Roskilde University

How density affects the ovigerous rate of the cyclopoid copepod Apocyclops royi

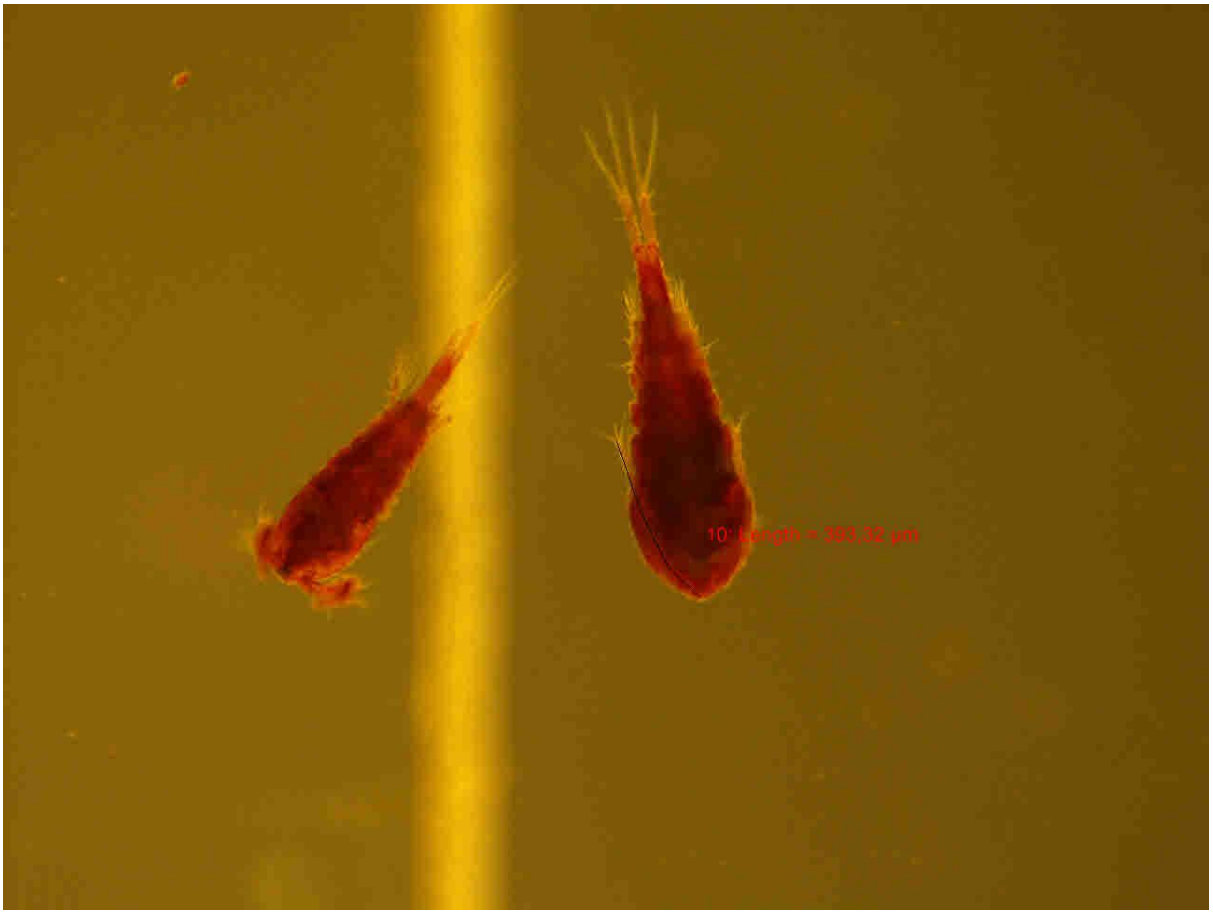

Figure 1. The picture shows two Apocyclops royi fixed in 1% Lugol. male on the left and female on the right, with length measurement of the female antenna; Antenna length = 393,32µm (Photo taken through a microscope, by the group, 2018).

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1.0 Abstract

The purpose of this study is whether there is a relation between density and the amount of ovigerous females in *Apocyclops royi*. The effect of the density was tested by two experiments of four days length; the first experiment analyzing densities between 50-1600 ind./L and the second experiment densities between 22-3000 ind./L, with focus on the difference between lower and higher ranges and what causes this difference. The varying densities were the only factor during the experiments.

The relation between ingestion rate and density was evaluated in relation to the ovigerous rate, and the results suggest a clear connection between these three factors.

The results of the study show that the ovigerous rate decreases in relation to an increasing density. The ovigerous rate is highest (17-36%) in the low density ranges (22-130 ind./L), dropping to an average ovigerous rate of 13% between 160-3000 ind./L.

The results from the study of ingestion rate in relation to density show that the ingestion rate is highest (120.000-400.000 cells/copepod/day) in the lowest densities (22, 70 and 130 ind./L). The ingestion rate decreases to a range of 8.100-76.000 cells/copepod/day in the higher densities of 160 ind./L and above.

With the conditions provided under the experiments, the optimal density range is between 22-130 ind./L for a stable ovigerous rate, which is around 20%.

Summing up all the results gathered, the study identifies that there is a relation between density and ovigerous rate in the cyclopoid copepod species *A. royi* in which the ovigerous rate is decreasing with increasing densities, as a result of increased competition.

2.0 Table of contents

3.0 Introduction

As the focus on and potential of aquaculture in recent years have received higher and higher demands, a response has been an increase in the interest and research on copepods as live feed. This is mainly due to the ever-increasing demand of food, especially as a source of protein supply for human consumption (FAO, 2016). Because of the larger amount of research on the topic, the properties the copepods possess as an efficient nutrient source have been documented further. This has especially caught the interest of fish hatcheries and the fish industry in general (Rasdi and Qin, 2016).

Currently, the main source of food for fish larvae is rotifers and *Artemia,* as they need live food to stimulate their feeding and support their dietary needs (Wu et al., 2011; Mangor-Jensen and Harboe, 1998). Rotifers and *Artemia* have a history of being used as live feed, due to their easiness of being produced at an industrial scale. They both have a high reproduction and are very easy to cultivate, which makes them ideal for quantitative production. Especially *Artemia*, as their life cycle contains very resistant and easily harvestable egg-cysts. This makes them easy to transport and removes the requirement of on-site cultivation (Gilbert, 2010). The drawback from their easy cultivation is on the other hand that both rotifers and *Artemia* are relatively poor in nutrients, especially the essential omega-3 fatty acids, that plays a key-role in proper development and pigmentation in many cultivated marine fish species. This is a main cause for low survivability among fish larvae, since they are more susceptible to diseases and other hazards when they do not get to grow properly (Rasdi and Qin, 2016). Malformed and misshapen fish are also not considered eligible on the fish market for human consumption and are treated as a waste product, which leads to a loss in commercial value. In an attempt of overcoming this problem, enrichments-methods have been developed to reach a sufficient amount of essential fatty-acids in rotifers and *Artemia*, but no method has solved the problem completely (Shields et al., 1999).

Since copepods have been proven to have a more sufficient amount of nutrients and essential fatty acids, a great effort is now being put into making them a reliable source of live-food for marine hatcheries (Næss et al., 1995). Despite this advantage, many of the natural strengths of rotifers and *Artemia* are the weaknesses of the copepods.

The reproduction rate of copepods are on average lower than rotifers and they are much more vulnerable to multiple factors when growing. This leads to a comparably slower reproduction rate and low survival rate for most copepods (Liao et al., 2001; Gilbert, 2010). Another disadvantage is that their rest stages and egg-cysts are not as adapted to draughts. This means that they have a much shorter durability when absent of water, making transport, storage and distribution of the copepods one of their main limitations (van der Meeren et al., 2014). In an attempt to solve these limitations of copepod as a stable live-food source, more in-depth research on a variety of copepod species is being done, in an attempt to get better insight in their properties and find a species that is optimal for future mass production (Blanda et al., 2017).

One of these species is the cyclopoid copepod *Apocyclops royi* Lindberg 1940, the subject of this report. The species is found in tropic and subtropic brackish waters, which also includes the estuaries around Taiwan (Blanda et al., 2015).

It is here the interest in the species has its roots, as scientists noticed that *A. royi* had some significant properties, compared to other local species. Even in studies where the interaction with *A. royi* have been very little, they have been recognized as a species with great potential for mass production and further research (Liao et al., 2001; Blanda et al., 2015).

A. royi has multiple times been acknowledged for their rapid reproduction and short-spanned life cycle, as well as their robustness, as they have a very high natural survival rate and can live under hostile circumstances (Lee et al., 2013). On top of this, further research on their feeding behavior suggests that they possess the ability to synthesize fatty acids themselves, meaning that even with food of poor quality, they will have a high amount of fatty acids (Pan et al., 2018).

Despite all these properties that makes *A. royi* such a promising species for cultivation, very little is still known about the species, since the scientific world have not done much in-depth work on the species so far. Therefore this study aims to establish further knowledge about the species *A. royi*, in this case their interaction between density and ovigerous rate.

4.0 Problem formulation

What is the relation between density as a stress factor and the presence of ovigerous female copepods, thus production of the copepod species *Apocyclops royi*.

Research questions

1. Does density have a direct influence on the ovigerous rate?
2. How significant is the grazing (ingestion and clearance) change that arises as a result of increased or decreased densities?
3. How do different initial densities affect population growth?

5.0 Theory

The introduction to the theory covers the basics of copepods, describing the fundamental information and taxonomy of the crustacean subclass Copepoda.
This study deals with the species Apocyclops royi, a member of the order Cyclopoida.
The order will be described in terms of basic morphology, reproduction, growth, development, behaviour, and feeding style.
These topics are all relevant to the study, since the traits are shared with the A. royi, thus being important for the experimental procedures. Furthermore, specific traits for the species A. royi will be briefly described.
The abiotic and biotic factors affecting A. royi will be defined, since it is critical for the experimental setup, the treatments in the experiment and for the processing and discussion of the results of the experiment.
The phytoplankton Rhodomonas salina will be briefly described, as it is used as feed during the experiment.

5.1 Copepods

5.1.1 Introduction

The name copepod derives from the Greek words *kope* (oar) and *podos* (foot), and it was described for the first time by Milne-Edwards in 1840 (Harris, 2008).

Copepods belong to the subphylum Crustacea (phylum Arthropoda) which are arthropods with an outer skeleton made of chitin, and consist of mostly aquatic species (Harris, 2008).

Most copepods are very small, but can vary in sizes from 0,2 mm to 30 cm(Harris, 2008).

In figure 2 the shape and length of a copepod of the species *Apocyclops royi*, can be seen.

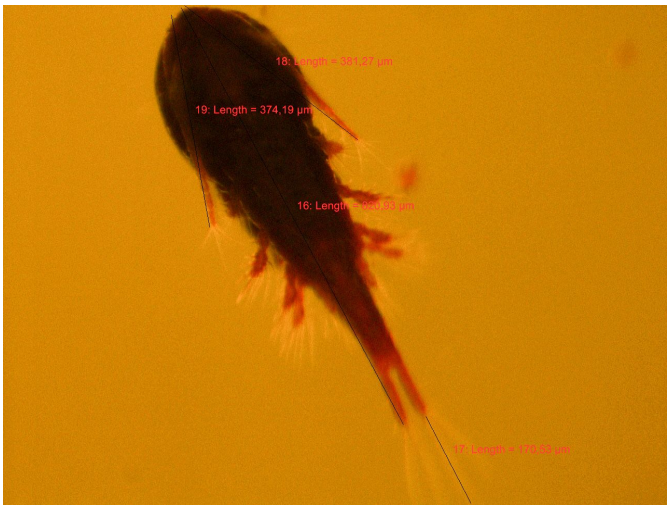

Figure 2. The picture shows an Apocyclops royi copepodite fixed in 1% Lugol with multiple measurements; Left antenna = 374,19µm, right antenna = 381,27µm, main body = 920,93µm, tail = 170,63µm (photo taken through a microscope, by the group, 2018).

Copepods can be found in all aquatic habitats, but are mainly found in marine habitats. They can be pelagic, commensal or parasitic. Free-living copepods have no gills, so oxygen is taken up by diffusion of oxygen saturated in the water. Because of this, most species have no heart or circulatory system (Harris, 2008). Copepods are purely heterotrophic and have an important role in ecosystems, especially in the marine systems. They play a role as a link in the food chain between the primary producers (phytoplankton) and the secondary consumers. Here they function as primary consumers, by controlling the phytoplankton production top down and as the principal food source of larval and juvenile fishes, whales and many invertebrate species (Harris, 2008).

