The role of sex-ratio on male reproductive investment of Calanoid copepod *Temora longicornis*

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Blindern, Oslo. Juni 2013

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Abstract

Copepods are said to be the key link between the primary producers and higher trophic levels. They inhabit all oceans and are found in almost all marine communities. In the ocean, it is common to observe skewed sex-ratios of copepods which fluctuates throughout the year. Still, how or if the sex-ratio is influencing male reproductive investment has yet to be investigated. The main objective of this study was to determine the influence of sex-ratio on the reproductive investment of the male copepod *Temora longicornis*. The study was conducted in May 2012 at Gulmarfjorden in west Sweden. *T. longicornis* was incubated for four days on a rotating wheel in three different treatments: male-skewed, female-skewed and gender balanced. The results indicated no adjustment of spermatophore production in any of the three treatments. However, a relationship between male body size and spermatophore production rates was observed. Spermatophore size and production rate were negatively correlated in the male-skewed treatment and positively correlated in female-skewed and gender balanced treatment. The conclusion is that sex-ratio has no influence on the spermatophore production. However, adjustments in reproductive investment cannot be excluded on the basis of the spermatophore production only.

1 Introduction

1.1 Sex-ratio and reproductive investment

Copepoda is one of the most abundant organism groups in the world, they are found in almost all marine environments (Riisgard and Larsen 2010). These crustaceans play a key role in marine ecosystems transferring energy from the primary producers to higher trophic levels such as fish, marine mammals and seabirds (Verity and Smetacek 1996, Hickman et al. 2008). In some systems, like the Baltic Sea, small herring and sprat are strictly zooplanktivorous in the autumn, feeding on just two species of copepods: *Temora longicornis* and *Bosmina maritima (Casini et al. 2004).*

In contrast to the land masses, the ocean have a three dimensional space. Therefore male copepods use most of their time searching for signals left by the female. These signals may be either chemical, e.g. pheromones produced by females, or hydro mechanical, e.g. synchronized hops in the water column (Bagøien and Kiørboe 2005a, b, Kiørboe et al. 2005, Kiørboe 2011b). Each species have their own specific mate-recognition system (SMRS) (Lonsdale et al. 1998). These systems involve several elements, such as biological and environmental cues, timing, signal-receptor mechanisms and genital morphology. *Temora longicornis* are so called "trail followers", females leave a discrete trail of pheromones with a distinct shape and dimensions dependent on swimming behavior (Doall et al. 1998, Goetze and Kiørboe 2008). The male use this

Figure 1. Female *Temora longicornis* with a spermatophore attached to her genital segment.

chemical trail to detect and locate the female. This indicates that mating is not necessarily a chance event. After reaching a female copepod, the male attaches a spermatophore containing spermatozoa on her genital segment (figure 1). Male fitness can therefore be set as a function of the efficiency to locate, recognition and capturing females. After successfully attaching the spermatophore, the spermatozoa empties into the genital antrum (Mauchline 1998). Some female copepods can store sperm in a storage organ called spermathecae, this makes them able to continuously fertilize eggs without the refill of new sperm from males (Mauchline 1998).

Kiørboe (2006) found that the sex-ratio of copepods communities was affected by their reproduction systems. Copepods lacking sperm storage organs, such as *T. longicornis*, tend to be closer to an equal gender based ratio. Sex-ratio in *T. longicornis*, although fluctuating, has been observed from previously experiments to be close to 1:1 (Harris and Paffenhofer 1976).

It is generally believed that males perform a trade-off between energy devoted to reproduction and growth (Stearns 1992), or predator avoidance (Kiørboe 2008). However, there are indications that the competition for females and the energetic cost of producing spermatophores have larger energetic requirements then commonly believed (Dewsbury 1982). He argues that even if the cost of spermatozoa production is low, it is the ejaculate or spermatophore that is the appropriate unit of consideration. The cost of a spermatophore is much greater than the individual spermatozoa. With an expensive spermatophore, it may be wise to time the production to the best suitable period. The sex-ratio may therefore change the reproductive behavior in different ways depending on the gender distortion. If so, it would be advantageous for males to either be selective in mate choice or differentiate its investment effort. In her review, Titelman et al. (2007) suggested that sexual selection may play a role in copepod mating behavior, and that sexual selection is density dependent with a higher mate choice under higher mate encounter rates.

Sex-ratio is said to be one of the key components in the evolution of life-histories (Milchtaich 1992). The ratio of sexually active males and females, at a given time, is known as the operational sex ratio (OSR). In a male based OSR, females can become more selective, and males tend to be more competitive. In a female based ORS the male have less competition

and can mate with more females, the females also tend to be less discriminative (Dur et al. 2012).

Adaptations to male competition do not only occur on the pre-copulatory stage, but is also seen in the post-copulatory stage when males attach themselves to the female. This is known as mate guarding, and will reduce the risk of both spermatophore displacement and make the female inaccessible for competing males (Jersabek et al. 2007). Burton (1985) also showed in a study on *Tigriopus californicus*, that males can have pre-copulatory mate guarding. The males clasped to females in younger developmental stages when faced with lower potential mates. In a high male-skewed environment, this is a strategy that can determine if one will have the opportunity to mate or not.

In the ocean, it is common to observe a skewed sex-ratio of copepods (Hirche 1991), and the sex-ratio will also fluctuate throughout the year (Dutz et al. 2012). How, or if the sex-ratio influencing the male investment in production is still unknown.

In my study, I investigated whether sex-ratio had any influence on the spermatophore production and if there are differences in the investment in female-skewed and male-skewed communities. The study setup was to incubating bottles containing six copepods in three different sex-ratios (male-skewed, female-skewed and gender balanced), with gender balanced treatments functions as the control.

I also tested spermatophore size compared to the production rate with regards to different sex-ratios treatments. This was to determine if there are different strategies between the treatments. I will also test if spermatophore production rate are affected by the size of the spermatophore.

These questions was the basis for my hypothesizes:

H1. Males in female-skewed treatments (2 males and 4 females) experience less intraspecific competition and have a higher chance of finding an unfertilized females, will invest more in the production of spermatophores.

