The mass cultivation potential of the cyclopoid copepod *Apocyclops royi*:

The effect of population density and food type on growth and reproduction

Image: Live adult female *Apocyclops royi* carrying two full egg sacs

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Abstract

Aquaculture has arisen in response to the global demand for fish. Most marine fish, however, need large amounts of live feed at the early life stages i.e. larval stage. Rotifers and *Artemia* are commonly used, but results in various difficulties in terms of cultivation and nutrient inadequacies. Another alternative could be free living copepods, but also here difficulties occur. Cultivation of copepods is a very time, money, and labour consuming job and efforts will have to be made to optimize these processes on an industrial level. This Master's thesis will aim, through experimental work, to find the optimal density that the copepod *Apocyclops royi* can be cultured at without it affecting its fecundity. Furthermore, it will focus on finding alternative food sources to microalgae, here *Rhodomonas salina*, which is a time, money and labour consuming food source to produce. Therefore, we fed the copepod *A. royi* inexpensive, easily maintained, and easily-accessible yeast cells (*Saccharomyces cerevisiae*), to evaluate if it could substitute algae as a food source. We found that fecundity is density dependent, with an optimum performance when the copepods are cultivated at a density of 660 ind. L^{-1}. There does not appear to be any effects on population composition as a result of population density. Furthermore, we found that food type has an effect on the growth rate, as yeast-fed copepods resulted in a significantly lower growth rate than the algae-fed copepods. However, feeding the copepods with algae or yeast did not have an effect on their survival rate. Furthermore, we found no statistical difference in the elemental (CHN) content of the feed or the algae- and yeast-fed copepods. Finally, we found that there was statistically more of the essential fatty acids ALA, ARA, DHA, and EPA in algae than in yeast cells, but no difference in the copepod tissues, except for DHA, which was statistically higher in the yeast-fed copepods. From this we can conclude that the yeast-fed nauplii stage of *A. royi* has the potential to replace the currently used rotifers as feed for first-feeding fish larvae, as they have a superior nutritional value and are of the right size. With this in mind we would recommend that this species is used for mass cultivation for fish larvae in aquaculture. Further studies into illuminating the mechanism behind *A. royi*'s trophic upgrade of short chain fatty acids as well studies on optimising cultivation conditions to maximise the population density for this species are still necessary.

Resume

Aquaculture er opstået som respons på den globale efterspørgsel på fisk. Dog har de fleste havfisk brug for store mængder levende mad i deres tidligste livsstadier dvs. larvestadiet. Hjuldyr og *Artemia* er almindeligt brugt, men resultere i forskellige vanskeligheder in form af kultivering og nærings utilstrækkeligheder. Et alternativ kunne være fritlevende vandlopper, men også her opstår der vanskeligheder. Kultivering af vandlopper er et meget tids-, penge- og arbejdskrævende job og der bør gøres en indsats for at optimere disse processer til et industrielt niveau. Dette kandidatprojekt vil stræbe efter, gennem eksperimentelt arbejde, at finde den optimale densitet som vandloppen *Apocyclops royi* kan kultiveres ved uden at det påvirker dens frugtbarhed. Ydermere vil dette fokusere på at finde en alternativ fødekilde til mikroalgen, her *Rhodomonas salina*, som er både tids-, penge-, og arbejdskrævende fødekilde at producere. Derfor fodre vi vandloppen *A. Royi* med billigt, nemt at vedligeholde og lettilgængeligt gær (*Saccharomyces cerevisiae*), for at kunne evaluere om det kan erstatte alger som fødekilde. Vi fandt at frugtbarheden er densitetsafhængig med optimum på 600 ind. L-1. Det ser ikke ud til at frugtbarheden er påvirket af populations kompositionen. Ydermere fandt vi at føde typen har en effekt på vækstraten, da de gærfodrede vandlopper resulterede i en signifikant langsommere vækstrate end de algefodrede vandlopper. Dog havde det ingen effekt på overlevelsesraten at fodre vandlopperne med enten alger eller gær. Ydermere fandt vi ingen statistisk forskel i det elementelle (CHN) indhold af hverken fødekilderne eller de alge- og gærforede vandlopper. Endeligt fandt vi at der var statistisk mere af de essentielle fedtsyre ALA, ARA, DHA og EPA i algerne end i gæren, men ingen forskel i vandloppernes væv, med undtagelse af DHA, som var statistisk højere i de gærfodrede vandlopper. Af dette kan vi konkludere at nauplii stadiet i de gærfodrede vandlopper af *A. royi* har potentialet til at erstatte det nuværende brug af hjuldyr som føde til første-spisende fiskelarver, da de har en overlegen næringsværdi og den rette størrelse. Med dette på sinde vil vi anbefale at denne art bliver brugt til masse kultivering af fiskelarver i aquaculture. Videre er det nødvendigt at der bliver lavet studier i at oplyse mekanismerne bag *A. royi*s trofiske opgradering af kort fedtsyrekæder såvel som optimere kultiveringsforholdene til at maksimere populationsdensiteten for denne art.

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1. Introduction and Theory

Since 1970 the population sizes of mammals, birds, reptiles, amphibians, and fish have been cut in half on average on a global plan. In terms of the fish population size, the more popular edible fish have been affected even more negatively. This already has a great impact on the human race and will continue to do so, as approximately 3 billion people are dependent on fish as their main source of protein and source of income (WWF, 2015). This has led to the intensification and continued development of aquaculture, commonly referred to as fish farming, to ensure that fish remains a global food and income source. In fact, aquaculture contributes to 58% of the global fish consumption and so far ensures that the average cost of fish does not increase (WWF, 2015). One could imagine then that aquacultures are the solution to the world's overfishing problem, but that is not the whole truth. Aquaculture is the business of "farming" live marine organisms like fish, which require a food source. This introduces the issue of cultivating appropriate live feed for the majority of the marine fish and especially fish at the larval stage (Dhont et al., 2013). Two types of live feed are commonly used; rotifers and the brine shrimp *Artemia*. Rotifers are very suitable due to their size, but have been shown to be problematic, as their cultivation is laborious and unreliable. *Artemia* are very easily accessible and storable but require enrichments in terms of vitamin content and fatty acids. This has led to the introduction of copepods, which have shown to fit the bill in terms of nutrient requirements but are problematic in the sense that the cultivation of large enough quantities is proving difficult and furthermore the food production for the copepods is reported to be very laborious and problematic (Dhont et al., 2013). However, there is steady progress being made towards overcoming these challenges (Abate et al., 2015 Abate et al., 2016).