5.1.2 Taxonomy

Current estimates on how many copepod species there are revolve around 10.000 species, divided into 200 families and 1650 genera. Despite this it is very likely there are even more, as they make up the most numerous animals (metazoans and multicellular) on Earth (Harris, 2008; Mauchline, 1998).

Copepoda consists of 10 taxonomic orders, out of which 9 have marine representatives.

The orders are as follows: Calanoida, Cyclopoida, Gelyelloida, Harpacticoida, Misophrioida, Monstrilloida, Mormonilloida, Platycopioida, Canuelloida *ordo. nov*., Siphonostomatoida (Khodami, et al., 2017).

The most relevant orders are the Calanoida, Cyclopoida and Harpacticoida, as they are the only orders including free-living copepods. The species examined in this study, the *Apocyclops royi,* belongs to the order Cyclopoida and family Cyclopidae.

5.2 Order Cyclopoida

The order Cyclopoida was described for the first time by Burmeister in 1834 in *"Beiträge zur Naturgeschichte der Rankenfüsser"* (Mauchline, 1998).

The name Cyclopoid derives from cyclops, since copepods of this order have only a single eye, sensible to light (Marten et al., 2007).

Cyclopoida includes copepods that live in seawater and freshwater and they can be pelagic, parasitic or commensal. There are 700 known species of freshwater cyclopoids in the world (Mauchline, 1998; Marten et al., 2007).

5.2.1 Growth, development and reproduction

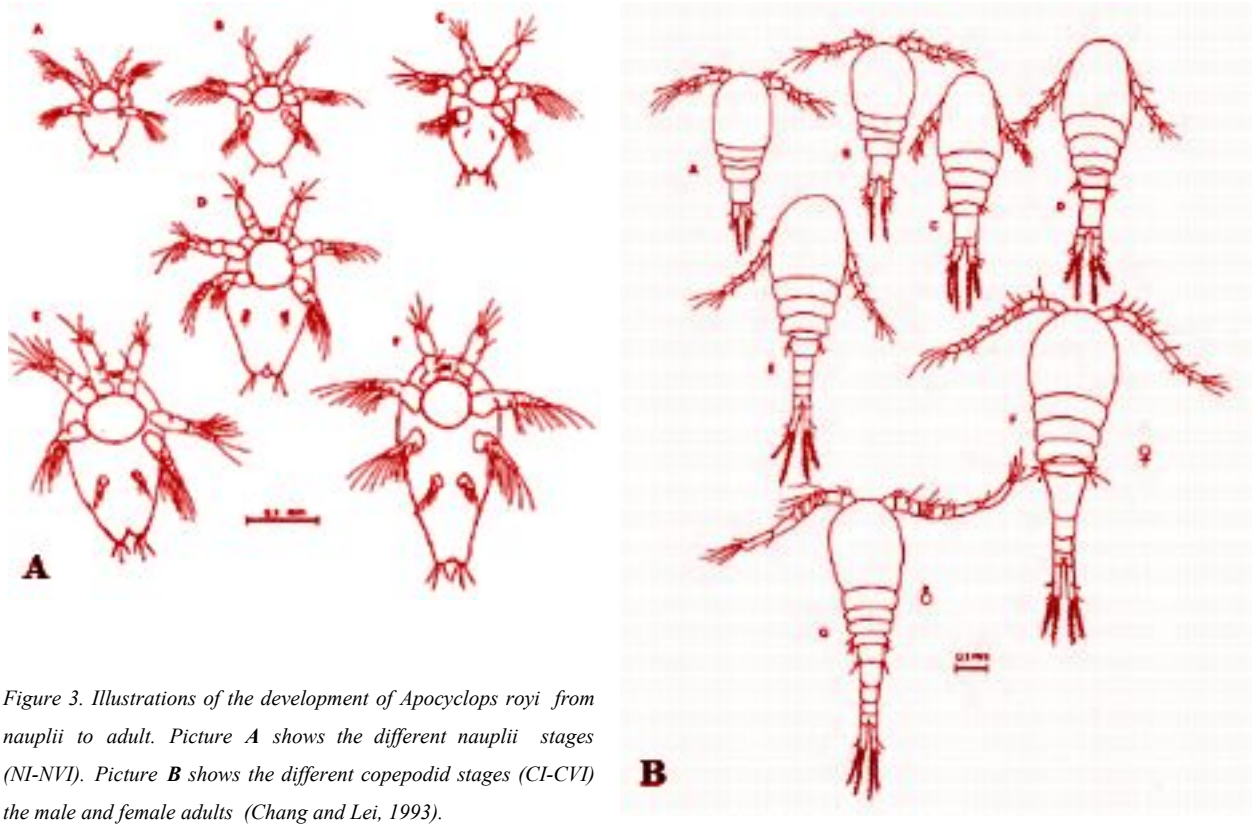

*Figure 3. Illustrations of the development of Apocyclops royi from nauplii to adult. Picture **A** shows the different nauplii stages (NI-NVI). Picture **B** shows the different copepodid stages (CI-CVI) the male and female adults (Chang and Lei, 1993).*

The copepods life cycle consists of several different stages, as illustrated in figure 3. Every stage consists of a molting change, since the outer shell is rigid and can not expand with the body. The first 6 stages are the naupliar stages (NI-NVI). The first stage, NI, is spawned from the egg, and goes through five molts until reaching the NVI stage.

Stages 7-12 are the copepodite stages (CI-CVI) where the copepods develop adult morphological characteristics including the sexual characteristics (Harris, 2008).

The cyclopoid copepod's development from egg to adult copepod can last for anywhere between a couple of days to a few weeks. Cyclopoid copepods reproduce sexually. The male matures before the female. As soon as the male is mature, the males use their antennules to hold on to the female while they transfer a package of sperm to the female by attaching the sperm package to the female (Harris, 2008). The female stores the spermatic liquid to fertilize new clusters of eggs every 3-6 days for the rest of its life (Marten et al., 2007).

Some species can produce 1-2 eggs during each fertility, others, like the *A. royi,* can produce a countless number of eggs which are kept in the egg sacs until the nauplii are hatched (Mauchline 1998).

Cyclopoids carry their eggs in a single or double egg sac. *A. royi* carry their eggs in a double sac. The eggs are usually spherical and range in size from 0,2 mm to 0,6 mm. The development of copepod eggs depends on temperature and the species, as egg-sac carrying species take a longer time to fully develop eggs than the free spawners (Harris, 2008).

The order Cyclopoida has a metamorphic larval development and the embryos are attached to the first abdominal stomite, in the egg sacs (Lowry, 1999).

Some cyclopoids have the ability to go into diapause by slowing down their metabolism. This interrupts their natural development, and allows them to survive periods of unfavorable conditions, such as periods with drought. This is often observed in freshwater cyclopoids (Marten et al., 2007).

5.2.2 Dispersion in water

An interesting behavioral characteristic of the planktonic copepods is the vertical migration. During the day they stay in the depths, and during the night they migrate up towards the surface. Light is the main environmental component controlling vertical migration. The migration into depth during the day helps copepods reduce the mortality from predators. The migration to the surface during the night is associated with feeding. Vertical migration involves mobile swimming by using their appendages. Nauplii use for example the antennules, the antennae, and the mandibles for this purpose (Harris, 2008).

5.2.3 Feeding

Cyclopoids can be herbivorous, carnivorous or omnivorous (Adrian, 1993).

The herbivorous specimens can be filter feeders, using their antennas for filtering, or raptorious in which they use their appendages to catch prey.

Figure 4 illustrates an example of a copepod with a raptorious feeding style.

Carnivorous copepods have spines on the mouth parts, and also use the antennules to detect prey. The copepods usually grab the prey with 3 pairs of grabbing mouth parts, and bites the prey with the mandibles (Marten et al., 2007).

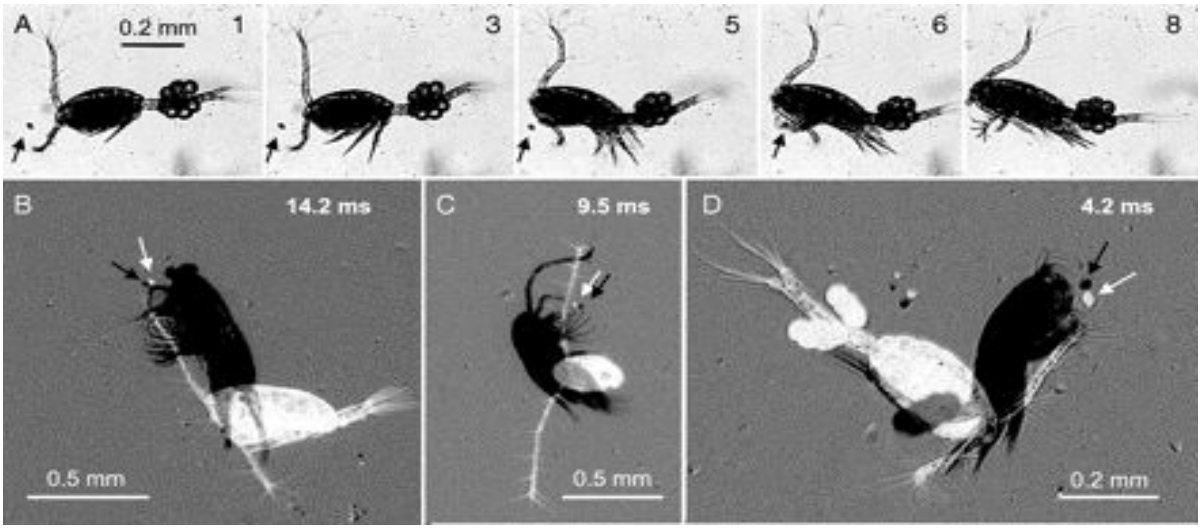

Figure 4. The pictures show A. tonsa (B+C), O. davisae (A+D).In (B,C,D) capturing a prey, in an example of raptorious feeding. Both are not cyclopoid copepods but the raptorious feeding style shown above are representative of a general raptorious feeding style (Kiørboe, et al., 2009).

Most of the Cyclopoids are omnivorous, which means that they consume phytoplankton and zooplankton. Recent studies reveal that carnivorous feeding is not always required for completing post embryonic stages and reproduction (Makino and Syhuei, 2000).

Some cyclopoid nauplii and early copepodite stages are herbivorous, but in the later copepodite stages can become predatory (Makino and Syhuei, 2000).

Species of Cyclopoida that are of a larger size are inclined to be more carnivorous than the smaller species. The body size of the female is related to the carnivorous diet for survival and reproduction of some species, however this condition is not present in all Cyclopoids (Makino and Syhuei, 2000; Marten et al., 2007).

5.3 *Apocyclops royi*

Table 1. The taxonomy of Apocyclops royi (Chad and Walter, 2018).

Taxonomy of *Apocyclops royi*	
Kingdom	Animalia
Phylum	Arthropoda
Subphylum	Crustacea
Class	Hexanauplia
Subclass	Copepoda
Order	Cyclopoida
Family	Cyclopidae
Genus	Apocyclops
Species	*Apocyclops royi*

Apocyclops royi is found in estuaries and brackish ponds in tropical and subtropical regions (Su et al., 2007). *A. royi* varies in length between 110-265 μm for nauplii and 425-1034 μm for copepodites, where female copepods are larger than males (Arnofsky, 1996; Su et al. 2007). Figure 5 illustrates the different measurements of an *A. royi* copepodite, nauplii, ovigerous female and adult of undetermined gender.