H2. Males in male-skewed treatments (4 males and 2 females) have lower chance of finding unfertilized females and will invest less in the spermatophore production.

1.2 Biology of *Temora longicornis*

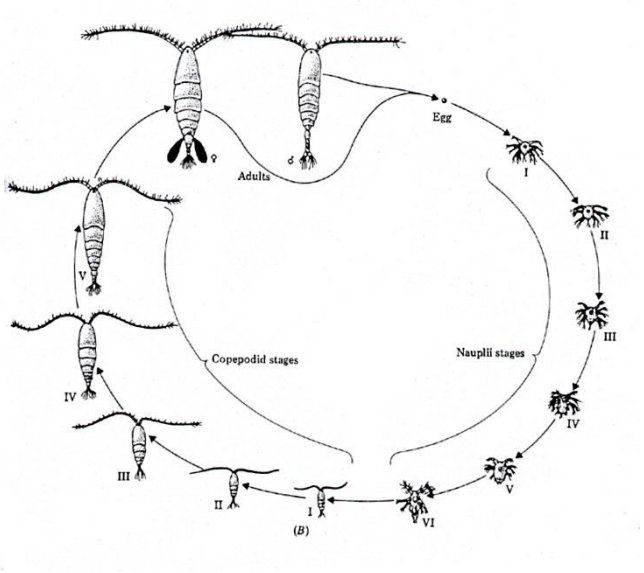

Figure 2: Generalized drawing of Calanoid copepoda life-cycle showing stages from egg to fully developed copepodid stage C6 (Nybakken 1982).

T. longicornis is a coastal species living in the North Atlantic Sea and the Polar Sea, where they inhabit the top layer of the water column (www.iobis.org). *T. longicornis* are suspension-feeders, however feeding strategies differ between sexes. Female tends to move slower and using nearly all their time on feeding, whereas males alternate between feeding and swimming at high speeds (Kiørboe 2008). Suspension-feeders make a feeding current and filter small particles like algae. This is why copepods are said to be the link between suspended phytoplankton and higher trophic levels.

Usually the copepod life-cycle is divided into three developmental stages: The nauplii stage, the copepodid stage and the adult stage. The nauplii and the copepodid stages are further divided into six and five stages (N1- N6 and C1 – C5), when including the adult stage (C6), it gives a total of 12 stages (figure 2). As male *T. longicornis* reach their adult stage, they develop into mature males and will be fertile for just a short amount of time. The males have around eight days to search and mate as many times as possible (Sichlau and Kiørboe 2011). When autumn arrive the female copepods will lay high densities of benthic resting egg (Naess 1996). The eggs will hatch in the spring.

Temora longicornis tracks and follows females by searching for pheromone trails that can last up to 21.4 seconds and be 61.6 mm long (Goetze and Kiørboe 2008). When the males are close, 0.3 to 2.4 mm from the female, he lunges to capture her. The sexual dimorphism of *T. longicornis* involves several characteristics, all which are adapted for transfer and placement of the spermatophore on the captured female (Mauchline 1998). In a study done on the close cousin of *T.longicornis*, *Temora stylifera*, Corni et al. (2001) found that male differs from the

female by having a hinge on the right antennules, copulatory appendages evolved from thoracic legs and five distinct somites on the urosome.

Since the *T. longicornis* lacks a storage organ for storing spermatozoa and fertilize the eggs (Mauchline 1998), it must mate several times (Sichlau and Kiørboe 2011). This makes *T. longicornis* ideal for mating experiments. Unlike some copepod species that carry the fertilized eggs attached to the genital somite until hatching into nauplii, *T. longicornis* are broadcast spawners and releases the fertilized egg freely in sea (Drif et al. 2010). Broadcast spawners have little investment after releasing the eggs, and it is believed that broadcast spawning have evolved in Calanoid copepods as an adaption to having a pelagic existence in contrary to Harpacticoid and Cyclopoid copepods who carry their egg in egg sacks throughout their development (Webb and Weaver 1988).

2 Material and Methods

2.1 Experiment and general procedure

This thesis is part of a collaboration study investigating the male reproductive investment of *Temora longicornis*. The experiment consists of three different aspects effecting investment, the effect of risk (Bjærke, unpublished), food accessibility (Bækkedal, unpublished) and sex-ratio (this study). The collaboration ended after the experiment was finished.

2.1.1 Sampling and study site

Both the sampling and the experimental studies were executed at the University of Gothenburg field station, Sven Lovén Center for Marine Sciences Kristineberg in Fiskebäckskil. Kristineberg field station is located in the east parts of Gullmarsfjord (the largest and only true fjord in Sweden) on the western coast of Sweden (figure 3). Sampling was continuously done every day from 04/05 to 26/05 2012. The two sampling locations (figure 3) are both coastal water but differ in depth (sampling location 1 = 50 meters and sampling location 2 = 20 meters). Not only is the location ideal for zooplankton studies, the field station was also equipped with a wet-laboratory and climate control room for executing experiments. The experiments were carried out with water pumped up from 32 meter depth just outsider the field station. In the period when the experiments were carried out, the temperature and salinity on 32 meters depth was 8.4 ± 1 °C and 32.3 ± 0.9 PSU, and surface water was 14.5 ± 4.5 °C and 19.6 ± 2.1 PSU (http://www.weather.loven.gu.se/en/ date between 2012-05-01 and 2012-05-31). The chryptophyte *Rhodomonas salina* was used as food in both culture and experiments. It was grown in aerated batch cultures at the facility. Every day some of the *R. salina* cultures were replaced with a B1 medium and vitamins solution. All animals in both cultures and experiment setups were provided food in excess.

The amount of excess food was 15000 cells per mL. To calculate this, a sample was taken from the *R. salina* culture and counted using the particle counter Elzone 180 XY. The number of cells mL^{-1} was then extrapolated using the formula $C_2 * V_2 = C_1 * V_1$, where C is the concentration and V is the volume.

