, two other sites, I and K, in eastern Long Island Sound and near the GLP site were included in the analysis (Site I: 41.1375N, -72.655W; Site K 41.2343N, -72.2658W). These sites are part of a zooplankton monitoring data set for the Department of Energy and Environmental Protection of the State of Connecticut. Two vertical plankton tows were taken at each site on a monthly basis, preserved in the field as above, and analyzed within 1 week of collection. Subsamples were taken from each replicate tow so that 30-40 females were counted from each of the three tows (50 females for each of the two tows for stations I and K) and the number of spermatophores on each female was recorded. The number of males in each subsample was also noted so that average sex ratios for each date could be calculated. Images of both female and male prosome lengths were taken as above, as well as images of every female's spermatophore(s). Spermatophore volume was calculated based on its length and width assuming an ellipsoid shape (Ceballos and Kiørboe, 2010). Regression analyses were used to evaluate inter- and intra-annual trends in spermatophore frequencies and

environmental/population variables (sex ratio, population density, temperature, male and female sizes).

Fitness costs and benefits of double matings

Copepods were sexed and separated during the 4th copepodite stage to ensure that recently matured (within 24 hours) and virgin individuals were used in experiments. 150 virgin females were then placed individually with 5 virgin males in 70mL petri dishes at high-food conditions to ensure mating. After 24 hours, females were checked for a single copulation event as evidenced by the presence of a single attached spermatophore. A third of the females that had mated were placed in new petri dishes with food solution and held alone as the "single mated" treatment. The remaining females were given new males and allowed to mate for an additional 24 hours to obtain females that had mated twice. After 24 hours, these females were placed in new dishes and held alone as the "double mated" treatment. Each day, females were transferred to new food solutions and dishes with eggs were kept to be counted for nauplii four days later, which is sufficiently long to ensure 100% hatching of viable eggs at the experimental temperature (Ueda, 1981; Holste and Peck, 2006). The length of time that females remained fertilized was recorded as the last day that females produced a nauplius. The length of time that spermatophores remained attached was also noted. Since fitness depends both on survival and reproductive success, female longevity was also recorded. Student's T-tests were used to test for differences in means. Survival (l_x) was calculated as the number of individuals alive at time x, n_x, out of the number alive at the start of the experiment, n_0: $l_x = n_x/n_0$. Hazard analyses showed that survivorship data were non-proportional and so differences in survival curves between singly and multiply mated copepods were tested using the Gehan–Breslow test (Lee and Wang, 2003).

Results:

Male-mate choice for female reproductive status in laboratory populations

Acartia hudsonica

There were significant differences in mating frequencies between all reproductive states, with males mating preferentially with virgin females (chi-square=22.485, P<0.001) (Fig. 1A). 36.8% (25 mated of 68 pairs) of males mated with virgin females, compared with 15.7% (n=70) with females that had mated but dropped their spermatophore, and only 6.4% (n=78) with females that still had a spermatophore present.

Acartia tonsa

Similar results were found for *Acartia tonsa* males. Males mated much more frequently with virgin females compared with females that had already mated (Z-test: z-stat= 3.044; P=0.002) (Fig. 1B). 22.4% (n=49) of pairs mated in the virgin treatment compared with only 2.9% (n=69) in the recently mated female treatment (Fig. 1B).

Male-mate choice for female reproductive status in field populations

Similar frequencies to those measured in the lab of females carrying multiple spermatophores were found in field populations for both *Acartia hudsonica* and *Acartia tonsa*.

Acartia hudsonica

For all three sites and over both the 2013 and 2014 seasons, the majority of female *Acartia hudsonica* had mated (Figs. 2, 3A). The percentage of unmated females (i.e. without a spermatophore present) at stations I and K, ranged from 6.5% to 15.1% (Fig. 2A) and 19.3% to 45.6% (Fig. 2B), respectively. At site GLP in 2013, the percentage of unmated females ranged from 11.6% in early January to 34.8% in mid-February (Fig. 2C). During 2014 at GLP, the

lowest frequency of unmated females (12.6%) was in early May, while the highest (50%) was in January (Fig. 2D). On average, more females were unmated at station K (average = 37.6 ± 4.8% S.E.) compared with stations I (10.5 ± 1.4%), GLP in 2013 (22.6 ± 1.2%), and GLP in 2014 (24.7 ± 2%) (Fig. 3A).

At all three stations, the majority of females were carrying a single spermatophore (Figs. 2, 3A). The frequency of females with a single spermatophore ranged from 58.9% to 83.2% at station I (Fig. 2A), from 47.8% to 79.4% at station K (Fig. 2B), from 49.9% to 80.8% at GLP 2013 (Fig. 2C), and from 50% to 78% at GLP 2014 (Fig. 2D). On average, over 60% of the female population at each site had a single spermatophore present (station I: 73.2 ± 3.5%; station K: 60 ± 4.8%; GLP 2013: 64.6 ± 1.6%; GLP 2014: 61.5 ± 1.6%) (Fig. 3A).

Few females at any site were carrying more than one spermatophore (Figs. 2,3A). At station I, 7% to 26.2% of females carried two spermatophores (Fig. 2A). Station K had much lower frequencies of females carrying double spermatophores, with some months being zero, and the highest month having only 7.6% of the population (Fig. 2B). At GLP in 2013, frequencies ranged from 3.9% in mid-May to 22.3% in mid-April (Fig. 2C). During 2014, January had the lowest frequencies (0%) and mid-April had the highest frequencies (30.5%) (Fig. 2D). The seasonal averages of double spermatophore frequencies for station I and GLP were similar (station I: 15 ± 3.4%; GLP 2013: 12 ± 1%; GLP 2014: 12.5 ± 1%) (Fig. 3A). Station K had a very low average over the season, with just 2.1% (± 1 S.E.) of females carrying double spermatophores (Fig. 3A). Less than 5% of females during any month and at any site were carrying 3 or more spermatophores (Figs. 2, 3A).

Linear regressions of the frequencies of females carrying double spermatophores on several predictors were performed from the weekly GLP tow data for 2013 and 2014. During

2013, the frequency of females carrying double spermatophores was unrelated to population density, sex ratio, female-to-male size-ratio, and time. However, there was a positive relationship found between the average size of females (mm) and multiple spermatophore frequency ($p = 0.0017$; $F = 12.44$; $R^2 = 0.3414$, $n = 26$) (Fig. 4A). This trend was similar when average size of males (mm) was used as the predictor ($p = 0.0021$, $F = 11.89$, $R^2 = 0.3313$, $n = 26$) (Fig. 4C). There was also a positive, but weaker, relationship between spermatophore volume and frequency of spermatophores ($R^2 = 0.2156$; $p = 0.0194$; $F = 6.32$, $n = 26$) (Fig. 4B). Water temperature was negatively related to double spermatophore frequency ($R^2 = 0.3132$, $p = 0.003$, $F = 10.95$, $n = 26$) (Fig. 4D). A multiple linear regression was also performed using the four variables that had significant predictive power. However, female size, male size, and spermatophore volume were all collinear. Thus, female size (highest R^2 value of the three collinear variables) and temperature were used as predictors of double spermatophore frequency in the model. Forward, backward, and stepwise selection models all agreed that the temperature variable did not increase the predictive power of a model containing only female size. Therefore, female size alone was the best predictor of double spermatophore frequency out of all other combinations of variables. During 2014, there was no significant relationship between double spermatophore frequency and density, sex ratio, temperature, or time. The significant trends that were found were weaker than in 2014. Double spermatophore frequency had positive relationships with female size ($p = 0.02$, $F = 6.6$, $R^2 = 0.22$, $n = 26$), male size ($p = 0.055$, $F = 4.1$, $R^2 = 0.15$, $n = 26$), spermatophore volume ($p = 0.004$, $F = 10$, $R^2 = 0.3$, $n = 26$), and female-to-male size-ratio ($p = 0.006$, $F = 9$, $R^2 = 0.27$, $n = 26$) (Fig. 5). Again, female size, male size, spermatophore volume, and female-to male size ratio are all collinear. Thus, for 2014, the best predictor of double spermatophore frequency was the spermatophore volume (highest R^2).

Acartia tonsa

For all three sites and over both the 2013 and 2014 seasons, the majority of *Acartia tonsa* females had mated (Figs. 3B, 6). The percentage of unmated females from sites I and K ranged from 7.2% to 27.8% (Fig. 6A) and 16.7% to 30.8% (Fig. 6B), respectively. At station GLP in 2013, the percentage of unmated females ranged from as low as 7.2% in mid-June to as much as 34.9% in mid-November (Fig. 6C). Averaged over the season, all three stations had similar frequencies of unmated females (site I: 15.6 ± 2.5% S.E.; K: 22.4 ± 1.3%; GLP 2013: 19.7 ± 1.2%) (Fig. 3B).

As with *Acartia hudsonica*, the majority of females that were mated bore a single spermatophore (Figs. 3B, 6). Frequency of females with a single spermatophore ranged from 72.2% to 91.1% at station I (Fig. 6A), from 65% to 83.3% at station K (Fig. 6B), and from 64.2% in mid-November to 93.6% in September at GLP (Fig. 6C). On average, over 75% of the female population at each site had a single spermatophore present (station I: 82 ± 2.1%; station K: 76.2 ± 1.7%; GLP 2013: 75.7 ± 1.1%) (Fig. 3B).

Similar to what was found for *Acartia hudsonica,* few females of *Acartia tonsa* at any site were carrying double spermatophores (Figs. 3B, 6). Percentages at stations I and K were similar, with ranges of 0- 6.3% and 0- 4.2%, respectively (Fig. 6A, B). At GLP in 2013, the percentage of females with two spermatophores ranged from 0% at the end of August to 12.4% in mid-August (Fig. 6C). The seasonal averages of double spermatophore frequencies was highest for GLP (4.2 ± 0.6%); stations I and K were similarly low (station I: 1.9± 0.8%; K: 1.4± 0.6%) (Fig. 3B).

At station I, less than 1.5% of the population ever had more than 2 spermatophores (average: 0.6± 0.2%) (Figs. 3B, 6A), and at station K, no females carrying 3+ spermatophores

were found in any month (Figs. 3B, 6B). At station GLP in 2013, the frequency of females carrying more than 2 spermatophores was never greater than 5% for any month (Fig. 6C), and was on average 0.4% of the population (± 0.2%) (Fig. 3B).

Using frequencies of double spermatophores from GLP in 2013 as the dependent variable, linear regressions were performed using the following independent predictors: population density, sex ratio, female to male size-ratio, female size, male size, male and female body size variance, percentage unmated, spermatophore volume, and temperature. No significant relationships were found using these variables. However, there was a negative relationship between time (week) and double spermatophore frequency (Fig. 7, n=26; R^2= 0.41; P<0.001; F=19.17). Because the relationship between the two variables may have been nonlinear, we fit logarithmic, exponential and polynomial curves to the data as well. However, the best fit (highest R^2) was still the linear model.

Fitness costs and benefits of double matings

There were no obvious fitness benefits or costs associated with mating multiple times for either of the species, *Acartia hudsonica* or *Acartia tonsa* (Table 1). There was no difference in the number of nauplii produced from females that had mated once compared with those that had mated twice: *A. hudsonica:* 109 nauplii (± 12) for single mating versus 100 nauplii (± 11) for twice mated. *A. tonsa*: 246 ± (16) for single mating versus 264 ± 24 for twice mated (*A. hudsonica:* Mann-Whitney Rank Sum test: U-stat= 1108, P= 0.991; n=60 single, n= 37 double; *A. tonsa:* T-stat= -0.64; P = 0.52).

Females that mated twice dropped their spermatophores significantly earlier than those that mated once, and usually both at the same time (*Acartia hudsonica:* Mann-Whitney Rank

Sum test: U-stat= 1202, P= 0.018; n=62 single, n= 52 double; *Acartia tonsa:* Mann-Whitney Rank Sum test: U-stat= 509, P= 0.03; n=41, n=35). For *A. hudsonica,* double spermatophores remained attached for an average of 3.1 (± 0.3) compared with 5.3(± 0.6) days. *A. tonsa:* 5.5 ± 0.6 for double spermatophores versus 8.0 ± 0.8 days for single spermatophores. However, this did not result in a difference in the average amount of time females remained fertilized if they mated once or twice (*A. hudsonica:* once= 8.1± 0.6 days, twice=6.8 ± 0.7 days; U-stat= 1323, p= 0.132; n=69 single, n= 46 double; *A. tonsa:* once= 13.6± 0.7 days, twice=15.2 ± 1.0 days; t-stat= -1.4, p= 10.152; n=51 single, n= 37 double) (Table 1).

There was also no difference in survivorship curves for females of either species that mated once or twice (*Acartia hudsonica:* Gehan-Breslow test stat= 0.341, p=0.56; *Acartia tonsa:* Gehan-Breslow stat= 0.1, p= 0.75) (Table 1). *A. hudsonica* females that mated once survived for an average of 18.6 (±0.7) compared with 18.1 (±0.9) days for those that mated twice. *A. tonsa:* 17.9 ± 1 for single mating versus 17.4 ± 0.8 days for twice mated.

Discussion:

This is the first study to show an effect of female reproductive status on mating in a calanoid copepod. Results from both laboratory and field data, for both *Acartia hudsonica* and *Acartia tonsa,* indicate that males are capable of distinguishing between female reproductive states and that they prefer to mate with virgin females over females that have already mated. Our findings suggest that female reproductive status is important for mating success in these two coastal copepod species and that, since there are no fitness costs to females for mating a second time, this is most likely due to male-mate choice.

Female reproductive status affects mating

While it is possible that a female's willingness to mate may change with her mating status, it is more likely that most, if not all, of the "choice" in our experiments was made by the males. Since the process of mating is very short in both of these species, and since mate searching can be done at the same time as food searching (Kiørboe, 2006), rejecting a mate because of her reproductive state is likely not costly for males. In addition, since population sex ratios are largely female-biased, and males can encounter up to 30 potential mates in a day, but only mate with one (Bagøien and Kiørboe, 2005), males likely never suffer from a missed mating opportunity as a result of rejecting a female mate. Thus, males can afford to be selective in their choice of mates. Females, on the other hand, should be willing to mate with the first male encountered since males are few and far between. Because females continue to produce eggs even if unfertilized, unmated females likely suffer a cost in terms of lost reproductive effort. As a result, females should be less selective than males when it comes to choosing a mate, so as to decrease the length of unfertilized periods between matings.

The process of mating itself is likely of little cost for females (no increased predation risk, little energy used for courtship, food acquisition is not interrupted, etc.) (Kiørboe, 2006). Hence, there is little incentive for females that had mated not to mate again. In addition, females in our experiments were given 24 hours to recover between mating events, making it unlikely that females were not able to re-mate a second time. For males, in contrast, mating may involve significant energetic investment, since spermatophore-production rates are so low (Mauchline, 1998). Because males can only produce about one spermatophore a day and thus have a limited number that can be produced over their lifetime (unpublished data), sexual selection should favor males that mate with virgin females over females that have already mated, because the latter are

less likely to use the second male's sperm. While our experiments were done on females of the same age, virgin females in the field are likely younger than mated females. Since egg production decreases significantly with female age, by mating with *young and virgin* females, a male's offspring production may increase (Ceballos and Kiørboe, 2011, Sichlau and Kiørboe, 2011). Moreover, offspring quality also decreases with increasing female age, which would increase the fitness benefits even more for males that mate with young, virgin females. Both of the above observations may explain why differences in mating frequencies between the two groups were more pronounced in field populations than in the laboratory.

Finally, while it is possible that the differences between mating frequencies of virgin females and those with a spermatophore present are due to female unwillingness to mate, the mating differences measured in the "attached" versus "dropped" spermatophore groups can only be due to male-mate choice. In *Acartia hudsonica*, females that have mated but dropped their spermatophore should be just as willing (or unwilling) to re-mate as females that still have an attached spermatophore because the length of time females remain fertilized is independent of female reproductive state (Table 1). Therefore, the differences in mating frequencies between these two reproductive states are likely due to male choice. It is possible that the presence of the spermatophore on the female is a cue that males use to determine if females have mated. However, because the frequencies of mating between the females that had dropped the spermatophore and virgins are also different, spermatophore presence alone is not the only cue that males use when deciding to mate. It is possible that some substance (sperm, etc.) in the attached spermatophore is detected by future male mates. If this is the case, then the concentration of the substance would be greatest in females with the spermatophore still attached, and weaker in females that had dropped their spermatophore. Thus, it would become

harder for males to determine if females had mated once they dropped their spermatophore, and this would explain the higher frequency of males mating with those females compared with the ones that had an attached spermatophore. In *Oithona davisae,* males can distinguish between virgin and mated females, and it is hypothesized that the cue is waterborne (Heuschele and Kiørboe, 2012). Many copepod species use chemical cues to find mates (Lonsdale et al., 1998; Bagøien and Kiørboe, 2005; Kiørboe, 2006, 2007), but the importance of chemical signals for mate-recognition and location have not been demonstrated for copepods in the genus *Acartia*. Instead, these copepods rely on hydromechanical signals to locate mates (Bagøien and Kiørboe, 2005), so it is unlikely that males are capable of detecting subtle changes in chemical cues produced by females before and after mating (Lonsdale et al., 1998). Therefore, the cue is likely a very strong chemical signal (another male's sperm) or a hydromechanical signal. Since males during mating physically grasp females near the spot where the spermatophore is attached, this may be close enough for males to detect the presence of another male's sperm. It is possible that mated females change their speed or pattern of swimming and that this can be detected by males. Again, though, if this was the case, then we should not have seen a difference in mating frequencies between females that had a spermatophore present and those that had dropped it, since these females were mated and still fertilized.

Mate choice for female reproductive status is evident in field populations

Field results over two seasons and at multiple stations in Long Island Sound gave similarly low frequencies for females with double spermatophores as those measured in the lab experiments for both *Acartia hudsonica* and *Acartia tonsa*. On average, the majority of females were mated (>75% for both species) at all stations and most of them had only a single

spermatophore present. Few females in the population carried multiple spermatophores (~15% for *A. hudsonica*, and <5% for *A. tonsa*) (Figs. 2-6). These results indicate that female reproductive status is important for mating in field populations of these copepods. Because the sex ratios and population densities did not correlate with the frequency of double matings for either species, mate choice appears to be fairly constant and fixed in these copepods. Since males are always limiting in these populations, this may explain why there was no correlation between double spermatophore frequency and the sex ratio. The populations were never male dominated, and so most males would have been able to find virgin females to mate with, resulting in consistently low frequencies of multiple matings. Interestingly, the frequency of multiple matings (double spermatophores) was positively correlated with male and female sizes in both 2013 and 2014 and with size ratio during 2014. This is not surprising given that male and female sizes covary, and that males are at their peak sizes during periods of high food (Durbin et al., 1992). We know that large males produce more spermatophores than small males (Ceballos and Kiørboe, 2010), and that males also produce more spermatophores under high-food conditions compared with low-food conditions (chapter 3). Thus, the positive relationship between females that had mated twice and male size can be explained by larger males eating more food and producing more spermatophores. With more mating opportunities, the operational sex ratio (ratio of reproductively ready males to reproductively ready females) is actually closer to equality than the ratio of males to females would imply. Double spermatophore frequency was negatively correlated with temperature in *Acartia hudsonica* during 2013, likely because this is a cold water species. Water temperatures increase throughout the season, with the highest temperatures at the end of the season when individuals in the population would be old and perhaps less healthy. These individuals have low mating rates because spermatophore production is reduced (Uchima,

1985; Kelly et al., 1998; Choi and Kimmerer, 2008 and 2009; Rodriguez-Grana et al., 2010; Ceballos and Kiørboe, 2011; Sichlau and Kiørboe, 2011). A similar pattern is also present for *A. tonsa*, with a negative relationship between spermatophore frequency and time during the season.

Since some females drop spermatophores soon after they have been attached (Fig. 1, Table 1), it is possible that the spermatophore frequencies measured in the field do not reflect the actual mating frequencies of the different groups. The *relative* frequencies of spermatophores, however, are likely accurate and would suggest that mate choice for female reproductive status is important for mating. As measured for both species (Table 1), double spermatophores fell off together and slightly earlier (about 1-2 days) than single spermatophores. Single spermatophores remaining attached for an extra day could not account for the extreme observed differences in single and double spermatophore frequencies. Similarly, because multiple spermatophores fell off at the same time, the frequency of single spermatophores would not be inflated. However, it is possible, since the double spermatophores fell off simultaneously, that females without a spermatophore present (and thus counted as "unmated" in the field samples) had actually mated once or multiple times. To test this possibility, 50 females without spermatophores collected on triplicate weekly tows were held alive in filtered seawater and checked over 4 days for the production of nauplii. Fewer than 10% of those females produced nauplii, meaning that at most (if we assume that all of those had double spermatophores that fell off), double spermatophore frequencies would be off by 2% (since unmated females accounted for about 20% of the population). This is not enough to change the observed difference between females with single and double spermatophores. Because spermatophores remained attached for 3-5 days in *Acartia hudsonica* and 6-8 days in *Acartia tonsa*, if males were preferentially re-mating with already

mated females over virgin females, then we should have seen much higher frequencies of females carrying multiple spermatophores, since the process would be additive. This was not the case for either species, and in all instances less than 5% of females carried 3 or more spermatophores. Finally, since the relative frequencies were fairly constant from week to week and from site to site for both species, this is further evidence that the frequencies are in fact, realistic. Thus, the relative frequencies are likely reliable for describing mating success between the different female reproductive states.

Benefits and costs associated with mating multiple times

Theory suggests that multiple matings by females exist either because 1) the benefits outweigh the costs, 2) it is more costly to refuse than accept a mating attempt, or 3) because males coerce females into mating (Holland and Rice, 1998; Arnqvist and Nilsson, 2000; Byrne and Roberts, 2000). Our results suggest, at least for the fitness-related variables measured, no detrimental effects for females that mate twice in a period of two days. Hence, females should always be willing to mate with any male of high quality that is encountered. Yet, there were no obvious fitness benefits for females to mate multiple times. There was no difference in the number of nauplii produced, the length of time females remained fertilized, or longevity between females that mated once or twice (Table 1). However, for both species, double spermatophores dropped off earlier than single spermatophores (Table 1). We observed both spermatophores being emptied over the course of the experiments. Thus, if the rate of sperm use by a female is constant, then it is possible that females use sperm from –presumably- two males, and perhaps that the amount of sperm use is the same for females that mate once or twice. If so, since there was no difference in the length of time females remained fertilized from 1 or 2 spermatophores,

less sperm is used from each of the two spermatophores compared to a single spermatophore. While females do not necessarily gain more sperm from each mating, they may derive a benefit, unmeasured here, in the genetic diversity of their offspring. Finally, the sperm that is not being used to fertilize eggs is either being released into the surrounding water (and used as a cue by males to determine mating status) and/or the remaining substances in the sperm packets may be used to increase reproductive output or longevity, as is the case for some insects (for a review see: Boggs, 1995; South and Lewis, 2012). While sperm is not the only material present in the spermatophore, specifics about the other substances and their uses are mostly unknown (Raymont et al., 1974; Hosfeld, 1994). If males are providing other substances that can be incorporated into developing oocytes or female somatic tissue, this may explain why spermatophore-production rates are so low in these male copepods. To conclude, this work suggests that there is no cost to females in mating multiple times; yet there may be a genetic benefit in terms of offspring diversity. As a result, it is likely that females are always willing to re-mate regardless of their mating status. Since females that mate multiple times do not produce more nauplii than those mated once, there is no added benefit to males that mate only once per day to do so with a non-virgin female.