This leads us to the issue and consequently the aim of this master thesis, which is to optimize the cultivation density of a tropical model, the copepod species *Apocyclops royi*, without affecting the fecundity and finding a cost and labour effective alternative food sources for the copepod. This will hopefully lead to cultivation of copepods on an industrial level to accommodate the need of aquacultures and to make the cultivation of both copepods and their feed less time, labour, and money consuming.

1.1 Aquaculture

The purpose of aquacultures or fish farming is to breed, rear, and harvest fish and other water-bound organisms, and to restore and rebuild population sizes of fish that are at risk of becoming endangered. This technology has been developed as a response to vast overfishing and environmental devastations done to the global bodies of water, which have led to immense decreases in fish population sizes (WWF, 2015; NOAA, accessed 2018). The farming of fish can be done in the open ocean or in ponds, where fish are kept in net pens or tanks, and the farming of fish is done by both large-scale industrial companies but also on small scale by local fish farmers (FAO, accessed 2018; NOAA, accessed 2018). The importance of keeping fish populations, as well as other water-bound organisms, at a healthy size cannot be stressed enough, as fish are an important part of peoples' nutritional intake and thereby its benefits on people's health, their livelihood and income, and for tourism (FAO, accessed 2018; WWF, 2015). Aquacultures therefore have a vast impact on billions of people's life by helping to ensure a healthy fish size population and avoiding extinction of certain fish species, as to enable people to profit from the catching and farming of set fish (FAO, accessed 2018; WWF, 2015).

1.1.1 Essential Fatty Acids

When rearing and breeding fish larvae in aquacultures, some requirements must be met in terms of nutrients. Some of the most important nutrients, which are used as a measurement as to whether or not the feed meets the nutrient requirements, are vitamin E and C, proteins, phospholipids, and fatty acids, more specifically the fatty acids; Alfalinolenic acid (ALA), Arachidonic acid (ARA), Docosahexaenoic acid (DHA), and Eicosapentaenoic acid (EPA) (Izquierdo & Fernandez-Palacios, 1997; Nielsen & Gøtterup, 2017).
ALA, as well as linoleic acid, acts as a precursor to ARA, EPA, and DHA and is therefore an important fatty acid to be present in the live feed for the fish, to enable the fish to further produce ARA, EPA, and DHA (Izquierdo, 2005).
EPA and ARA have been shown to directly affect whether female fish produce pheromones that stimulate male sexual behaviour and thereby potential fertilization. This means that the succession of new fish larvae and that these larvae further on are capable themselves of ensuring the production of new generations can be attributed to these two fatty acids (Izquierdo, 2005). DHA and ARA have been shown to be very important in salinity changes, thereby making them

important for osmotic regulation in the fish larvae (Izquierdo, 2005). EPA and DHA have been shown to increase overall survival, growth, and pigmentation in fish larvae. A specific ratio of 1:5:10 of ARA, EPA, and DHA has been proven very favourable for some fish larvae and shows to be of great importance in neurological activity, membrane functionality, and cell signalling. Furthermore, a ratio of 2:1 or more of DHA:EPA have shown to be the optimal general rule, when assessing the nutritional value of live feed and its suitability for fish larvae (Payne & Rippingale, 2001; Rayner, 2017).

Although these are just a few of the biological factors that are affected by these fatty acids, it is apparent how important fatty acids are for the fish larvae and further how they can be used as a measurement tool for assessing the viability of the fish larvae. These fatty acids, ALA, ARA, EPA, DHA, and the DHA:EPA ratio will therefore be the focus in this Master's thesis as a tool for assessing the fatty acid profile of copepods fed different food types.

1.1.2 Live feed - the standard feed Rotifers and *Artemia*

When working with aquacultures and the rearing of fish larvae there are a number of challenges. These include the issue of creating an artificial food web as fish are removed from their natural surroundings and supplies, which requires the cultivation of a food source. This food source has for the last almost 50 years been composed of either rotifers or *Artemia* or both along with various types of dry feed (Dhont et al., 2013; NOAA, accessed 2018a).
Rotifers have been very favourable when dealing with feeding fish at the larval stages, which is due to a number of factors. These include the size of the rotifers, the ability to cultivate them at high densities (20,000,000-160,000,000 ind. L^{-1}) (Yoshimatsu & Hossain, 2014), their high reproductive rate, their tolerance to fluctuating temperatures and salinities, their ability to be easily and quickly enriched with other nutrient supplements, and lastly that they swim slowly which ensures easy capture by the fish larvae (Dhont et al., 2013). However, rotifers have a downside as they are very susceptible to infections by birnavirus and fungii, which means that they have to be disinfected prior to their usage as feed. Furthermore, this susceptibility to infection often results in the entire culture's demise. This issue has been resolved to some extent by having several cultures in different tanks, but unfortunately also results in the need to cultivate the rotifers all year round, even in the periods when larvae are not reared and has therefore become very costly (Dhont et al., 2013).

Artemia have, like rotifers, been a live feed favourite in aquaculture. They are easily cultivated in high salinity in tanks or lakes at densities ranging from 10,000-18,000 *Artemia* larvae L^{-1} (Lavens & Sorgeloos, 1991), and they can be stored for long periods of time. But as with rotifers, they need some enrichment in terms of fatty acids and vitamins, though with today's enrichment technologies this is a lesser issue. However, a downside to *Artemia* is the fluctuating prices of the cysts, which is the dormant pre-embryo of *Artemia*. This fluctuation is due to periodical shortages of the species and therefore results in that the farmers might have to pay extra or change the diet of their fish larvae altogether. These issues with rotifers and *Artemia* have consequently led to the exploration of using copepods as future live feed in aquaculture (Dhont et al., 2013).