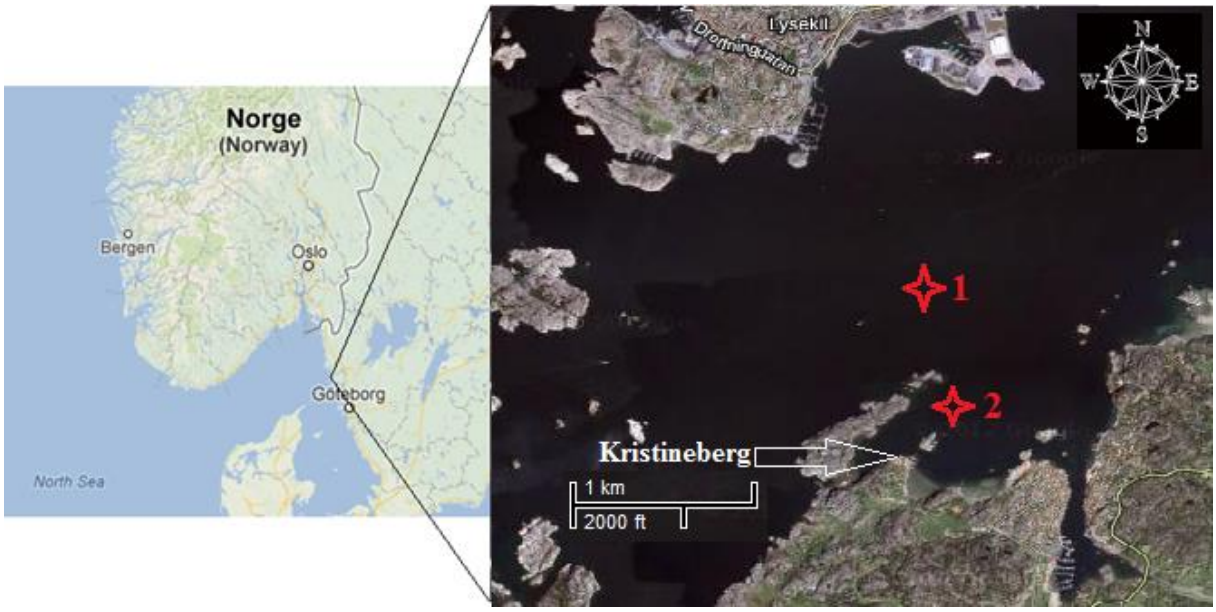

Figure 3: Study area and sampling stations. Kristineberg = University of Gothenburg, Sven Lovén Center for Marine Sciences, Kristineberg, N 58° 15.132‘, E 11° 27.096‘. 1 = Sampling done by RV Oscar von Sydow, 2 = sampling done by row boat (Map made from Google Maps).

All animals were obtained using WP2 plankton net (200µm) (figure 3). The RV Oscar von Sydow, a 12 meter long research vessel was used every third or fourth day. On a daily basis samples were collected using a small rowing boat. Animals were collected every day in between 09:00 and 12:00. All animals were sampled in the top 20 – 30 meters of the water column. The animals were placed in thermo containers on the boats for safe transport to the laboratory.

2.1.2 Sorting and culture

Samples were placed in an aerated 50 L container to give the animals more space. *T. longicornis* were sorted and verified under a light microscope. The males in the copepodid stage C4 and C5 was individually placed in wells on a 6-microwell plate and left to molt into

the adult C6 stage. This was also done to insure that only virgin males were used in the experiment. The sorting was done using a Leica light microscope. Since younger stages of copepods are harder to gender-determine, they were kept in a beaker until their gender could be determined. The culture was kept in an aerated 50 L container and was used as an additional supply of animals if needed.

2.1.3 Incubation

After molting into C6 virgins, the animals were collected and placed in 250 mL turn-cap-bottles free of bubbles on a rotating wheel for four days, the total volume of the bottles when filled to the top is 320 mL. Three different treatments were chosen to test for sex-ratio effect (table 1). Male-skewed having a male to female ratio of 2:1, female-skewed having a male to female ratio of 1:2 and a gender balanced treatment that also functioned as a control. The three different treatments were randomly spread out over the whole experimenting period. The total number of individuals was 270 (134 males and 137 females), this was divided in 45 bottles (gender balanced (n = 14), female-skewed (n = 16) and male-skewed (n = 15)).

Table 1: Table showing the three different treatments and the numbers of males and females in each treatment.

Sex\Treatment	**Control**	**Male-skewed**	**Female-skewed**
Males	3	4	2
Females	3	2	4

In the incubation period *T. longicornis* was kept in a climate control room at a temperature of 15 degrees Celsius, this have earlier been shown to be the optimal temperature (Maps et al. 2005, Record et al. 2012). The incubation had a light-dark cycle of 12 hour light and 12 hour dark. Replicates were randomly placed on a rotating wheel which rotated at 1/7 rotation per minute (0.15 rpm). This is to prevent the algae from falling to the bottom and simulate the sea. The water was changed after two days on all treatments to prevent anoxia, monitor the condition of the animals and to keep the concentration of food stable at 15000 cells per mL. If necessary, weak or dead females were replaced with new ones. The water from two days of

incubation was thoroughly inspected by emptying the content through a 30 µm sieve and examined under a light microscope. All spermatophores, eggs and nauplii were counted. Copepods produce ~1 spermatophore per day (*Acartia tonsa ~1 per day* (Ceballos and Kiørboe 2010), *Centropages typicus* <1 per day (Miralto et al. 1995) and *Temora stylifera 0.7 per day (Ianora and Poulet 1993))*. To insure the incubated animals experienced the effect of the treatment, the incubation was set to a period of four days.

2.1.4 Counting and preserving

All spermatophores, eggs and nauplii were counted, and as many spermatophores as possible were placed on cryo tubes and shock-frozen in nitrogen gas containers (Dewar container). The containers were later transported back to the University in Oslo. Counting was done by emptying bottle through a 30 µm sieve. Each bottle was rinsed two times to insure that everything was accounted for. The substance was then released from the sieve into a checkered petri dish and counted under a light microscope. A sharpened glass pipette was used to collect the counted spermatophores. Both males and females used in the experiments were placed in eppendorf tubes and preserved with Lugol. Shrinking of prosome length by preserving on Lugol is not accounted for in the measurements (Jaspers and Carstensen 2009). Preserved animals were brought back to the University in Oslo where they were photographed and measured.