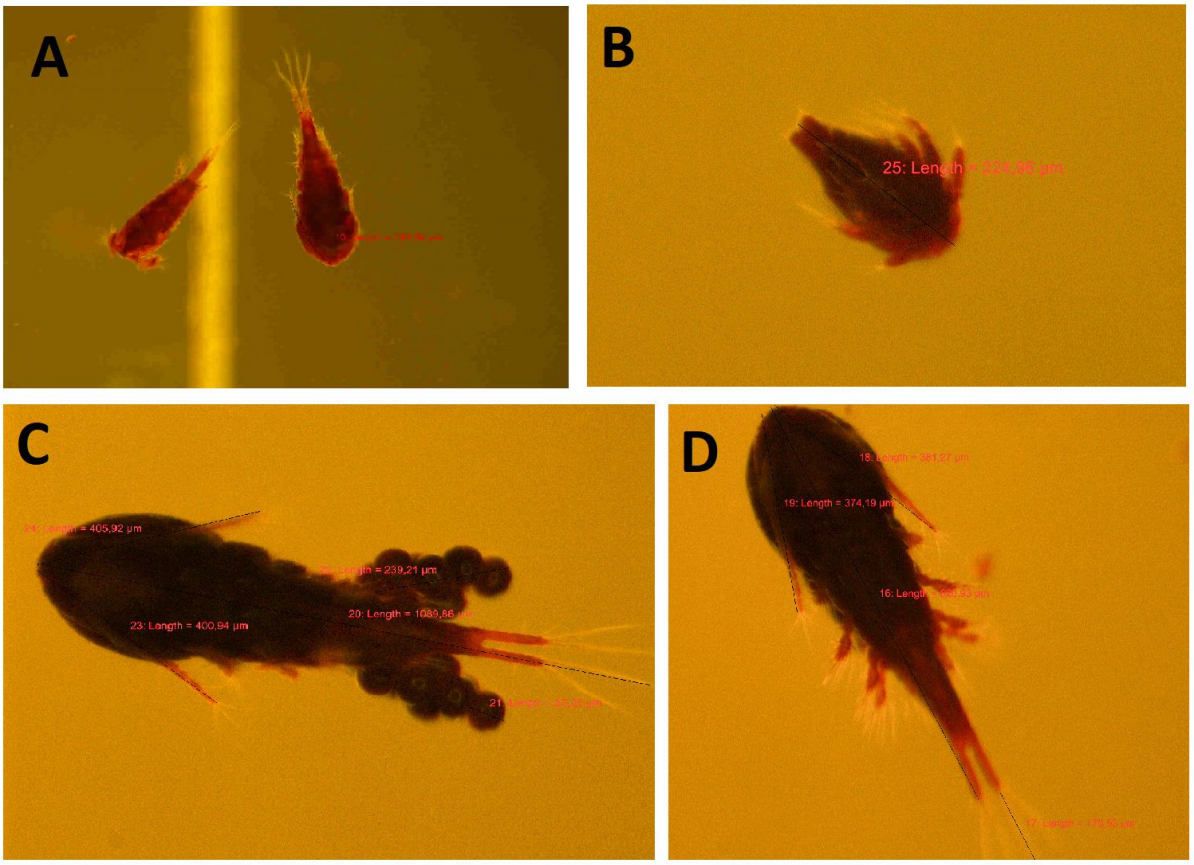

Figure 5. All pictures show the cyclopoid copepod Apocyclops royi fixed in 1% Lugol. Pictures show ***A:*** *Two A. royi copepodite, male on the left and female without egg sacs on the right, with length measurement of the female antenna; Antenna length = 393,32µm.* ***B:*** *An A. royi nauplii, with body length measurement; Body = 224,96µm.* ***C:*** *A female A. royi with egg sacs, with multiple measurements; Top antenna = 405,92µm, bottom antenna = 400,94µm, body length = 1089,86µm, top egg sac = 239,21µm, bottom egg sac = 87,28µm.* ***D:*** *An A. royi copepodite, with multiple measurements; Left antenna = 374,19µm, right antenna = 381,27µm, main body = 920,93µm, tail = 170,63µm (All photos taken through a microscope, by the group, 2018).*

A. royi shares similar characteristics with other copepods of the same genus. The body is oval and wider than the abdomen and consists of five thoracic segments, while the abdomen is narrow with caudal furca extended from the urosome (Arnofsky, 1996; Bledzki and Rybak, 2016). A mature *A. royi* is shown in figure 5.D. They have 11 segmented antennules, 3 segmented antennae and have 5 pleopods with the fifth pair reduced (Arnofsky, 1996).

A. royi reproduce similarly to many other copepods and the females produce two egg sacks with approximately 16-24 eggs per female as shown in Figure 5.C, but the number of eggs depends on abiotic and biotic factors (Pan et al., 2016; Pan et al., 2018).

A. royi undergoes development similar to other copepods with 6 nauplii stages and 6 copepodites stages. The copepod reaches maturity (from NI to CI) in 4-5 days, and it takes

7-8 days for *A.royi* to develop from nauplii to adult under optimal conditions (Su et al., 2007).

A. royi are omnivorous, raptorious feeders. Adults, especially ovigerous females, prey on heterotrophic foods in periods with low abundance of autotrophic foods, and prefer live phytoplankton over artificial feed (Dhanker and Hwang, 2013).

5.3.1 Abiotic and biotic factors

Table 2: Optimal growing conditions and maximum observed densities in cultures of *Apocyclops royi:*

Temperature	**25-30 °C** (Su et al. 2007)
Salinity	**10-20 ppt** (Su et al. 2007; Pan et al., 2016)
pH	**8.0-8.7** (Blanda et al., 2015; Blanda et al., 2017)
Maximum observed density (laboratory conditions)	**17-37 ind./mL** (Su et al. 2007; Lee et al., 2013)
Natural densities	**Ranges from approximately 4 ind./L** to **approximately 120 ind./L** (Blanda et al., 2015; Blanda et al., 2017).

Different abiotic factors can contribute to a difference in population growth, population composition, and reproduction.

Temperature, oxygen levels, and pH have been measured in tropical Taiwanese outdoor aquaculture ponds, where *A. royi* are commercially cultured as live feed. Surface oxygen levels were measured to be between 16,3 mg L^{-1} in April and 7,9 mg L^{-1} in January, while measurements 0,5 m below the surface measured values below 4 mg L^{-1} (Blanda et al., 2017; Blanda et al., 2015). The pH values were measured at an average at 8.2 (Blanda et al., 2017). Surface water temperature in the ponds varied seasonally with the highest temperature measured at 32 ℃ in July-August and lowest measured at 20 ℃ in January, which

corresponded to a higher biomass of *A. royi* in July-August and lower biomass in January (Blanda et al., 2017; Blanda et al., 2015). The optimal temperature has been shown to be 30 ℃ (Su et al., 2007). The natural densities in outdoor aquaculture ponds with varying abiotic and abiotic factors was estimated according to *Blanda et al. (2015)* and *Blanda et al. (2017),* to be from approximately 4 ind./L to approximately 120 ind./L (Blanda et al., 2015; Blanda et al., 2017).

Light has shown to have an effect on the swimming behavior of *A. royi* in cultures. Like other cyclopoid copepods, *A. royi* has a diel vertical migration. (Blanda et al., 2015; Wu et al., 2011). In the presence of light *A. royi* has shown to swim at a slower speed and in low light conditions/absence of light *A.royi* has shown to swim at a higher speed compared to in the presence of light (Blanda et al., 2015; Wu et al., 2011).

While *A. royi* can adapt to different salinity levels, it has been shown that different salinity levels can affect population growth, population composition and reproduction. Salinity between 10 and 20 ppt has been shown to be the optimal salinity level for population growth (Pan et al., 2016). Both higher and lower salinity (0 -5 ppt and 30-35 ppt respectively) has been shown to decrease population growth, the number of nauplii and daily production of nauplii per female (Pan et al., 2016). Higher salinities (30 ppt and higher) have been shown to increase the development time for nauplii to reach maturity (Su et al. 2007).

Different food-types can contribute to the population growth, population composition, reproduction rate and survivability. The survival rate of *A. royi* when fed on live phytoplankton has shown to be 77.8-89.6% (Su et al. 2007; Liao et al., 2001). The phytoplankton size must not be too big, since the nauplii can not feed on the bigger phytoplankton (Pan et al., 2018).

When *A. royi* has been cultured under optimal conditions, similar to the abiotic factors mentioned above, *A. royi* has been observed to live at densities of 17-37 ind./mL (Su et al. 2007; Lee et al., 2013; Liao et al., 2001).

5.4 Phytoplankton *Rhodomonas salina*

The phylum Cryptophyta, are a group of unicellular plankton between 2-30 μm diameter and can vary in color from blue green to red and brown (Archibald et al., 2017; Evert et al. 2013). *Rhodomonas salina* is 5-12 μm in diameter (Vu et al., 2016).

Cryptophyta has an asymmetrical, flattened cell shape and two slightly unequal flagella. They have four-bound membranes, with a nucleomorph between the middle membranes (Archibald et al., 2017). *R. salina* has chloroplast which contains the pigments chlorophyll a, chlorophyll c and phycoerythrin (Archibald et al., 2017; Vu et al., 2016).

They live in a range of aquatic environments, but many species live in colder aquatic environments (Evert et al., 2013).

They are an important part of aquatic ecosystems, as many of them are primary producers and are an important food source for aquatic life (Archibald et al., 2017). According to *Vu et al. (2016),* the optimal growing condition for *R. salina* to obtain highest production has been shown to be at irradiance between 60-100 μmol photons m^{-2} s^{-1} and an excess of inorganic nutrients with maximum cell density at 3,2-5,3 □10^6 cells mL^{-1} (Vu et al., 2016).

6.0 Method

The method covers the different approaches and tests performed throughout the study. The general circumstances and approaches will be presented first, followed by how densities were established. The main culture and the environmental factors surrounding it will be described. The first experiment, the interface and the second experiment will then be presented in said order, with focus on the experimental approach and the running evaluation of the methods and equipment used. At last the method for the calculations used in the data-treatment will be presented as well. Throughout the course of the study, many changes were made to the method, due to changes in the main culture or new realisations by the group. The approach to this is also explained chronologically.

6.1 Approach/objective

The study's overall objective was to identify and describe the relation between density and ovigerous rate of the copepod species *Apocyclops royi*. Through data from the experiments, an establishment of what effect a change in density has on a population of *A. royi* and distinguish what would cause a change, should there be any. The experimental setup from *Rayner et al. (2016)* was used as a source of inspiration when designing our own experiment and procedure.

In this study, determination of female copepods was solely based on the observation of egg-sacs on adults, as in the case illustrated in figure 5.C.

Potential loose egg-sacs were observed and used in the estimation of the number of ovigerous females. This was done under the assumption that two egg-sacs equals one female, while any odd number would mean that a female dropped one or both egg sacs, but only one was collected. What will be deemed "adults" and "nauplii" from now on, are in this study distinguished as copepods with a developed urosome, as seen in figure 2 and figure 5.B. This means that the copepodite stage is divided upon both.

6.2 Main culture

The study was performed on the species *Apocyclops royi.* The strain of the cyclopoid copepod *A. royi* originates from Tungkang Biotechnology Research Center in Taiwan. This culture has later been transferred to the LOG-Marine Station of Wimereux, France where the *A. royi* was obtained from (Pan et al., 2018). Before this study, the culture has been running for 12 months in the plankton laboratory at Roskilde University.

The main culture was kept in a climate room set to 27,3˚C. The culture was raised in a dark bucket containing 50-55 litres of seawater (0,22 µM filtered) with a salinity of 33-35 ppt. The culture had a constant supply of air from a tube, making sure the water was oxygen-saturated.

The copepods were fed with the phytoplankton *Rhodomonas salina*, as this species was the only type that was accessible. The exact amount varied, but the culture was always fed with a surplus of algae, as the concentration should be above 50.000 cells/L. This estimated optimal concentration was determined based on a study by *Berggreen et al.* (1988), which states that the growth rate is no longer limited by the concentration of food when the cell concentration is more than 50.000 cells/mL (Berggreen et al., 1988).

Before and during the first part of the experiment, the culture was fed daily. After the first experiment the feeding schedule of the main culture was changed to only every second day, since they would still be fed with a surplus of food.

6.3 Establishment of copepods per litre

Before beginning an experiment an estimate of the density of adults in the main cultures needed to be established. In order to find a reliable sampling method, scooping and pipetting were compared. Scooping is done by taken in a desired amount of water containing copepods using a fitting beaker, while pipetting is done the same way, but with a pipette of a desirable size. The size of the sample was chosen to be 50 mL amount, this was chosen based on it being easy to scale up and collect. The two methods' reliability was estimated by taking samples and comparing the number of copepods caught and how close to 50 mL volume each sampling was. Sampling was done by stirring the water, in order to disperse the copepods evenly and then taking the 50 mL sample. For each estimation, four samples of 50 mL were taken from the main culture and the average numbers of copepods was used to establish the efficiency. Scooping showed the best consistency and was chosen to be used throughout the first experiment.