Summary

Taken together, our results show that female reproductive status affects mating in both field and laboratory populations, with higher mating frequencies for virgin females over females that have already mated. In addition, our work indicates that this is probably due to male-mate choice, since there are no costs to females for mating a second time. The consistently low field frequencies of females carrying multiple spermatophores suggest that mate preferences in the

field are constant and fixed. The ability to distinguish between different female reproductive states may increase male reproductive success by ensuring that its sperm will be used. This is the first study to examine mate choice for female reproductive state in calanoid copepods, and now these mate preferences have been shown for all groups of copepods: benthic, parasitic, pelagic, harpacticoid, cyclopoid, and calanoid, and in groups in which only the male searches for a mate and in those in which mate-searching occurs in both sexes. Theories of sexual selection often assume that males should not be choosy for mates. In copepods, even in species in which both sexes search for sexual mates, males may be more selective than previously thought. Since copepods have a wide variety of mating techniques and needs, they are an important group on which to test theories of sexual selection and the evolution of mate choice.

Table and Figure legends:

Table 1: Fitness measures of *Acartia hudsonica* and *Acartia tonsa* females mated either once or twice.

Figure 1: Male-mate choice for female reproductive status in *Acartia hudsonica* (A) and *Acartia tonsa* (B). Males of both species mated significantly more frequently with virgin females (*A. hudsonica*: 36.8%, n=68; *A. tonsa:* 22.4%, n=49) than females that had already mated (*A. hudsonica:* 6.4%, n=78; *A. tonsa:* 2.9%, n=69) ($P<0.01$ for both). In *A. hudsonica*, males also distinguished between females that had dropped their spermatophore (n=70) and those with it still present, mating more frequently with the former (15.7% vs. 6.4%).

Figure 2: Spermatophore frequencies for *Acartia hudsonica* at three sites in Long Island Sound: Site I (A), Site K (B), Site GLP 2013 (C), Site GLP 2014 (D). All frequencies were fairly constant over the season. Frequencies of unmated females (unfilled circles) ranged from 6.5 to 15% at station I, 19.3 to 45.6% at station K, 11.6% to 34.8% at Station GLP 2013, and 12.6% to 50% at GLP 2014. The majority of females in both species carried a single spermatophore (filled circle), with between 58.9- 83.2% at station I, 47.8-79.4% at station K, 49.9- 80.8% at GLP 2013, and 50% to 78% at GLP 2014. Few females had double spermatophores attached (filled triangles) (site I: 7-26.2%, site K: 0-7.6%; GLP 2013: 4-22.3%; GLP 2014: 0-30.5%). Fewer than 5% of females during any month were carrying 3 or more spermatophores (unfilled triangles). Data points are averages of two (sites I and K) or three (GLP sites) replicate plankton tows with SE bars.

Figure 3: Average seasonal spermatophore frequencies for *A. hudsonica* (A) and *A. tonsa* (B) at station I (black bars), station K (dark grey), GLP 2013 (light grey), and GLP 2014 (white). All stations showed similar patterns of spermatophore frequencies for both species. On average for both species and all sites, the majority of females in the population carried a single spermatophore (over 60% for *A. hudsonica*, and over 75% for *A. tonsa*). Fewer than 20% of female *A. hudsonica* and less than 5% of *A. tonsa* had double spermatophores, and almost no females were found with 3 or more spermatophores attached. Bars are standard error.

Figure 4: Linear regressions for *Acartia hudsonica* at site GLP in 2013 of double spermatophore frequencies against female size (A), spermatophore volume (B), male size (C), and temperature (D). Female size, spermatophore volume, and male size all were positively correlated with spermatophore frequency, while temperature had a negative correlation. All regressions are significant at $\alpha = 0.05$ (detailed information given in text).

Figure 5: Linear regressions for *Acartia hudsonica* at site GLP in 2014 of double spermatophore frequencies against female size (A), spermatophore volume (B), male size (C), and size-ratio (D). Female size, male size, spermatophore volume, and size-ratio were all positively correlated with spermatophore frequency. All regressions are significant at $\alpha = 0.05$(detailed information given in text).

Figure 6: Spermatophore frequencies for *A. tonsa* at three sites in Long Island Sound: Site I (A), Site K (B), Site GLP(C) in 2013. Frequencies of unmated females (unfilled circles) ranged from 7.2- 27.8% at station I, 16.7- 30.8% at station K, and 7.2-34.9% at Site GLP. Females carrying a

single spermatophore (filled circle) ranged between 72.2 and 91.1% at station I, 65 and 83.3% at station K, and 64.2 and 93.6% at Site GLP. Females with double spermatophores attached (filled triangle) ranged from <6.5% at Stations I and K up to 12.4% at Site GLP. Fewer than 5% of females during any month were carrying 3 or more spermatophores (unfilled triangles). Data points are averages (±SE) of two (sites I and K) or three (GLP site) replicate plankton tows.

Figure 7: Linear regression for *Acartia tonsa* at site GLP in 2013 of double spermatophore frequency by time (week). The frequency of females in the population carrying two spermatophores decreased linearly as the season progressed. The regression is significant at $\alpha = 0.05$(detailed information given in text).

Tables:

Table 1

	Single mating ±S.E.	n	Double mating ±S.E.	n	Test statistic	p-value
Average nauplii produced						
Acartia hudsonica	109±12	60	100±11	37	U=1108	0.99
Acartia tonsa	246±16	51	264±24	37	t=-0.6	0.52
Average days spermatophore attached						
A. hudsonica	5.3±0.6	62	3.1±0.3	52	U=1202	**0.02**
A. tonsa	8.0±0.8	41	5.5±0.6	35	U=509	**0.03**
Average days fertilized						
A. hudsonica	8.1±0.6	69	6.8±0.7	46	U=1323	0.13
A. tonsa	13.6±0.7	51	15.2±1.0	37	t=-1.4	.15
Average longevity in days						
A. hudsonica	18.6±0.7	54	18.1±0.9	37	G.-B.=0.3	0.56
A. tonsa	17.4±0.8	51	17.9±1.0	37	G.-B.=0.1	0.75

Figures:

Figure 1:

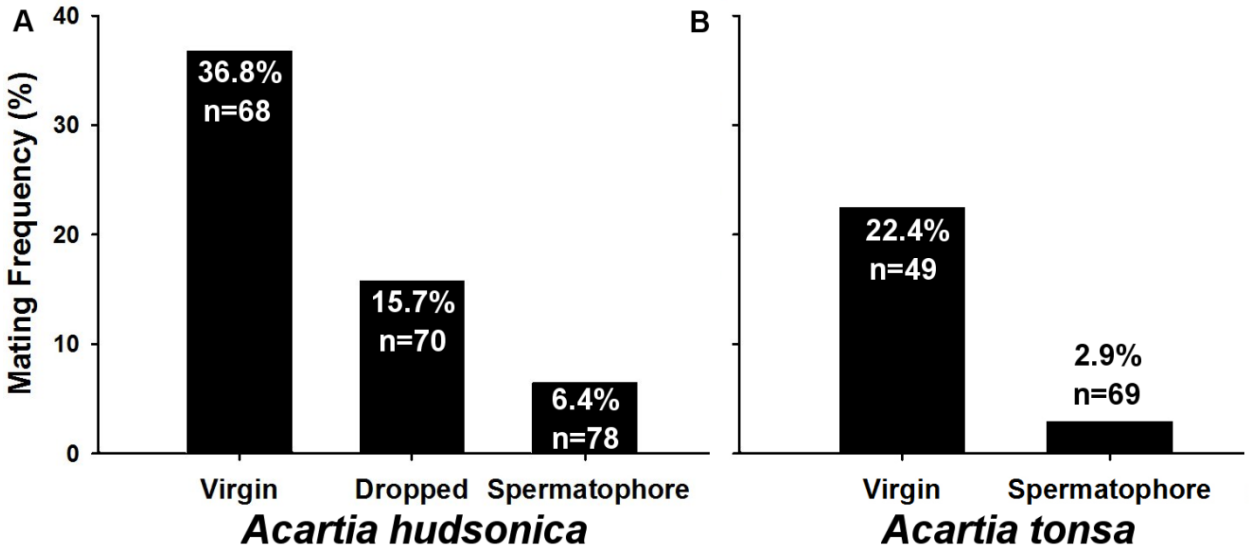


Figure 2:

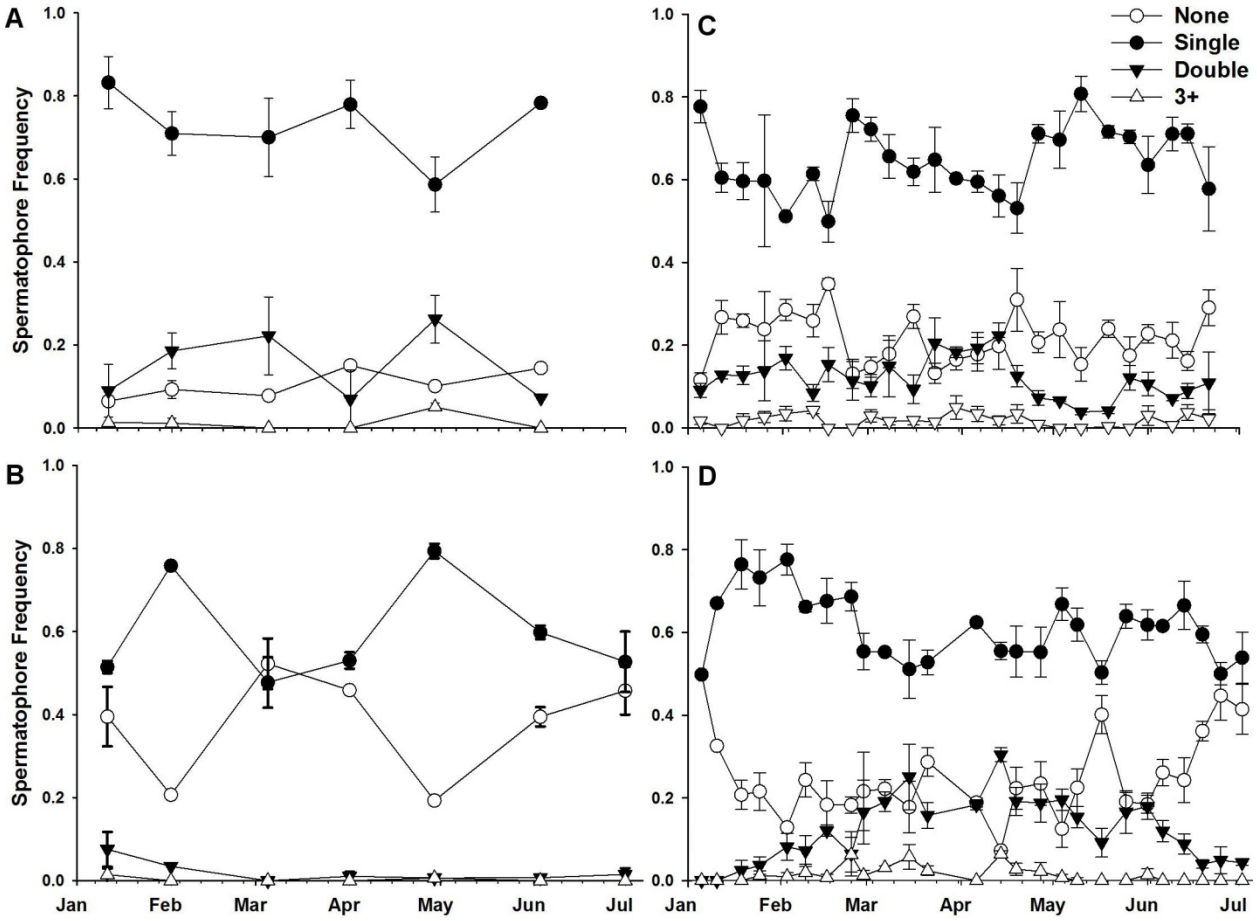

A
B
C
D
Spermatophore Frequency
Spermatophore Frequency
None
Single
Double
3+
1.0
0.8
0.6
0.4
0.2
0.0
Jan
Feb
Mar
Apr
May
Jun
Jul

Figure 3:

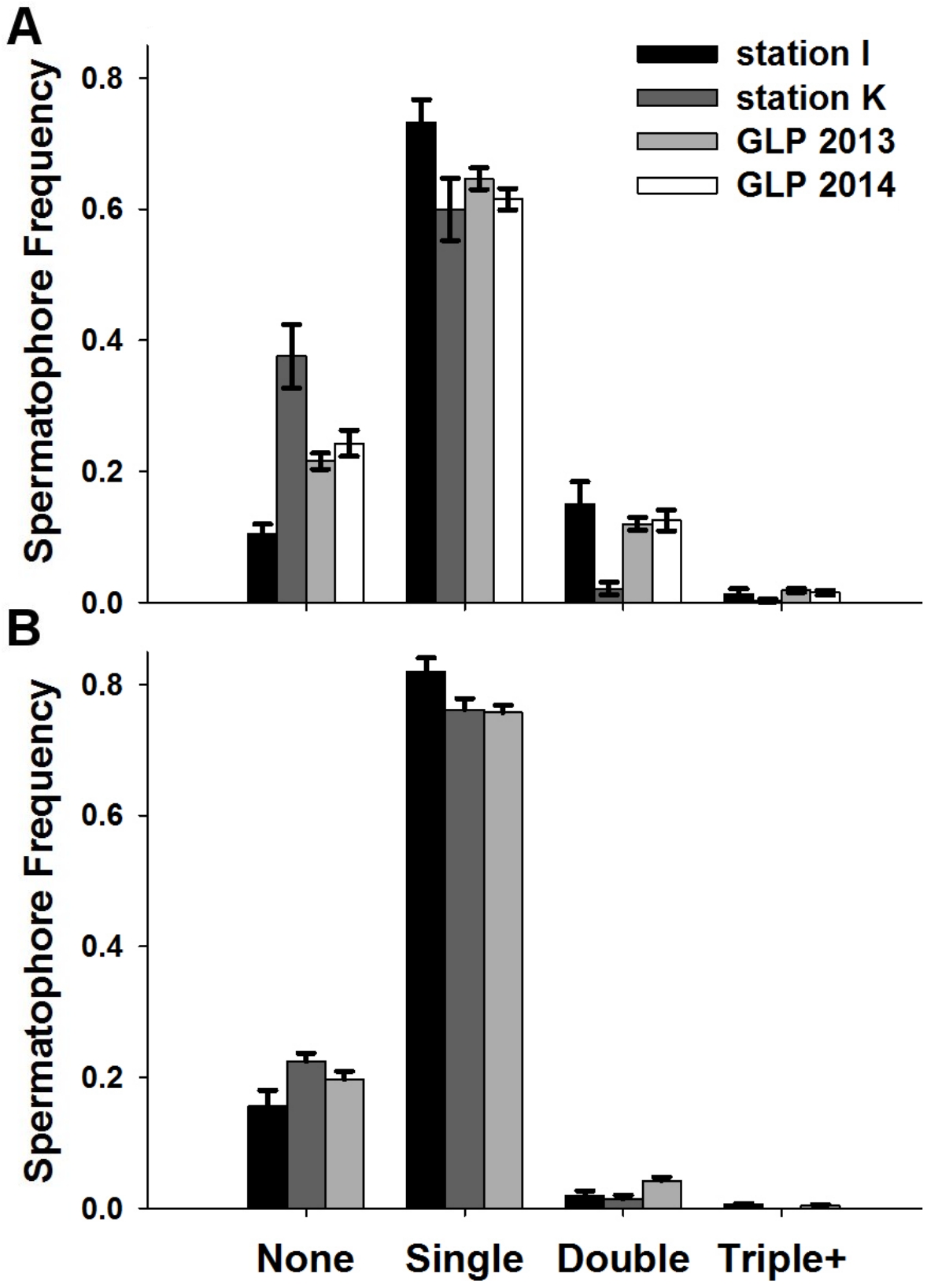

A
B
Spermatophore Frequency
0.0
0.2
0.4
0.6
0.8
station I
station K
GLP 2013
GLP 2014
None
Single
Double
Triple+

Figure 4:

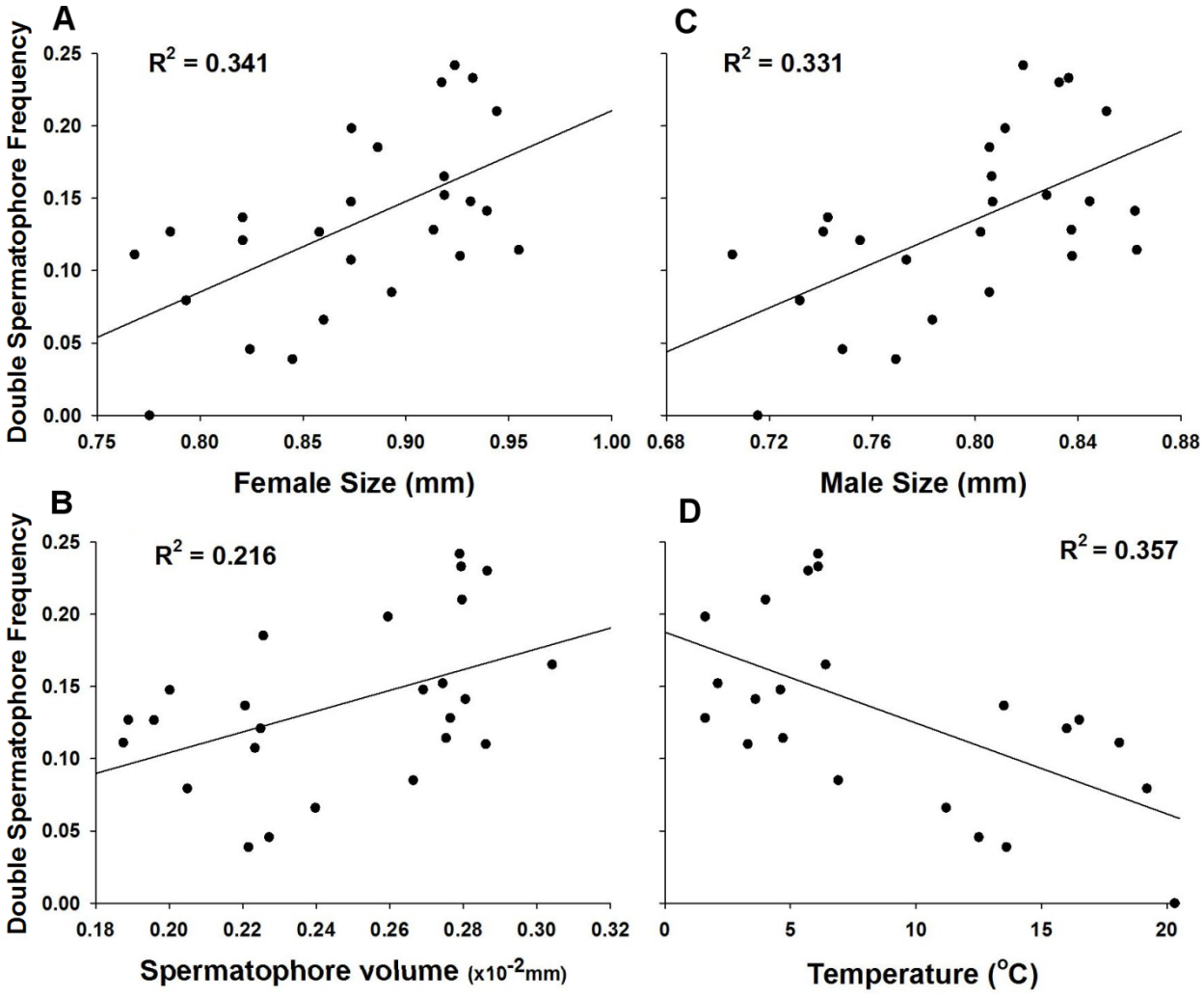

A
R^2 = 0.341
Double Spermatophore Frequency
0.25
0.20
0.15
0.10
0.05
0.00
0.75
0.80
0.85
0.90
0.95
1.00
Female Size (mm)
C
R^2 = 0.331
0.68
0.72
0.76
0.80
0.84
0.88
Male Size (mm)
B
R^2 = 0.216
Double Spermatophore Frequency
0.18
0.20
0.22
0.24
0.26
0.28
0.30
0.32
Spermatophore volume (x10^{-2}mm)
D
R^2 = 0.357
0
5
10
15
20
Temperature (°C)

Figure 5:

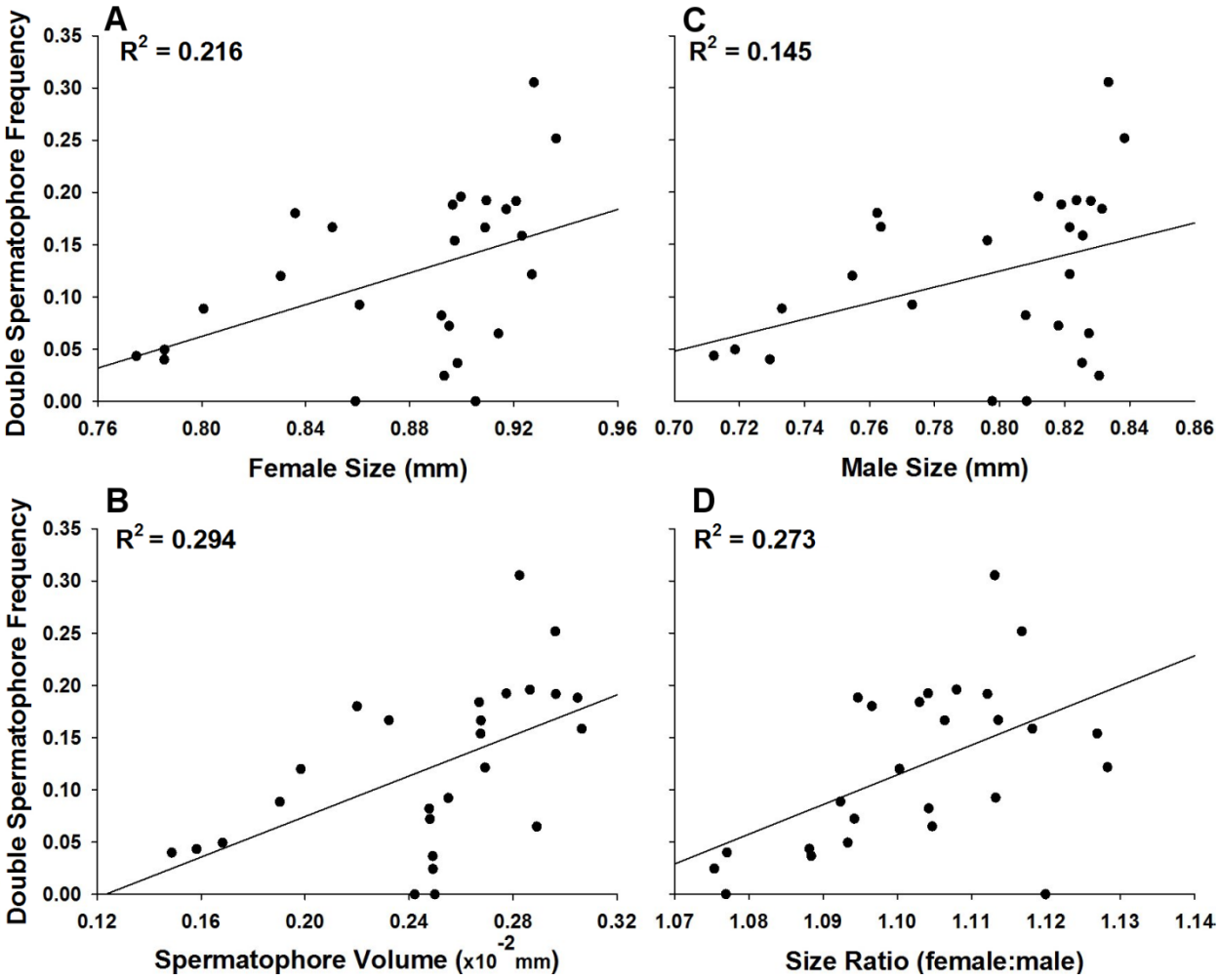

A
R^2 = 0.216
Double Spermatophore Frequency
Female Size (mm)
C
R^2 = 0.145
Male Size (mm)
B
R^2 = 0.294
Spermatophore Volume (x10^{-2} mm)
D
R^2 = 0.273
Size Ratio (female:male)