2.1.5 Pictures and length measurements

All measurements were done in the laboratory in the University in Oslo. Measurements were conducted by photographing the *T. longicornis* while lying in an angle that shows the full length of the prosome (figure 4a). The picture was taken using a Canon EOS 7D camera with a MP-E 65mm 1:2.8 Canon macro photo lens. The camera was mounted on a camera stand before the pictures was taken. The photographed animals were then measured in the free multi-platform image-analysis software Image J (Schneider et al. 2012). It uses the pixel in the image and a known distance provided by measuring a picture of a calibration slide to calculate the length of the animal. The frozen cryo tubes containing spermatophores was

defrosted and the cryo tubes was rinsed several times. This was done by carefully spraying water into the cryo tubes using a micropipette containing 200 µL of water. The length of the spermatophores (figure 4b) was measured with a Leica DMLS microscope.

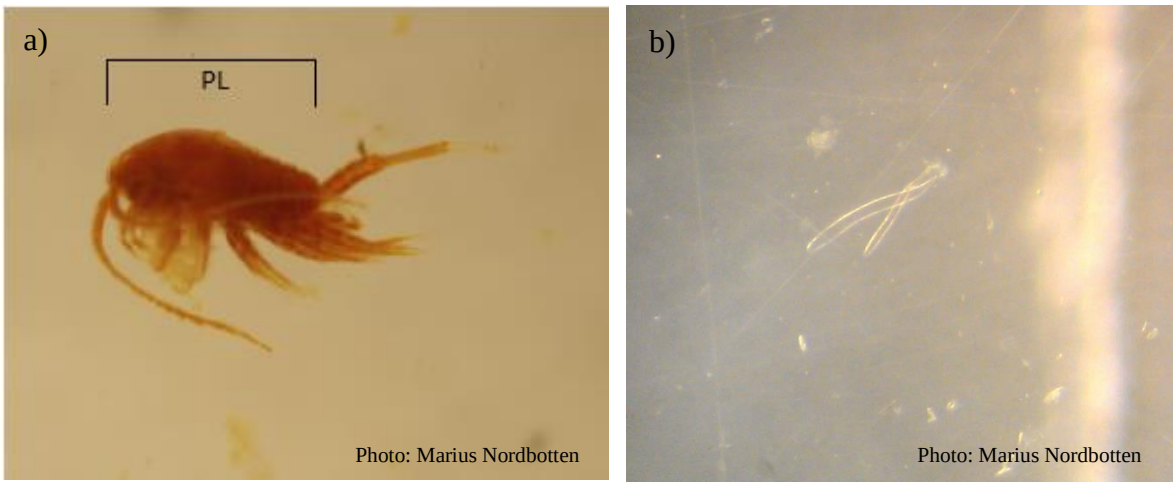

Figure 3a, b: a) Picture of adult male *Temora longicornis* prepared on Lugol. PL = prosome length. b) Spermatophores.

2.2 Statistical analysis

Statistical analyses were performed with the R language and statistical programming environment version 2.11.1 (www.R-project.org). A generalized linear model (GLM) with a quasipoisson distribution to predict the results in the data set. Poisson regression is common to use when dealing with counted numbers. Poisson models can underestimate the variance, when using quasipoisson instead of poisson it allows for overdispersion in the data set and gives prediction from the model.

2.2.1 Spermatophore and egg production

To test the sex-ratio on spermatophore and egg production in the GLM models, the males or females are set as an offset. The offset adjusts for the different number of males or females producing the observed spermatophore or egg count in the different treatments such that the model prediction will be log (spermatophores / male or egg / female).

To test the effect of male size on the production rate a scatter plot showing production rates and the prosome length was made. The prediction line used to estimate production per size is taken from the GLM model.

2.2.2 Spermatophore size vs. production rate

To test for difference in the spermatophore size with the production rate regarding the sex-ratio, males are divided in to treatments, male-skewed treatment and a treatment combining gender balanced and female-skewed. A scatter plot displays the 26 individuals and their number of produced spermatophore and its size. A linear model predicts the regression slopes of the two treatments.

3 Results

3.1 Effect of skewed sex-ratio

The three different gender-skewed treatments showed no clear difference in production rate (tables 3 and 4). Male prosome length (PL) had a high influence on the spermatophore production rate (table 3). The same was seen in the egg production, where larger females produced significantly more than the small ones (table 4). Treatments showed a high variation, however both spermatophore and egg production had a higher, but non-significant mean production in the gender-skewed treatments than the gander balanced treatment (table 2). Spermatophore size from male-skewed treatments was positively correlated with production rate, while males form the combined gender balanced and female-skewed treatment showed a negative correlation between spermatophore size and production rate (figure 8).

Table 2: Table of descriptive statistics. Number of replicates, sex-ratio, spermatophore production and egg production per four days (mean ± SD). Each replicate is one 320 mL bottle containing six copepods, making a total of 270 individuals. The gender balanced treatment (1:1) functioned as control.

Replicates	**Sex-ratio (Male : Female)**	**Spermatophore male^{-1} mean ± SD**	**Egg female^{-1} mean ± SD**
14	1:1	3.19±2.0	19.71±12.9
16	1:2	3.94±2.9	26.56±16.7
15	2:1	3.72±1.6	30.43±19.1

3.1.1 Spermatophore production

The results after four days incubation showed that sex-ratio has no significant influence on the production (table 3). The highest observed mean spermatophore production was in the female-skewed treatment, while the lowest was in the gender balanced treatment. Males in the gender balanced treatment had a 19 % lower production the female-skewed treatment and 14% less the male-skewed treatment (table 2). The largest variance in production was in the female-skewed treatment, which varied from 0 - 10 spermatophores per male per four days (figure 5). The results of spermatophore production predicted from the generalized linear model (GLM) showed that the effect of sex-ratio was not significant (t_{45} = 0.106, DF = 43, p = 0.92 [GLM, quasipoisson distribution]). Size of the male, represented by its prosome length (PL) had a clear effect on production rate (t_{45} = 2.727, DF = 43, p = 0.01 [GLM, quasipoisson distribution]).