Once a sample had been collected, it was filtered through a 120 μm mesh in order to isolate adults, so they could be accounted for in order to establish the adult density. The copepods were then flushed out of the mesh and into a petri dish using a handheld water pump containing 35 ppt salt water. A couple of drops of 1% Lugol were added with a pipette to the petri dish in order to kill the copepods, making it easier to count and store them. The total number of copepods in the samples was counted twice to quantify the counting. The distinguishing described in section 6.1 were used to determine which were adults with unknown gender, ovigerous females and nauplii. Only adults with unknown gender and ovigerous females were accounted for when counting. The average of adult copepods sampled was used to determine the average in 50 mL in the main culture. By scaling this number up to one litre, the copepods per litre for the main culture were established.

6.4 First experiment - range finding

The first experiment was conducted over a 4 day period, aimed towards establishing a range in which future work would proceed.

The experimental setup consisted of eight 2-litre glass beakers each filled with 1 litre of seawater (33-35 ppt salinity). The beakers were placed in the same climate room as the main culture.

The beakers got a supply of air from a LP-60 Air-Pump, which was transferred through a plastic tube connected to a glass pipette, placed in the water in each beaker. The amount of air was determined by looking at the air bubbles in the beaker and trying to make it similar in all the beakers. This was done since there was no other way to adjust the machine.

Tinfoil was then placed over the top of the beakers to function as a lid to avoid contamination and limit evaporation.

How the experiment was set up can be seen in figure 6.

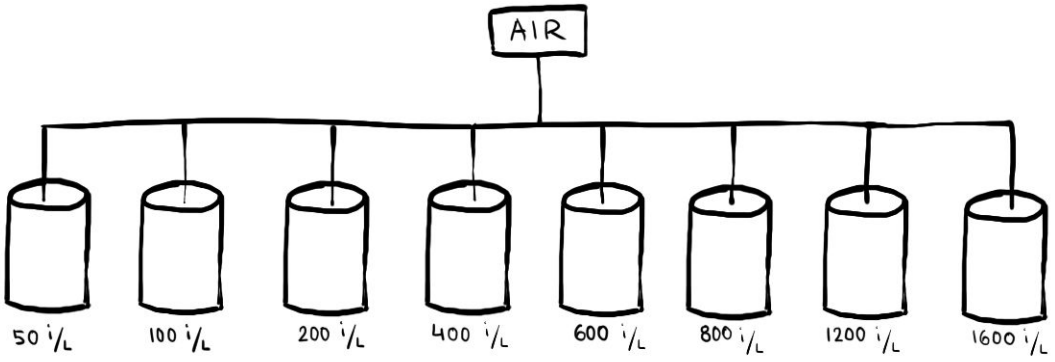

Figure 6. Simplified drawing of experimental setup. The AIR represents the pump, the lines represents the tubes dispersing the air in the beakers. (Knigge, 2018)

The copepods were fed daily during the experiment to a concentration of 50.000 cells/mL.

To achieve the correct concentration, the cell concentration in the beakers and of the algae-feed had to be measured on the *Multisizer 4e Coulter Particle Analyzer*.

First the concentration was estimated in each beaker by taking 2 samples from each (1 mL of water for each sample), as well as 3 samples from the algae-feed.

10 mL of 0,2 µM salt water was added to each sample, to dilute the samples, and was tested on the *Multisizer 4e Coulter Particle Analyzer*. The dilution was necessary for the machine to work, since the filter in the machine would otherwise get clogged and stop the sample. With the results from the samples, each concentration was estimated by reading the number of cells (n) of the graph in the algae peak LC=5,770 µm to UC=9,880 µm.

The cell concentration was then calculated with the following equation: $c = (n \cdot 11\ mL)/2$
When the concentration for each beaker was estimated, it was possible to calculate the volume of algae-feed that should be added to the beaker to achieve the concentration of 50.000 cells/mL. The formula used for this is: $v_2 = (c_1 \cdot v_1)/c_2$

Eight densities were to be tested during the first experiment: 50, 100, 200, 400, 600, 800, 1200 and 1600 ind./L. The selection of densities was based on a combination of the densities that *Blanda et al. (2015)* found in the pond cultures in Taiwan, and the study by *Rayner et al. (2016)* where densities between 100-1200 ind./L were studied.

The densities were created by using the dilution formula. $v_1 = (v_2 \bullet c_2)/c_1$
Assumed that v_2 = 1 L (in the beaker), c_1= density of main culture (ind./L), c_2 = the wanted density, then v_1 is the volume needed from the main culture to create the density.
An example could be for the density of the 50 ind./L beaker. Here v_2 = 1 L, c_1= 700 ind./L (see appendix 1) and c_2 the wanted 50 ind./L. Putting these numbers into the formula gives:

$$v_1 = (1L \bullet 50\ ind./L)/700\ ind./L \quad = \quad v_1 = 0{,}071\ L \text{ or } 71mL$$

These calculations were used to determined how much water from the main culture, needed to be transferred to each beaker, as seen in table in appendix 1 and 2.
To divide the correct v_1-volumes for the initial volumes in each beaker, a subsampler and/or a plankton splitter, which can be seen in figure 7, was used. The subsampler consisted of a two litre plastic bottle with a Kipp dispenser head attached, either a 10 or 15 mL kip-automate (NS29,2/32; Buch & Holm, Witeg, Germany).

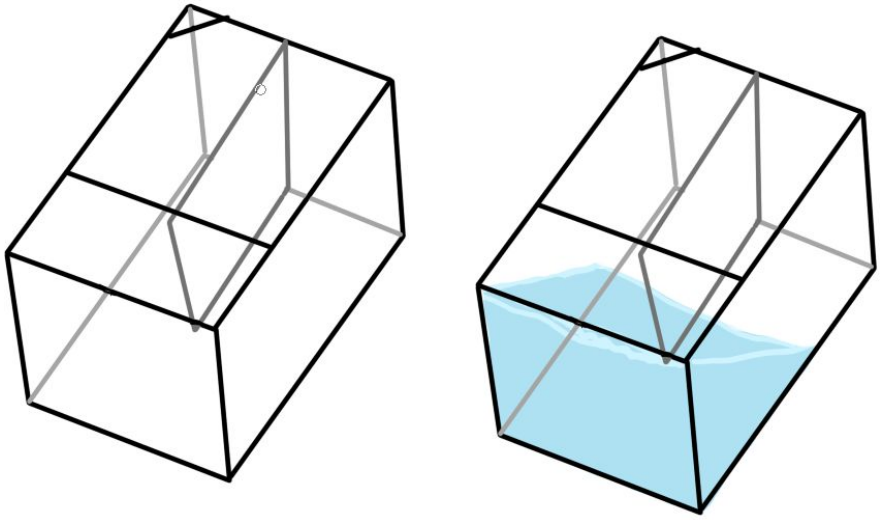

Figure 7. Plankton splitter (Knigge, 2018)

The subsampler was used by filling the bottle with water from the main culture, collected by filling the 2 litre plastic bottle. The bottle was then sealed and turned slowly 7 times to distribute the contents evenly. The 10 or 15 mL top, for measuring out a specific volume, was then added to the bottle, and the water could then be filtered through a 250 µm filter, whereafter the copepods were transferred to the assigned beakers.

The plankton splitter was only used when more than 500 mL was needed from the main culture. The plankton splitter works by halving the initial volume by a wall dividing it inside, containing half of the water on one side.

Exactly 1 litre was taken from the main culture and transferred to the plankton splitter (the plankton splitter had a limit of 1 litre), which would become roughly 500 mL by holding the plankton splitter upright, allowing us to pour half of the water onto a filter, and then transferring the copepods to the assigned beakers.

Samples from the beakers were taken each day during the experiment to observe the development, except on the first day where the densities were created. The samples were taken by scooping with beakers and noting the volume of each sample, then filtering with a mesh (100-120µm) and lastly transferring the copepods to petri dishes. The copepods were in the petri dishes fixed with a few drops of 1% Lugol and the total number of copepods and the number of egg-carrying females were counted.

On the fourth and final day (day 3 in data) the number of copepods in each beaker was counted.

In all densities except 1200 and 1600 ind./L, all the contents of the beakers were filtered and split out onto petri dishes, and all adult copepods were counted.

Subsamples from 1200 and 1600 ind./L were taken to estimate the total population, again counting the number of adult copepods and egg-carrying females.

6.5 Interface

In between the two experiments that make up the study, two changes happened in the main population.

A contamination of an invading copepod species *Tisbe holothuriae* (strain code: RUC.THI) was discovered in the main culture (Hansen et al., 2018). In an attempt to remove the invading species, the bucket and water of the main culture was changed and the population was filtered in an attempt to reset it to only containing *A. royi*. Before removing the water, parts of the water were filtered in an attempt to obtain some copepods for the new culture. Since *T. holothuriae* is benthic associated, whereas *A. royi* is semi-pelagic, filtering was only done in the pelagic phase of the contaminated culture. A filter with a 120 µm mesh was used for filtering the copepods.

A week after inoculation of the new culture, when the second experiment were to be started, the main culture's density dropped drastically from about 700 adult ind./L before the reset, to about 120 adult ind./L. In an attempt to establish why this had occurred, biotic and abiotic factors were checked in the new culture. A portable handheld refractometer was used to eliminate salinity as the cause. A handy polaris 2 hand-held meter was used to control oxygen saturation, excluding that as well. Looking into the algae composition, an unknown amoebe was observed, but in such a low degree that it could not be a reasonable explanation either. At last the population composition was looked into further, by sampling 50 mL water, but this time filtering with a smaller mesh of 40µm. The sample, filtering and counting procedure described in section 6.3 were used, but this time counting everything, including nauplii. This revealed that the main culture contained a lot of nauplii, suggesting that when the culture had been reset, the nauplii that would be grown up at this time had been flushed out. The second

experiment was pushed for a week, at which time the culture had reached adult density of 275, doubling in one *A. royi* life cycle. From this point on, it was decided to use a 40 µm filter for filtering the copepods, as the incidence had shown the importance of taking eggs and nauplii into consideration.

6.6 Second experiment - Further data collection

The aim was to achieve a more complete understanding of how density could be related to ovigerous rate, based on the knowledge gathered from the first experiment.

The data from the second experiment was meant to "fill in the gaps" in the data from the first experiment and thus making the possible relation between density and ovigerous rate more visible and to get a clearer result of the most ideal density, under these circumstances. In the second experiment two higher densities were also used, to give a clearer contrast between higher and lower densities.

From these considerations the following eight densities were chosen: 20, 70, 130, 230, 300, 2200 and 3000 ind./L.

The second experiment was performed under similar conditions as the first experiment. Eight 1 L beakers containing copepods were kept in the climate room at 27,3 C^o, but this time they were only filled with 500 mL 35 ppt. seawater, in order to have a smaller proportion to work with. All eight beakers had a supply of air from a LP-60 Air-pump, fixed the same way as described in the first experiment, and were fed daily to a cell concentration of 50.000 cells/mL.

In the second experiment it was also decided to study the copepods clearance and ingestion rate more in depth, than simply taking note of changes in algae concentrations. This addition to the experimental procedure, came from looking at the results of the development in the first experiment. In the first experiment a strange pattern in how the algae concentration changed from day to day was seen, as seen in appendix 3, which was aimed to elaborate on.

For observing the algae, three replicate 1 L beakers with 500mL saltwater 35 ppt containing algae were set up together with the copepods density, making a total of 11 beakers being used. An initial algae-concentration of approximately 50.000 cells/mL was calculated using the same calculation as seen in section 6.4. The algae beakers were equipped with an airtube and tinfoil as with the other beakers.

Measurements of the algae concentration were taken by using the *Multisizer 4e Coulter Particle Analyzer* initially (0 hours), first on after ½ hour, and then one on every hour until the 6th hour after start-up, and then the last on was 24 hours after start-up, in order to establish the natural growth(k) at time Δt. From this the actual amount of food available in each beaker could be calculated by using calculations of *Frost (1972)*, which is explained more in depth in section 6.7.