Figure 6:

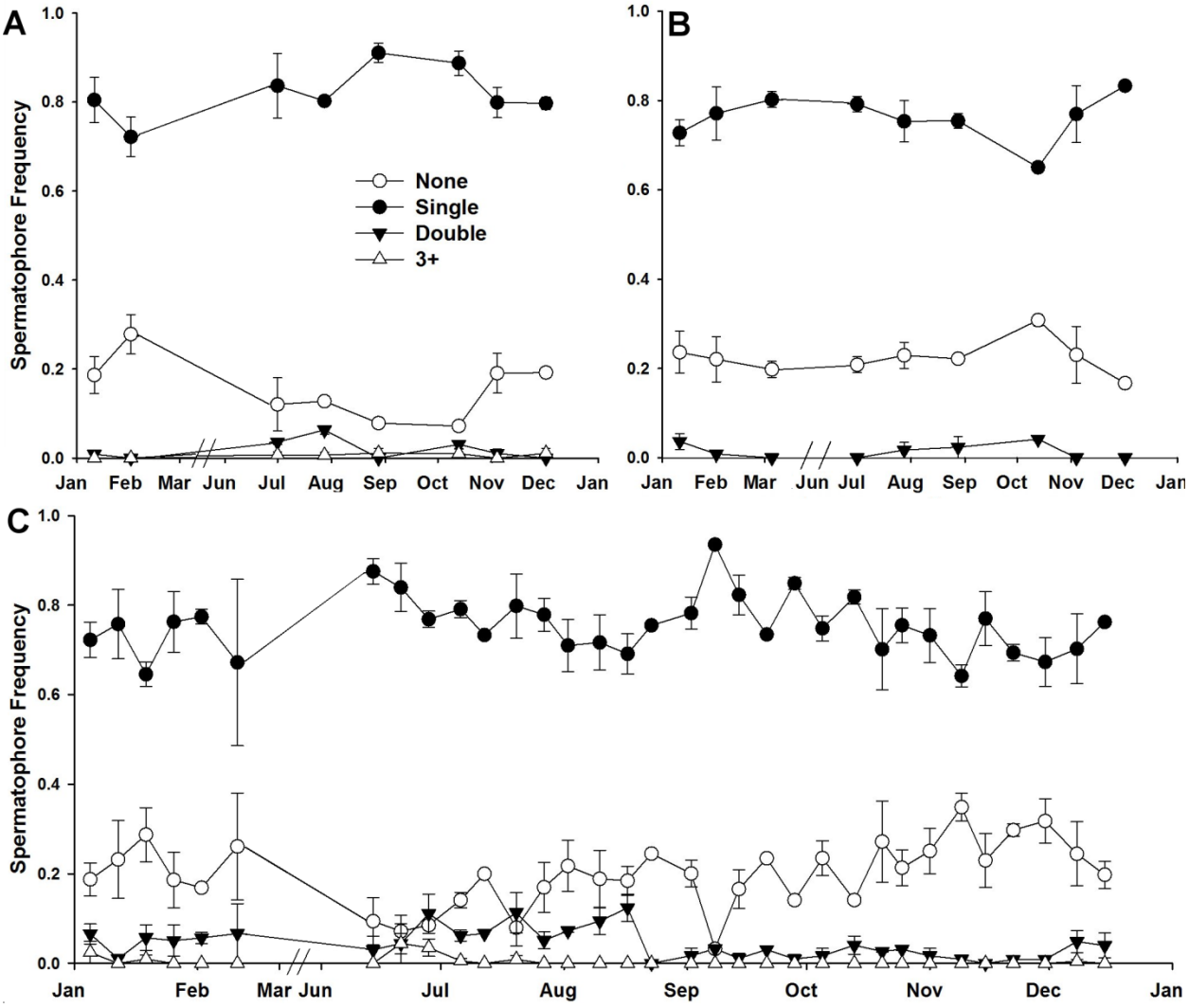
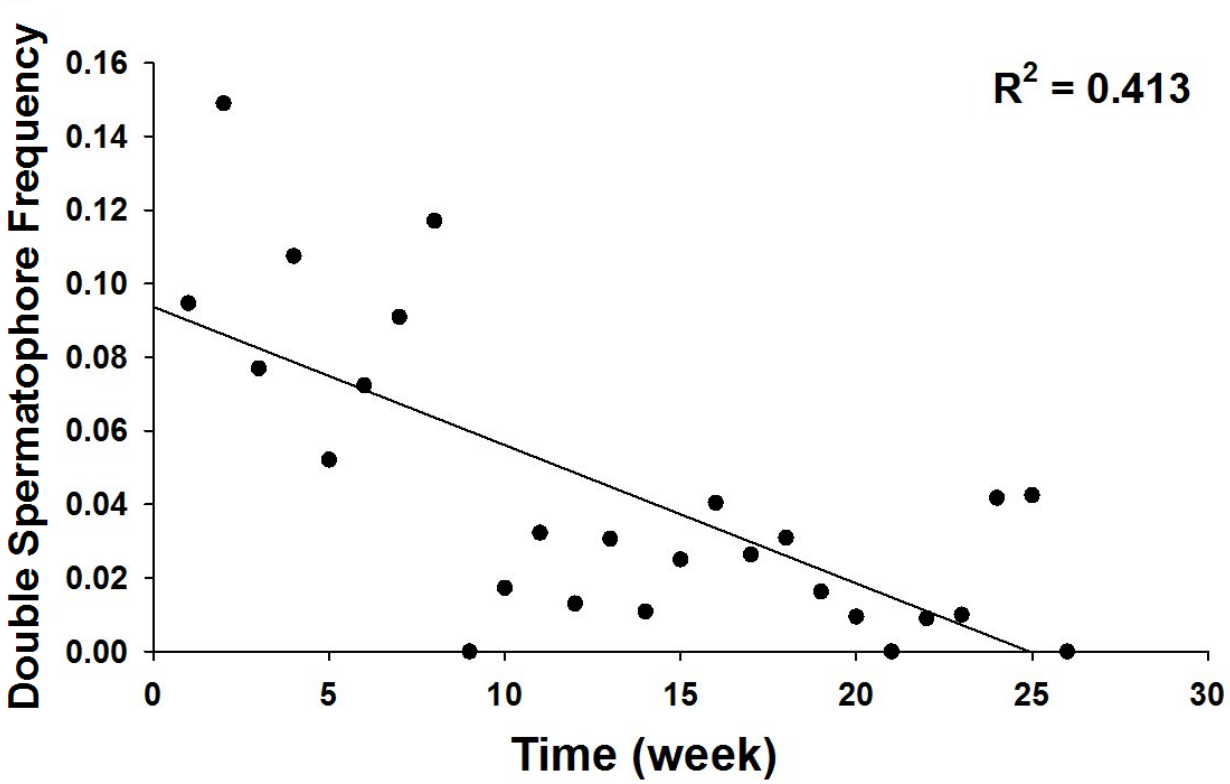

A
B
C
Spermatophore Frequency
1.0
0.8
0.6
0.4
0.2
0.0
None
Single
Double
3+
Jan
Feb
Mar
Jun
Jul
Aug
Sep
Oct
Nov
Dec
Jan

Figure 7:


Double Spermatophore Frequency
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
0
5
10
15
20
25
30
Time (week)
R2 = 0.413

Chapter 5:

Mate choice as a function of resource and mate availability, and previous social experience in the copepods, *Acartia tonsa* and *Acartia hudsonica*

Abstract:

Copepods, the most abundant metazoans in the oceans, exhibit sexual selection through mate choice. Mate choice may depend on resource and mate availability, mate quality, and the ability of an individual to assess potential mates. We measured the relative strength of mate choice for body size in male and female individuals of the copepod species *Acartia tonsa* and *Acartia hudsonica* as a function of food availability, encounter time, and previous social experience with the same and opposite sex. Mate choice in *Acartia tonsa* females depended on food availability and previous exposure to the opposite sex, whereas exposure to the opposite sex and encounter time affected mate choice in males. For *Acartia hudsonica*, previous social experience with the same and opposite sexes and encounter times were important for mate choice in females, and food availability and encounter time affected mate choice in males. The results suggest that mate choice responds to resource and mate availability and social experience, but that the responses are species and sex-specific even within the same genus.

Introduction:

Sexual selection, a form of natural selection due to differences in mating success (Darwin, 1871), can often explain a species' morphology, behavior, and population biology (Andersson, 1994). While sexual selection has been studied extensively in numerous terrestrial and even marine taxa, there have been few studies on pelagic copepods (Titelman et al., 2007; Ceballos and Kiørboe, 2010) even though they are the most abundant metazoans in the ocean. Copepods exhibit several behaviors that may be examples of sexual selection (i.e. pre-copulatory mate guarding, copulatory dances, mate coercion, and mating avoidance) (Titelman et al., 2007); moreover, the strength of sexual selection is likely different from one copepod species to the next since mating patterns vary among species (brood vs. free-spawn, single mating vs. obligate repeated mating to remain fertilized, feeding vs. non-feeding males, etc.). Thus, copepods may be informative for studies of sexual selection (Ceballos and Kiørboe, 2010).

Sexual selection, by way of mate choice, is starting to be recognized as important in the reproductive biology of pelagic copepods. Recent work has provided evidence that copepods may choose mates based on a variety of traits including size, age, and reproductive state. For instance, both sexes of *Acartia tonsa* prefer to mate with large mates (Ceballos and Kiørboe, 2010). Since size is heritable in copepods (McLaren, 1976; McLaren and Corkett, 1978), and since larger individuals produce larger and more offspring, an individual can increase its fitness by mating with a large mate (Weatherhead and Robertson, 1979, Ceballos and Kiørboe, 2010). Similarly, both sexes of *Temora longicornis* mate preferentially with young individuals likely because offspring number and quality decrease with parental age (Ceballos and Kiørboe, 2011; Sichlau and Kiørboe, 2011). Additionally, males of many species (*A. tonsa, Acartia hudsonica , Oithona davisae,* and numerous parasitic copepods) distinguish between the reproductive states

of females, and most mate with virgin females over those females that have already mated (Anstensrud, 1990, 1992; Heuch and Schram, 1996; Heuschele and Kiørboe, 2012; Burris and Dam, in press). In summary, members of either sex or of both sexes simultaneously can choose for a specific trait and select among available mates.

While these studies are valuable in providing information about whether or not a trait is important for mate choice, relatively little work has been done in the field where mate choice is subjected to the effect of multiple time-dependent parameters. Mate choice in natural populations can change temporally depending on different ecological conditions as either more or less of the population is being selective (Emlen and Oring, 1977; Wilson and Hedrick, 1982; Jennions and Petrie, 1997; Forsgren et al., 2004). For instance, the strength of mate choice can vary simply because the potential benefits of mating with a particular mate is context-dependent and influenced by factors like the availability of mates (Shuster and Wade 2003), the quality and variation in quality of those potential mates, and the ability of an individual to assess potential mates (Jennions and Petrie, 1997, Kokko and Monaghan, 2001; Shuster, 2007). As these conditions vary over time, the strength of mate choice will also likely vary. In field populations of copepods, these parameters can vary significantly between and within a season (Mauchline, 1998). Availability of potential mates, for instance, can be influenced by a number of population parameters, including population density and sex ratio. In coastal copepods, encounter rates can be very high during peak densities, when up to 30 potential mates may be encountered on a daily basis (Kiørboe, 2007). Since males can only mate about once a day and females remain fertilized for up to ten days (Ceballos and Kiørboe, 2010; chapters 3, 4), these high densities exceed the mating needs of both sexes and likely allow individuals to be selective when choosing a mate. In contrast, at the beginning and end of the growing season for these populations,

densities can be low enough that a potential mate is encountered only every few days (see chapter 6). Here, the benefits of being selective may no longer exist, as copepods run the risk of not finding a mate, or experience increased costs as they search for another mate (Real, 1990). This inference is supported by numerous models (Hubbell and Johnson, 1987; Crowley et al., 1991; Kokko and Monaghan, 2001; Hardling and Kaitala, 2005; Kokko and Mappes, 2005, Heuschele et al., 2013).

Variation in sex ratio can also influence mate availability, and thus mate choice, by changing the density of members of the opposite sex as well as that of the same sex (Kokko and Rankin, 2006). Competition and aggression among individuals of the abundant sex for mating opportunities with the rarer sex is reported in some copepod species (see Ahnesjö et al., 1993). In this case, mate choice may be influenced by previous exposure to members of the same sex prior to mating. Moreover, copepod sex ratios vary considerably over the season and are often highly skewed toward females (review: Kiørboe, 2007). Therefore, individuals may adjust mate choice in response to the rate at which they encounter other individuals of the same sex. Many animal species across many taxa vary their choice of mate according to their previous social experience. For instance, a high-quality female that encounters a low-quality female may become more selective in her initial choice of a mate, since she may have a lower risk of failing to mate (Petrie and Hunter, 1993).

Similarly, mating preferences are influenced by the variation in trait quality between potential mates in a number of invertebrate species (Hebets, 2003; Dukas, 2005; Fincke et al., 2007; Hebets and Vink, 2007). In this case, a female's decision to mate may be influenced by the quality of the previous *male* she has encountered. When male size is variable, a female may refuse small males since the potential benefits of mating with a large male are much greater

(Parker, 1983; Getty, 1995; Real, 1990). Conversely, females may be less selective in choosing a mate when male body size varies little. There is little information on variation in mate quality in copepods, but body size has been shown to be an important trait for mate choice in *Acartia tonsa* (Ceballos and Kiørboe, 2010) and *A. hudsonica* (chapter 6). Copepod body size is a relatively easy trait to measure, and is variable within and between seasons in copepod populations, by up to a factor of two in field populations (Bergreen et al., 1988; Dam and Peterson, 1991; Arendt et al., 2005; unpublished observations). Body size can be used as a proxy for mate quality since it reflects reproductive potential: female fecundity and spermatophore size are positively correlated with adult size in copepods (Kiørboe and Hirst, 2008; Ceballos and Kiørboe, 2010). Additionally, size may indicate 'good genes' (Andersson, 1994) by reflecting the ability to obtain food: adult size is related to juvenile feeding rates (Arendt et al., 2005). Body size can be influenced in field populations by a number of factors, including temperature and food availability (Durbin et al., 1971). Therefore, variance in body size, in addition to mate availability, may be important in determining the strength of mate choice in these copepods.

Finally, in addition to trait quality, the short-term *condition* of an individual also influences mate choice in invertebrates. For instance, hungry females mate less selectively and often more frequently than well-fed females in a number of insects (Proctor, 1991; Poulin, 1994; Simmons, 1994). Copepods likely experience instances of food limitation in the field (Durbin et al., 1992; Bergreen et al., 1988; Müller-Navarra and Lampert, 1996; Gulati and Demott, 1997; Sterner and Schulz, 1998). Therefore, changes to food availability may also influence mate choice in these copepods.

Individuals make mate choices. Yet, there have been no experimental studies on how changes to population-level parameters may impact *individual*-mate choice in pelagic copepods. In the present study, variation in mate-choice strength was measured experimentally in the lab as the availability and quality of potential mates were manipulated in two pelagic copepod species, *Acartia tonsa* and *Acartia hudsonica.* The effect of resource (food) availability on mate choice was also studied. In particular, we manipulated four variables: the amount of time before two potential mates were allowed to encounter one another (a proxy for population density), the presence/absence of an individual of the *same* sex of either low or high quality before mating, the presence/absence of an individual of the *opposite* sex of either low or high quality before mating, and food availability. We hypothesized that both sexes would be less selective (i.e., mate more often) as the time between encounters increased. In addition, we predicted that those held with another individual will be less selective (mate more frequently) than those held alone, and that copepods held with similarly sized copepods would mate less readily than those held with smaller copepods. Finally, we predicted that under limiting-food conditions, male and female mating preferences would become weaker.

Methods:

Maintenance of copepods

Live animals were collected by surface tows off of Groton Long Point, Long Island Sound, USA (Latitude: 41.3271 N, Longitude: 72.00150 W) using a conical plankton net with a 200-µm mesh and solid cod end. Tows were conducted each year in October for *Acartia tonsa* and April for *Acartia hudsonica;* adults were sorted from the tows and kept at 15°C (the water temperature in the field) in an environmental chamber on a 12:12 light:dark cycle and fed on a standard diet of

the diatom *Thalassiosira weissflogii* and the green flagellate *Tetraselmis sp.* (>700 µg C L^{-1}; 50% of each by carbon). Algae were grown at 18°C as semi-continuous cultures on F/2 medium (Guillard, 1975). Every week, eggs were separated from adults and raised to adulthood under the same conditions, so that differently aged cohorts could be obtained.

Mate pairing of copepods

In all experiments, individuals were sorted as juveniles by sex, placed in pretreatment conditions for up to 24 hours, then paired with an optimally sized partner for 24 hours, and finally, checked for mating (Fig. 1).

All individuals, whether to be tested or used in pre-treatments or as potential mates, were sorted by sex as stage C4 juveniles from the same cohort and held either individually in 70 mL petri dishes or with individuals of the same sex in 8L containers, depending upon the experiment, and fed as above. Within 24 hours of maturation, each copepod was photographed using a camera mounted on a dissecting microscope and its prosome length (a correlate for body size as per Mauchline, 1998) was measured to 1/1000 of a millimeter, using the program ImageJ. Copepods were paired so that the average body size for each sex was the same for each treatment. During the measurement phase, individuals were held alone in a well of a 12-well plate with high-food solution as above (about 6 mL). For any pre-treatment that lasted more than 2 hours (i.e. food availability treatments, density treatments), individuals were transferred to 70mL petri dishes with replete food. If pre-treatment lasted for more than 24 hours, then water and food were changed each day. Pre-treatment conditions are explained for each experimental variable in the next section.

Since copepods can likely only perceive one potential mate at a time, they must choose with each individual encounter whether to accept or reject mating advances (Ceballos and Kiørboe, 2010). Thus, for all experiments, the ***final pairing*** for an individual being tested was with only **one** mate of **optimal** size. To control for size differences, the 'optimal size' for a mate resulted in an average 0.09 mm difference between female and male body lengths for each treatment, with females being the larger sex. These optimal mates were always held alone during the pre-treatment time of the test copepods. For all experiments, these pairs were allowed to interact for 24 hours after which they were checked for mating. Since mating in these copepods lasts for only a few seconds (Bagøien and Kiørboe, 2005), it could not be reliably observed directly, so the presence of an attached spermatophore on a female was used to indicate a mating event. These spermatophores remain attached for more than 24 hours, and so are useful proxies for mating if checked within 24 hours of mating (Chapter 3).

Since all treatments had the same final optimal mate-pairings, any difference in mating frequencies between the treatments could be attributed to changes in mate-choice strength as a result of the pre-treatment exposures. Thus, for each treatment, a single 'mate choice' value was calculated as the proportion of matings that occurred out of the total number of pairings for that treatment. Therefore, each treatment gave a single mating frequency (i.e. if there were 40 replicate pairings in a treatment and 10 pairs mated, then the mating frequency was 10/40 or 0.25). Differences in frequencies between treatments were tested using either Chi-square tests (for three or more treatments) or Fisher's exact tests (for 2 treatments). These mate choice frequencies are relative values, and indicate whether copepods become more selective in mating (indicated by a lower mating frequency relative to the other treatments or control) or less selective (more willing to mate as indicated by a higher mating frequency) as the experimental

variables are manipulated. In addition, since these experiments were conducted on both sexes at the same time, the strength of mate choice can be compared between the sexes as well as within each sex.

Pre-treatment Conditions

Encounter time

Changes in population density (of the opposite sex) were simulated by varying the amount of time before which two potential mates were allowed to encounter each other. After copepods from the cohort matured, we held individuals of each sex alone for 0 hours (directly after maturation, corresponding to instances of high density since a mate is encountered immediately), 24 hours, 48 hours, and 72 hours. The latter corresponds to low densities that would occur during the beginning or end of the growing season. For example, from Kiørboe and Bagøien (2005): the product of search volume rate (0.008 to 0.05m^3d^{-1}; Choi and Kimmerer, 2009) and low male densities (<10 m^{-3}) would give <0.5 potential mates per day. After each pre-treatment, copepods were placed with an optimal mate that had recently matured (< 24 hours old; time 0). Thus, only the pre-treated copepods were of different ages due to the time treatments. These differences in copepod age between treatments (3 days different at the most) should not affect their mating success since "age effects" do not become apparent until 10 days for males and 20 days for females (Rodríguez-Graña et al., 2010; Ceballos and Kiørboe, 2010, 2011; Sichlau and Kiørboe, 2011). In addition, it is unlikely that the "optimal mates" can distinguish between individuals differing by only 3 days in age, for similar reasons. Therefore, any differences in mating frequency between treatments were considered to be due to mate choice by the sex that had been time-manipulated. As such, "female choice" was measured when females

were the sex held alone for different times and paired with time-0 males, and "male choice" was measured when males were held alone for different times and paired with time-0 females. Finally, to determine if there was a difference in mating when **both** males and females were being selective, an additional treatment was added at each time point. Here, both males and females that had been held alone for the same amount of time were paired together and allowed to mate. Thus, for each experimental time, we had a 'female-choice' treatment (i.e. female held alone for 72 hours, paired with a newly matured male), a 'male-choice' treatment (i.e. male held alone for 72 hours, paired with a newly matured female), and a 'both-sexes-choosing' treatment (i.e. female held alone for 72 hours, paired with a male held alone for 72 hours). There were between 25 and 44 replicates per treatment, and a total of 10 treatments for each species. To determine if female-choice changed as treatments varied, a Chi-square test was run using the four female-choice mating frequencies. This was also done for male-choice and 'both-sexes-choosing' treatments.

An additional study was performed to determine if the results in the above experiments were due to males taking a long time to produce their first spermatophore rather than just being selective. For both species, newly matured males were placed with five females and allowed to mate for 24 hours. If these time-0 males were being selective, then by increasing the number of potential optimal mates, we should see high rates of mating. In contrast, if the results of our above experiments were due to young males not producing spermatophores, then we would expect to find few males that had mated and few males with spermatophores in their bodies. Therefore, after 24 hours, females were checked for mating and males were preserved in 10% buffered formalin and immediately examined for the presence of a spermatophore in their body.