1.1.3 Live feed - Copepods

Copepods, or Copepoda as the subclass is called, is comprised of 10 orders, where *A. royi* is of the Cyclopoida order. These small aquatic creatures are crustaceans and are found in both freshwater, brackish water and saltwater. They range in sizes from 0.2mm - 17mm, depending on which of the 11,472 species they are. Depending on their size and life stage, their morphological appearance also differs tremendously. Their diet consists primarily of microalgae cells and in turn they themselves are eaten by juvenile fish and other planktivores (Walter & Boxschall, 2018). Copepods also vary in their way they produce eggs, as some, like *Acartia tonsa*, shed their eggs freely in the water column (Rayner et al., 2016) and others, like *A. royi*, keep their eggs attached to the females in egg sacs where from the nauplii larvae hatch. Copepods are therefore already a substantial part of the natural food web in oceans, lakes, and ponds of the world. This, combined with their high nutritional value, has led aquaculture farmers to investigate the possibilities of utilizing this small crustacean as a live food source for their fish larvae (Dhont et al., 2013).

There have been successful experiments with feeding various fish species diets consisting fully or partially of copepods for several decades. For example, the turbot (*Scophthalmus maximus*), a species of flatfish, showed enhanced feeding rates and hence improved survival and growth when fed a basic rotifer diet supplemented with copepod nauplii as compared to a pure rotifer with *Artemia* diet (Støttrup & Norsker, 1997). The orange-spotted grouper (*Epinephelus coioides*) also showed improved growth and survival, as well as higher feeding incidences, when fed a diet supplemented with rotifers and copepods. This species has been difficult to raise in large numbers

due to the low and unstable rates of survival amongst the grouper larvae, likely due to the smaller mouth size of the larvae that makes it difficult for them to ingest standard feed (Toledo et al., 1999). Copepod nauplii were also shown to be a superior choice of feed in halibut and cod species when compared to rotifers and *Artemia* nauplii on their own (Nielsen et al., 2017; Støttrup et al., 1998).

There are several reasons why a pure copepod diet, or at least a copepod-supplemented diet, leads to these increases in survival among a variety of fish species. Firstly, in wild-caught fish species there is the issue of some not accepting non-living feed and so are at risk of starvation (Live Copepods and Phytoplankton - AlgaeBarn, 2018). The addition of copepods to the diets of these species would in such cases increase the survival of the fish both as a source of nutrition and due to being a suitable size and shape moving in ways that the species can recognise as food. First feeding fish larvae are raptorial in nature but are not yet experienced at recognising prey items. They require prey, such as copepods, of the right size and with the right movement in a manner that triggers their instincts to hunt (Nielsen et al., 2017). Furthermore, an experiment was conducted where it was observed that the actual size preferred by fish larvae was the smallest nauplii in the size range 80-100µm (Højgaard, 2018: unpublished manuscript 4)

Secondly, it is also partially due to the increased levels of polyunsaturated fatty acids (PUFAs) present in copepod tissue. The quality and quantity of n-3 PUFAs in feed has been shown to improve survival and growth in marine fish larvae (Toledo et al., 1999; Watanabe et al., 1983), but these lipids are not present in as high amounts in the standard feed species – rotifers and *Artemia* (Rainuzzo et al., 1997). As such, these nauplii are usually enriched with marine oil emulsions to ensure they are nutritionally adequate. However, the selective catabolism of fatty acids by *Artemia* reduces the efficiency of this method by degrading and converting the oils, and so this enrichment process needs to be done by the user in 12-hour intervals prior to feeding to the fish larvae (Sorgeloos et al., 2001). Still, rotifers and *Artemia* are very easy and relatively cheap to cultivate in large numbers as they tolerate high population densities, so they are currently preferred by the aquaculture industry (Støttrup et al., 1998).

1.1.4 Aquaculture cultivation - current and ongoing challenges

Aquaculture is a rapidly growing industry, and since 2014 provides half of the fish that humans consume (FAO, 2016). With the industry moving away from fishing of wild population and towards more sustainable aquaculture practices, there is a greater chance that we can continue to provide sufficient levels of seafood to an ever-growing population. However, the survival and feeding of first-feeding fish larvae is proving to be one of the supply bottlenecks. Copepods, along with krill, have been considered as a feed supplement due to their high PUFA levels. They could potentially replace the current standard live feed items, rotifers and *Artemia* enriched with fishmeal and fish oil, as this option steadily grows more expensive. However, copepods are currently considered too costly to add to the fish feed as a substitute (FAO, 2016). There are a number of issues still preventing the wholehearted adoption of copepods by the aquaculture industry. These include, but are not limited to; the cost of producing live algae as copepod feed, the work necessary to train individuals in copepod rearing and how to harvest the correct copepod size fractions, and technical challenges such as the maintenance of water quality, salinity, and temperature, and the transport of the copepods, most likely as stored eggs, to the end user (Drillet et al., 2011; Lee et al., 2008; Nielsen et al., 2017).

Due to copepods having a proven nutritional value and size availability that suits different fish larvae, the interest in cultivating copepods commercially has been growing steadily over the last few decades. Live algae as food for the copepods is one of the issues delaying this commercialisation (Drillet et al., 2011; Vu et al., 2017). Wild copepod populations, as well as most other zooplankton species, feed off all algae present in the water column, including the algae produced in algal blooms. At least 20 different species of algae have been found to be suitable for copepods in culture due to their high growth rates, salinity tolerance, and nutritional profile (Brown, 2002). While most primitive copepod production sites induce wild algal blooms in ponds, optimisation of the copepod production will require a similar optimisation of algal production. Other types of feed, such as lipid emulsions, flour, yeast and bacteria, have been used with mixed success depending on the species of copepod (Drillet et al., 2011) and currently live algae is seen as the most reliable and common type of food despite the costs involved (Brown, 2002; Nielsen et al., 2017).