The cell concentration was measured by taking 1 mL of water from the beakers with a pipette and adding it to a measuring cup. Two samples were taken from each copepod density beaker, and two samples were taken from the algae control beaker. Samples from the algae-feed were also taken to use in the calculations for feeding the copepods.

A new method for handling the copepods when filtering them was used. The method consisted of lowering the filter into a beaker filled with seawater, thus making sure the copepods were always in water and that the disturbance from flushing were lower.

The density of the main culture had to be estimated once again to create the wanted densities. This was done with the same procedure as described in section 6.4, with small variations, such as taking 4 samples which were then filtered through a 40µm mesh, using the method described in section 6.5.

When the density of the main culture was estimated, the correct densities could be made. Three methods were used for this: handpicking, subsampling and plankton-splitter.

The sampling method had been altered for the lower densities. Handpicking using a pipette was used for the lowest densities (20, 70 and 130 ind./L) to make sure the right number of copepods was collected, since individual handpicking was deemed more precise than scooping of smaller densities. It was noticed how many handpicked ovigerous females that were added. One ovigerous female was added to both 20 ind./L and 70 ind./L, and two ovigerous females was added to 130 ind./L. The subsampler, with a 10, 15 or 25 mL top, and plankton-splitter was used like in the first experiment, using the same approach as described in section 6.5.

For this second experiment the copepods were only counted on the final day. The whole content of the beakers were counted, except for the densities 2200 and 3000 ind./L, where scooping was used to collect three subsamples in 50 mL beakers, after which the counting procedure in section 6.4 was used to determine the population composition.

During this experiment the total number of adult copepods (CI-CVI) were observed , number of ovigerous females, loose eggs, nauplii, and observed specimens of the invading *Tisbe* copepods. All these were accounted for, as can be seen in appendix 8.

6.7 Calculating clearance rate and ingestion rate

Calculations from *Frost (1972)* were used to calculate the clearance rate and the ingestion rate of the copepods.

First the algae growth constant (k) is calculated. To do this, it is needed to know the cell concentration in the control beakers without copepods (c_1 and c_2, cells/mL) at .

$$k = \ln\left(\frac{\frac{c_2}{c_1}}{t_2 - t_1}\right)$$

The grazing coefficient (g) is found after calculating k. To calculate g, it is needed to know the algae growth constant (k) and the concentration in the beakers with copepods (c_1* and c_2*, cells/mL) at time t_1 and t_2.

$$g = \ln\left(\frac{\frac{c_{2*}}{c_{1*}}}{t_2 - t_1} - k\right)$$

The clearance rate F (mL copepod^{-1} day^{-1}) is the volume cleared by each copepod per. time unit. To find this, it is needed to know the volume of the beaker (V), the grazing coefficient, and the number of copepods in the beaker (N).

$$F = Vg/N$$

g and k are then used to calculate the average cell concentration in the beaker (c) at time (t_2-t_1),

$$(c) = \frac{c_1[e^{(k-g)(t_2-t_1)}-1]}{(t_2-t_1)(k-g)}$$

(c) (average cell concentration) and F (clearance rate) is then finally used to calculate the ingestion rate I (cells copepod^{-1} day^{-1}), the cells eaten per. copepod pr. time unit.

$$I = (c) * F$$

7.0 Results

The result section presents the data that was gathered over the course of the study. This is presented chronologically, as the further steps of the results often build on the former. The first experiment, the range finding, is presented first, along with the population change and female concentration, that are used for the further assumptions. The change in algae-data from both first and second experiment is compared to distinguish a pattern. Next, the calculated data from the second experiment are shown, comparing the average food available, clearance rate, ingestion rate and ovigerous rate. Finally, the population dynamics and developments in the second experiment are presented.

7.1 First experiment - Range finding

The graphs in figure 8 show the development of density in each beaker during the experiment. The general picture is, that the densities increases during the experiment, though many graphs show a decline at some point. All density-ranges (50-1600 ind./L) generally show an increase in density on day 3, where densities increased from 50 ind./L to 108 ind./L and from 1600 ind./L to 2987,5 ind./L respectively (see Appendix 4).

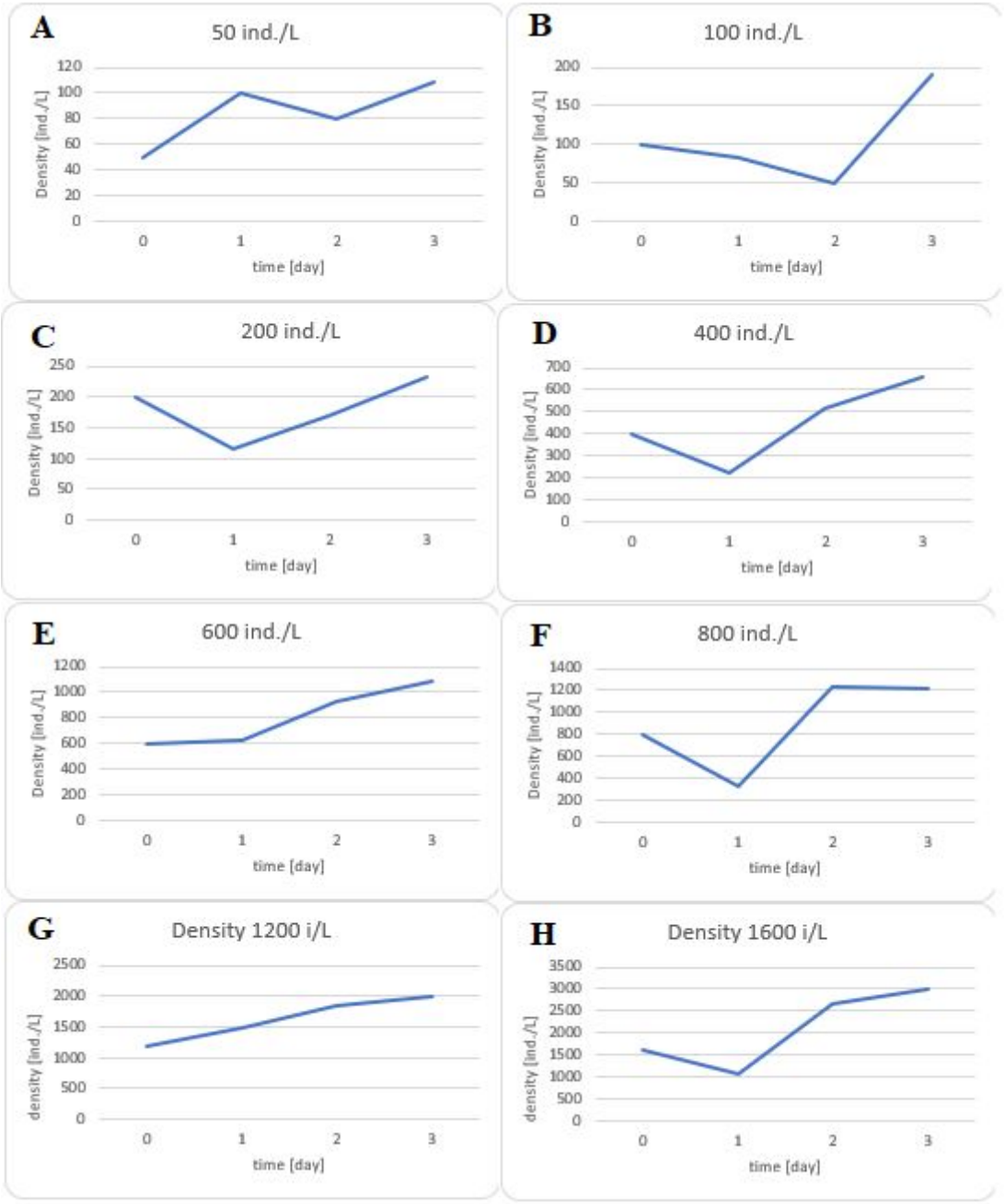

***Figure 8**:Graphs shows the density changes of Apocyclops royi over time in days with different initial densities 50, 100, 200, 400, 600, 800, 1200 & 1600 ind./L. Results are from the first experiment (see appendix 4) **A:** Initial density: 50 ind./L **B:** Initial density: 100 ind./L **C:** Initial density: 200 ind./L **D:** Initial density: 400 ind./L. **E:** Initial density: 600 ind./L **F:** Initial density: 800 ind./L **G:** Initial density: 1200 ind./L **H:** Initial density: 1600 ind./L*

Figure 9 shows the relation between percentage ovigerous females and density in the first experiment. There is a weak tendency showing a decrease in ovigerous rate along with the increasing density, where the highest percentage ovigerous females (approximately 17,4%) is found at 100 ind./L (see appendix 11).

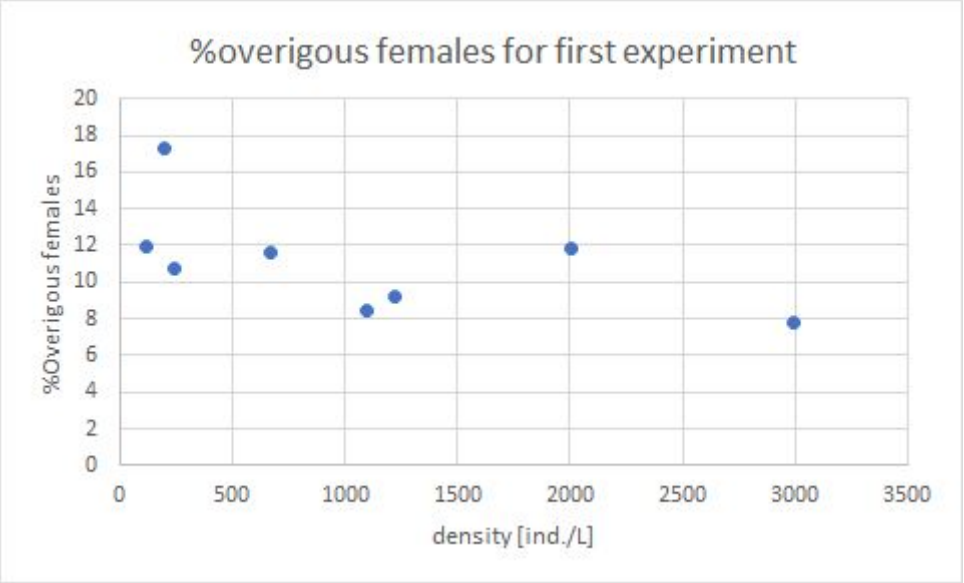

Figure 9. The graph shows the final percentage of ovigerous females of the final densities at different initial densities for Apocyclops royi . The results are from the first experiment with initial densities: 50, 100, 200, 400, 600, 800, 1200 & 1600 ind./L.(see appendix 11)

7.2 Algae results from all experiments

The results of the control algae experiment are shown in figure 10. The figure shows, that the algae concentration decreases exponentially over time. The results are used in the calculations of clearance rate and ingestion rate.

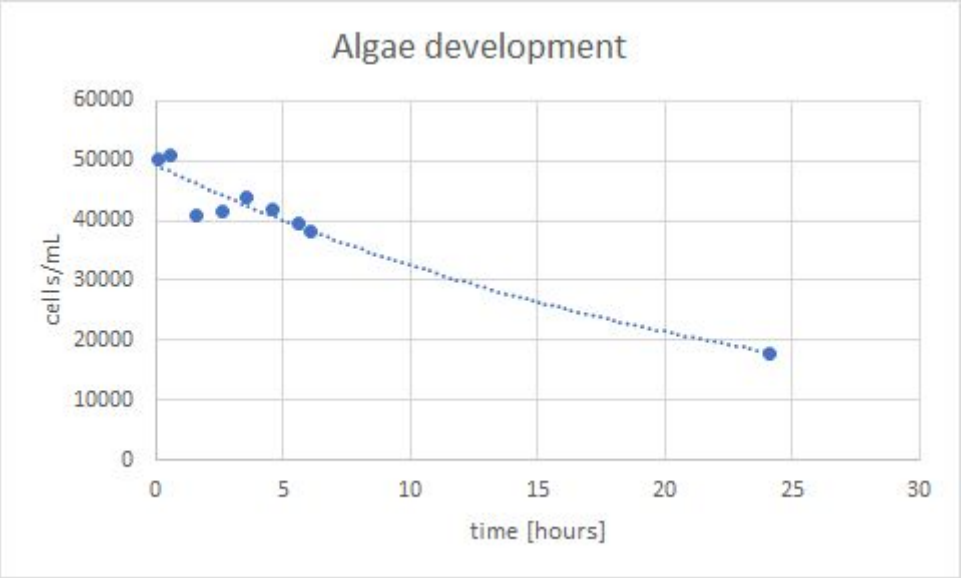

Figure 10. The graph shows the development of Rhodomonas salina during the control experiment over time (in hours). At time: 0 hours the initial concentration of R. salina was 50.000 cells/mL (see appendix 10).