Same-Sex Exposure

Variation in the population sex ratio results in changes to both the encounter rate with the opposite sex (see density experiment above) and the encounter rate with the ***same*** sex. For instance, if males are the more common sex, then a male will likely encounter more males before finding a female than he would if females were the common sex. Thus, here we are testing the effect of an encounter with an individual of the same sex on the subsequent mate choice of a copepod. In addition, because the quality of the same sex individual may influence mate choice, copepods were exposed to individuals of different sizes (large individuals are considered high quality since both sexes prefer to mate with large mates; Ceballos and Kiørboe, 2010). For each sex, copepods were placed in one of three pre-treatments for 24 hours: held with a copepod of the same sex and of smaller size than itself (average 0.1 mm difference for females, 0.085 for males), held with a similar-sized copepod, or held alone. After pre-treatment, copepods were then placed with a mate of optimal size for 24 hours and allowed to mate. Here, 'female choice' was measured in the treatments where females were pre-treated with other females, and 'male choice' was measured in treatments where males were exposed to other males. Mating frequencies were then compared among treatments. There were 37-38 replicates per treatment for each sex. At the start of the experiment, individuals were one day old.

Opposite-Sex Exposure

An individual may become more or less selective in mating depending upon the number and quality of other potential mates it has encountered. Thus, these experiments are the same as the sex-ratio experiments, except that instead of using an individual of the same sex during the pre-treatment period, individuals of the opposite sex are used. Therefore, for each sex,

copepods were held with either a low-quality copepod of the opposite sex (females were placed with small males for an average size difference of 0.2 mm; males were placed with small females for a difference of 0.004mm), an optimal (high-quality) mate, or held alone. Since these copepods were paired with the opposite sex during the pre-treatment, to ensure that mating did not occur during this phase, pairs were held together for 2 hours, and females were checked for mating before the final optimal pairing and removed from the trial if mating had occurred (<3% of pairs mated during pretreatment). To make sure that the pairs encountered each other during this short time period, they were held in12-well plates (6mL). For the final optimal mate pairings, the individuals to be tested were then placed into 70mL petri dishes with their mate and allowed to mate for 24 hours. There were 44 replicates in each treatment and mating frequencies were then compared for each sex between the three treatments. At the start of the experiment, individuals were recently matured (day 0).

Food availability

These experiments tested the effect of food abundance on the strength of mate choice for both species of copepods. Copepods of each sex were pre-treated for 24 hours under three food regimes: Food replete: ~30 mL of >700 μg C L^{-1}; food limiting: ~100-200 μg C L^{-1}; no food: filtered sea water). The different conditions were chosen from functional relationships of food versus ingestion rates (Besiktepe and Dam, 2002). These copepods were then placed in high food with either an optimal mate fed at replete conditions, or one fed at the same food condition, in order to determine the effect when both sexes are selective. Mating frequencies among treatments were tested for differences using chi-square tests. At the start of the experiment, individuals were two days old.

Results:

Encounter time

Male choice

For both *Acartia tonsa* and *Acartia hudsonica* males, the strength of mate choice was significantly affected by being held alone for different amounts of time (Fig. 2). Males of both species were most selective directly after maturation (*A. tonsa:*1 in 46 pairs (2.2%) mated; *A. hudsonica:* 1 in 44 pairs (2.3%) mated) compared with the other experimental times (*A. tonsa:* day 1= 24%, day 2 = 41%, day 3= 47%, all N=34; *A. hudsonica:* day 1= 3.3% of 30 pairs, day 2 = 28% of 25, day 3= 18% of 22) (Fig. 2). For *A. tonsa*, males became statistically less selective (were more willing to mate) as time held alone increased (chi-square = 25.36, $p < 0.001$, power = 1.0) (Fig. 2A). *A. hudsonica* males did not show the same increase in mating with time; instead, there was little mating the first two days and significantly higher mating the following two days (chi-square = 14.04, $p = 0.003$, power = 0.9) (Fig. 2B).

Female choice

In order to measure female-mate choice, females were paired with recently matured males, which were the most selective in mating. Thus, for both *Acartia tonsa* and *Acartia hudsonica*, male-mate choice was much stronger than female-mate choice. Because of this, few pairs mated in any treatment (< 9% for *A. tonsa* and < 6% for *A. hudsonica*) and as a result any female-mate choice that was occurring was swamped out by male choice. However, data from the 'both-sexes-selecting' treatment enables us to compare day-0 females with day-1, day-2, and day-3females. For example, mating frequencies of day-0 females that mated with day-1 males can be compared with those for day-1 females that mated with day-1 males. Since the males for both treatments have been held alone for the same amount of time, any differences in mating are due to female choice. To standardize for the effect of the male treatment (since mate choice in

these males is affected by being held alone for 1, 2, and 3 days), we can calculate a proportional increase in mating for each time treatment. For example, the mating frequency of day-1 *A. tonsa* females held with day-1 males (0.143) is divided by the mating frequency of time-0 females held with day-1 males (.235). A value of 0.61means that day-1females mated only about 2/3 as frequently as day-0 females after controlling for male choice (Table 1). After doing this for the other 2 treatments, we find that day-0 females mated 16.5% and 13.7% more than day-2 and day 3 females, respectively (Table 1). Therefore, time-0 females of *A. tonsa* were the least selective (most willing to mate), followed by day-3 females, then day-2 females, and ending with the most selective, day-1 females. For *A. hudsonica*, proportionally, day-1females had much higher mating frequencies than the other female treatments after correction for male-mate choice (day-1 females mated 6 times more frequently than day-0 females) (Table 1). However, day-2 females had the lowest mating frequency after correction, with day-0 females mating 55% more frequently than day-2 females. Finally, day-3 females were less selective than day-0 and day-2, but more selective than day-1females. They mated 3.4 times more frequently than day-0 females.

Interaction

When both sexes of *Acartia tonsa* and *Acartia hudsonica* were held for the same amounts of time and then mated, the strength of mate choice was also significantly affected by treatment time (Fig. 2). In *A. tonsa*, couples became significantly less selective as the time held alone increased (chi-square: 21.89, $p < 0.001$, power = 0.99) (Fig. 2A). Day-3 females and males were the least selective, with 41% of pairs mating (N=32), followed by 34% of day-2 pairs (N=32), 14% of day-1 pairs (n=35) and 2.2% of time-0 pairs mating (N= 46). While at every time point these frequencies were lower than for the male-choice treatments, the difference was not

statistically different (paired t-test: = 2.846, p = 0.065) (Fig. 2A). This suggests that male-mate choice accounts for most of the success in mating under these conditions. For *A. hudsonica,* day-3 males and females were also the least selective, with 63% of pairs mating (N= 27) (Fig. 2B). Day-1 pairs and day-2 pairs were much more selective, with 20% (N= 45) and 13% (N=24) of pairs mating, respectively. Day-0 pairs were the most selective, with 2.3% of 44 pairs mating (Fig. 2B). As with *A. tonsa,* the differences in frequencies between the treatments were statistically significant (chi-square: 38.45, $p < 0.001$, power = 1.0). Compared with the male choice frequencies, however, two of the three same-time treatments were significantly higher. This suggests that female-mate choice is stronger than male-mate choice in these instances.

The follow up study to determine if male-mate choice results were due to day-1 males being selective rather than unable to produce a spermatophore showed that males of both *Acartia tonsa* and *Acartia hudsonica* produced spermatophores within the 24 h mating period. In addition, by increasing the number of potential female mates, a significant number of males mated. For *A. tonsa*, 58.8% of 51 males mated, and over 45% of 50 *A. hudsonica* males mated as well. However, all but 3 of the remaining unmated males in *A. tonsa* (6 in *A. hudsonica*) had large spermatophores present in their bodies.

Same-sex Exposure

For *Acartia tonsa* female choice, in which all males were held alone and females were exposed to another female of the same size, one of much smaller size, or held alone, there were no significant differences in mating frequencies among treatments (chi-square= 0.056, p = 0.97) (Fig. 3A). Females pre-treated with another female of their size mated 58% of the time (N= 38), those held with a smaller female mated 57% of the time (N= 37) and those held alone mated 59%

of the time (N=37) (Fig. 3A). For females of *A. hudsonica*, in contrast, there was a significant difference in mating frequency for females that were held with small females (N=37, 42.9% mated) compared with females held with large females (N=36, 8.3% mated) or those held alone (N=42, 14.3% mated) (chi-square= 14.6, $p < 0.01$) (Fig. 3B).

There was no significant difference in mating frequency for males of either species when held with another male of different sizes or held alone (*A. tonsa:* chi-square= 0.95, $p = 0.62$; *A. hudsonica:* chi-square= 0.23, $p = 0.89$) (Fig. 3A,B). Those *A. tonsa* males held with a large male mated 57% of the time (N=37) (*A. hudsonica:* N= 37, 13.5% mated), and those held with a small male mated 49% of the time (N=37) (*A. hudsonica:* N= 37, 10.8 %). Neither was there a difference in being held alone or with another male (Fig. 3A,B).

Opposite-sex Exposure

For *Acartia tonsa* females, there was no significant difference in mating frequencies for those exposed to a small male (0.33, N= 42) versus those exposed to an optimal male mate (0.27, N= 62) before allowed to mate (chi-square = 0.18, $p = 0.67$) (Fig. 4A). However, while size of the male did not matter, its presence prior to mating resulted in higher mating frequencies for females when compared with females that were held alone (0.07, N = 56) (chi-square = 9.66, $p = 0.002$) (Fig. 4A). Similar results were found for females of *Acartia hudsonica*-- the size of the male did not affect mating frequency, but the presence of a male resulted in higher mating frequencies compared with females held alone (Fig. 4B). Females held with a small male mated more frequently (0.52, N = 44) compared with for females held with an optimal male (0.32, N =41) (chi-square = 2.88, $p = 0.09$). Females held alone mated statistically less frequently (0.02, N = 44) than those held with a male of either size (chi-square = 20.85, $p < 0.001$) (Fig. 4B).

Male choice for *Acartia tonsa* also varied significantly according to treatment (Fig. 4A). The size of the female was important for mating; males held with a small female mated more frequently (0.37, N= 41) than males held with an optimal female (0.18, N = 57) (chi-square = 3.6, p = 0.054). Similarly, the presence of the female was important for mating, with fewer matings (0.07, N= 56) when males were not exposed previously to a female, regardless of its size (chi-square = 13.52, p= 0.001) (Fig. 4A). For males of *Acartia hudsonica*, there was no significant difference in mating frequency when exposed to females of different sizes (Fisher's Exact test: p = 0.71) (Fig. 4B). Those exposed to an optimal female mated slightly more (0.12, N= 43) than those held with a small female (0.07, N= 43). There was neither a significant difference in mating when males were held alone (0.02, N= 44) or exposed to a female prior to mating (z=1.12, p = 0.26) (Fig. 4B).

Food Availability

Mating frequencies for males of *Acartia tonsa* were not influenced by food availability (Fig. 5A). Males in the high-food treatment mated slightly more frequently (0.46, N = 95) than those in the low-food (0.4, N = 88) or no-food treatments (0.44, N = 89), but these differences were not statistically significant (chi-square = 0.81, p= 0.67) (Fig. 5A). Female-mating choice, however, was influenced by food availability (chi-square = 7.09, p = 0.03) (Fig. 5A). Females in the low-food treatment had the highest mating frequency (0.56, N = 88), followed by those in the high-food treatment (0.46, N = 95) (Fig. 5A). The lowest mating frequency occurred in the no-food treatment (0.36, N =87). Mating frequency did not vary among food treatment when both males and females were part of a treatment (chi-square = 0.43, p = 0.80): 46% of 95 pairs mated

in the high-food treatment, 44% of 93 pairs in the low-food treatment, and 49% of 92 pairs in the no-food treatment (Fig. 5A).

Results for *Acartia hudsonica* were exactly opposite to those for *A. tonsa* (Fig. 5). Male-mate choice was influenced by food availability, but female-choice was not (Male: chi-square = 11.14, p 0.004; Female: chi-square = 3.27, p = 0.20) (Fig. 5B). Males in the high-food treatment mated more frequently (0.54, N = 35) than those in the low-food (0.26, N =34), or no-food treatment (0.19, N =37) (Fig. 5B). Females showed the same pattern of mating frequencies by food treatments (High: 0.54, N =35; Low: 0.48, N = 40; Starved: 0.34, N = 41), just not as extreme as males (Fig. 5B). There was also a significant effect of food when individuals of both sexes were part of a treatment, with the highest mating frequencies for the high-food treatment and the lowest for the no-food treatment (High: 0.54, N = 35, Low: 0.54, N =39, No-food: 0.24, N = 38) (chi-square= 9.41, p = 0.009) (Fig. 5B).

Discussion:

In both male and female copepods of the species *Acartia tonsa* and *Acartia hudsonica*, the strength of mate choice in laboratory experiments varied in response to changes in resource availability (food abundance), mate availability (encounter rates), mate quality (body size variation) or previous social experience (same or opposite sex encounters). This suggests that not only are both males and females being selective when mating, but also that mate choices are not fixed and uniform. Instead, our results suggest that mate choice is flexible in these two species and that the sexes are influenced differently by the factors above. A mate-sampling strategy that relies upon a fixed mate preference has advantages and drawbacks. An advantage is that individuals are not required to remember the previous potential mates encountered. The

drawbacks are that, at the extremes, individuals will not mate if their preferences are set too high, or, will mate with low-quality mates if their fixed preferences are too low (Jennions and Petrie, 1997). The flexible mate-sampling strategy would allow for an individual that is at either extreme to raise or lower their mate preferences based on information from their surroundings (food availability, mate density, sex ratio, trait-quality, etc.) in order to balance the benefits and costs associated with finding a preferred mate (Janetos, 1980; Real, 1990). Here, we summarize the general mate choice findings and then discuss which variable(s) may be most important for each sex in the field where all variables may be encountered simultaneously. In addition, we discuss some possible reasons for the differences between the two species and the two sexes in mate-choice responses.

Encounter time

In both *Acartia tonsa* and *Acartia hudsonica*, the strength of male-mate choice was affected by variations in the time they were held alone, and both species were most selective (least likely to mate) during the first 24 hours after maturation (Fig. 2). This was not due to recently matured males being unable to produce spermatophores (see methods). It is not surprising that the youngest adult males in our study were the most selective. They can enhance their fitness by mating with a higher-quality mate than by mating immediately with an inferior one, since female egg production rates increase significantly with female size (Weatherhead and Robertson, 1979; Ceballos and Kiørboe, 2010). *A. tonsa* males became increasingly less selective with increasing time held alone (i.e. as mate density decreased) (Fig.2), as predicted by density-dependent models (Hubbell and Johnson, 1987; Crowley et al., 1991; Kokko and Monaghan, 2001; Hardling and Kaitala, 2005; Kokko and Mappes, 2005). This decrease in

choosiness at low population densities is probably adaptive. By passing on a mating opportunity later in life in hopes of finding a higher-quality mate, an individual would risk never finding another mate, or delaying its reproductive output (Real, 1990). *A. hudsonica* males also appear to have a time-dependent reduction in mating preferences, but their threshold for finding a mate is longer than for males of *A. tonsa* (Fig. 2). Males of *A. hudsonica* mated very little during the first two days (days 0 and 1), but significantly increased their mating by days 2 and 3. Two days of not encountering a female may be the threshold value after which males significantly alter their mate choice criteria. The difference in the response between males of the two species may be due to differences in the length of time their spermatophores remain viable. For instance, spermatophores produced by males of *A. tonsa* may go bad earlier than spermatophores produced by males of *A. hudsonica*. If so, then this may explain why *A. tonsa* males were more willing to mate after only 1 day than *A. hudsonica* males. While there is no information on spermatophore viability in copepods, it may explain the species specific response to different mate encounter rates.

Female-mate choice in *Acartia hudsonica,* but not *A. tonsa,* was influenced by increasing the time it took to encounter a mate. After accounting for male choice, day-1 *A. hudsonica* females mated proportionally more than any others (Table 1), possibly because the benefit of waiting longer for a high-quality mate is not worth the trade-off of delaying offspring production (Janetos, 1980; Real, 1990). Since these females continue to produce eggs even when unfertilized, it may be energetically costly to remain unfertilized for extended periods.

In both species, when females and males were held alone for the same amount of time (interaction treatment), the strength of mate choice was also affected by treatment time. While at every time point the mating frequencies for the interaction in *Acartia tonsa* were lower than for

the male-choice treatments, the difference was not statistically different between treatments (Fig. 2A). This suggests that male-mate choice accounts for most of the success in mating under these conditions. In *Acartia hudsonica*, day-3 males and females were the least selective (most willing to mate) (Fig. 2B), which is what is predicted by mate choice models (Hubbell and Johnson, 1987; Crowley et al., 1991; Kokko and Monaghan, 2001; Hardling and Kaitala, 2005; Kokko and Mappes, 2005; Heuschele et al., 2013). Compared with the male-choice frequencies for *A. hudsonica*, however, two of the three interaction treatments were significantly higher for females. This suggests that female-mate choice is stronger than male-mate choice in this species, at least for females that have been held alone for more than 24 hours.

Contrary to what was predicted by a mate-selectivity model for copepods (Heuschele et al., 2013), mate choice was stronger in males than in females for recently matured individuals of both species, and also more affected by encounter time. Males of both species are restricted by the number of times they can mate, since they produce a limited number of spermatophores over their lifetime (chapter 3) at a low rate (only about 1 spermatophore day^{-1}) (Ceballos and Kiørboe, 2010; chapter 3). Therefore, when population density is high, males would encounter more females per day than they can mate with (up to 30 potential mates, see Kiørboe, 2007), which favors choosy males. By being selective, a male can increase its fitness considerably since large females have more and larger offspring (Ceballos and Kiørboe, 2010). Since females of species that are not capable of storing sperm, such as *Acartia tonsa* and *Acartia hudsonica* (Ohtsuka and Huys, 2001), can re-mate immediately after having just mated, and, theoretically, an unlimited number of times over a lifetime, males appear to have the more limited reproductive period. As such, males may experience a greater fitness penalty than females by making a poor mating decision, and so should always be more selective in choosing their partners than females.

Same-sex Exposure

Exposure to another member of the same sex, regardless of its quality, did not affect the strength of mate choice for either sex of *Acartia tonsa* or for males of *Acartia hudsonica* (Fig. 3). Mate choice in females of *Acartia hudsonica*, however, was affected by the quality of the previous female encountered. Large females held with small females mated significantly more frequently than those held alone or with other large females. This suggests that females may adjust their selectivity in response to assessment of their own quality (Burley and Foster, 2006). Since an individual's attractiveness to potential mates can limit its ability to obtain a willing mate, individuals may adjust the strength of their mate choice based on their own current mating quality. This may help them to avoid wasting energy courting an unwilling mate (Burley, 1977, 1981). Females without knowledge of other females (i.e. held alone) or with knowledge of similarly attractive females (held with large females) may deem themselves of low quality and decide not to waste time or energy in courtship that will may not lead to mating (Burley and Foster, 2006). Conversely, females held with low-quality females may be more willing to mate when high-quality males are encountered because they have assessed themselves to be of high-quality as well. It is not surprising that female choice is influenced more than male choice by previous same-sex social experience. In the field, there are often more females than males in the population and so both sexes will likely encounter more females than males. Thus, in the field, male-choice should be influenced more by the opposite sex, and female-choice by previous experience with the same sex.

Opposite-Sex Exposure

Neither *Acartia tonsa* nor *Acartia hudsonica* females showed differences in mating frequencies between those previously exposed to small or large mates (Fig. 4). However, previous exposure to a male regardless of its size increased mating significantly when compared with females held alone. It may be that females with no information about males are less willing to mate than those that have information because they are uncertain about the quality of the male (Dukas, 2006). For both species, there was also a trend (although not significant) of increased mating frequency in females exposed previously to small males compared with large males. It is possible that by encountering a low-quality male, females are more willing to mate with the next male (of high quality) because the difference in size is more extreme and thus quality is easier to determine (Janetos, 1980; Reid and Stamps, 1997). It may be easier for females to tell that this male is of high quality after exposure to a small male. Effects of previous exposure to males are known to influence female-mating choice in a number of animals, including birds (Collins, 1995), fishes (Bakker and Milinski, 1991; Brown, 1981; Downhower and Lank, 1994), crickets (Bailey and Zuk, 2009), and beetles (Roitberg et al., 1993). Copepods now join the ranks.

While previous exposure to females had no effect on males of *Acartia hudsonica,* male mate choice in *Acartia tonsa* was significantly affected (Fig. 4). The size of the female, as well as just her presence, was important for mating. As hypothesized, males held with a small female subsequently mated more frequently than males held with an optimal female, and males held alone mated less frequently than males held with a female. This suggests, as with the female choice results, that previous exposure of males to low-quality females may make high-quality females more attractive or their quality more easily distinguished. In some flies, males are capable of assessing relative sizes of females in order to mate with larger ones (Svensson and

Petersson, 1994). Therefore, it is conceivable that copepods use information obtained from a previous encounter to influence mate preferences. It is surprising that male mate choice in *A. hudsonica* was not affected by social experience, when male choice in *A. tonsa* was significantly influenced. It is possible that by allowing males to encounter multiple females over a short period of time, we mimicked high encounter rates. From the encounter rate experiment above, we know that males of *A. hudsonica* are most selective under "high encounter rates" (when allowed to immediately encounter a female), more so than males of *A. tonsa*. This suggests that for *A. hudsonica* males, mate choice is influenced more by female encounter rates than by the actual quality of the females encountered. If we had conducted this experiment after holding males alone for several days, instead of using males at their most selective, we may not have measured a difference in the response between the two species.

Food availability

Mate choice in *Acartia tonsa* was affected by food availability in females, but not males (Fig. 5). Females were less choosy in the low-food treatment and most selective (least willing to mate) in the no-food treatment. Egg production of *Acartia* decreases as food decreases and ceases in the absence of food after 2-3 days (Dagg, 1977; Tester and Turner, 1990; Finiguerra et al., 2013). Thus, in the absence of food, mating has little or no benefit for *Acartia* females as there are few or no eggs to be fertilized. Moreover, mating decreases the longevity of both sexes even under high-food conditions (Nakatsuru and Kramer, 1982). As a result, starved females, by mating, would only incur costs and none of the benefits associated with mating. Previous work has shown that males of *A. tonsa* are much more sensitive to lack of food than females (Finiguerra et al., 2013), and are thus less likely to survive long without food. This outcome is

consistent with the terminal investment hypothesis (Issac and Johnson, 2005). That is, males, instead of ceasing reproduction to increase survival (as females may do), continue to reproduce to increase offspring because their own survival is probably limited.

Contrary to what was found for *Acartia tonsa*, male-mate choice in *Acartia hudsonica* was affected by food availability, but female choice was not. Males were more selective in matings as food became less available. Females also showed the same pattern, but the differences between treatments were not significant. When both sexes were part of the same food treatment, individuals were more selective in mating as food availability decreased. It is possible that the differences observed in mate choice as a function of food availability between the two species are the result of a difference in energy requirements or tolerance to lack of food. *A. hudsonica* may be able to survive for longer periods of time under food limitation than *A. tonsa*, increasing the chance they will make it to a new food patch. If this were the case, then it may be more beneficial for males of *A. hudsonica* to delay mating now in order to invest in future reproduction.