In addition to cultivating copepods as live prey items for fish larvae, great efforts have been put into developing methods of storing the copepod eggs for later use. These have proved somewhat successful, with *A. tonsa* eggs being kept in anoxic cold (4°C) for 6-11 months and still achieving up to 70% hatching success (Drillet et al., 2006; Hansen et al., 2016; Hansen et al., 2016a). However, this method still needs some work, as storing eggs for longer than this period of time compromises the fatty acid profile in a negative way (Dhont et al., 2013). Furthermore, this method of storing is not directly transferable to *A. royi* as *A. tonsa* spawn their eggs in the water column prior to hatching unlike the egg-carrying *A. royi* that hatch their eggs directly from the egg sacs (Miller & Marcus, 1994; Støttrup, 2003).

Copepods that are cultivated as live prey for fish larvae can be cultivated via a range of methods. One such method has the copepods kept in the same ponds as the fish larvae and algae, called "endogenous" production. This method requires that the pond is fertilized, ensuring algae blooms which the copepods can eat (Lee et al., 2008). Although this method requires fewer facilities, as copepods, fish larvae and algae are produced and collected in the one pond, it does have some challenges in terms of external and abiotic factors easily affecting the ponds. This, for example, can refer to rain entering and lowering the salinity of the pond, or high temperatures that make the water evaporate and thereby enhancing the salinity, which can affect both the copepods productivity and fertility (Pan et al., 2016).

Another method used to produce large amount of specific copepods species, is the "exogenous" production, where the copepods are cultivated elsewhere in a controlled environment i.e. abiotic factors such as salinity, temperature, aeration, etc. are controlled. Furthermore, this method of cultivating copepods in large amount brings issues like water quality having to be kept at certain level, algae having to be cultivated and added to the copepods' tanks, which all adds to the amount of labour and therefore person-hours (Boyd & Tucker, 1998; Lee et al., 2008).

The final issues with cultivating copepods are the bulk harvesting and harvesting copepods in specific sizes. Different fish larvae need specific sizes of live prey and furthermore as the fish larvae grow the size of the life prey has to follow. Therefore, getting the copepods in the right sizes requires a vast knowledge of their life cycle, so that the harvest is done at the right time to acquire the largest amount of the right sized copepods (Højgaard, 2018).

Whether one chooses to utilise endogenous or exogenous production for copepod live prey cultivating for fish larvae, one is met with some challenges, challenges that will have to be considered before utilising the method best suited for the user's needs (Drillet et al., 2011).

Two of these challenges include the effects of high densities on the copepods and their fecundity, and the high costs and time involved in producing live algae as feed and therefore trying to find alternative less costly feed types. These two challenges will be investigated in this project.

1.2 The effect of population density on copepod fecundity:

One of the obstacles in aquacultures is the need for live feed for first feeding fish larvae. In addition, this live feed is required in sufficient quantities to accommodate the amount of fish larvae in industrial aquacultures. This means that efforts have to be made to find the highest possible densities that live feed such as copepods can be cultivated at without it causing excessive stress to the copepods that could lead to a drop in fecundity or a total population crash of the copepods (Nilsson et al., 2017; Rayner et al., 2016). Although the article "*Copepod swimming behaviour, respiration, and expression of stress-related genes in response to high stocking densities.*" (Nilsson et al., 2017) has shown that no significant stress is caused to the copepod species *A. tonsa* when kept in densities from 100 ind. L^{-1} gradually raised to 10.000 ind. L^{-1}, this may not apply to all species of copepods.
Besides the possibility of the copepods being stressed by being kept in too dense cultures, another issue could also occur in these high densities when accompanied by food shortage for the copepods; cannibalism. Cannibalism was shown in the article "*Cannibalism in omnivorous calanoid copepods*" (Daan et al. 1988) to account for 5-100% of nauplii mortality and thereby would affect the density and supply of copepods for the fish larvae in aquacultures. Ensuring the copepods are fed *ad libitum* may be a way to circumvent this issue but, as stated previously, live algal production is also costly (Drillet et al., 2011; Vu et al., 2017).

As outlined in the examples above, many issues like fecundity, stress, and cannibalism, will have to be addressed when trying to cultivate copepods in as high population densities as possible. Cultivating the copepods at the highest possible densities is necessary to accommodate the fish larvae's required food amount, and to bring the costs down in terms of labour, algae cultivation for

copepod feed, as they affect the overall profit of aquaculture production (Abate et al., 2015; Abate et al., 2016; Dhont et al., 2013; Lee et al., 2008).

This leads to the first aim of this Master's thesis; to cultivate *A. royi* in the highest possible densities without it significantly affecting the fecundity of the female copepods.

1.3 The effect of food type on copepod growth and the nutritional value of the feed and copepods:

Another obstacle that aquacultures encounter is to ensure that the live feed that is provided to the fish larvae has a nutritional value that ensures the fish larvae's survival and growth. As mentioned earlier (see 'Live feed - Rotifers and *Artemia*' and 'Live feed - Copepods'), the choice of live feed for the fish larvae has to be of a specific nutritional value, which has so far entailed nutritionally enriching rotifers and *Artemia*. Copepods, however, are much more suited in their nutritional value and therefore need no additional preparation when used as live feed. Furthermore, some species have also been shown to trophically upgrade alpha-linolenic acid (ALA) to long-chain polyunsaturated fatty acids when living on a nutrient poor food regime, thereby adding to and upgrading the source of fatty acids in an otherwise nutrient poor habitat (Nielsen & Gøtterup, 2017). These facts have been further established in the article "*Copepods enhance nutritional status, growth and development in Atlantic cod (*Gadus morhua *L.) larvae - can we identify the underlying factors?*" by Karlsen et al. from 2015 where it was shown that cod larvae benefited greatly in terms of nutritional value and growth when fed copepods as opposed to rotifers and *Artemia*.