The average food concentration (cells/mL) in the densities, as seen in figure 11, shows the average algae concentration that is available in the different densities. Figure 11 shows that there is a negative correlations between algae conc. (cells/mL) and densities, with less food available at higher densities. This can further be used to clarify if the different densities are food limited at some point.

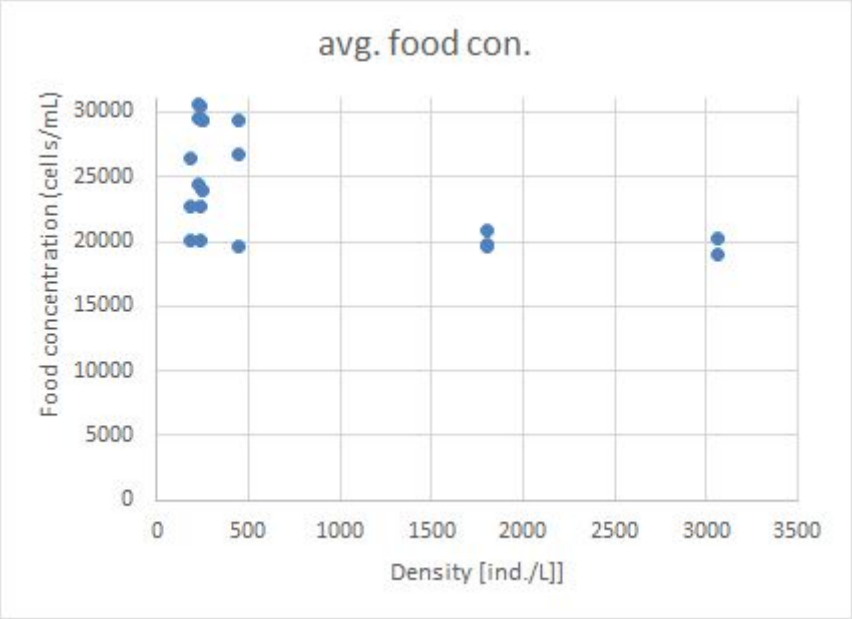

Figure 11: The graph shows the average algae concentration (cells/mL) available at different final densities. Final densities consist of both the adults and nauplii (ind./L).The results are from the second experiment with initial densities: 20, 70, 130, 230, 300, 2200 and 3000 ind./L (see appendix 14 for final densities (adults+nauplii) and see appendix 12 for the avg. algae conc.).

The algae consumption over time was examined at different initial densities. The results can be seen in figure 12. In the lowest densities (20 and 50 ind./L), a gradually increase (of about 21% of the initial consumption in 20 ind./L and 25% in 50 ind./L) in food consumption each day as observed, which is shown in figure 12A and 12B. In density at 2200 ind./L the food consumption slowly decreased from day 1 to day 3 at approximately 20%, which is illustrated in figures 12C and 12D.

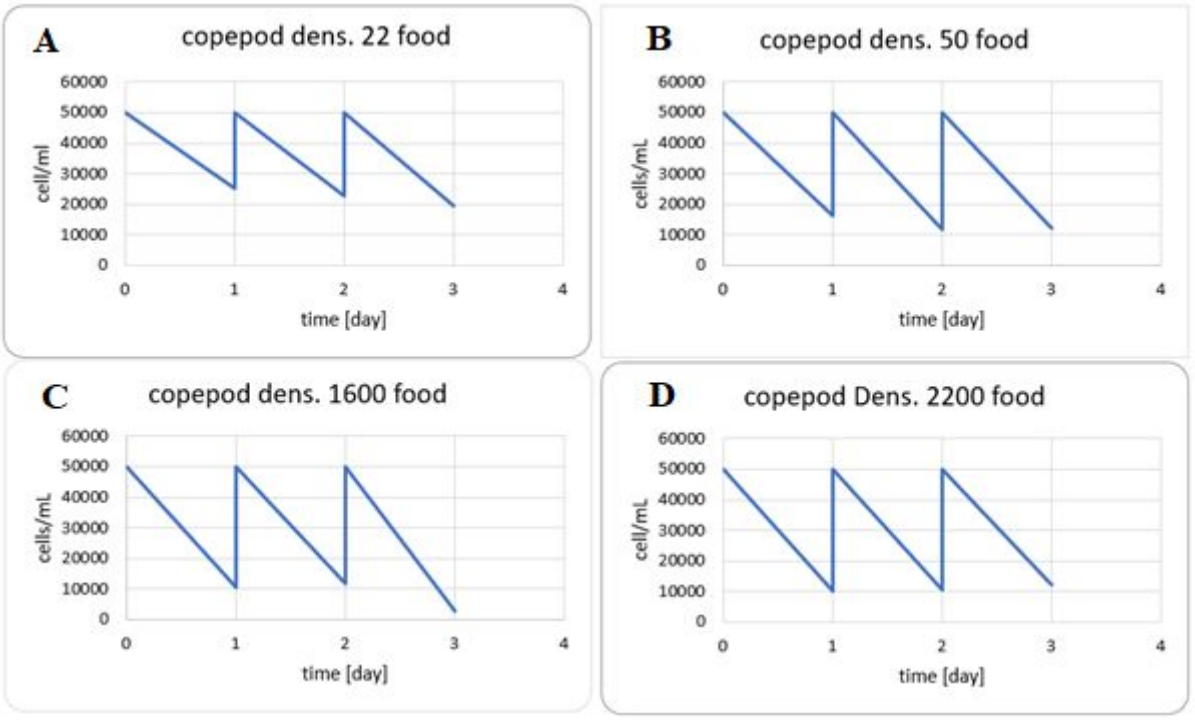

Figure 12: The graphs shows the algae consumption by Apocyclops royi at different initial densities over time (in days), when fed 50.000 cells/mL every day. ***A****: Initial density:22 ind./L. Results are from second experiment (see appendix 6).* ***B****: Initial density:50 ind./L. Results are from the first experiment (see appendix 3)* ***C****: Initial density: 1600 ind./L. Results are from the first experiments (see appendix 3).* ***D****: Initial density: 2200 ind./L. Results are from the second experiment (see appendix 6).*

7.3: Grazing as a limitation

Using the results from the algae control-experiment shown in figure 10, and the results from the algae consumption at different densities shown in figure 12, it becomes possible to calculate the effect density has on the grazing of the individual copepod.

The clearance rate show us how big of a volume in mL each copepod clears through per day. Figure 13 shows the relation between density and clearance rate. The general picture is, that the clearance rate decreases with increase in density. The highest clearance rates is in the densities from 22-138 ind./L at a mean value of 13 mL/cop./d. where it dropped in the range 182-1586,67 ind./L down to a clearance rate of 1,51 mL/cop./d.

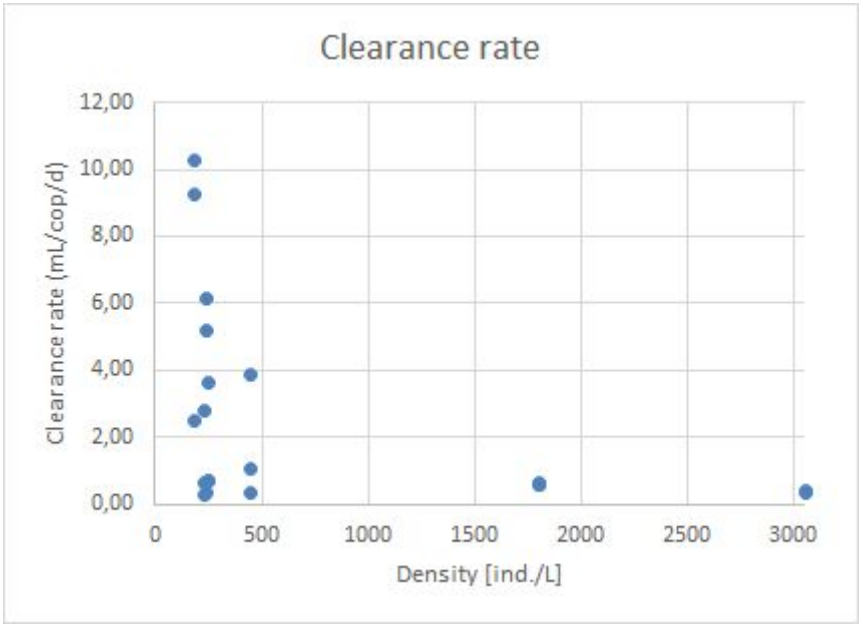

Figure 13. The graph shows the volume (in mL) cleared per Apocyclops royi copepod per day at different initial densities. Results are from the second experiment (see appendix 14).

Figure 14 illustrates the ingestion rate in relation to density.

The ingestion rate shows us how many cells each individual copepod ingests pr. time unit (day). The tendency of the ingestion rate in relation to density, shows that the ingestion rate decreases with an increasing density. The highest ingestion rate was at 212.879,68 cells/cop./d. at density 70 ind./L. The average ingestion rate at 130 ind./L was 85.498 cells/cop./d. The average ingestion rate at 160 ind./L was 44.485 cells/cop./d. and the ingestion rate at the higher densities keeps decreasing (see appendix 13).

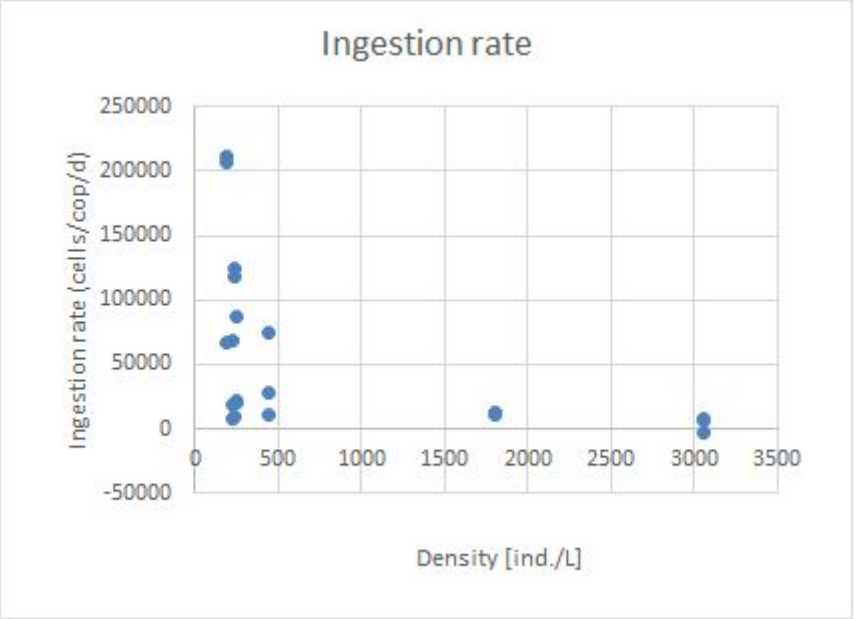

Figure 14. The graph shows the ingestion rate of Apocyclops royi (cells/copepod/day) at different final densities. Results are from the second experiments (see appendix 13).

7.4 Ovigerous females

The ovigerous rate shows the percentage of ovigerous females in the total final adult population, which can be seen in the graph in figure 15. The graph shows that in the higher densities (160-3000 ind./L), there are fewer ovigerous females (less than 15%), compared to the lower densities (22-130 ind./L).