Future Work

Our results suggest that not only are mate choices in different species affected by different factors, but that male and female choice within the same species are also affected differently. In field populations, copepods likely encounter many of these variables simultaneously and the interaction of changing variables may be important in determining whether or not an individual is selective during mating. While our study only measured mate preferences for a single variable at a time, it is the first evidence that individual-mate preferences can vary. Our laboratory findings can be used to make predictions and design future field studies to determine which

factors are important for mate choice in natural populations. For instance, the sizes of mated and unmated females in the field can be compared over a season(s) as a proxy for male-mate choice and then mate choice tested for correlation to hypothesized environmental factors. In addition to the variables tested in our experiments, other factors may be as important for influencing mate choice in these copepods. For instance, predation pressure or water turbulence may have an effect on mate choice, by changing the costs associated with being selective (Jersabek et al., 2007). Copepods may attract more attention from predators during mating because of increased conspicuousness as a result of courtship and copulation behaviors (Svensson, 1995). To minimize the costs of mating during times of high-predation pressure, copepods may decrease their swimming speeds or number and length of courtship bouts by mating with inferior mates. Similarly, the search costs of finding a mate may increase with increasing turbulence (Strickler, 1975; Hwang et al., 1994; Hwang and Strickler, 1994; Kiørboe et al., 1999; Lee et al., 2011); thus, having flexible mate preferences may be beneficial in a changing environment. Likewise, mate choice for other traits (age, reproductive state, etc.) may also respond differently to changes in ecological variables. Our understanding of copepod-mating dynamics would be aided by research focusing on these questions.

Table and Figure legend:

Table 1: Ratios of female-mating frequency by density treatment after standardization for male-mate choice.

Figure 1: Schematic of experiments conducted.

Figure 2: Mating frequency of males (black bars) and both sexes (grey bars) for *Acartia tonsa* (A) and *Acartia hudsonica* (B) as a function of time held alone. Males of both species (black bars) were most selective (lowest mating frequencies) directly after maturation compared with the other experimental times. *A. tonsa* males became statistically less selective (were more willing to mate) as time held alone increased (chi-square = 25.36, $p < 0.001$), whereas *A. hudsonica* males reached a peak in mating frequency (least selective) by day 1 (chi-square = 14.04, $p = 0.003$). When both sexes of *A. tonsa* (A) were held alone for the same amount of time, couples became significantly less selective as the time held alone increased (chi-square: 21.89, $p < 0.001$). Similarly, *A. hudsonica* day-3 males and females were the least selective (highest matings), and time-0 pairs were the most selective (chi-square: 38.45, $p < 0.001$). Lowercase letters above histograms represent significant differences.

Figure 3: Mating frequencies for females (black bars) and males (grey bars) of *Acartia tonsa* (A) and *Acartia hudsonica* (B) after being exposed for 24 hours to a large or small individual of the same sex, or being held alone. For *A. tonsa*, there were no significant differences in mating frequencies among treatments for males or females. For *A. hudsonica*, females held with small females mated significantly more frequently (*) than those held with either large females or

alone (47.9% compared with 8.3 and 14.3%, respectively) (chi-square= 14.6, $p < 0.01$). There was no difference between treatments for males.

Figure 4: Mating frequencies for females (left 2 bars) and males (right 2 bars) of *Acartia tonsa* (A) and *Acartia hudsonica* (B) after being exposed for 2 hours to a small or optimally sized individual of the opposite sex, or being held alone. For females of both *A. tonsa* and *A. hudsonica,* the size of the male did not affect mating frequency, but the presence of a male resulted in higher mating frequencies compared with females held alone. For males of *A. tonsa,* both female size and female presence were important for mating. Males held with a small female mated more frequently than males held with an optimal female. Similarly, there were fewer matings when males were not exposed previously to a female, regardless of her size. In males of *A. hudsonica*, neither the presence of a female nor her size was important for mating. Lowercase letters above histograms represent significant differences.

Figure 5: Mating frequencies for males (black bars), both sexes (grey bars), and females (light bars) of *Acartia tonsa* (A) and *Acartia hudsonica* (B) after being exposed for 24 hours to high, low or no-food conditions. Mating frequencies for males of *A. tonsa* were not influenced by food level, but female mating did significantly increase in the low-food treatment and decreased in the no-food treatment compared with the high-food treatment. When both males and females were part of a treatment, there was no difference in mating frequencies among treatments. In *A. hudsonica*, mating in both males and females decreased as food availability decreased, but this tendency was only statistically significant for males. When both sexes were part of a treatment, the no-food treatment had significantly lower mating frequencies than the other two.

Tables:

Table 1: Ratios of female-mating frequency by density treatment after standardization for male-mate choice.

Male : Female	*Acartia tonsa*	*Acartia hudsonica*
Day 0: Day 0	1.0	1.0
Day 0: Day 1	0.61	6.0
Day 0: Day 2	0.84	0.45
Day 0: Day 3	0.86	3.4

Figures:

Figure 1:

1. Sorted by sex as stage C4 juveniles
2. Within 24 hrs. of maturation, placed in pretreatment condition:
    a. Encounter time (held alone for 0, 1, 2, 3 days)
    b. Same sex exposure (held alone or with small or large individual for 24 hrs.)
    c. Opposite sex exposure (held alone or with small or large individual for 2 hrs.)
    d. Food availability (held in high, low, or no food for 24 hrs.)
3. Removed from pretreatment and placed with an optimally sized partner for 24 hrs.
4. Females checked for presence of spermatophore (indicates mating) and mating frequencies calculated for each treatment.

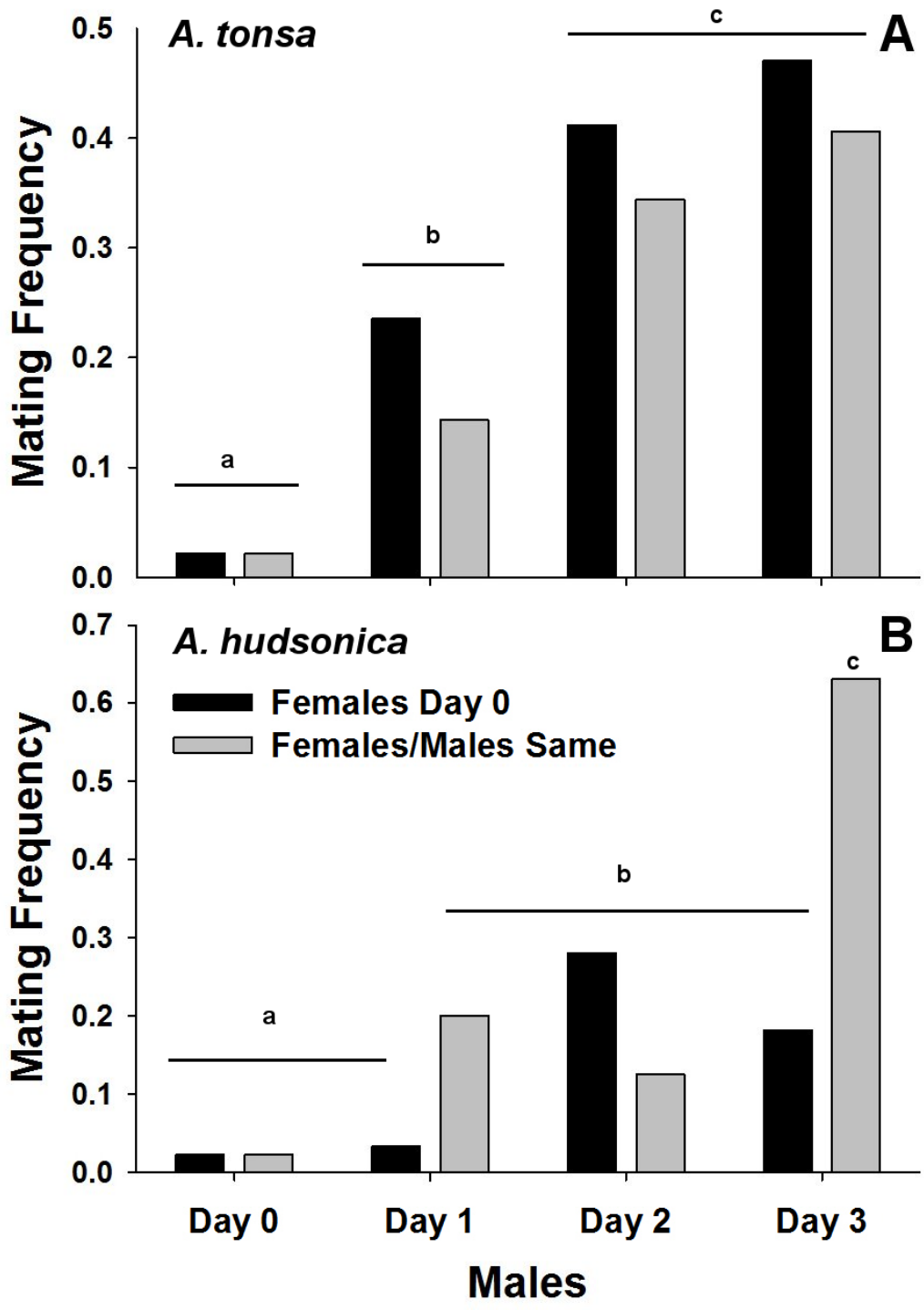

A. tonsa
A
c
b
a
Mating Frequency
0.5
0.4
0.3
0.2
0.1
0.0
A. hudsonica
B
Females Day 0
Females/Males Same
c
b
a
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Day 0
Day 1
Day 2
Day 3
Males

Figure 2:

Figure 3:

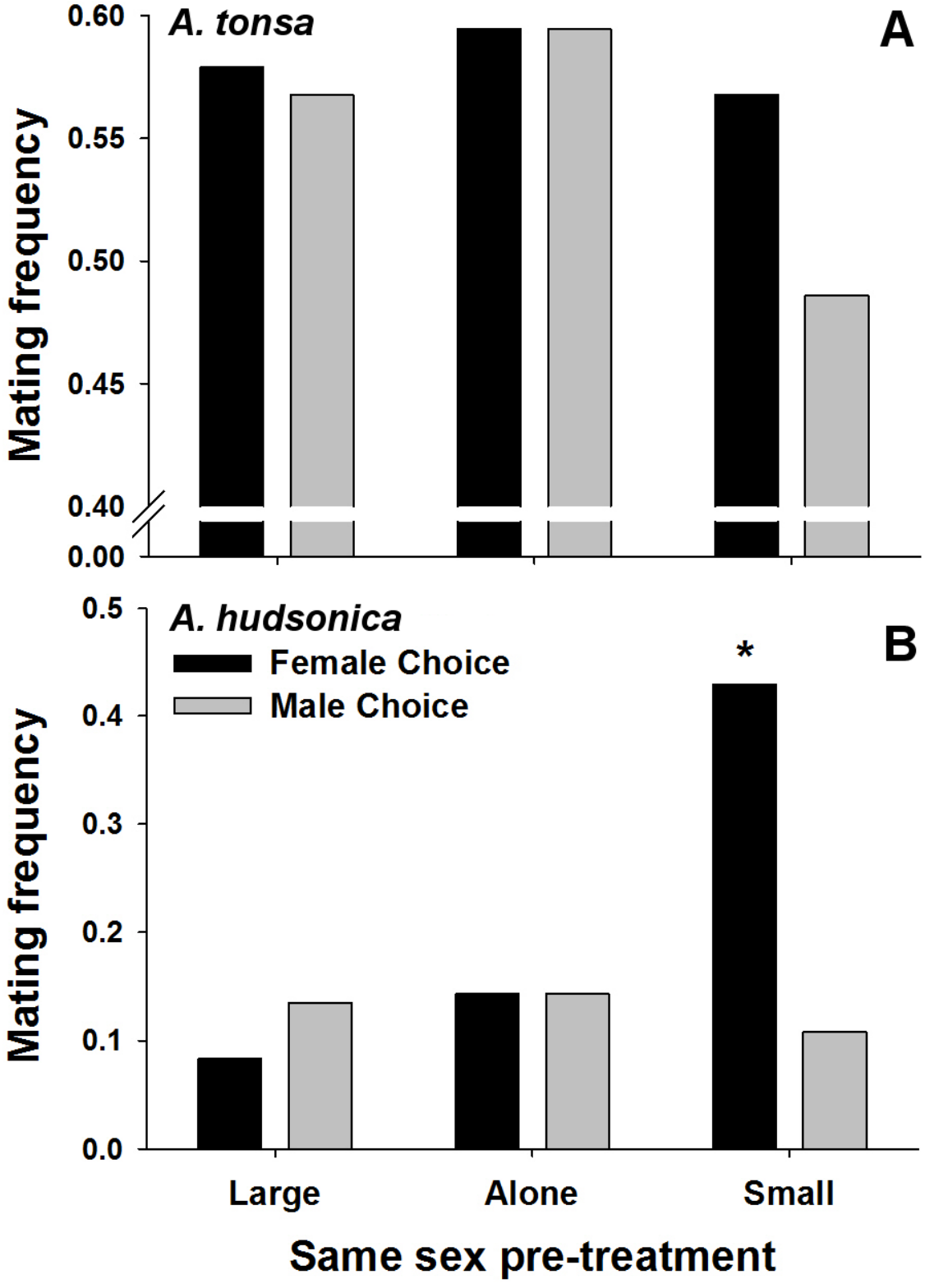

A. tonsa
A
Mating frequency
0.60
0.55
0.50
0.45
0.40
0.00
A. hudsonica
B
Female Choice
Male Choice
*
Mating frequency
0.5
0.4
0.3
0.2
0.1
0.0
Large
Alone
Small
Same sex pre-treatment

Figure 4:

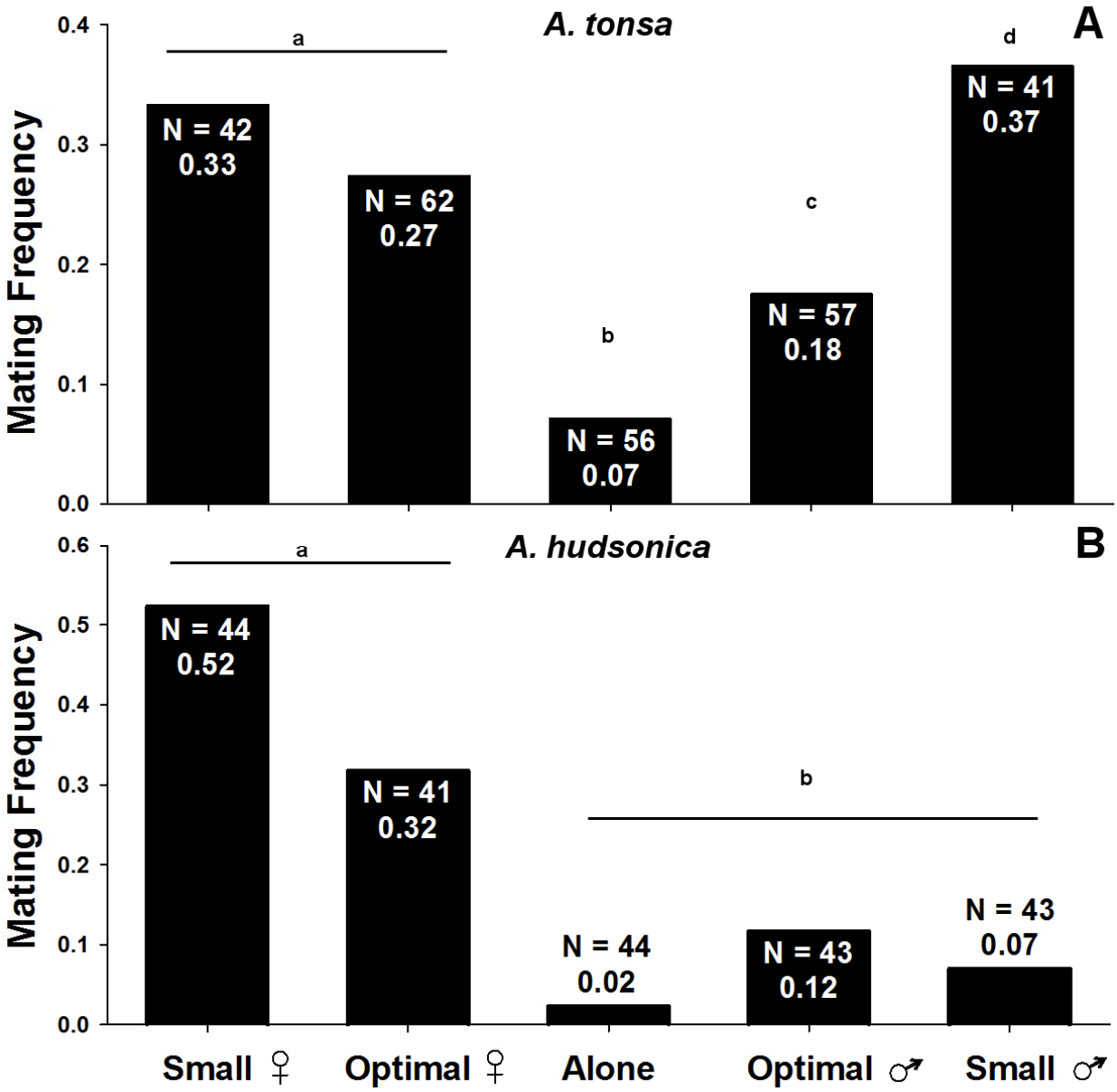
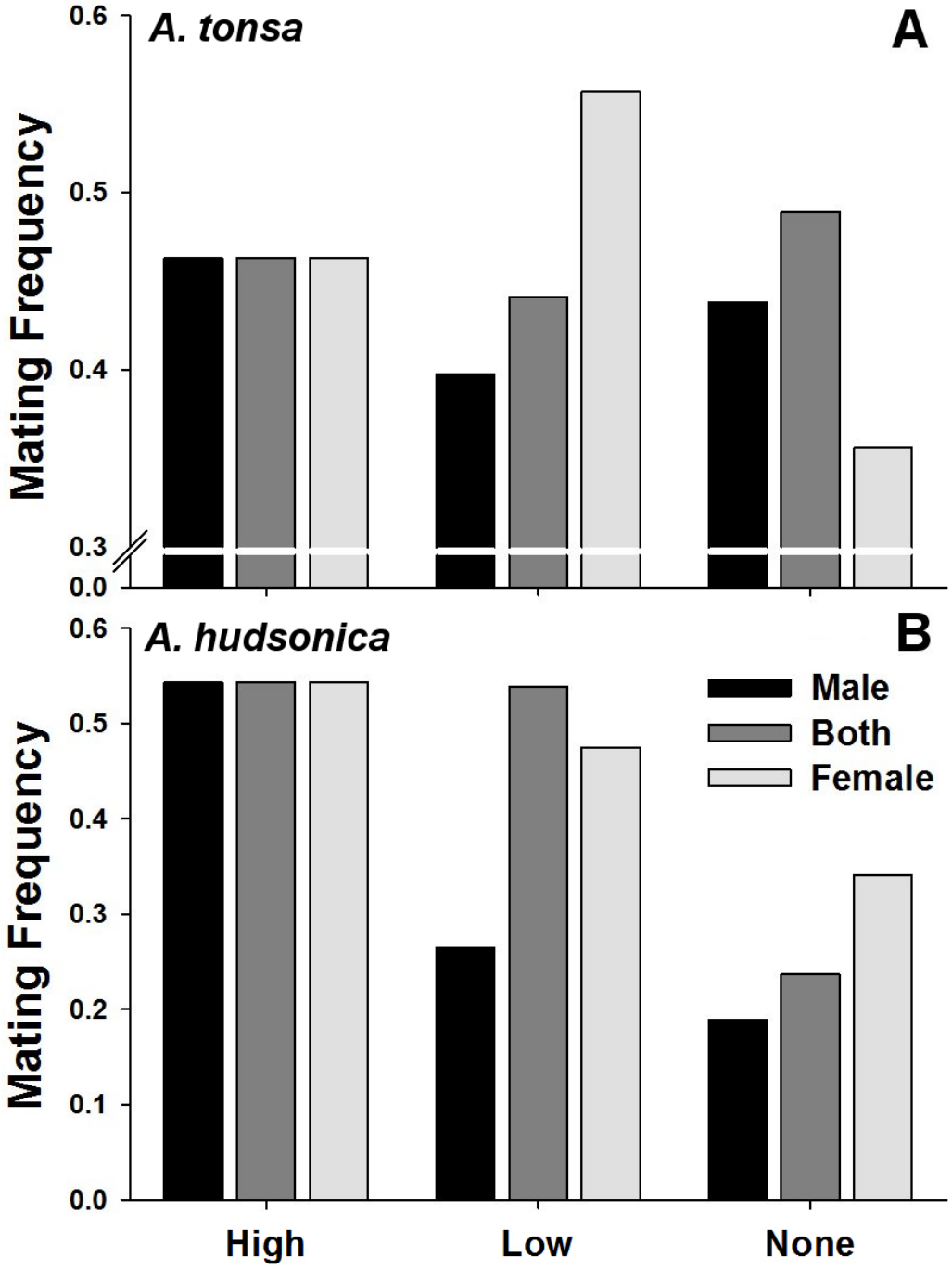

A. tonsa
A
Mating Frequency
0.4
0.3
0.2
0.1
0.0
a
d
c
b
N = 42
0.33
N = 62
0.27
N = 56
0.07
N = 57
0.18
N = 41
0.37
A. hudsonica
B
Mating Frequency
0.6
0.5
0.4
0.3
0.2
0.1
0.0
a
b
N = 44
0.52
N = 41
0.32
N = 44
0.02
N = 43
0.12
N = 43
0.07
Small ♀
Optimal ♀
Alone
Optimal ♂
Small ♂

Figure 5:


A. tonsa
A
Mating Frequency
0.6
0.5
0.4
0.3
0.0
A. hudsonica
B
Male
Both
Female
0.6
0.5
0.4
0.3
0.2
0.1
0.0
High
Low
None

Chapter 6:

Strength of mate choice for body size in a field population of the calanoid copepod *Acartia hudsonica*

Abstract:

Laboratory studies have shown that pelagic copepods exhibit mate choice for a variety of traits, and that mate-choice strength can be influenced by several ecological factors. In the field, however, the costs and benefits of being selective likely change as food abundance, mate density, and variation in mate size fluctuate concurrently over a season. Thus, field studies are necessary to determine if mate choice occurs in copepods and whether it is constant or variable. Body size of the coastal copepod species *Acartia hudsonica* was measured as a proxy of mate quality at weekly intervals during two seasons in Long Island Sound. Male-mate choice and the strength of size-assortative mate choice were calculated for each sampling date and tested for statistical relationship to 11 potentially influential variables. Male-mate choice for female body size was strong during the 2013 season, occurring during 50% of sampling dates, but weak in 2014, when many of the measured variables showed little variation. Multivariate regression analysis showed that the strength of male-mate choice was strongly and positively related to male and female sizes, and negatively related to temperature. The strength of size-assortative mate choice, measured by using the size of attached spermatophores as a proxy for the size of a female's mate, was weak for both years. The findings demonstrate that male-mate choice does occur, but not always, in field populations; moreover, male-mate choice can vary in complex ways both within and between seasons. The difference in findings between the two seasons demonstrates the need for multi-year studies in order to obtain even a basic understanding of mate choice in field populations.

Introduction:

Mate choice for body size occurs in a variety of animals, including birds (Price, 1984), amphibians, reptiles (Halliday, 1992, Olsson, 1993; Tokarz,1995), insects (Bonduriansky, 2001; Bateman et. al., 2001), fishes (Noonan, 1983), and crustaceans (Aquiloni and Gherardi, 2008). Mate choice for body size likely occurs because body size is often directly related to fecundity. In general, larger individuals produce greater numbers of gametes *(*MacDiarmid and Butler, 1999; Radwan et al., 2005; Lehmann and Lehmann, 2009;Gasparini et al., 2010). In copepods, the most abundant planktonic marine metazoans, a few species have been shown to select mates according to size as well. In *Acartia tonsa* and *Temora longicornis*, both males and females prefer large mates (Ceballos and Kiørboe, 2010; Sichlau and Kiørboe, 2011), presumably because egg production rates increase with increasing female size in copepods (Kiørboe and Hirst, 2008; Ceballos and Kiørboe, 2010), and increases in male size correlate to larger sperm packets (spermatophores) (Kiørboe and Hirst, 2008; Ceballos and Kiørboe, 2010; Sichlau and Kiørboe, 2011). In addition, adult body length is heritable in copepods (McLaren, 1976; McLaren and Corkett, 1978; Bergren et al., 1988; Arendt et al., 2005); thus, individuals that mate with large mates may produce more and larger offspring (Weatherhead and Robertson, 1979), a feature that may enhance fitness.