In addition to copepods being of appropriate nutritional value for the fish larvae, they also fulfil other needs; movement, visibility/colour and appropriate size. An important part of the live feed is to trigger the fish larvae's sight-based raptorial behaviour, being visible to the fish larvae, while also being the appropriate size for the fish larvae i.e. being able to fit in their mouth (Drillet, accessed 2018; Højgaard, 2018).
Copepods are in these regards a near perfect organism as live feed for the fish larvae as they are their natural prey item, but there is still the issue with the copepods having to be fed microalgae, which is time consuming and financially costly to cultivate. This has led to the exploration of alternative food sources to microalgae such as ordinary baker's yeast (Lee et al., 2005). Using yeast

as an alternative to microalgae would prove to be highly preferable to microalgae as it is easier to store, less time-consuming to produce, and overall involve fewer costs. A possible issue with this could be that the copepod species would be unable to sufficiently 'upgrade' the less nutritious yeast while achieving a similar growth rate and nutritional profile to copepods fed microalgae.

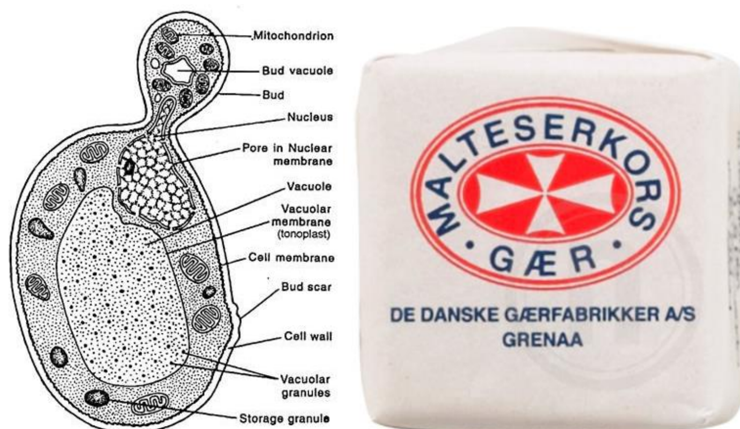
This leads to the second aim of this Master's thesis; the investigation of yeast as an alternative food source to the microalgae *R. salina*, and the effect on copepod growth and nutritional value.

1.3.1 *Rhodomonas salina*:

The algae *Rhodomonas salina* is a cryptophyte, which is a group that encompasses unicellular phytoplankton ranging from 2-30µm in diameter, of various colours, and are capable of photosynthesis due to the presence of chloroplasts in the cell (Khan et al., 2007). Being primary producers, they form an important link in the food web as a source of food for larger aquatic organisms (Azam et al., 1983). Cryptophytes such as *R. salina* have been found in a wide range of aquatic habitats, including marine, brackish, and freshwater areas (Hoef-Emden & Archibald, 2017), and have even been found to bloom in habitats with low light, temperature, and oxygen (Hammer et al., 2002).

R. salina contains the fatty acid precursor ALA and has a high quantity of the fatty acids DHA and EPA when compared to other microalgal species (Støttrup & Jensen, 1990). These fatty acids are a vital part of the copepods' diet as they are necessary for yielding ATP through oxidation and as components incorporated into their cells' phospholipids (Dalsgaard et al., 2003). In turn, the presence of essential fatty acids in the copepods are vital to fish larvae as they cannot synthesise polyunsaturated fatty acids such as DHA or EPA themselves. Since fatty acids form integral parts of the fish's cell membranes and is necessary for their somatic and neural development, the fatty acids must be taken up from their diet (Kainz et al., 2017).

1.3.2 *Saccharomyces cerevisiae:*



Picture 1: Illustration of *Saccharomyces cerevisiae* with budding cell under electron microscope (left) and common baker's yeast used in Denmark (right). (BiologyDiscussion, accessed 2018; Nemlig, accessed 2018)

Saccharomyces cerevisiae (Picture 1) is widely used as a model organism and is also utilised in diverse biotechnological industrial productions (Mapelli, 2016). Furthermore, on a more low-scale production level, *S. cerevisiae* is also often used in both brewing beer and baking bread, hence the common names; baker's or brewer's yeast (Onofre et al., 2017). Due to its multiple uses in different fields, it was suggested that its utilisation could also stretch to the field of aquaculture. More specifically, it could be used as feed for rotifers and copepods, which in turn are used as feed for fish larvae in aquaculture. This investigation into the nutritional value of yeast as feed instead of alga was in part done by James et al. from 1987, where they studied the effect of feeding the rotifer *Brachionus plicatilis* with two types of yeast. The addition of yeast as a supplement to algae at was attempted by Penglase et al. (2011). Their aim was to enrich the rotifers with selenium through the yeast, as to obtain better live feed for the fish larvae. It has therefore already been suggested and shown that yeast as an addition to other feed, has favourable qualities that will heighten the growth and survival rate of the fish larvae (Penglase et al., 2011).

Building on this idea, it should be possible to cultivate the copepod species *Apocyclops royi* exclusively on yeast, which due to the low cost of yeast production would be quite favourable in terms of production expenses and maintenance (Lee et al., 2005). The usage of yeast has also been hypothesized to be of sufficient nutritional food value, as some species of copepods like *A. royi* is thought to be capable of modifying fatty acid by 'upgrading' short chain or precursor fatty acids to the PUFAs that fish larvae require in their diet (Nielsen & Gøtterup, 2017). Yeast therefore does not

have to be of the highest nutritional value for an organism like *A. royi*, as it is capable of compensating and bringing itself, as further live feed for fish larvae, to a sufficient nutritional value that the fish larvae requires.

Yeast is a very versatile organism as its chemical composition and nutritional value, containing essential amino acids, selenium, chromium, nickel, lithium, and both saturated and mono-unsaturated fatty acids, is considered very favourable, so it is not a far stretch to imagine its value as feed for rotifers and some species of copepods such as *A. royi* which is thought to be capable of 'upgrading' less nutritious feed compared to live algae (Lee et al., 2005; Onofre et al., 2017). Although yeast is used in both enrichment processes for *Artemia* and rotifers and as feed in itself for copepods, it has not yet shown to result in copepods with superior nutritional value in comparison to copepods being fed alternative diets such as algae (Dhont et al., 2013; Lee et al., 2005; Penglase et al., 2010).