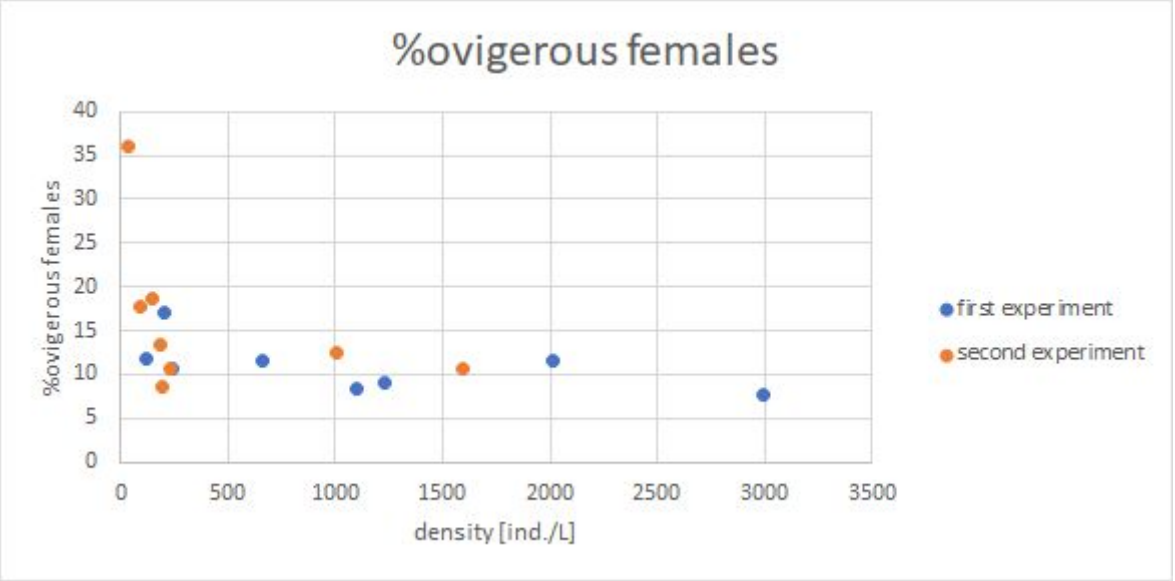

Figure 15:. The graph shows the percentage of the ovigerous females at different final densities (ind./L). All the densities are for adult copepods. The blue datapoint represent the results from the first experiment at different initial densities: 50, 100, 200, 400, 600, 800, 1200 & 1600 ind./L.. The orange datapoint represent the results from the second experiment at different initial densities: 20, 70, 130, 230, 300, 2200 and 3000 ind./L (see in appendix 11) .

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7.5 Densities

The development in the different densities is shown in figure 16, which illustrates the growth of adults, ovigerous females and nauplii at each initial density in the second experiment.

The initial number of nauplii was unknown in the densities from 160-3000 ind./L, and therefore the initial number of nauplii was set to 0 ind./L, which meant that the nauplii density increases at all densities, though with varying end numbers. The initial number of ovigerous females was also unknown in the densities from 160-3000 ind./L, and therefore set to 0 ind./L, but in 22 ind./L and 70 ind./L there was one ovigerous female and in 130 ind./L there was 2 ovigerous females. The number of ovigerous females also increases in all densities, though with varying end numbers.

The total population density increased at all initial densities, but it stagnates at the highest population densities at 2200 ind./L and 3000 ind./L.

The lower densities (22, 70 and 130 ind./L) has more nauplii compared to the total population, than the higher densities (160 ind./L and up). The lower densities (22 and 130

ind./L) also has more ovigerous females compared to the total population, than the higher populations (160 ind./L and above).

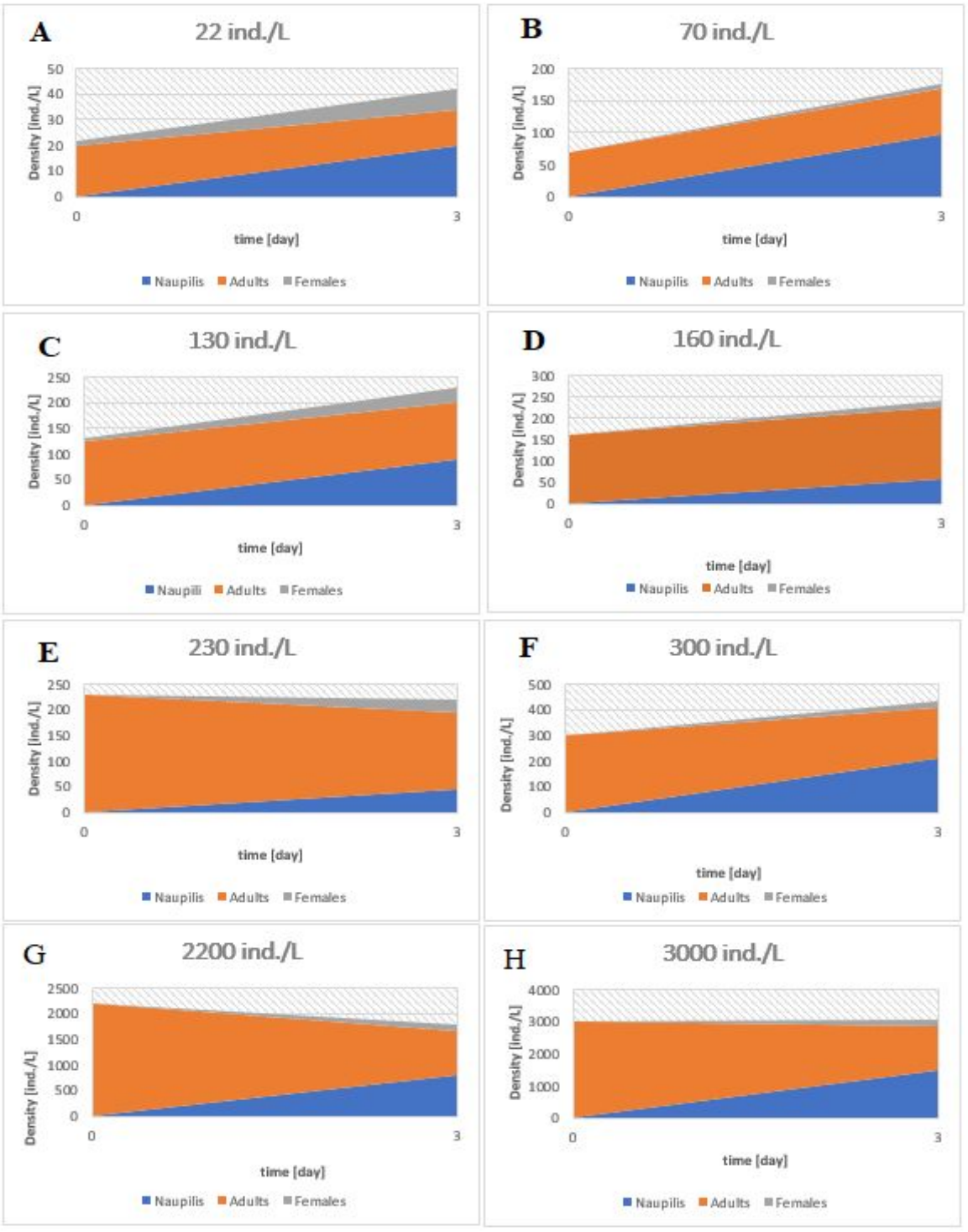

*Figure 16: The graphs shows changes in densities and population composition from day 0 to day 3, divided in nauplii, adults and ovigerous females. The densities are only from the second experiment and contains the densities 22, 70, 130, 160, 230, 300, 2200 & 3000 ind./L. All initial densities below 130 ind./L: 0 nauplii and a known number of adults and ovigerous females. At initial densities above 130 ind./L : the exact population composition is not known.. **A**:22 ind./L. **B**: 70 ind./L. **C**: 130 ind./L. **D**: 160 ind./L. **E**: 230 ind./L. **F**: 300 ind./L. **G**: 2200 ind./L. **H**: 3000 ind./L.*

8.0 Discussion

The discussion discusses in depth the methodology and the results from our experiment. First, a general discussion of factors and circumstances that covers the entire study. Afterwards, a chronological approach is taken, covering the first experiment, the interface and the second experiment in said order. In each part, circumstances to take in consideration are discussed along with the results gathered. In each the strengths, weaknesses and general influences they have on the experiment, are reviewed. Finally, the results and deductions as a whole will be discussed to sum up what the study has shown.

8.1 General discussion

The study is built up around two experiments, of which the second is built upon the knowledge gathered in the first, which altogether illustrates how density limits the ovigerous rate in *A. royi*.

In a similar study on the ovigerous rate of the calanoid copepod *Pseudodiaptomus annandalei*, in relation to density, a decline at around 385 ind./L is shown (Rayner et al., 2016). This indicates at which density it becomes a limiting factor for the species *P. annandalei*, that *A. royi* shares the Taiwanese ponds with. Very similar patterns in ovigerous rate were seen in *A. royi* as in *P. annadalei*, although a much more rapid decline in ovigerous rate in this study was observed. This makes an estimate of optimal density difficult, leading to a similar pattern, but with the decline earlier around a density of 130 ind./L. The decline suggests that they are affected by similar limitations from density, which seems reasonable since they share habitats. This assumption will make a basis for comparison between the study on *P. annadalei* and this study on *A. royi.*

Albeit the results fit former research on similar species, no observations from literature this study found, seems to explain this early and rapid drop in ovigerous rate, suggesting that it is either bound to the true nature of the species or an error in the experiment.

As mentioned in section 6.2, the feeding of the main culture, meant that food was always abundant and starvation did not occur, eliminating food limitation as a limiting factor for the main culture itself. This could mean that the populations were reacting more strongly to being

held in circumstances where food was not abundant, with the way the populations were fed when undergoing the experiment, as expressed in section 7.2 in figure 11 where at the higher densities, the algae concentration gets much lower than the full saturation level at 50.000 cells/mL.

A wide array of factors and limitations from the setup and execution of the experiment could lead to disturbances in the collected results. A problem with the sampling of the species was encountered, as both gathering and sorting of *A. royi* proved rather difficult. The chosen method of gathering, scooping, was chosen based on initial tests between scooping and pipetting of the water, as well as the experience level of the group. In these first tests described in 6.3, the mobility of the copepods proved to make pipetting very inconsistent, as they often would escape before they were gathered. Scooping works best under ideal circumstances; that the copepods are always equally dispersed in the water and that potential disturbance, behavior and other factors do not change this. Because of the motile nature of the *A. royi* they would naturally work against all these assumptions.

Aside from their natural movement, both when feeding and escaping attempts during gathering, numerous observations suggested their behavior was very influenced by light. Papers, such as *Blanda et al., (2015)* and *Wu et al., (2011)* suggest that the migrational behavior of *A. royi* is highly influenced by light and they are very influenced by its presence, further supporting this argument. Since the culture that the copepods were gathered from was usually kept in a dark enclosed container, whenever the lid was opened to gather samples, it would mimic daylight and make them migrate downwards, away from the surface where the scooping would take place.

Despite the numerous factors that could influence whether the gathered sampled contained the intended amount of copepods, all sampling was made under the ideal assumptions. Recounting at initial sampling by scooping was considered, but discarded since further disturbance and removing of copepods for counting, especially in the lower concentrations, would cause more harm than good to the results. A measure taken in a attempt to reach this optimum however, was to stir the water whenever a sample were taken. The consequence from countermeasures was that the copepods would be exposed to the physical stress of the water movement, potentially causing damage and stress to them, thus making this method undesirable but unavoidable in lack of better methods.

Some environmental factors seem to have a role in the composition of the adult-to-ovigerous female and adult-to-nauplii ratio. One such is the salinity, which was kept at a rather high ratio of 33-35 ppt. throughout the experiment. *Pan et al.* (2016) states that 20 ppt. is the most optimal salinity for population growth in *A. royi*, showing a significant drop in population size at salinities of 30 ppt and higher. Despite this, most densities in this study still increase over the course of the experiment, with exceptions mainly among the higher densities. This weak impact could be because the main culture had been living in this salinity for some time, as mentioned in section 6.2, and might have gotten more used to this salinity level. Even though the main culture have been in the high salinity for some time, it could still have an influence at the population composition, population growth and ovigerous rate, as according to *Pan et al.* (2016), *A. royi* were not cultivated under ideal salinity. The results from this study and *Pan et al.* (2016) contradict each other on this issue and therefore there cannot be reached a conclusion, and further studies would be needed.

Another factor from this is, that *Pan et al. (2016)* suggests that high salinity makes the composition of the population more aligned towards adults, thus increasing the presence of ovigerous females. Since the females were chosen solely on whether they were carrying eggs or not, this would mean that despite the females not carrying eggs not being counted, the estimate would still be a good indicator for how many females were present.