In addition to being heritable, body size in copepods varies with temperature (Landry, 1976; Vidal, 1980) and food abundance (Mullin and Brooks, 1970a,b; Paffenhofer and Knowles, 1978) in the field, and changes in these factors likely influence individual-mate choice for this trait. In the abundant coastal copepod *Acartia hudsonica*, body size has been shown to vary both predictably and substantially over the season (Durbin et al., 1992), thereby making this species a good candidate for experiments exploring the relationship between mate choice and body size.

Recent laboratory studies (Chapter 5) have shown that in *A. hudsonica*, the strength of mate choice for body size can be influenced by changes in environmental and population-level variables, and that males and females may be influenced differently by changes in these factors. In females of *A. hudsonica*, variation in previous social experience, in body size, and encounter times influenced the strength of choice, whereas male choice responded most strongly to changes in encounter time and food availability. In field populations, copepods may face simultaneous changes in several variables. Thus, while laboratory experiments have proven useful, field studies are needed to understand how mate choice responds to multiple variables: population density, sex ratio, and variation in body size may influence mate choice in field populations.

At peak-population densities, *A. hudsonica* likely encounters more potential mates than can be mated with since males mate less than once per day (Chapter 3), and females remain fertilized for about a week after each mating (Kiørboe, 2007). Thus, at high densities, both sexes would experience high encounter rates (very little time in-between potential mate encounters). In contrast, at low densities, individuals would experience increased time in between mate encounters. Models predict that individuals should become less selective as density decreases (Hubbell and Johnson, 1987; Crowley et al., 1991; Kokko and Monaghan, 2001; Hardling and Kaitala, 2005; Kokko and Mappes, 2005), since the cost of mate-sampling increases (chapter 5). Thus, in field populations, we would expect the greatest mate choice for body size under high-population densities.

Similarly, adult sex ratios can be a proxy for previous social experience since deviations of the ratio from unity increase mate-availability for one sex at the expense of the other. Thus, under female-skewed sex ratios males would likely encounter a female before encountering another male. Adult sex ratios in the field are known to vary widely (Conover, 1956; Deevy,

1960;Lee and McAlixe, 1979), so this variable may be important for influencing mate choice in natural populations of *Acartia*.

Finally, variation in body size over a season may be important in determining the strength of mate choice. For instance, when males of different sizes are available, a female may refuse small males since the potential benefits of mating with a large male are much greater (Parker, 1983; Real, 1990; Getty, 1995). Conversely, females may be less selective in choosing a mate when male body size varies little. Copepod body length over a season in the field can vary by a factor of around 1.5 for both males and females (Bergren et al., 1988; Dam and Peterson, 1991; Arendt et al., 2005). Thus, body size and its variation may be useful for measuring mate choice in field populations.

This study aimed to measure mate choice for body size in a field population of *Acartia hudsonica* in Long Island Sound. Since males only deposit a single spermatophore during mating (see Chapter 3), female mating status can be determined by the presence (i.e. mated) or absence (i.e. unmated) of an attached spermatophore. Thus, the sizes of mated and unmated females were compared to determine if mate choice for body size occurred in males. Under strong mate choice we would expect mated females to be significantly larger than unmated females. In addition, since spermatophore size increases with male body size (Ceballos and Kiørboe, 2010), the spermatophore that a female is carrying can serve as a proxy for the size of its recent male mate. Spermatophore size is most strongly dependent on male size, with factors such as food availability having a limited effect (see Chapter 3). Thus, if size-assortative mate choice is occurring (large mate with large and small mate with small), then we should observe a strong positive relationship between female body size and the size of its attached spermatophore.

Both measures of mate choice were tested for correlation to 11 variables that potentially influence mate-choice strength.

Methods:

Field collection and preparation

Three replicate horizontal plankton tows were taken weekly, using a conical plankton net with a 200-μm mesh and solid cod end, on incoming tides off of Groton-Long Point Bridge, Long Island Sound, USA (Latitude: 41.3271 N, Longitude: 72.00150 W) from January to July in 2013 and 2014, months in which *Acartia hudsonica* is present in Long Island Sound (Peterson, 1986). The volume of water sampled by the net was calculated from a calibrated flow meter attached to the mouth of the net. Water temperature and salinity data were recorded at a depth of 0.3m using a YSI meter (total water column depth varied between 1-1.5 meters). Tow contents were preserved immediately upon collection with 10% buffered phosphate formalin, for a final concentration between 3-5%. This preservative does not cause copepods to drop their spermatophores (chapter 3). Furthermore, it is unlikely that females drop spermatophores during the tow because spermatophores are attached by a cement-like substance (Hammer, 1978; Buskey, 1998). Moreover, there is no evidence that females can remove spermatophores once attached. Thus, attachment is most likely controlled by the quality and quantity of the male's glue than by the female herself. Although females without a spermatophore present (and thus counted as "unmated") may have actually mated and simply dropped their spermatophore, fewer than 10% of "unmated" females in these samples produced nauplii (i.e. had mated) (Burris and Dam, 2014). Thus, females carrying one or more spermatophores were considered "mated," and those without a spermatophore "unmated."

Subsamples were taken from each of the three replicate tows so that 30-40 *Acartia hudsonica* females from each were counted (for a total of 90-100 for each tow date) and the number of spermatophores attached to each female was recorded. The number of males in each subsample was also noted so that average sex ratios for each date could be calculated. Individuals from the three tows were pooled, and images of both female and male prosome lengths (a proxy for body size, Mauchline, 1998) as well as each spermatophore were taken using a camera mounted on a microscope. Each copepod was then measured in ImageJ to 1/1000 of a millimeter. Spermatophore volume was calculated using its length and width assuming an ellipsoid shape (Ceballos and Kiørboe, 2010). Data on 11 variables were obtained for each sampling date as either single values (temperature, proportion of females mated in the population, female to male size ratio, week), as averages from the three replicate tows (population density, sex ratio) or as an average of the 90-100 individuals grouped together from the three tows (female size, male size, variation in female size, variation in male size, spermatophore volume). These variables were used in the analysis because they either have been shown to affect mate choice in copepods (female and male size, variation in male and female sizes, ratio of female size to male size)(Ceballos and Kiørboe, 2010; Sichlau and Kiørboe, 2011; Chapter 5), or are hypothesized to affect mate choice (spermatophore size, sex ratio, density, proportion of females mated) (Kiørboe, 2007; Sichlau et al., 2014), or have the potential to affect mate choice by having a direct impact on the previous variables (temperature, time).

Although shrinkage in body size from preservation in formalin occurs in copepods until after three months of preservation (Bottger and Schnack, 1986), analysis was always done within one week of preservation for all samples; hence, shrinkage was considered to be small and

uniform for all samples, and no correction for shrinkage of body length or spermatophore size was done.

Sample analysis

Two techniques were used to quantify the strength of mate choice for body size and how it changes over the season. The first measures male-mate choice and the second measures the effect of both male and female choice.

Male-mate choice

The hypothesis that males prefer larger females (Ceballos and Kiørboe, 2010) was tested. If male copepods do not choose their mates based on size, then one would expect equal size of mated and unmated females. Thus, average size of unmated and mated females were compared on each sampling date using one-tailed T-tests or Mann-Whitney rank sum tests. The strength of mate choice was defined as the ratio of the average size of mated to unmated females. A ratio significantly greater than one indicates males choose larger females; the larger the ratio, the stronger the preference for larger females.

Size-assortative-mate choice

Since spermatophore size increases with male body size (Ceballos and Kiørboe, 2010), the size of the spermatophore that a female is carrying can be used as a proxy for the size of the male with which she mated. Thus, if size-assortative mate choice is occurring (large males mating with large females and small males mating with small females), then we should observe a strong positive relationship between spermatophore volume and female body size. Thus, for each sampling date, a linear regression was performed using arcsine transformed spermatophore volumes and the associated female sizes. Positive slopes that are significantly different from

zero indicate that assortative mate choice occurs. Next, the strength of mate choice can be approximated by the correlation coefficients, r, obtained from each regression. Positive values significantly different from zero suggest strong mate choice, whereas values near zero suggest weak or absent size-assortative mate choice.

For both methods, to determine which of the 11 independent variables were important for predicting mate choice, multiple regression analysis was performed using either the ratio from the male-mate choice section or the correlation coefficients from the size-assortative mate choice section as the dependent variable. Data for all variables were checked for normality and uniform variance, and were transformed when appropriate. SAS 11.0 was used for all statistical tests and model building.

Results:

Seasonal Patterns

In both 2013 and 2014, female and male sizes, as well as spermatophore volumes followed parabolic curves, with peak in prosome length between February and April (Fig. 1A,B) and peak in spermatophore volume between March and April (Fig. 1C). Females and males were much larger at the start of the season than at the end during both years (Females: start 2013: 0.873mm ±0.0085, 2014: 0.823mm ±0.009; end 2013: 0.775mm ±0.0134, 2014: 0.775mm ±0.004; 2013: U=593, 2014: U=1012, P<0.001 for both; Males: start 2013: 0.77mm ±0.012, 2014:0.798mm±0.005; end 2013: 0.741mm ±0.004, 2014: 0.712mm±0.004; 2013: U=.036, 2014: t=13.64, p<0.05 both). The season was longer in 2014 than in 2013, starting two weeks earlier and ending one month later. Males and females reached significantly larger sizes during 2013 than 2014 (females: U=3015, males: t=4.88; P<0.001 for both), and males in 2013 also reached

significantly smaller sizes than males in 2014 ($t=2.48$, $p<0.001$). For females in 2013, sizes ranged from 0.781mm (±0.003) to 0.955mm (±0.005), and in 2014 from 0.786mm (±0.004) to 0.936 (±0.004). In 2013, average male size varied from 0.707mm (±0.002) to 0.87 mm (±0.004) (Fig. 1B), whereas in 2014 males varied from 0.71(±0.004) mm to 0.838(±0.003) mm. Similarly, during both years, the peak in average spermatophore volume was almost double that of the average minimum spermatophore volume (2013: 0.188 ±0.01 mm at the end of July and 0.304 ±0.01mm in mid-April; 2014: 0.158 ±0.005mm at the end of July and 0.307 ±0.011mm in mid-March) (Fig. 1D). During 2013, between 65 and 89% of females in the population had mated, while the range was greater during 2014 (50% to 95%). There was no apparent seasonality in the proportion of mated females (Fig. 1C).

The adult male-to-female sex ratio varied much more in 2013 than in 2014 (Fig. 2 A), with a peak in 2013 of 1.25 males to every 1 female, and a minimum of about 1 male to every 7 females. In 2014, males were much scarcer and the sex ratio was always strongly female biased (peak of 1 male to every 2 females, minimum of 1 male to every 6 females). The ratios of female to male size were similar during both years, and average female sizes were always larger than males (range: 1.06 to 1.114) (Fig. 2B). Ratios of size closer to one occurred at the tail ends of both seasons. During 2013, there were two peaks (in April and June) in population density, while in 2014 there was only a single peak in June (Fig. 2C). During both years, as expected, the temperature increased over the course of the season, but lower values and a sharper climb in temperature occurred during 2013 (Fig. 2D).

Male-mate choice

For *Acartia hudsonica* during 2013, on 50% of dates (11 out of 22), mated females were significantly larger than unmated females (Table 1) (Fig. 3A). On average, these mated females were 0.04 mm (S.E. ± 0.004) (or about 5%) longer than unmated females. The majority of the dates on which mated females were larger than unmated ones occurred in the middle of the season, and none occurred after the second week of April. This coincided with a peak in female sizes between the end of February and April, with mated females at their largest (average size of 0.958 mm± 0.004). Sizes decreased almost linearly after the beginning of April, with the smallest mated females (ave.: 0.764 ± 0.02 mm long) at the end of June. Unmated females reached their peak size in mid-April (0.93 ± 0.01mm) and sizes also decreased after April until June when they reached their smallest sizes (0.777± 0.02 mm). Averaged over the season, mated females (0.88 mm ± 0.01S.E.) were significantly larger than unmated females (0.86 mm ± 0.01 S.E.) (Paired T-test= -3.165, p=0.004).

In 2014, fewer significant differences were found between mated and unmated female sizes (Fig. 3B). Mated females were significantly larger than unmated females on only three of 27 dates (Table 2) (Fig. 3B). As in 2013, these dates occurred during the period when average sizes were still increasing from week to week. The peak in mated female size occurred midway through the season as in 2013, with the largest females occurring during the month of March to the first week of April (largest in mid-March: 0.936 ±0.004 mm; smallest in July: 0.775 ±0.004 mm). The largest unmated females occurred in early May (0.92 ± 0.009mm) and the smallest in July (0.776 ±0.0067mm). Averaged over the season, mated females were also significantly different in size (0.877±0.009mm) than unmated females (.872 ±0.009mm) (Paired T-test= -2.102, p=0.045).

Ratios of size of mated female to unmated female were calculated as a predictor of mate-choice strength (Fig. 3). Ratios greater than one indicate strong male-mate choice for larger females. In 2013, these ratios ranged from 0.981 to 1.063 (in 2014: 0.972 to 1.063), with mated females being much larger than unmated females (ratios > 1) in mid-March (Fig. 3).

Regressions for Male Choice

Using the 25 (2013) and 28 (2014) weekly ratios of mated to unmated female sizes as the dependent variable (Fig. 3), linear regressions were performed on 11 independent variables (average female size, average male size, female size standard deviation, male size standard deviation, time, density, sex ratio, ratio of female to male size, temperature, proportion of females that have mated, and spermatophore size) to determine which were predictive. In 2013, five variables were not useful (statically significant) in predicting mate-choice strength: the ratio of female size to male size ($p=0.99$; $F=0$), proportion of mated females ($p=0.919$, $F=0.106$), standard deviation of female size ($p=0.95$; $F=0$), standard deviation of male size ($p=0.95$; $F=0$) and time of sampling ($p>0.05$; F-stat = 4.09). Thus, these variables were not included in future analyses. Of the six remaining variables, four were positively related and one was negatively related to mate-choice strength (Fig. 4). Male size ($r^2=0.472$; $F=20.52$, $P=0.0002$), female size ($r^2=0.37$, $p=0.001$, $F=13.59$), spermatophore size ($r^2=0.21$; $p=0.022$; $F=6.02$), sex ratio ($r^2=0.19$; $p=0.03$; $F=5.27$), and temperature ($r^2=0.34$; $F=10.31$, $P=0.004$) all had predictive power. As male size, female size, spermatophore size, and the sex ratio in the population increased, mate choice also increased, whereas as temperature increased, mate-choice strength decreased. The final variable, density, had a parabolic relationship with mate-choice strength ($r^2= 0.28$; $p=0.028$; $F=4.22$) and so the combined variable (density + denisty^2) was used in further analyses. Of these

six predictors, the spermatophore size and female size variables were removed from further analysis because they were both found to be collinear with male size (regression of spermatophore size on male size see Fig. 6; regression of male size on female size: r^2=0.93, p<0.001.; F=328.73). Thus, for the multiple linear regression, only four variables were tested: male size, proportion of males, density, and temperature. Using forward, stepwise, and backward selection techniques, a model including only male size was found to be the best. Adding any combination of other variables did not significantly increase the regression fit; hence, the predictive value.

For 2014, there were fewer significant relationships between variables and those that were significant were weaker than in 2013. Of the 11 predictors, eight were not useful in predicting the strength of mate choice: the ratio of female size to male size (p=0.52; F=1.89), proportion of mated females (p=0.249, F=1.39), standard deviation of female size (p=0.075; F=3.47), standard deviation of male size (p=0.47; F=0.54) , male size(p=0.085; F = 3.217), female size (p=0.319, F=1.03), sex ratio (p=0.55, F=0.36), and spermatophore size (p=0.51, F=0.44). Thus, these variables were not included in the model. The remaining three were all negatively related to mate choice (Fig. 5): density (r^2=0.27, p=0.005, F=9.62), time of sampling (r^2=0.33, p=0.001, F=12.77), and temperature (r^2=0.22, p=0.013, F=7.12). Again, time and temperature were collinear, since as time increased so did temperature, and thus temperature was removed from the model. Using forward, stepwise, and backward selection models all agreed that adding population density to a model that already contained time did not add any significant predictive power. Thus, for 2014, the most important variable for describing mate-choice strength was time of sampling.

Size-assortative-mate choice

In both 2013 and 2014, average spermatophore size was significantly and positively related to average male size (2013: $F=37.064$, $p<0.001$, $r^2=0.63$; 2014: $F=109.4$, $p<0.001$, $r^2=0.82$) (Fig. 6).

For 2013, only four of the 24 weeks showed significant relationships between female size and spermatophore volume (Table 3). In 2014 (Table 4), only five of 26 dates showed significant relationships, and all but one occurred early in the season. Using the correlation coefficients of the regressions as the dependent variable of mate-choice strength, regressions were performed using the same 11 independent predictors as above. In 2013, seven variables were not helpful in predicting mate-choice strength: sex ratio ($p=0.31$, $F=1.06$), density ($p=0.31$, $F=1.06$), ratio of female size to male size ($p=0.16$, $F=0.13$), standard deviation of female size ($p=0.81$, $F=0.06$), standard deviation of male size ($p=0.39$, $F=0.76$), spermatophore size ($p=0.1$, $F=3.0$), and proportion of mated females ($p=0.47$, $F=0.55$). Of the variables shown to be predictive, both male and female sizes had negative relationships with mate-choice strength (Female: $p=0.026$, $F=5.67$, $r^2=0.18$; Male: $p=0.018$, $F=6.66$, $r^2=0$ .182), whereas temperature and time of sampling had positive relationships (temperature: $p=0.037$, $F=4.9$, $r^2=0.25$; time: $p=0.036$, $F=5.0$, $r^2=0.18$) (Fig. 7). Using forward, backward, and stepwise selection models, a model including solely temperature was more predictive than any combination of other variables (Table 4). In 2014, only the female to male size ratio had a significant relationship with mate-choice strength ($p=0.038$, $F=4.86$, $r^2=0.174$); as females size increased out of proportion to male size, the strength of mate choice decreased (Fig. 8). None of the remaining variables showed significant relationships with the mate choice variable (all $p>0.05$).

Discussion:

For the first time, mate choice in field populations over an entire season has been measured (twice) for a copepod species. Results for the abundant coastal copepod *Acartia hudsonica* reveal three main findings: First, mate choice for body size exists in field populations; second, the strength of mate choice is variable both within and between seasons; third, mate choice depends on several variables (male size, sex ratio, density, etc.), to different degrees.

Male-mate choice for female body size was more prevalent in 2013 than in 2014, but even then did not occur over the entire season. During 2013, mated females were much larger than unmated females on over 50% of sampling dates, suggesting that males were selecting large females over small females for mates. In this species, since both sexes contribute equally to the acts of courtship and mating, unlike many other copepods (Bagøien and Kiørboe, 2005; Kiørboe, 2007), both sexes have to be willing to mate in order for mating to be successful. Thus, large females were willing to mate with large males. It is possible that our results were due to small females being unwilling or selective in mating, and not to male-mate choice itself. In theory, however, small females should never pass up a mating attempt since their low quality implies that only small males would otherwise mate with them (Real, 1990). Thus, our results are most readily explained by males selecting large females as mates.

The strength of male-mate choice for body size during the 2013 season was positively related to both male and female sizes, spermatophore size, and the population sex ratio, and negatively related to temperature. As male and female sizes increased, the proportional difference in body size between mated and unmated females also increased. This suggests that large males are more selective than small males, which is in agreement with theoretical predictions (Real, 1990). Since body size is positively related to fecundity in copepods (Ceballos

and Kiørboe, 2010, 2011), by mating with a large female that will produce more and larger gametes, males can increase their fitness. Similarly, large males produce larger spermatophores that contain more spermatozoa than those produced by small males (Ceballos and Kiørboe, 2010, 2011; this study). Thus, a large male that mates with a small female may experience a fitness penalty because much of its sperm may be wasted since she will produce fewer eggs than a large female. Conversely, a small male will likely fertilize the same number of eggs regardless of a female's size because his sperm quantity is limiting. As a result, a small male should not be selective during mating (i.e. mate with the first female encountered), whereas a large male should delay mating until a high-quality female is found. This may explain the observed relationship between increasing male size and increasing mate-choice strength, as well as spermatophore size and female size. The latter is not surprising since, as mentioned above, male size is also correlated to spermatophore size. Similarly, female size is collinear with male size throughout both seasons, and thus may only be useful in predicting mate-choice strength because of this fact. The observed parabolic seasonal pattern of male and female size is mediated by temperature and particularly food abundance (Durbin et al., 1990). In both years, animal size starts small and increases over several months as temperatures warm and food becomes more abundant. The peak in sizes occurs around the time or soon after the spring phytoplankton bloom (Durbin et al., 1990). Since size in these copepods is dependent on food acquired as juveniles, the largest sizes should occur during times of high food. The positive relationship between female size and mate-choice strength may just be a result of collinearity with male size. However, if this were the case, then the slope and R^2 of the regression of female size vs. mate choice and male size vs. mate choice should be similar. The two were different, suggesting that the positive relationship between female size and mate choice were due to the benefits accrued to

females by choosing larger males. Thus, when most females in the population are small (and producing few and small gametes) it may not be cost-effective for males to be size selective. Conversely, when the population is composed of mostly large females that produce many, large gametes, it may be more beneficial to pass up a small female for the possibility of mating later with a large female.

The positive relationship between the strength of male-mate choice and the ratio of males to females in the population is difficult to explain. The expectation is that males should be selective when the ratio of males to females is <1, and the strength of male-mate choice should increase as that sex ratio becomes smaller (more biased toward females). Indeed, a strongly female-biased ratio was observed on most sampling dates (on average 1 male to every 2 females). Yet, the opposite pattern was observed; i.e., the strength of male-mate selection increased as the sex ratio became more biased towards males. One possible explanation is that the ratio of males to females and population density are hyperbolically related. Hence, the ratio of males to females does not increase in direct proportion to density. As the latter increases, there are also more females per male in the population, which leads to an increase in the strength of male-mate choice. The correlation between density and mate-choice strength was much stronger than for the sex ratio variable, suggesting that density may be more important as a predictor than sex ratio. Models predict that at high densities, mate choice may decrease if individuals become 'overwhelmed' with choice, and at low densities, mate choice may also decrease since opportunities for mating are so low (Heuschele et al., 2013; Mahjoub et al., 2014). As expected, then, the strongest mate choice in our study was observed at mid-densities.