1.4 The copepod *Apocyclops royi*:

The cyclopoid copepod *Apocyclops royi* is an omnivorous pelagic arthropod that lives in estuaries, brackish water, and freshwater areas (Balian et al., 2008) in both tropical and subtropical regions (Marinespecies.org, 2018). They are capable of surviving in a wide range of salinities and temperatures and are currently one of the only species of copepods being cultured commercially for the aquaculture industry as live feed in Taiwan (Dhanker & Hwang, 2013; Su et al., 1997). It is a highly important organism in certain tropical aquatic ecosystems as a food source for various fish larvae species (Lee et al., 2013; Pan et al., 2016). *A. royi* themselves consume a wide variety of microalgae that are present in their natural environments.
A. royi is also a very important organism in natural environments. This is due to its ability to inhabit nutrient poor environments, which lacks sources of certain fatty acids. This is because they are able to upgrade these short chain fatty acids to long-chain PUFAs, as mentioned earlier (See 'The effect of food type on copepod growth and the nutritional value of the feed and copepods' and '*Saccharomyces cerevisiae*'). It has actually been shown that even in the absence of algae that contain the long-chain fatty acid DHA (C22:6, ω-3), these copepods contained a significant percentage of DHA (19-27% of total fatty acids) in their body. This strongly suggested they were able to synthesize LC-PUFA´s from the short chain fatty acid ALA (C18:3, n-3) that was present in their food source (Nielsen & Gøtterup, 2017; Pan et al., 2017). This has sparked a great interest in

the cultivation of copepods like *A. royi* in high densities and in various sizes to accommodate the different size needs of live prey that the fish larvae in aquacultures require (Dhont et al., 2013).

1.4.1 Life stages:

Like other cyclopoids, *A. royi* is an egg-carrying copepod whose development comprises of six nauplii stages, five copepodite stages, and the final adult stage (Støttrup, 2003). These life stages are divided into morphological differences, length differences and duration of the life stages as seen in Table 1 and 2 (Chang & Lei, 1993).

Table 1: The average duration of the nauplii and copepodite stages, which have been kept and cultivated in a laboratory (modified from Chang & Lei, 1993).

Nauplius stage						
Instar	I	II	III	IV	V	VI
Duration (day)	few minutes	0.5	0.5	0.5	1	1
Copepodite stage						
Instar	I	II	III	IV	V	VI (adult)
Duration (day)	2	2	2	2	2	2

Table 2: The prosome length and full body length (prosome + urosome) of the copepods at different life stages (modified from Chang & Lei, 1993).

Development stage		**Prosome length (µm)**	**Prosome + urosome length (µm)**
Nauplius	I	110.0 ± 8.9	-
	II	122.5 ± 2.9	-
	III	152.5 ± 19.5	-
	IV	209.5 ± 23.2	-
	V	240.3 ± 30.0	-
	VI	265.0 ± 18.2	-

Copepodite	I	294.0 ± 26.1	424.8 ± 42.7
	II	344.3 ± 18.4	513.9 ± 36.1
	III	415.2 ± 45.0	647.9 ± 81.2
	IV	521.1 ± 29.5	846.0 ± 54.7
	V	588.4 ± 11.8	976.5 ± 22.6
(Adult)	VI	618.0 ± 16.9	1034.1 ± 35.6

As is depicted in Table 2, nauplii only have numeric values under "prosome length" as the nauplii only consist of the prosome, otherwise known as the carapace. Copepodites and adults also contain a urosome, which ends in caudal furca (Chang & Lei, 1993). In the present project, when length measurements are described the full body length is used; prosome length for nauplii, and prosome and urosome length but excluding caudal furca for copepodites.

As arthropods, they develop by moulting to shed their rigid chitinous exoskeletons. They have a short generation time compared to other copepod species (≥13 days), and a relatively fast growth rate that appears to be closely linked to temperature, food type, and food availability (Lee et al., 2005). This species reproduces sexually, and females can use the spermatic liquid received from males to fertilise egg clusters that hatch 10-15 nauplii per day (Pan et al., 2016). The females carry the eggs in double egg sacs attached to their first abdominal somite (Støttrup, 2003). The high growth rate and high egg production are some of the reasons why *A. royi* is of interest as live feed for aquaculture.

2. Problem Formulation:

In order to optimise the mass cultivation of the cyclopoid copepod *Apocyclops royi*, further knowledge on important parameters influencing copepod growth, survival, and reproduction are necessary. For this species, it is currently poorly understood;

1. How *A. royi* population density influences fecundity and
2. How food type influences the growth and nutritional value of *A. royi*.

To further our understanding, a number of research questions were investigated for this report.

2.1 Research Questions:

1. Does population density influence fecundity in *A. royi*?
2. What is the highest population density that *A. royi* can be cultivated at without significantly affecting fecundity?
3. Does the population composition change with density?
4. How does food type influence the survival rate of *A. royi*?
5. How does food type influence the growth rate of *A. royi*?
6. How does food type influence the nutritional value of *A. royi*?
7. Can baker's yeast (*Saccharomyces cerevisiae*) be used as a food source for the copepods as compared to the microalgae *Rhodomonas salina*?

3. Methods:

This chapter will provide an overview of the general setup as well as focus on the methods used in our experiments. This will include the effect of population density on fecundity (density experiment), the effect of food type on growth (growth experiment), and the effect of food type on the copepods' nutritional value (nutrition experiment).

3.1 General setup:

3.1.1 Experimental organisms:

The origin of the copepod stock culture used in this current report's experiments is from Tungkang Biotechnology Research Centre in Taiwan, obtained through the LOG-Marine Station of Wimereux in France. All copepods were kept in 0.2µm filtered seawater (FSW) in a climate-controlled room at 25°C and under a 12:12 light/dark cycle. The *R. salina* was sourced from a stock culture at Roskilde University and kept in plastic bags filled with approximately 10-15L of 20 or 32 psu FSW containing F/2 growth media in a climate-controlled room at 20°C with constant light between 60-100 µmol PAR photons m^{-2} s^{-1} (Vu et al., 2016). A detailed cultivation and maintenance protocol is specified in Appendix A. The *S. cerevisiae* was sourced from the yeast production company De Danske Gærfabrikker A/S through a local supermarket and stored in a fridge.