Table 3: Estimates, standard error and the significant value from the generalized linear model. Log (females) is the influence of sex-ratio on the spermatophore production rate and log (PL males) is the influence of the male prosome length (PL) on production rate.

	Estimate	**Std. Error**	**value Pr (>|t|)**
log (females)	0.03	0.29	0.92
log(PL males)	3.96	1.45	0.01

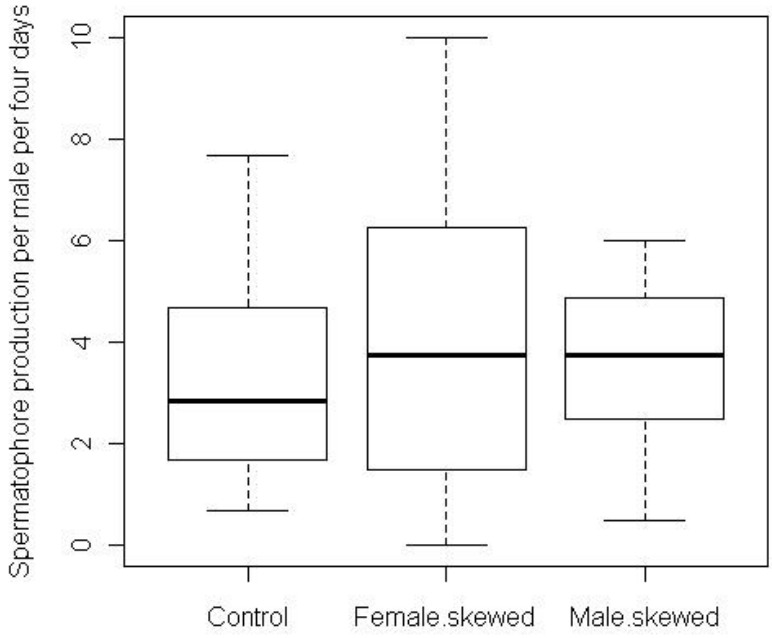

Figure 5: Boxplot showing production of spermatophores after four days. Spermatophore production per male per four days in individual replicates (y-axis), and the treatments (x-axis): Control is gender balanced, female-skewed has a male to female ratio of 1:2 and male-skewed has a male to female ratio of 2:1.

3.1.2 Effect of prosome length on production rate

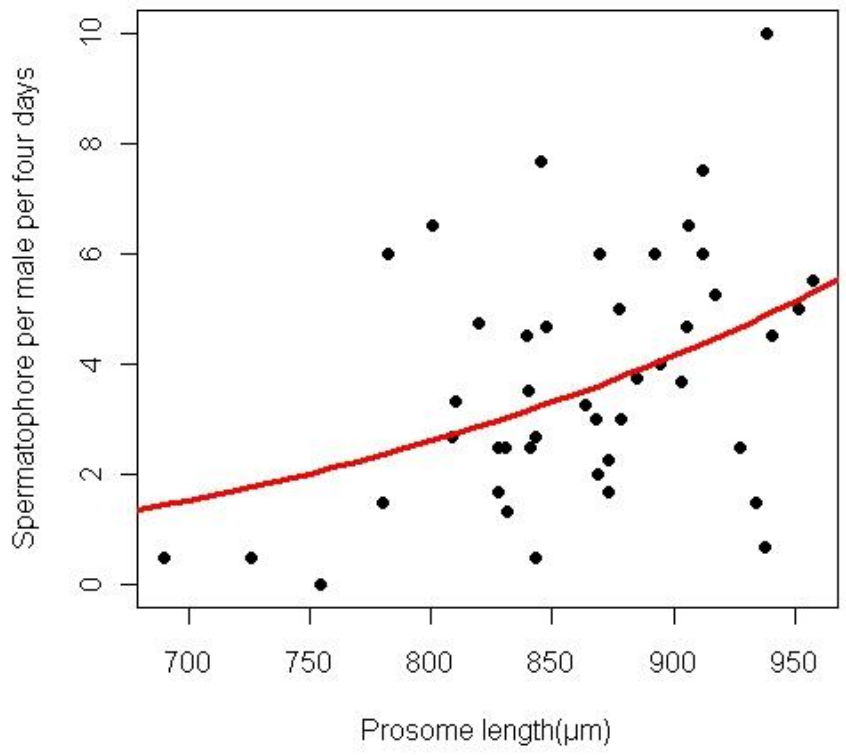

Figure 6: Scatter plot showing spermatophore production after four days versus prosome length in µm (n = 45). Points representing individual production and the best fit predicted production rate (red line) calculated from the GLM model (table 3).

The spermatophore production was strongly influenced by the size of the male *Temora longicornis* (t_{45} = 2.727, DF = 43, p = 0.01 [GLM, quasipoisson distribution]). Figure 6 shows the prediction line estimated from the GLM model (table 3). It predicts production rate increases as the prosome length increases. By using the formula (Log (spermatophore) = -25.54 + 3.96 Log (PL) (table 3, GLM model)), the calculated increase is: 10% increase in prosome length gives a 46% increase in spermatophore production ($1.1^{3.96} = 1.46$).

3.1.3 Egg production

The highest egg production was seen in the male-skewed treatment, and the lowest in gender balanced treatment (table 2), the gender balanced treatment produced 35.2% less than the male-skewed and 25.8% less than female-skewed. The GLM modeling showed that sex-ratio had no significant effect on egg production (t_{45} = -0.070, DF = 42, p = 0.94 [GLM, quasipoisson distribution]). The prosome length (PL) of the females had a significant influence on egg production (t_{45} = 3.97, DF = 42, p = 0.00 [GLM, quasipoisson distribution]), such that a 10% increase in prosome length gives a 60% increase in egg production ($1.1^{4.93}$ = 1.60).

Table 4: Estimates, standard error and the significant value of the generalized linear model. Log (females) is the influence by sex-ratio on the production rate and log (PL females) is the influence of the female size on production rate.