Temperature was shown to have a negative relationship with the strength of male-mate choice during 2013. This finding may be the result of changes in physiological mating rates with

temperature. While there is no information on the effect of temperature on male mating rates, increases in temperatures are known to increase a number of other physiological processes in copepods, including feeding and swimming rates (Kiørboe et al., 1982; Dam and Peterson, 1988; Larson et al., 2008). Thus, it is possible that males at higher temperatures may be producing spermatophores faster and finding mates sooner because they are swimming faster. Mating capacity, then, may increase with temperature, and as a result, increase the number of opportunities a male may have for mating. Under high temperatures each mating, as a result, may be relatively less important, allowing males to be less selective when choosing a mate (Edward and Chapman, 2011). Temperature is also collinear with time of sampling (week), and so another possibility is that as temperature increases over the season, individuals in the population are less healthy. Thus, early in the season when the temperature is low and individuals healthier, there is stronger male-mate choice. This may explain why mated females were never significantly larger than unmated females late in the season.

The final regression model for the 2013 season was composed solely of male size, suggesting that while other variables may be important, male size is the main determinant of mate-choice strength. The fact that male sizes were much smaller in the 2014 season may explain the finding of fewer and weaker significant relationships between variables than in 2013. In 2014, evidence of male-mate choice for body size in the field was almost nonexistent: mated females were larger than unmated female on only about 11% of sampling dates. In fact, most variables measured during the 2014 season did not reach the maxima measured during the previous season. Female sizes, male-to-female sex ratio, and ratio of female size to male size were much lower in 2014 than 2013. While the peak in population density was greater in 2014, it occurred much later in the season. This may be due to a large bloom of ctenophores, voracious

predators of copepods, that started much earlier in 2014 and lasted much longer than in 2013. The negative relationships found in 2014 between strength of male-mate choice and population density, time, and temperature, albeit significant, were weak. All three variables were collinear, since the peak in population density occurred so late during the year. Thus, these variables may simply be describing weaker male-mate choice as the population nears the end of the season. It may be that different cohorts of copepods respond in different ways to the variables, and late cohorts respond less to the variables than early cohorts. These findings suggest that during seasons of low-population density, sex ratios highly skewed toward females, and smaller ranges in animal size, male-mate choice for female size in field populations may be very weak.

Size-assortative-mate choice

In both 2013 and 2014, average spermatophore size was significantly and positively related to average male size in the population. Of the variables measured in this study (including temperature and female size), average male size was the most important factor in predicting average spermatophore size (R^2= 0.63 and 0.82), implying that average spermatophore sizes may be useful as a proxy for average male size. In 2013, four of 24 sampling dates showed significant relationships between female size and the size of the spermatophore she was carrying, while in 2014, only five of 26 dates were significant. These findings suggest that either there was weak mate choice on the part of both sexes (large males mating with large females; not just male choice as is the case above), or that spermatophore volume is not a helpful predictor of *individual* male size. While *average* male size explained up to 82% of the variation in *average* spermatophore volume in field populations, this does not tell us how well it describes individual spermatophore volumes. In a laboratory study by Ceballos and Kiørboe (2010), individual

variation in spermatophore volume was large, and individual male size only accounted for 44% of individual spermatophore volume. Thus, while male size may be useful in predicting average spermatophore volume in field populations, the relationship is too variable for comparing small differences in individual sizes.

It is possible, though, that the relationship between spermatophore volume and male size does reflect the strength of size-assortative mate choice in these field populations. It is possible, especially coupled with the results for the strength of male-mate choice, that size-assortative mating is simply weak in these populations. In 2013, male and female sizes had negative relationships with the strength of size-assortative mate choice, and temperature and time had positive relationships. This means that as male and female sizes increased, large males were not mating with large females, or small males were not mating with small females, or both. While not intuitive at first, if we remember the results of male-mate choice, these findings actually make sense. Recall that as male and female sizes increased, male-mate choice increased. As a result, fewer small females mated. Since this method of measuring size-assortative mate choice **only** takes into account individuals that have mated, these small females are not being included in the analysis. Thus, when male-mate choice is occurring, this method is only looking at a biased sample of large females. Differences in female sizes then are much smaller and as a result harder to discern. When male-mate choice is not occurring (when body size is small), then all females in the population are included in the analysis, and differences can be measured more easily. Similarly, in 2014, only the ratio of female size to male body size was an important, although weak, predictor of the strength of size-assortative mate choice. The lack of significance for other variables may be explained by the smaller range in sizes for both males and females, which makes it difficult to discern differences. It makes sense that as female size increased out

of proportion to male size, the strength of mate choice decreased. In a few species of freshwater copepods, peak mating occurs during instances of specific size ratios, possibly as a way to decrease the chances of one species mating with other co-occurring species (DeFrenza et al., 1986). In *Acartia hudsonica* it appears that individuals prefer a specific size ratio. In *Acartia tonsa*, Ceballos and Kiørboe (2010) showed that when both males and females are being selective during mating, there was an optimal difference in size that met the standards of both mates.

In summary, male-mate choice does occur, but not always, in field populations; moreover, male-mate choice can vary in complex ways both within and between seasons. The difference in findings between the two seasons demonstrates that multi-year studies need to be done in order to obtain even a basic understanding of mate choice in field populations. While most studies of copepod mate choice have been done in the laboratory under controlled conditions, individuals in the field must simultaneously assess multiple traits under varying ecological conditions when making a mating decision. The multivariate analysis carried out here is one way to discern the relative effects of these variables in determining the strength of mate choice in the field.

Table and Figure legend:

Table 1: Statistics of T-tests or Mann-Whitney tests for mated versus unmated female sizes for each sampling date during the 2013 season. Significant dates are in bold.

Table 2: Statistics of T-tests or Mann-Whitney tests for mated versus unmated female sizes for each sampling date during the 2014 season. Significant dates are in bold.

Table 3: Regression statistics of female size by spermatophore volume for each date during the 2013 season. Significant dates are in bold.

Table 4: Regression statistics of female size by spermatophore volume for each date during the 2014 season. Significant dates are in bold.

Figure 1: Average female size (A), average male size (B), proportion of females mated in the population (C), and average spermatophore volume (D) over the 2013(dark circles) and 2014 (light circles) seasons for *Acartia hudsonica*. Female size, male size, and spermatophore volume all followed similar parabolic curves. Bars are standard error (n= 25 to 111).

Figure 2: Average male –to-female sex ratio (A), ratio of female size to male size (B), and density (C) of *Acartia hudsonica,* and temperature (D) data over the 2013 (dark circles) and 2014 (light circles) seasons. Bars are standard error (n=3).

Figure 3: Average size for mated (light circles) and unmated (dark circles) *Acartia hudsonica* females over the 2013 (A) and 2014 (B) seasons. Dotted lines show the ratios of mated to unmated female size at each date. Asterisks mark dates when mated females were statistically larger than unmated females, and hence when male-mate choice occurred. Error bars are standard error (n=25 to 111).

Figure 4: Linear regressions of significant predictor variables on the strength of mate choice for *Acartia hudsonica* in 2013. Female size (A), male size (B), proportion of males (C), and spermatophore volume (D) all had significantly positive relationships with mate choice, whereas temperature (E) had a negative relationship.

Figure 5: Linear regressions of significant predictor variables on the strength of mate choice for *Acartia hudsonica* in 2014. Temperature (A), week (B), and density (C) all had significantly negative relationships with mate choice.

Figure 6: Average spermatophore volume by average male size of *Acartia hudsonica* over the 2013 (A) and 2014 (B) seasons. In both years, average spermatophore volume increased linearly with average male size. Males and corresponding spermatophores were larger in 2013 than in 2014.

Figure 7: Strength of mate choice for *Acartia hudsonica* based on attached spermatophore size as a function of female size (A), male size (B), temperature (C) and week (D) in 2013. Female

and male sizes had significantly negative relationships with mate choice, whereas temperature and week had significantly positive relationships.

Figure 8: Strength of mate choice for *Acartia hudsonica* based on attached spermatophore size as a function of female-to-male size ratio in 2014. Size ratio was the only significant predictor and had a negative relationship with strength of mate choice.

Figures:

Figure 1:

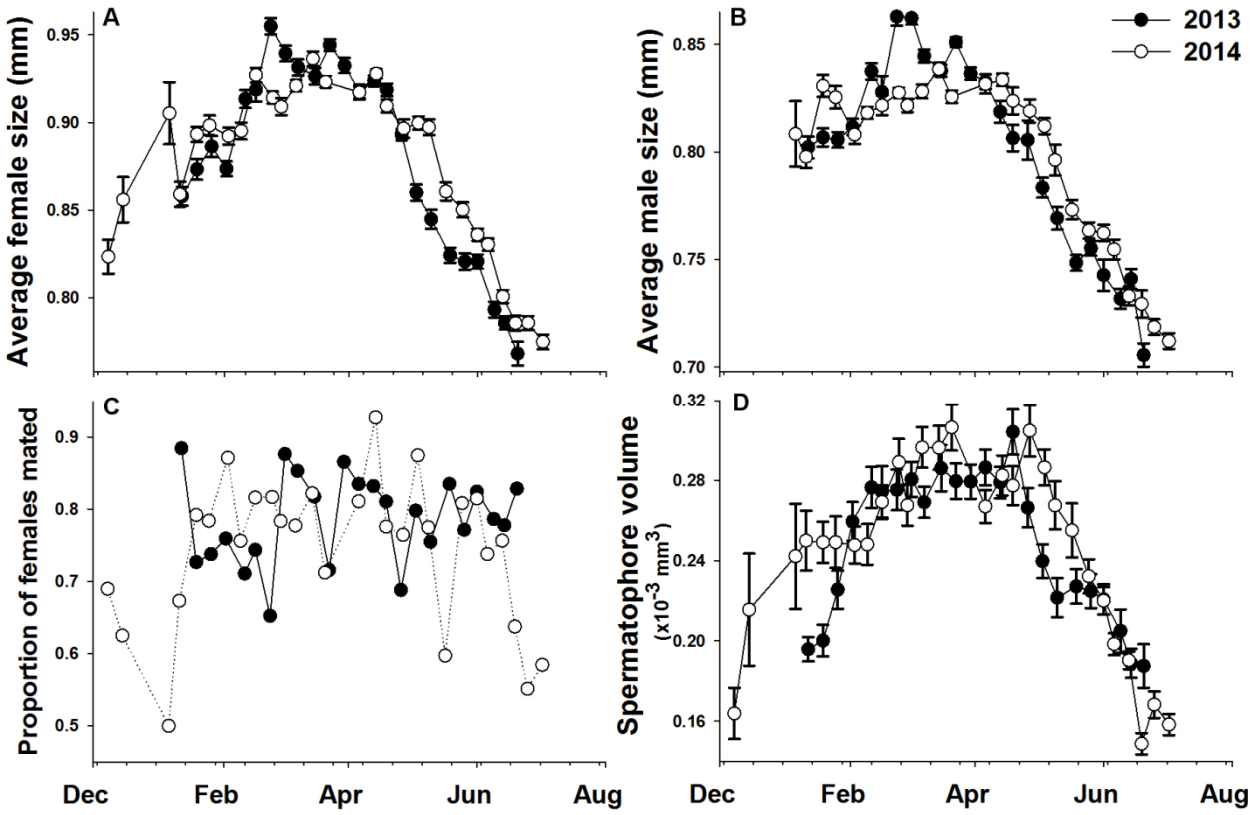


Figure 2:

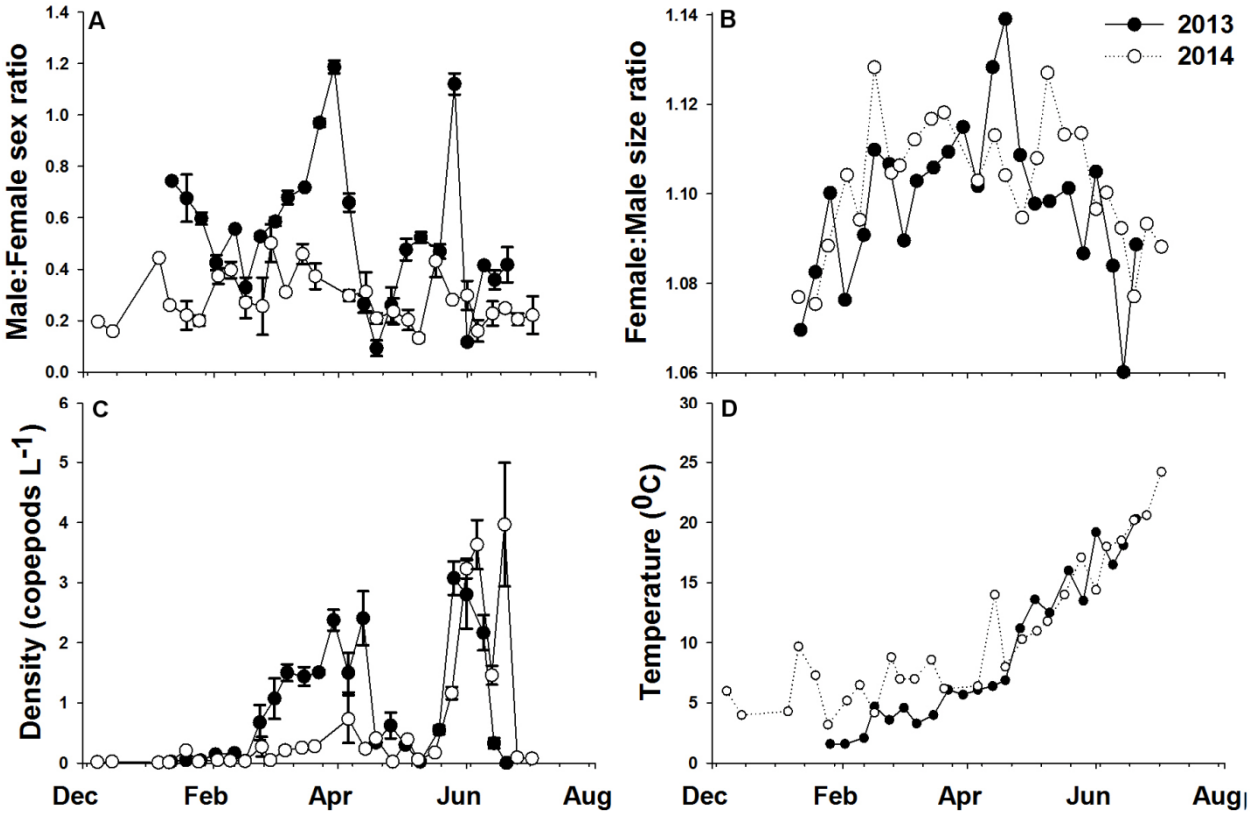

A
Male:Female sex ratio
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
B
Female:Male size ratio
1.14
1.12
1.10
1.08
1.06
2013
2014
C
Density (copepods L-1)
6
5
4
3
2
1
0
D
Temperature (0C)
30
25
20
15
10
5
0
Dec
Feb
Apr
Jun
Aug

Figure 3:

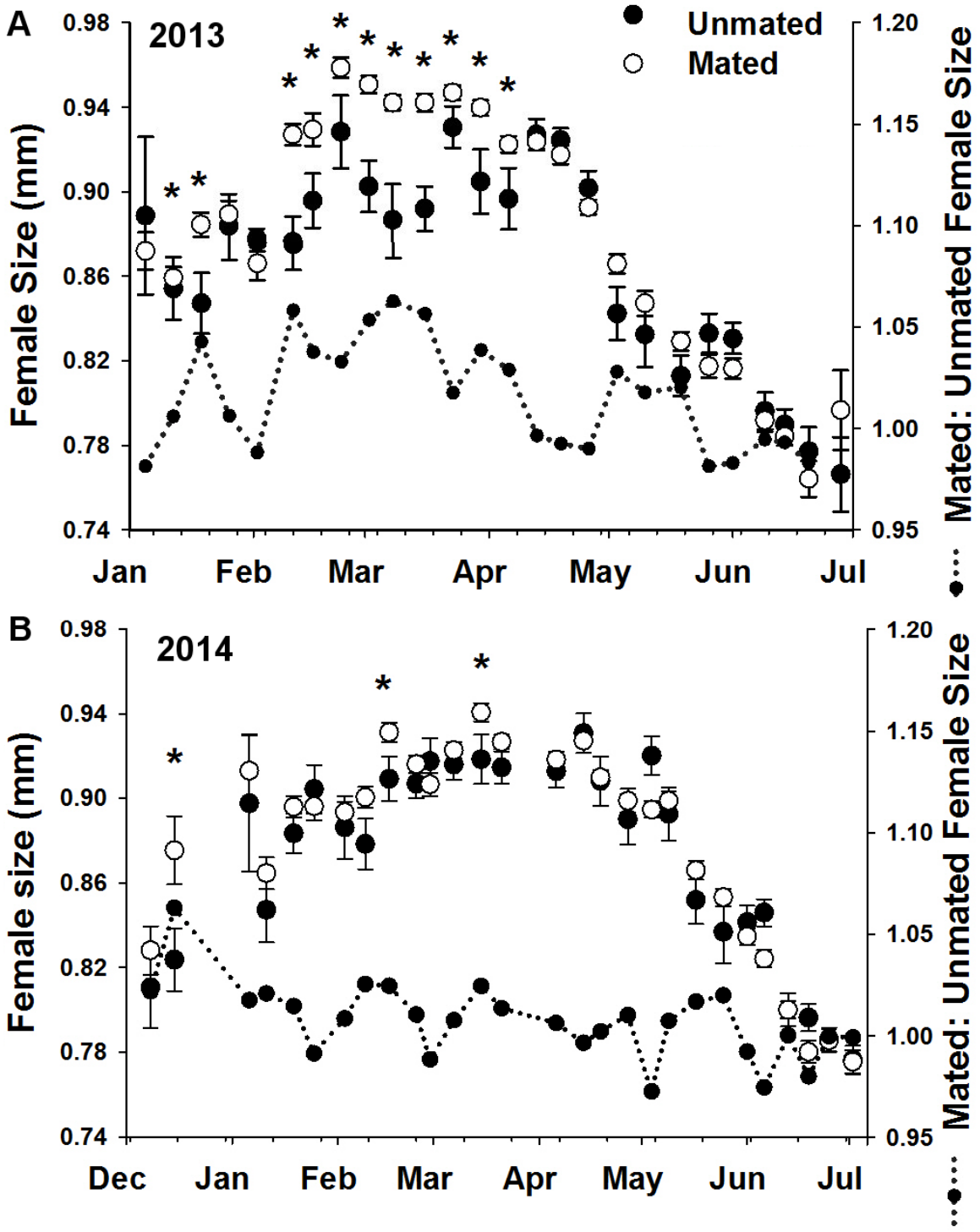

A
2013
Unmated
Mated
Female Size (mm)
Mated: Unmated Female Size
0.98
0.94
0.90
0.86
0.82
0.78
0.74
1.20
1.15
1.10
1.05
1.00
0.95
Jan
Feb
Mar
Apr
May
Jun
Jul
B
2014
Female size (mm)
Mated: Unmated Female Size
Dec
Jan
Feb
Mar
Apr
May
Jun
Jul

Figure 4:

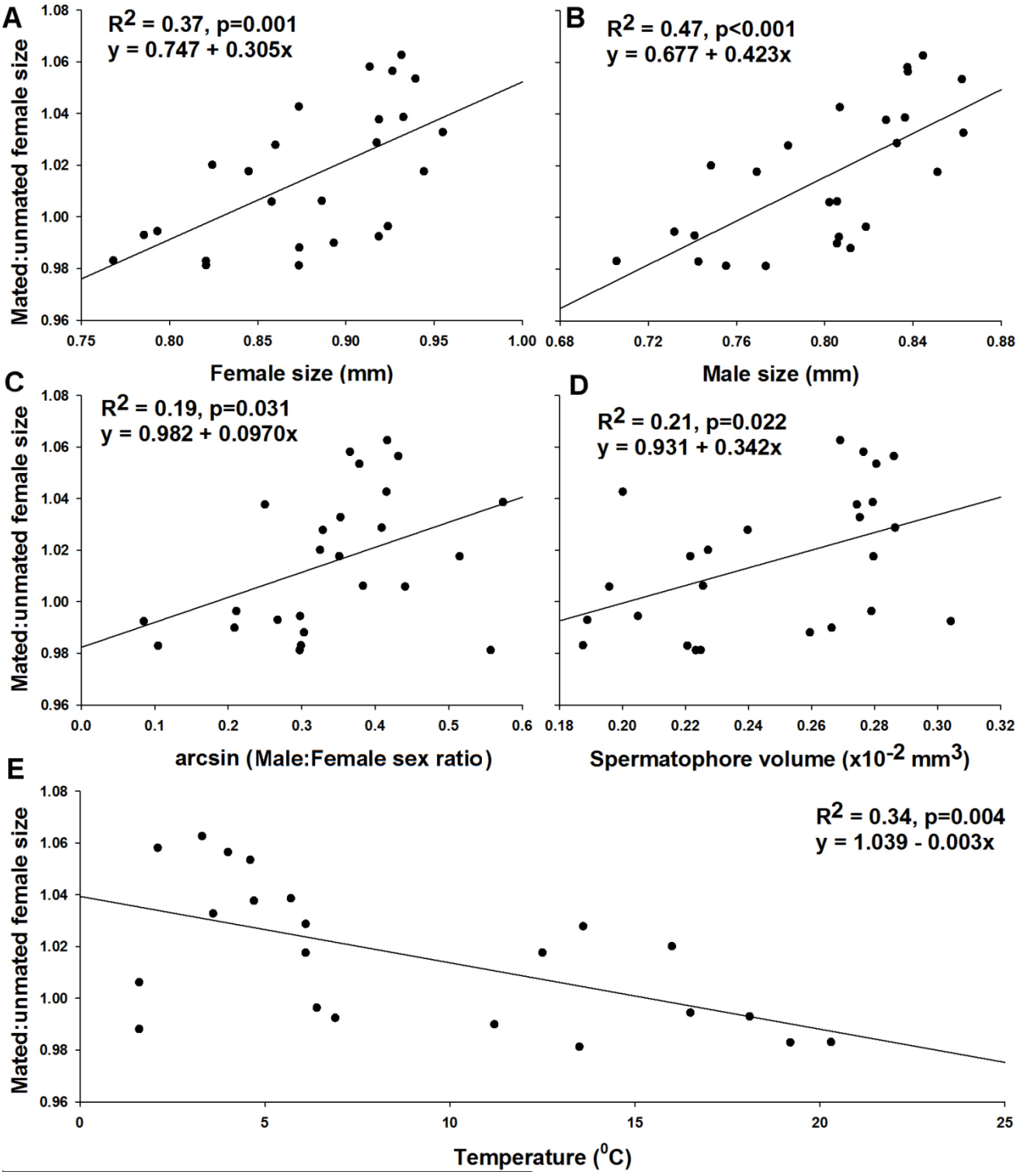

A
R^2 = 0.37, p=0.001
y = 0.747 + 0.305x
Mated:unmated female size
Female size (mm)
B
R^2 = 0.47, p<0.001
y = 0.677 + 0.423x
Male size (mm)
C
R^2 = 0.19, p=0.031
y = 0.982 + 0.0970x
arcsin (Male:Female sex ratio)
D
R^2 = 0.21, p=0.022
y = 0.931 + 0.342x
Spermatophore volume (x10^{-2} mm^3)
E
R^2 = 0.34, p=0.004
y = 1.039 - 0.003x
Temperature (^{0}C)

Figure 5:

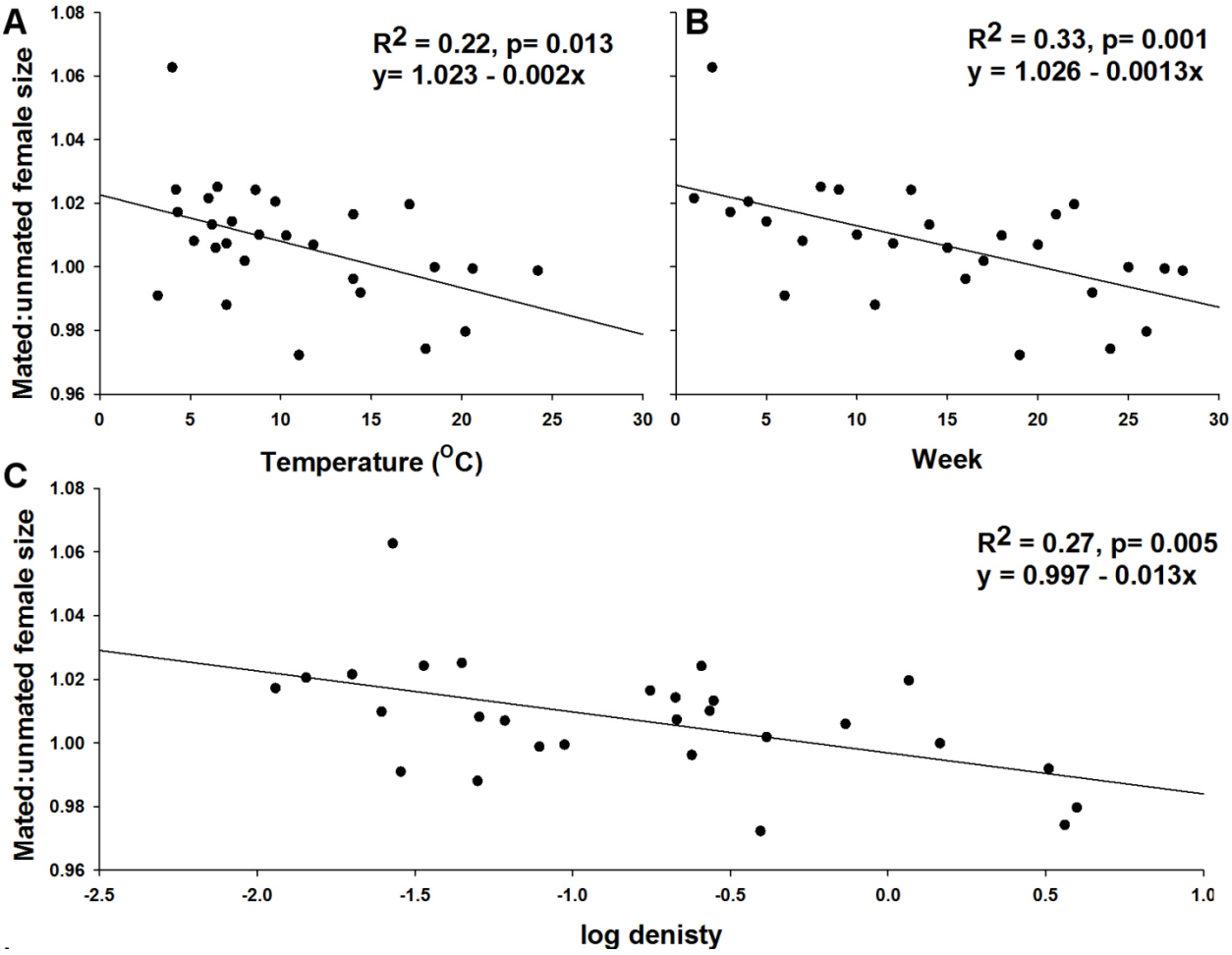

A
R^2 = 0.22, p= 0.013
y= 1.023 - 0.002x
Mated:unmated female size
Temperature (°C)
B
R^2 = 0.33, p= 0.001
y = 1.026 - 0.0013x
Week
C
R^2 = 0.27, p= 0.005
y = 0.997 - 0.013x
Mated:unmated female size
log denisty

Figure 6:

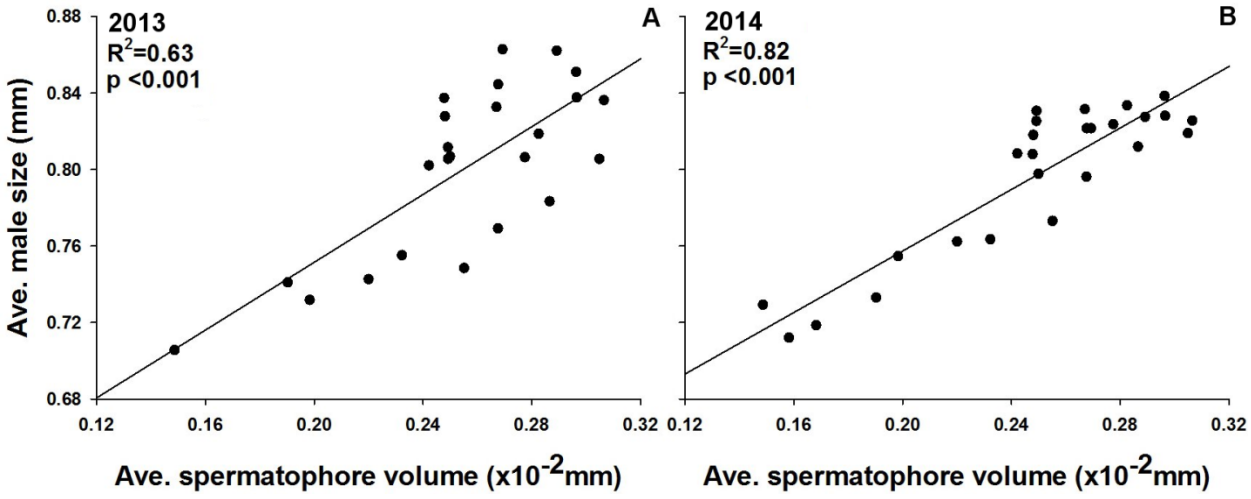

2013
R^2=0.63
p <0.001
A
2014
R^2=0.82
p <0.001
B
Ave. male size (mm)
0.88
0.84
0.80
0.76
0.72
0.68
0.12
0.16
0.20
0.24
0.28
0.32
Ave. spermatophore volume (x10^{-2}mm)
Ave. spermatophore volume (x10^{-2}mm)

Figure 7:

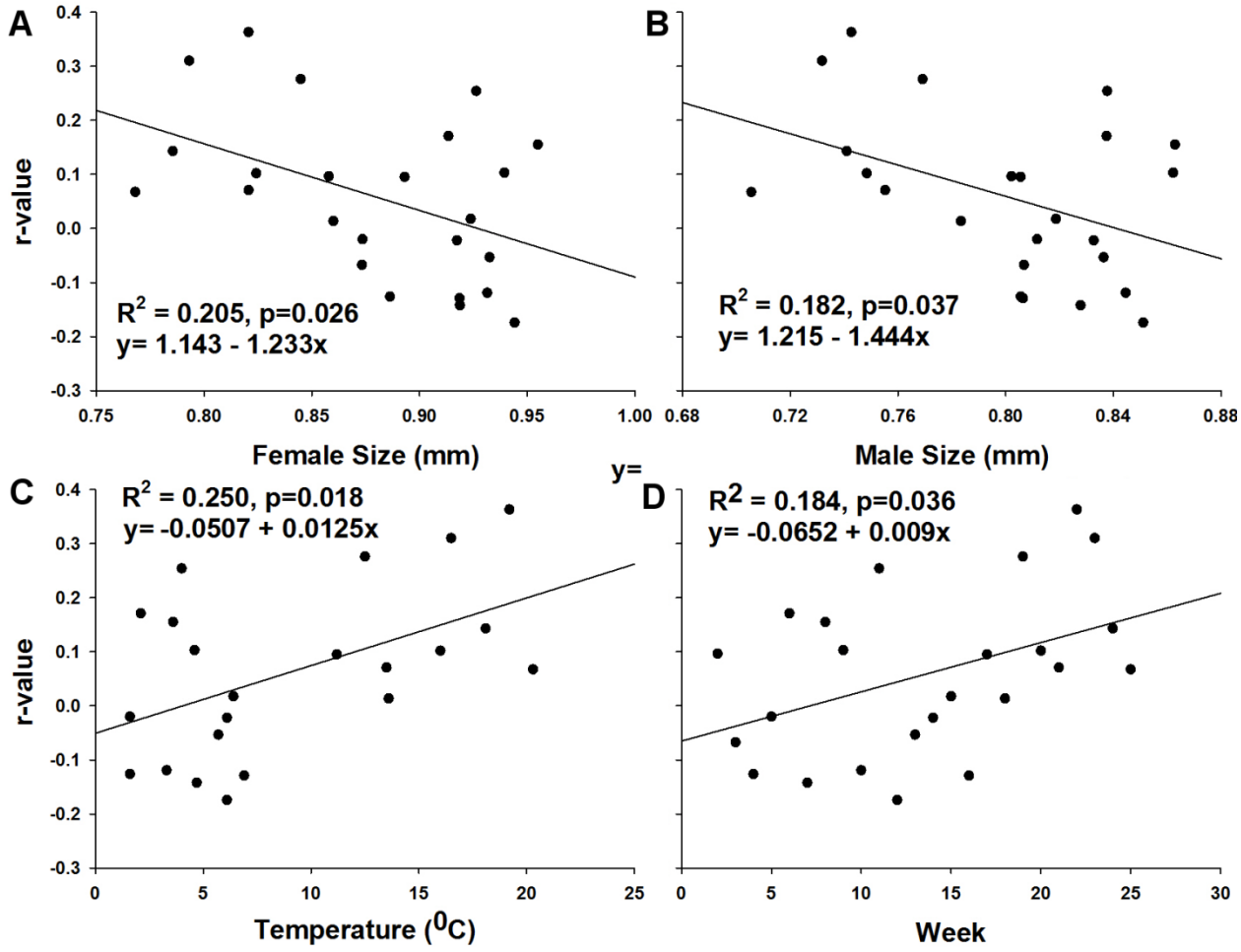
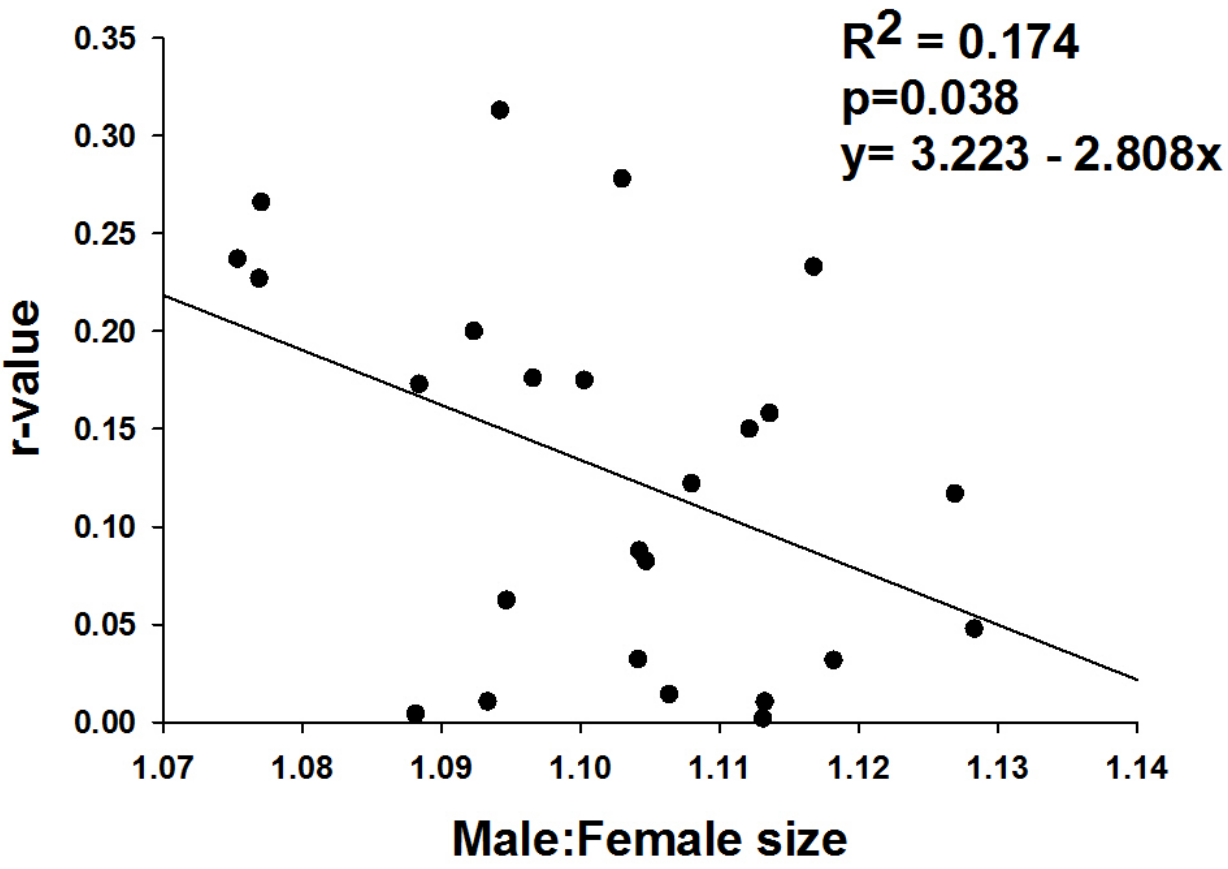

A
r-value
R2 = 0.205, p=0.026
y= 1.143 - 1.233x
Female Size (mm)
B
R2 = 0.182, p=0.037
y= 1.215 - 1.444x
Male Size (mm)
C
R2 = 0.250, p=0.018
y= -0.0507 + 0.0125x
r-value
Temperature (0C)
y=
D
R2 = 0.184, p=0.036
y= -0.0652 + 0.009x
Week

Figure 8:


R2 = 0.174
p=0.038
y= 3.223 - 2.808x
r-value
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
1.07
1.08
1.09
1.10
1.11
1.12
1.13
1.14
Male:Female size

Tables:

Table 1: Statistics of T-tests or Mann-Whitney tests for mated versus unmated female sizes for each sampling date during the 2013 season. Significant dates are in bold.

2013	Stat	p-value
1-12	**U= 1731.5**	**0.032**
1-19	**U= 1199**	**0.004**
1-26	U=734.5	0.25
2-2	**U=934.5**	**0.057**
2-11	**U=608**	**<0.001**
2-16	**T=-2.36**	**0.0105**
2-23	**U= 406.5**	**0.0385**
3-2	**U= 560**	**<0.001**
3-8	**U= 513**	**0.001**
3-16	**U= 480**	**<0.001**
3-28	**U= 1540.5**	**.007**
4-6	**U= 717**	**0.056**
4-13	0.422	0.337
4-19	0.918	0.18
4-26	1.038	0.15
5-3	U= 850	0.11
5-10	U= 567.5	0.164
5-19	U= 1179	0.177
5-26	1.337	0.092
6-1	1.61	0.11
6-9	0.452	0.326
6-14	0.37	0.355

Table 2: Statistics of T-tests or Mann-Whitney tests for mated versus unmated female sizes for each sampling date during the 2014 season. Significant dates are in bold.

2014	Stat	p-value
12-15	**-2.161**	**.024**
1-6	-0.421	0.34
1-11	-1.14	0.13
1-19	-1.186	0.115
1-25	0.624	0.27
2-3	-0.538	0.3
2-9	491.5	0.06
2-16	**614**	**0.02**
2-24	-1.07	0.14
2-28	0.939	0.18
3-7	-0.875	0.19
3-15	**427**	**0.03**
3-21	752.5	0.10
4-8	615	0.41
4-14	0.488	0.31
4-19	804	0.49
4-27	583.5	0.47
5-4	529	0.002
5-9	681.5	0.76
5-17	598	0.07
5-25	650.5	0.24
6-1	0.791	0.22
6-6	2.896	0.06
6-13	848	0.30
6-19	1.45	0.075
6-25	1080	0.354
7-2	838.5	0.456

Table 3: Regression statistics of female size by spermatophore volume for each date during the 2013 season. Significant dates are in bold.

Date	slope	se	p-value	R	n
1/12/2013	0.114	0.066	0.361	0.0964	92
1/19/2013	-0.0911	0.079	0.568	-0.0674	74
1/26/2013	-0.206	0.095	0.359	-0.126	55
2/2/2013	-0.0432	0.096	0.881	-0.0201	58
2/11/2013	0.335	0.084	0.15	0.171	72
2/16/2013	-0.242	0.105	0.33	-0.142	49
2/23/2013	0.36	0.104	0.172	0.155	79
3/2/2013	0.239	0.082	0.397	0.103	69
3/8/2013	-0.26	0.071	0.322	-0.119	71
3/16/2013	**0.698**	**0.097**	**0.052**	**0.254**	**59**
3/23/2013	-0.448	0.087	0.13	-0.174	77
3/30/2013	-0.136	0.088	0.687	-0.0535	59
4/6/2013	-0.0559	0.088	0.864	-0.022	63
4/13/2013	0.0444	0.091	0.883	0.0175	73
4/19/2013	-0.328	0.097	0.339	-0.129	57
4/26/2013	0.285	0.09	0.458	0.0952	63
5/3/2013	0.0255	0.076	0.914	0.0136	66
5/10/2013	**0.435**	**0.082**	**0.013**	**0.276**	**80**
5/19/2013	0.177	0.068	0.4	0.102	70
5/26/2013	0.102	0.067	0.588	0.0707	61
6/1/2013	**0.591**	**0.063**	**0.008**	**0.363**	**53**
6/9/2013	**0.641**	**0.081**	**0.055**	**0.31**	**39**
6/14/2013	0.189	0.047	0.275	0.143	60
6/20/2013	0.0903	0.053	0.784	0.0674	19

Table 4: Regression statistics of female size by spermatophore volume for each date during the 2014 season. Significant dates are in bold.

Date	slope	se	p-value	R	n
12/8/2013	**0.466**	**0.07**	**0.047**	**0.359**	**31**
1/11/2014	0.447	0.102	0.124	0.227	47
1/19/2014	**0.537**	**0.101**	**0.026**	**0.237**	**88**
1/25/2014	0.369	0.103	0.212	0.173	54
2/3/2014	-0.174	0.085	0.466	0.0878	71
2/9/2014	**0.665**	**0.08**	**0.019**	**0.313**	**56**
2/16/2014	1.0959	0.084	0.69	0.0479	72
2/24/2014	-0.283	0.098	0.59	0.0825	45
28-Feb	0.0294	0.087	0.924	0.0144	47
3/7/2014	0.437	0.089	0.274	0.15	55
3/15/2014	0.681	0.096	0.099	0.233	51
3/21/2014	-0.0798	0.071	0.828	0.0319	49
4/6/2014	**0.611**	**0.069**	**0.043**	**0.278**	**53**
4/14/2014	0.008	0.104	0.988	0.002	55
4/19/2014	-0.082	0.076	0.813	0.0323	56
4/27/2014	0.143	0.112	0.69	0.0625	43
5/4/2014	-0.008	0.081	0.325	0.1222	67
9-May	0.354	0.109	0.395	0.117	55
17-May	0.0335	0.106	0.948	0.0106	41
25-May	0.307	0.071	0.208	0.158	65
1-Jun	0.325	0.06	0.18	0.176	60
6-Jun	0.234	0.05	0.135	0.175	74
13-Jun	0.317	0.054	0.107	0.2	66
19-Jun	**0.279**	**0.037**	**0.045**	**0.266**	**57**
6/25/2014	-0.0137	0.053	0.943	0.0107	47
7/2/2014	0.00435	0.038	0.98	0.00435	37

Chapter 7:

Summary

In this dissertation I aimed to gather information on aspects of copepod mating biology that have been ignored previously. The main findings of my research are:

1) Many field-caught females of *Acartia tonsa* produced offspring with significantly skewed sex ratios. Since offspring were raised under standardized conditions, and because there was no evidence of sex-change as juveniles, the results suggest that in *A. tonsa*, biased sex ratios at birth may explain the common observation of skewed adult sex ratios in the field (Chapter 2).
2) The rate of spermatophore production depended on food in males of *Acartia hudsonica* but not in males of *Acartia tonsa*. In both species, males experienced significant decreases in spermatophore production with age. Most importantly, even during the peak time for production (early in the male's life) spermatophore-production rates for both species were low and the production period was short. In these two copepod species— in which adult sex ratios are often highly skewed toward females and females must re-mate multiple times to stay fertilized- availability and fertility of males can severely limit female fertilization, and hence population growth (Chapter 3).
3) In both *Acartia tonsa* and *Acartia hudsonica*, males mated more frequently with virgin females than with females that had already mated, suggesting that female-reproductive status is important for mating and mate choice. Frequencies of females carrying double spermatophores in the field were consistently low, implying that mate choice for female-reproductive status is present in natural populations as well. In *A. hudsonica*, the frequency of females carrying double spermatophores was positively related to spermatophore volume and male and female sizes. This can be explained by larger males producing more spermatophores, and thus mating more frequently than small males. No

such relationships were found for *A. tonsa*. However, the frequency of females carrying double spermatophores decreased as the season progressed. In both copepod species, there was no difference in the number of nauplii produced, length of fertilization, or lifespan between females that had mated twice compared with those that had mated only once. Hence, there was no particular cost or benefit to mating a second time (Chapter 4).

4) The strength of mate choice in laboratory experiments was not constant for either *Acartia tonsa* or *Acartia hudsonica* as ecological factors were manipulated. In addition, mate choice affected by different factors in males and females. For females of *A. tonsa*, the most important factors were food availability and previous exposure to the opposite sex, whereas mate choice in males was affected by exposure to the opposite sex and encounter time. For *A. hudsonica* females, previous social experience to the same and opposite sexes and encounter times were important for mate choice, whereas mate choice in males responded most to changes in encounter time and food availability (Chapter 5).
5) In field populations of *Acartia hudsonica* followed over two seasons, male-mate choice for body size and its strength were evident, but variable both within and between seasons. Male-mate choice for female body size was strong during the 2013 season, occurring during 50% of sampling dates, but weak in 2014, likely because most variables that influence mate choice showed little variation over this season. In addition, the results indicate that several variables (male size, sex ratio, density, etc.) can be useful in predicting the strength of mate choice in a given season. The strength of male-mate choice was strongly and positively related to male and female sizes, and negatively related to temperature (Chapter 6).

Population success depends largely on the fitness of its members; in other words, on an individual's ability to leave offspring that survive to adulthood and reproduce successfully. There are many factors that affect copepod reproduction : egg production as a function of age, food availability, food quality, and temperature have been extensively studied in copepods. However, information on the factors that may be important for mating success has been understudied in comparison. This dissertation explored the importance of some of these factors (adults sex ratios, male mating capacities, and mate preferences in both field and laboratory populations) in two coastal copepod species, *Acartia tonsa* and *Acartia hudsonica*. Information on mate choice and mating dynamics in copepods may help to explain the evolution of sexual-size dimorphism (females are generally larger than males). Stronger preferences among males for large females (mate choice) may explain the observation that sex ratios in calanoid copepods are relatively constant and female-biased (Clutton-Brock and Harvey, 1977; Maly 1984). Differences between species in morphology, behavior, and population biology may be explained by the strength of sexual selection (Trivers, 1972). Through mate choice, sexual selection may occur in copepod populations, especially since copepod mating strategies are diverse (Ceballos and Kiørboe, 2010). Finally, interest in optimizing copepod yields from cultures for use in ecotoxicological tests and as feed in larval fish aquaculture provides an economic reason for studying copepod mating dynamics. Thus, understanding how mate choice impacts mating and reproductive success as a function of culture conditions (food abundance, density and sex ratio) will help improve culturing techniques.

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