3.1.2 Procurement of copepods from stock cultures:

Prior to each experiment, the copepods were removed from the stock culture bucket by sieving the stock population through a 10 µm mesh to catch all individuals or acquired in the size fraction of 10 µm-100 µm to catch nauplii, which was done by having a 10 µm sieve attached under a 100 µm sieve. The caught individuals were gently transferred to a 1L flask filled with aerated FSW. A 10 ml Kipp (NS29, 2/32; Buch & Holm, Witeg, Germany) was added to the top of the flask, the copepods were evenly distributed in the water by gently upending the flask at least three times, and three replicates of the Kipps were measured out to calculate the average number of copepods per Kipp. As this species is pelagic, there may be some daily vertical migration, but as the copepods are gently swirled to evenly distribute them in the containers, this should not have an effect on sampling. Acid Lugol solution in a final concentration of 10% was added to each replicate, and the

individuals were counted. Seawater was added or sieved out to achieve concentrations that allowed for even addition of the Kipps to achieve the desired copepod concentrations throughout the experimental setups. It should be noted that there is an approximate 10% error margin in density determination when using the Kipp method (Hansen et al., 2009).

3.1.3 Determination of food particle size and concentration:

The cell count and cell volume for both the yeast and algae were calculated using a Coulter Counter (Multisizer 4e Particle Analyzer, Beckman Coulter Counter) for the purposes of calculating the amount of feed for the copepods and for preparing samples for the CHN and fatty acid analyses of the feed particles.

3.1.4 Counting and imaging:

The copepods in the replicates were fixed with acid Lugol, as the copepods were too fast to allow accurate counting without immobilising them. The densities of copepods were calculated by systematically counting all individuals in etched petri dishes with a clicker counter under a dissecting microscope. Population composition was conducted for all individuals and copepod size was conducted for 25 individuals per sample when possible were counted and measured under Nikon SMZ18 camera which uploaded the images to a NIS Elements laboratory imaging software version 4.40 system.

3.1.5 Aeration:

Each replicate was provided with an air hose with a glass pipette end to gently aerate the water and to ensure suspension of the food particles. The beakers for the density experiment were covered with a clear film lid with a hole to let out CO_2 due to their large openings to prevent contamination, while this was left off for the much smaller 77 mL cell culture treatments flasks used in the growth experiment.

3.1.6 Salinity

The copepods in the density experiment were kept in 32 psu 0.2µm FSW, while the copepods in the growth experiment were kept in 20 psu 0.2µm FSW.

3.2 Effect of population density on fecundity (density experiment):

At the beginning of the experimental process a number of trial runs were done in order to familiarise ourselves with the methods and techniques necessary. A number of difficulties arose during the course of these trial runs, the details of which are included in Appendix B for clarification but will be excluded in this chapter for the purpose of brevity.

3.2.1 Densities:

The purpose of this experiment was to investigate the effect of copepod density on the fecundity of *A. royi* adult females over a period of 96 hours. To this end, five different initial copepod densities were chosen: 200, 400, 800, 1200, and 1600 individuals L^{-1}, with four replicates per copepod concentration for a total of 20 replicates. The copepods were kept in 800mL glass beakers containing 500mL of FSW at 32psu (Picture 2). These densities were chosen in order to be in line with previous research on the co-occurring calanoid copepod *Pseudodiaptomus annandalei* (Blanda et al., 2015; Rayner et al., 2016) on the effect of density on copepods, to see if *A. royi* is subjected to similar density limitations. One of the replicates was fixed on the initial day, to determine the initial copepod density. The final three replicates were incubated for 96 hours and finally fixed with approximately three drops of 10% acid Lugol. For all replicates, the total number of individuals were counted for all replicates, as well as life stage composition counts for the number of nauplii, copepodites, males, females without egg sacs, females with egg sacs, and free egg sacs.

Picture 2: The experimental setup testing the effects of copepod density on fecundity, with three replicates for each concentration provided with air hoses.

3.2.2 Feeding:

The copepods were fed with *R. salina* each day of the experiment. Three samples of the *R. salina* stock culture were taken and diluted 20 times and placed in the Coulter Counter to determine the algae concentration and to calculate the required amount of algae to add to the experimental beakers.

Previous studies show (Berggreen et al., 1988; van Someren Gréve et al., unpublished) that the copepod egg production reaches a maximum when fed at least 50,000 cells (*R. salina*) mL^{-1}. Each day a minimum amount of cells roughly equivalent to 50,000 cells mL^{-1} were added to each beaker to feed the copepods after water was removed via a 10µm sieve from the beakers, as to keep the volume at 1L throughout the experiment's duration. This amount was found to be excessive at the

lower concentrations and insufficient at the higher concentrations during the trial runs. Based on these trial run observations, the amount of feed was adjusted to the various copepod concentrations, which were fed amounts that resulted in minimal amounts of sedimentation of algae after 24 hours, while still achieving a light red colour to the water. This technique ensured sufficient microalgae availability for the copepods, i.e. *ad libitum* feeding.

The experiment was repeated with other copepod concentrations (50, 100, 400, 600, 2000, and 4000 ind. L^{-1}) as to examine a wider spectrum of concentrations and to see if the more extreme low and high concentrations would have a greater effect on the fecundity of the adult female copepods.

3.3 Effect of food type on growth (growth experiment)

No trial runs were necessary at the start of the feeding experiment, due to sufficient handling experience from the previous experiment.