	Estimate	**Std. Error**	**value Pr (>|t|)**
log(males)	-0.02	0.29	0.94
log(PL females)	4.93	1.24	0.00

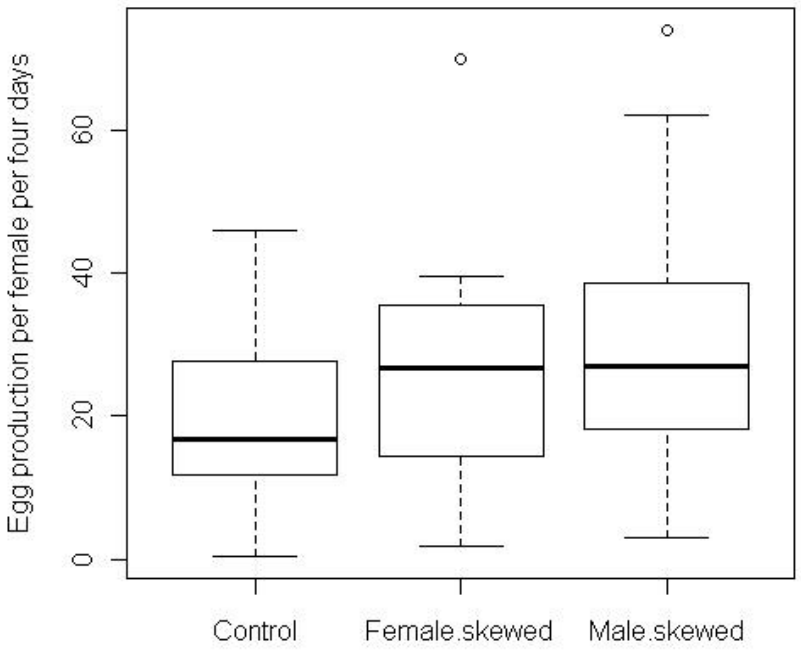

Figure 7: Boxplot of egg production rates after four days. Eggs production per female per four days incubation (y-axis) and the treatments (x-axis): Control is gender balanced, female-skewed has a male to female ratio of 1:2 and male-skewed has a male to female ratio of 2:1.

3.2 Analysis of spermatophore size with production rate

The qualitative production analysis, showed that spermatophore size and spermatophore production is negatively correlated with males from male-skewed treatments (figure 8, red line), and positively correlated with males form either gender balanced or female-skewed treatment (figure 8, black line). Males from the male-skewed treatment produced smaller spermatophores as the production increased. While the opposite was shown in males from gender balanced or female-skewed treatments, here males produce larger spermatophores as the production increases. The different correlation cannot be explained by the production of spermatophore per male (t_{26} = 0.799, DF = 22, p = 0.43 [LM]). The male-skewed treatment and the combined female-skewed and gender balanced treatments were significantly different

in terms of spermatophore size and production rate (t_{26} = 2.157, DF = 22, p = 0.04 [LM]). The different correlation was not explained by combining production per male per four days whit the treatment interaction (t_{26} = -0.939, DF = 22, p = 0.08 [LM]), therefore the model can be simplified by removing the interaction section.

Table 5: Table showing estimates, standard error and the significance value from the linear model explaining correlation between spermatophore size and production rate. Spermatophore production per male per four days (n = 26). Different treatments being if males are in: Male-skewed treatment(n = 15) or males from the combined female-skewed and gender balanced treatment (n = 11). Spermatophore production per male per four days with the different treatment interaction.

	Estimate	**Std. Error**	**Pr(>|t|)**
Spermatophore production male^{-1} four days^{-1}	0.25	0.32	0.43
The different treatments	5.31	2.46	0.04
Spermatophore production male^{-1} four days^{-1} with the treatment interaction	-0.94	0.51	0.08

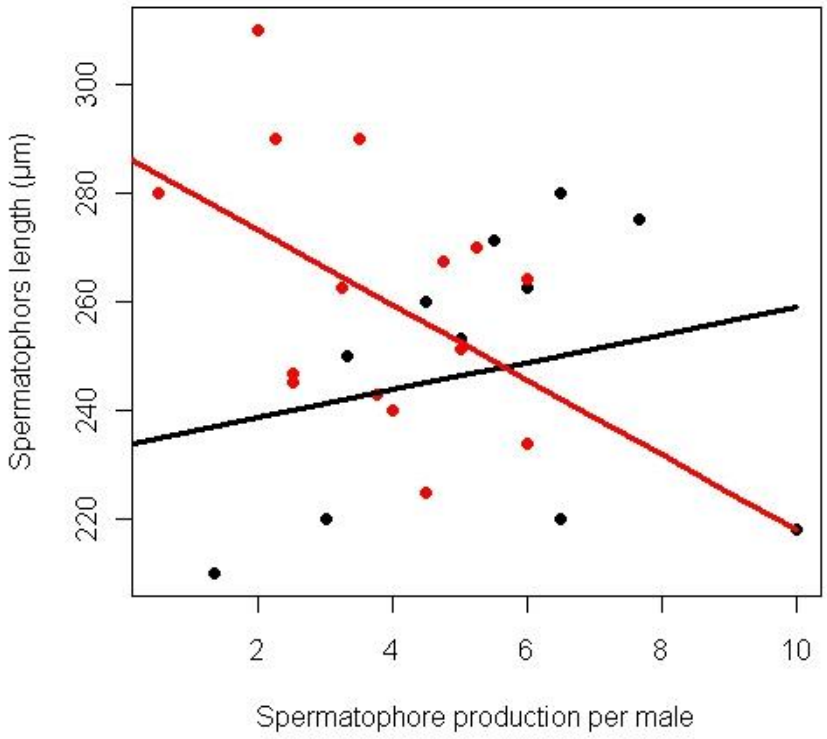

Figure 8: Scatter plot of spermatophore production rate as a function of spermatophore length (µm). Red dots and line reprisenting spermatophore production in male-skewed treatment (n = 15), black dots and line repriset the spermatophore production of the combined two other treatments: gender balanced and female-skewed (n = 11).