8.2 First experiment

As described in section 6.4, the objective of the first experiment was mainly to test different densities and how they would evolve. Our assumption was to see a drop in ovigerous rate as the densities increased.

The general picture of the density evolvement created in the first experiment is, that the density increases. This was to be expected, since the tested densities was far from carrying capacity of the species (Lee et al., 2013).

In section 7.1 in figure 8, it is noticeable that all densities (except 50, 1200 and 1600 ind./L) had a drop in the population from day 0 to day 1. Some densities also showed a significant increase in density between day 1 and day 3, where at day 3 the whole final population was counted instead of being sampled.The drop in the density on day 1 could be explained with the stress inflicted by the handling on day 0, due to the sampling and filtering approach.

This suggests that the sampling and/or handling of the copepods played a role in the outcome of the experiment, something that would have to be changed in the second experiment.
Another possible problem is, that the date for density on day 0 is based on the wanted density instead of an actual observed density. This could also explain the differing densities on day 1, since the initial density could be higher or lower than the wanted.
When the final densities were counted only the adults and the ovigerous females were counted and the number of nauplii was not observed in the first experiment. The nauplii show the actual "additions" to the population size, whereas the ovigerous females only show the reproductive potential, because it is uncertain if all eggs turn into nauplii. The end populations in all the densities during this experiment therefore only give an insight in how the population might grow, but not how it has grown.
The population development over time could be more precisely described if the nauplii growth also had been observed in accordance to the total population. The nauplii production over time of each density would have given a new image on the optimal density for production of copepods.

The tendency of the ovigerous rates in the first experiment wasn't as clear as it could have been expected. We expected to see a tendency showing a high ovigerous rate in the low densities, and a decreasing ovigerous rate in the higher densities based on the results of *Rayner et al. (2016)*. As seen in figure 9 in section 7.1 a decrease in ovigerous rate at increasing densities could somehow be observed in the first experiment, but only a weak tendency. The maximum ovigerous rate observed in the first experiment was at 17,4 % at initial density of 100 ind./L.
Where as the results of *Rayner et al. (2016)* showed a ovigerous rate of approximately 40% at densities of 100-300 ind./L (species *P. annandalei*), and a clear drop after density 270 ind./L and further, our results for *A. royi* didn't show a similar tendency. The reason for this could be as stated earlier in section 8.1 and 8.2.

8.3 The interface

After summarizing what information that could be gathered from the first experiment, many changes had to be considered in order to make up for the very uncertain results. Moderate

success in finding a range to work on, as well as a establishment of which parts of the experimental execution needed to be changed were the main points that could be brought in when designing the second experiment.

New population densities were chosen in order to give better insight in what the difference in low and high densities was. Since nothing suggested we were approaching the carrying capacity of *A. royi* in the first experiment and did not see a clear drop in the ovigerous rate, but still a tendency towards a higher ovigerous rate in smaller populations, this lead us to choose the initial population densities in the second experiment as described in section 6.6. Compared to the results in *Rayner et al. (2016)*, it makes a lot of sense that this change would be needed to achieve more fitting results, as *A. royi* are smaller than *P. annandalei,* making it quite sensible that their carrying capacity in ind./L is higher.

To solve the problem of the uncertainty in sampling, as well as not stressing the copepods, new precautions had to be taken. After consultations with the supervisor and colleagues, new methods of sampling and filtering were established, in which physical stress were taken much more in consideration. It was decided to use hand-picking in the smaller populations (22, 70 and 130 ind./L), since they are much more susceptible to errors in sampling. Overall, the filtering was changed to a method where the filters always were submerged in saltwater to reduce the physical stress caused by being poured and filtered directly through the filter while in free air, which had been observed as one of the main reasons that females discard their eggs when filtered. Also, daily sampling was changed into start and end sampling, since it was decided that it would be more beneficial to reduce stress from handling, and relying only on the results from the true counting on the last day, instead of potentially unreliable numbers from sampling everyday.

Aside from the changes in the experiment, the circumstances in our main culture had changed in between, which also needed to be taken on consideration. As mentioned in section 6.5, the main culture was reset between the two experiments due to the contamination of *T. holothuriae* (Hansen et al., 2018). As described in section 6.5 the filter used to transfer the copepods to the new bucket was not suitable for catching the nauplii and eventual loose eggs, which meant that an entire generation of nauplii was potentially lost.

A week later, when the second experiment was scheduled to start, large decrease in density had occured from 700 ind./L to 130 ind./L, as mentioned in section 6.5, was observed and the

experiment was delayed in order to investigate the potential reasons for the decrease. First, abiotic factors were measured, with air saturation was around 98% and salinity was normal so nothing was out of the ordinary as mentioned in section 6.5. The presence of an invasive and unknown dinoflagellate was observed and was worrisome at first, as a sister culture in the same institute had succumbed to a toxic algae. Although, an unknown dinoflagellate was observed, it was in very low abundance and did not seem to have any visible effect on the copepods, but it is yet unknown if they had a negative effect on the culture. With the nauplii loss from the reset of the main culture in mind, it was decided to recount our samples, as described in section 6.5, but also including nauplii. The outcome of the recounting showed that the true density of the main culture was 545 ind./L, when including the nauplii, which fits the former population counts much better. This strongly suggests that the lost generation of nauplii was the cause in this drop, as they would have been counted with the adult at this stage in their life cycle. Since this now gave a much clearer picture of the true compositions of the populations, filtering of nauplii and eggs was chosen to be included from now on by using the 40 μm mesh for this purpose and in the second experiment.

8.4 Second experiment

As described in section 6.6 the new densities were chosen to achieve a more clear understanding of the relation between ovigerous rate and density.

We wanted to establish the possibility of grazing being a factor for the females' egg production under the assumption that if the grazing is worse due to a specific density range, it affects the uptake of nutrients, and this could affect the production of eggs. Therefore we measured the algae growth, so that the grazing of the copepods could be calculated at each density.

Figure 11 shows that there is a decrease in the algae concentration in the control experiment. This decrease could be a response to a limitation of nutrients in the beakers in addition to a space limitation and the sedimentation of algae in the beakers, since the samples were taken in the free water mass. The copepods seem to be limited in how much food they can filter through, based on the average food concentration seen in figure 11. Over the span of time between feedings, once a day, no algae concentrations were seen to be measured much lower than roughly 20.000 cells/mL, suggesting that the total algae consumption(figure 12) across

all densities were capped around 30.000 cells/mL daily. Comparing this observation to the decrease in individual ingestion rate (figure 14) and clearance rate (figure 13), it would seem that the interspecies competition from increased density at a point between 600 ind./L and 2200 ind./L comes to a halt.

In all densities we saw a growth in population and a growth in ovigerous females and nauplii, as shown in figure 16. Because we don't know the exact initial population composition and the exact initial population size, including nauplii, at densities above 130 ind./L, the population growth and composition may be overestimated in the higher densities, as densities sampled by scooping likely have a large input of nauplii, due to the weaknesses in this method, discussed in 8.1.

The development of the adults in the higher densities seems to be troublesome, as seen in section 7.4. In figure 16, there is a tendency of more nauplii in the higher densities and also fewer ovigerous females, than in the lower densities. The slow growth in the high densities can most likely be explained with the low volume of algae in the beakers at the end of every day. When there isn't plenty of food constantly accessible, the population will become more competitive over the food.

The results from figure 16 do not seem to suggest a clear relation between the percentages of the ovigerous females and the nauplii, as a high percentage of nauplii is observed in the high densities with a low percentage of ovigerous females.

As mentioned in section 6.6 we hand-picked 1-2 ovigerous females. The reason for this was that we wanted to make sure that the low densities could increase, due to the ovigerous female. By adding these females, the result have been manipulated, and because of that, the three lower densities could have had an advantage due to the ovigerous female/females.

The higher percentage of nauplii and females in densities 22, 70 and 130 ind./L, seen in figure 16, could be due to the use of hand-picking in the lower densities, while the rest were subsampled. In the lower densities, it generally seemed like there were more ovigerous females than in the higher densities. In hindsight, we should have added one or two ovigerous females to all of the densities or none of them, for a truer result.

The development in the densities where hand-picking was performed was much clearer, since there were no nauplii added, and the amount of mature and ovigerous females was known. By

using this method, the only unknown part was whether or not the adults were males or females without egg sacs.

When counting the fixated copepods after using the new method described in section 6.5, more females were carrying egg sacs and we observed fewer dropped egg sacs compared to our earlier observations (only observations) and the eggs accounted for after the 40 µm mesh were used (appendix 7). This indicates that the changes in procedure that were made in order to lessen the stress from handling the copepods worked, but still could be improved with further efforts in decreasing handling stress.

8.5 Summary of study

In both experiments we saw a general growth in the populations and densities, up until a point between 1600 ind./L (figure 8) and 2200 ind./L (figure 16), when under the specific conditions as described in section 6.2. Overall the results suggest that an interaction between density, food availability and competition, together determine the ovigerous rate and thus growth potential in the populations. As shown in figure 13 and 14 there is a relation between food availability, thus individual ingestion rate, and the populations density. Comparing this relation with the ovigerous rate from figure 15, there is a connection between density in the populations, individual food availability and final ovigerous rates. Since food is controlled in the experiments, only achieving a level of optimal saturation when they were resupplied with food, there would be a general incentive for food competition in-between feeding. The experiments shows that the copepods in the larger densities have both the lowest individual feeding and lowest ovigerous rates. Something to deduct from this pattern is that the increased density causes a greater interspecific competition. Since the total daily algae consumption reached the maximum every day in the high densities, as seen in figure 12, these densities would starve and most likely end up collapsing at some point, if we kept the experiment running for more days. This interspecies competition seems to be the main interaction that determines the ovigerous rates under the conditions the experiment were run under. However, since the set environmental factors such as salinities and feeding are not differentiated, meaning that the actual significance of these factors cannot be deducted.

9.0 Conclusion

The purpose of this study was to determine the relation between density as a stress factor and the presence of ovigerous female copepods, thus production of the copepod species *A. royi*.

The study shows that there is a negative relation between density and ovigerous rates, meaning that increased densities can slow down the reproduction in *A. royi* as it reaches the capacity that the species has in its set environment. Albeit there is a relation, the stress caused by this is not only by density, but through the indirect effect from increased competition from the higher population per area. The increased population per area further increased the competition for other factors, such as room to forage on and through that how much food is available to an individual copepod. This is a potential cause for starvation, which in turn decreases the ovigerous rate among starved females. Overall, species density is a regulating factor for interspecies competition which in turn limits the potential for growth by limiting food per copepod.

By all mentioned above, the study concludes that the stress factor increases in the population, when the density increases and the stress factor has an effect on the amount of ovigerous females in the population, which therefore decreases when densities increases.

10.0 Perspectivation

Despite that the experiments in the study achieve an answer to the problem formulation, a lot can be done to further improve the quality of the study. If the experiment would be performed again, it should be done under optimal environmental conditions for *A. royi*, as an instance where it can be compared to the optimal deducted from known literature, such as *Su et al. (2007*) and *Pan et al. (2016).*

Under the conditions mentioned in section 5.3.1, *A. royi* maybe would have a higher population growth and a better reproduction performance. To quantify the data, it would be interesting to replicate the experiment under other environmental factors. To test the influence of food availability, a replicate where food is always abundant could also be interesting.

In this study the phytoplankton *Rhodomonas salina* was the only algae species used, as mentioned in section 6.2. It would be interesting to do a study where the *A. royi* is feed a

different kind of algae or a mixture of different kinds of algaes could give some interesting results. As mentioned in section 5.3 and according to *Pan et al. 2018,* it suggests that there is a relation between different kind of feed and population growth and fecundity.

During the experiments it became clear that the methodology could be improved and streamlined, instead of figuring out what to do along the way. The most precise way to collect and make the different densities was to hand-pick the copepods. It would be beneficial to be able to see the difference between the males, non-ovigerous females, nauplii and copedite and not just the ovigerous females, “adults” and “nauplii”, as described in section 6.1. By knowing these differences and doing it by hand-picking, the amount of every size and gender in the different densities can be known for sure. Furthermore, duplicates and controls would solve the big problem of inconsistencies, seen in sampling and certain populations in both experiments.

11.0 References

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