4 Discussion

The natural fluctuations in sex-ratio of the calanoid copepod *Temora longicornis* throughout the year have earlier been documented (Dutz et al. 2012). Which implications this can have with regards to the reproduction investment is still unknown. The goal for this thesis was to investigate how the sex-ratio influenced reproduction investment in male *T. longicornis*. My results are in consensus with Ceballos et al. (2013). Stating that sex-ratio have no influence on spermatophore production.

Studies on copepod reproduction have traditionally focused on the female production of eggs (Halsband and Hirche 2001, Jonasdottir et al. 2009, Drif et al. 2010, Dutz et al. 2012). It is just recently that researcher have started focusing on the production made by male copepods (Dur et al. 2012, Ceballos et al. 2013). Even though there is no clear indication that spermatophore production differ with the sex-ratio (tables 3, 4), both gender-skewed treatments exhibited higher mean production rate than the gender balanced treatment (table 2).

In the male-skewed treatment, spermatophore size is negative correlated with the production rate and shows a decrease in size as the production rate increased (figure 8, red line). The opposite was seen for males in female-skewed and gender balanced treatment where spermatophore size and production rate were positively correlated (figure 8, black line).

4.1 Male reproductive investment with regards to skewed sex-ratio

In the light of the results of this study, where males have no difference in their investment of reproduction, I have rejected my first two hypotheses H1 and H2. The first hypotheses (H1) was how male investment when incubated in a female-skewed environment. Here I predicted an increase in spermatophore production as a result of the high chance finding unfertilized females. Hypotheses 2 (H2) predicted that male incubated in a male-skewed treatment have

lower chance of meeting an unfertilized female and will therefore invest less in production of spermatophores. None of the predictions were not observed.

Sperm limitation, in terms of energy requirements, is for most animal groups not a problem. This is because of their small size and apparent low cost of production (Charnov et al. 1981, Dewsbury 1982), but this is an assumption which lack overall consideration. Male copepods must invest time and energy on mate searching, risk of predation and time spent on eating (vanDuren and Videler 1996, Kiørboe 2011a). Therefore, the energy put into reproduction has to be timed carefully.

In my hypotheses I assume that there is a cost to producing spermatophores, therefore there is a paradox why males do not adjust their production according to when it is beneficial to reproduce. With the chance of finding an unfertilized female being affected by the sex-ratio, there should be observed a difference of number of produced spermatophore in the three treatments. With the lack such observation, the invested energy can be allocated to other areas such as an adjustment of the search behavior.

In a study done on *Oithona davisae*, Heuschele and Kiørboe (2012) observed changes in search behavior from males incubated with virgin females. These males swam significantly faster, which can benefit both males and females by lowering search time and thereby the risk of predation. As there was no observed adjustment in spermatophore production in any of the treatments, the same search behavior may also have been observed in this study. Changes in behavior like this is more important for species such as *Oithona davisae* as they can store sperm and females only have to mate once (Ceballos and Kiørboe 2011). *Temora longicornis* lack this ability and must therefore mate multiple times (Barthelemy et al. 1998). Males in female-skewed treatments with a high chance of finding unfertilized females should therefore invest more on search time and mate whenever possible.

If the change in investment in terms of skewed sex-ratio were to be explained by an adjustment in search behavior, one of the limiting factors in the study could be the encounter rate. If sex-ratio is based on how many times males meet females, males need to have enough encounters in order to make investment productive. By using the model for encounter rates for *Temora longicornis* (Doall et al. 1998, Kiørboe and Bagøien 2005), and transferring it to each treatment, it predicts an estimate of how many times each males encountered females.

$$E = \beta C_M C_F$$

Encounter rate (E) is estimated from search volume rate (β) and the concentration of males (C_M) and females (C_F). β are dependent on the species method of tracking females.

In the case of *T. longicornis*, females release a pheromone trail which males follow (Doall et al. 1998). β are explained by being an estimate of male search volume rate per liter (L) per day (Kiørboe and Bagøien 2005). The estimated search volume rate for *T. longicornis* is 117 L per day (Doall et al. 1998), and the volume used in the experiments is 320 mL.

Table 6: Table showing male to female encounters per male per day.

	Encounters
Gender balanced (Control)	112.32
Female-skewed	149.76
Male-skewed	74.88

In table 6 the encounter rate is estimated to be the encounter rate for each individual male per day. It shows that males have more than enough encounters with females in all treatments, thus encounter rate is probably not an inhibiting factor for spermatophore production.

The concept of males competing for females is well established (Begon et al. 2006). But how male copepods experience the operational sex ratio (OSR) and the presence of other males are uncertain. If males have the ability to determine sex ratio, it would be reasonable to assume that their behavior would adapt to fit the best strategy. Studies on skewed sex-ratio behaviour done on insects using spermatophore as a reproduction instrument showed that males under the in male-skewed environments undergo changes in reproductive investment (Gao and Kang 2006). They found that in Chinese bushchricket, males in a male-skewed environment invest in fresh ampulla weight and sperm number, which in a high competing circumstance will increase fertilization potential and ejaculate.

In the study, there were observed a high variance of produced spermatophores per male in all treatments, especially in the female-skewed treatment (figure 5). One possible reason for the observed high variance could be the low number of males (two males and four females). High individual variation in spermatophore production has been seen in precious studies on *Acartia tonsa* (Ceballos and Kiørboe 2010) and *Temora stylifera (Ianora and Poulet 1993).* The average production for *A. tonsa* and *T. stylifera* was one and 0.7 spermatophore per male per day, but the individual variation ranged between 0 - 4 for *A. tonsa* and 0 - 2.3 *T. stylifera*.

The results from this study showed similar variation, the production rate of males in female-skewed treatments varied from 0 – 2.5 spermatophore per male per day (figure 5). High individual variance can indicate that there are some males that function as the dominant donors to the gene pool and many others that contribute very little.

One explanation for the lack of observed adjustment in spermatophors production according to sex-ratio is that producing spermatophore is low in cost. Or that excess food has dilutes the effect of adjusting production. If there is no cost in producing a spermatophore, but rather a production limitation in terms of the time it takes to produce one. One might think that they would produce a spermatophore just in case they find a female. This might explain the observed production of ~1 each day conclusive in several studies (Ianora and Poulet 1993, Miralto et al. 1995, Ceballos and Kiørboe 2010), including this.

In a study on pacific white shrimp (*Litopenaeus vannamei),* sperm quality was higher in regenerated spermatophores (Ceballos-Vazquez et al. 2004), Alfaro and Lozano (1993) also concluded when studying *L. vannamei*, that spermatophores which are not transferred to a female or manually ejaculated will eventually degenerates of natural processes. This can indicate that males produce a new spermatophore instead of saving the already produced spermatophore if food is unlimited, as it was in this study. This would explain the low number of observed females with attached spermatophores (females found carrying spermatophores had a high number of attached spermatophores, figure 9a) and the high number of spermatophores found lying on the bottom of the bottle (own observations). However females are known to remove discharged spermatophores (Ohtsuka and Huys 2001). There were also found males carrying spermatophore (figure 9b). There is no profit in placing a spermatophore on a male, except the disability it would be for the competing male they have.

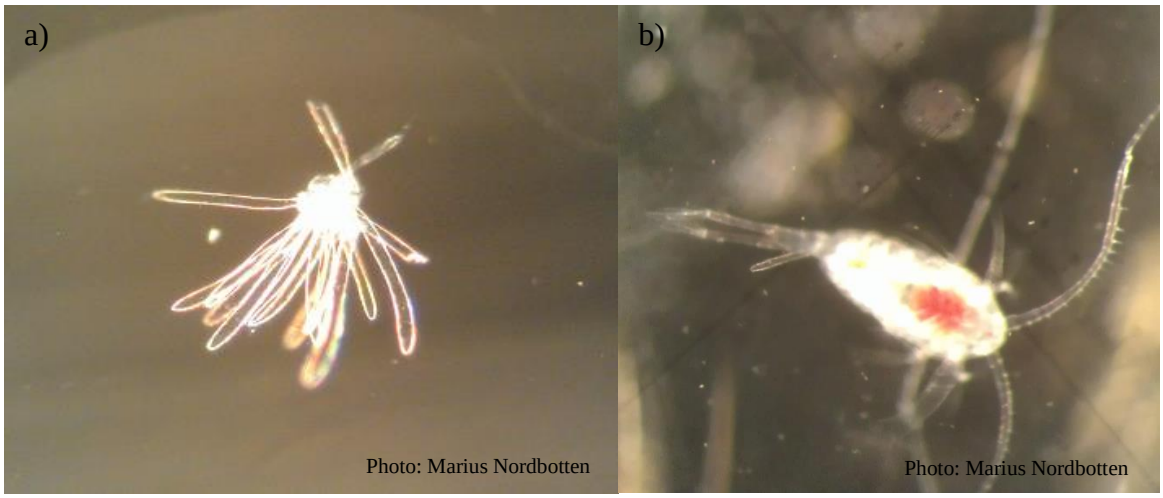

Figure 9a. and b: a) Photo of spermatophore in a cluster. b) Spermatophore attached to a male *Temora longicornis.*

The prosome length of males is known to affect the spermatophore size in copepods (Ceballos and Kiørboe 2010, Sichlau and Kiørboe 2011). In this study I observed that males not only produce larger spermatophores but also have a higher production rate. With larger males both producing larger spermatophores and also have a higher production rate, one could expect that the selection would drive copepods to becoming larger. However, larger copepods are more likely to be eaten by visual predators (Fortier et al. 2001).

4.2 Egg production in a gender-skewed environment

Female production rate has earlier been proven to be linked with the size of females (Sichlau and Kiørboe 2011), the same was seen in this study. The production rate with regards to the different treatments was not significant, showing investment is unaffected by sex-ratio. Spermatophores contains sperm cells and seminal liquid, and the sperm cell lack flagellum (Bozzo et al. 1998). The number of sperm cells in a spermatophore is more or less constant and independent of the size of the spermatophore (Sichlau and Kiørboe 2011). It is still uncertain what the seminal liquid consist of. It has been speculated that it can be a nuptial gift for the female. In a study on spermatophores size in bushcrickets, Wedell (1993) found that males invest in mating effort, this being the nuptial gift, rather than parental effort.

4.3 Spermatophore size and production rate

In the study I also tested the spermatophore size as a function of production (figure 8). The results showed that males in a male-skewed treatment have a negative correlation between size and production, and males in a female-skewed or a gender equal treatment showed a positive correlation.

The number of sperm cell in a spermatophore from *Temora longicornis* is on average 1000 – 1300 sperm cells per spermatophore (Sichlau and Kiørboe 2011). This is five times more than the average egg production in one single mating event. Males that produce many small spermatophores have the opportunity to fertilize more females in a short amount of time. This may be an adaption to a high male-skewed environment. Sexual selection has been pointed out as an influencing factor to the reproductive behavior (Titelman et al. 2007, Ceballos and Kiørboe 2010, Ceballos and Kiørboe 2011). In a male-skewed environment, females have the opportunity to choose from multiple mating partners. Sichlau and Kiørboe (2011) saw that females, if given the opportunity, prefer larger males (large males produce large spermatophores). Selective females may benefit from the males that investing in larger spermatophores. However, results showed a negative correlation between spermatophore size and production rate. This could be interpreted as some male trying their luck with several females by making many small spermatophores, or investing in a few larger spermatophores.

5 Concluding remarks

The results from this study show that sex-ratio is no influence on the spermatophore production from males. As a result, both hypothesis (H1 and H2) was rejected. If sex-ratio has any influence on reproduction investment, this investment is allocated elsewhere then spermatophore production. Spermatophores size are known to be positively correlated with prosome length, in this study I observed that larger males also have a higher spermatophore production rate then smaller males. The high variance in production rate shown in the study was explained as being a result of high individual differences. This can indicate that some alpha males dominating the fertilization of females.

In conclusion, spermatophore production seems not to be effected be the sex-ratio in *Temora longicornis*. However, sex-ratio is not ruled out as to influence the reproduction investment. Further studies on male reproductive investment are needed to fully understand these remarkable animals

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