The purpose of this experiment was to investigate the effect of feeding *A. royi* with either algae (*R. salina*) or yeast (*S. cerevisiae*) on survival and growth. To this end, 70 x 77 mL cell culture treatments flasks were set up in a climate-controlled room at 25°C. These were divided into two treatments of 35 cell culture treatments flasks per treatment; one treatment where the copepods were fed algae and one treatment where the copepods were fed yeast. In each treatment, five flasks were grouped together in a lot resulting in 5x7 lots per treatment (Picture 3). In each cell culture treatments flask, 50-60 nauplii were added through sieving the size fraction 10µm-100µm, i.e. the nauplii life stages. The densities were measured out using a 10 mL Kipp, as described above in the "Density-dependent fecundity setup". The copepods were acclimated to and kept in FSW at 20 psu for this experiment. All equipment for the two treatments were kept separate and cleaned thoroughly and regularly to avoid cross-contamination with food particles.
Each day a set of five cell culture treatments flasks per treatment were fixed with 10% Lugol and poured into petri dishes for a total of 10 dishes per day. 25 pictures of the copepods per petri dish were taken and the length of their bodies were measured (prosome only for nauplii and prosome+urosome for copepodites) to determine copepod growth. The total amount of copepods in the petri dish was counted using a clicker counter in the different treatments over six days (144 hours) to record mortality during the experiment.

3.3.1 Feeding:

Each day the cell culture treatments flasks had 25 mL of water removed (half the total volume), which was done with a disposable 50 mL syringe connected to an air hose that had a 15 µm mesh attached to the end. To avoid removing copepods during this process, a slightly higher volume was extracted using the syringe, to enable us to dislodge any copepods on the filter by reversing the water flow. The removed water was replaced with 25 mL fresh 20 psu 0.2µm FSW mixed with algae or yeast. We had found from previous articles that algae-fed copepods were to be fed a minimum of 50,000 cells mL^{-1} per day, so we calculated from a CHN analysis of yeast and algae that to provide the copepods fed on yeast with the same amount of carbon as those of the copepods fed algae, we should make a solution of 1L 20 psu 0.2µm FSW with 0.068g of yeast to achieve an equivalent level of carbon content (Appendix C).

Picture 3: The experimental setup for testing the effect of food type on copepod growth, with the division of the two feeding treatments; yeast (top left corner) and algae (lower right corner). The picture also shows the air hoses in the cell culture flasks as well as the syringe with a 15 µm mesh attached to the end, which was used to remove and add water and food.

Due to lack of experience in identifying live vs. deceased copepods before adding 10% acid Lugol, a mortality rate count was not carried out, and the eventual decrease in total amount of copepods per petri dish could therefore be due to handling, mortality, or both. However, both treatments were treated the same way so the decrease between treatment groups should represent a valid internal logic in the experiment.

3.4 Effect of food type on nutritional value (nutrition experiment):

To investigate the nutritional value of feeding the copepod *A. royi*, with either yeast (*S. cerevisiae*) or algae (*R. salina*), samples were taken directly from the stock cultures that were kept at RUC for the duration of this project. These samples were made for an elemental and lipid analysis, as described further in the following section.

3.4.1 Elemental analysis (CHN)

The CHN samples were done with the intent to determine the carbon content of the algae and yeast. This was done to ensure the same carbon content in the growth experiment, i.e. that the same amount of carbon in the particle volume was fed to the copepods fed with yeast as the copepods fed algae. Furthermore, it was used to determine the CHN profile of copepods fed yeast vs. copepods fed algae to see if the CHN profile of the yeast-fed copepods had changed in comparison to the algae-fed copepods.

A known number of food cells were filtered down on a muffled 1.28 cm GF/C filter. The cell number was given by counting cell concentration on the Coulter Counter. From there the known volume (mL) of algae and yeast was filtered down on the filter in three replicates. We ensured that we had at least 0.5 mg C of algae cells or yeast cells on each filter (See Appendix D).
When the algae or yeast were filtered, they were packed into a tin capsule (5 x 9mm). First, we packed the filter by folding it, and therefrom placed it into a tin capsule without closing them. They were then placed into a 96 multiwell and dried for 24 hours in an oven at 60°C to remove all excess water. After the samples were dried, the tin capsules were placed into a premade metal block. The tin capsules were then closed using tweezers and a special tool that is made to completely seal the tin capsules, which were put back into the 96 multiwell. Hereafter the 96 multiwell was placed into a desiccator until further processing was made in the CHN machine (Thermo Scientific Flash 2000 Organic Elemental Analyser), that burns the samples, releasing and converting carbon, hydrogen and nitrogen into gasses, which are detected by the machine. A standard curve was hereafter made by the lab technicians and the amount of C, H, and N were read from the standard curve by the lab technicians and the final results were provided to us.

3.4.2 Lipid analysis:

The fatty acid samples were done with the intent of having comparable data to the growth experiment to evaluate if feeding with yeast had any effect on the fatty acid profile of the copepods compared to the fatty acid profile of copepods fed algae. Identical protocols were conducted to prepare the feed samples; algae and yeast.

We made three samples, containing a specific number of individual copepods for each treatment, yeast-fed and algae-fed, and three samples of a specific number of cells of algae and of yeast (See Appendix E). We also prepared 2x3 samples of 20 psu 0.2µm seawater as controls. All samples were filtered onto a 25mm GF/C filter, which were all put in individual pre-muffled 7.5 mL Pyrex vials. The samples were then freeze dried in an Alpha 1-2 LDplus Entry Laboratory Freeze Dryer for Aqueous Samples, Buch & Holm for 24 hours (and until pressure is ≤ 0.06 mbar) to remove any excess water and to crush the cells, releasing the fatty acids. Hereafter we added chloroform: methanol (2:1) until the filter is covered by the liquid (3 mL), which extracts both free and bound fatty acids from the cells. We then added 20µL internal standard (C23-methyl tricosanoate dissolved in chloroform (C23:0)): 2,0 mg C23+43,4 ml chloroform = 46,1 µg mL^{-1}), which would assist us later on when analysing the fatty acid samples by adjusting for lost substrate. This internal standard was kept in the -20°C freezer. The test tubes were capped and kept at -20°C for 24 hours in order to extract lipids. We then transferred as much liquid as possible to a Gas Chromatography (GC) 2mL glass vial. We then dried out all chloroform:methanol solvent by applying a nitrogen gas flow from a needle in the mouth of the GC vial for about 10 min, while sitting in an aluminium block that was set to 60°C. The end of the needle were cleaned and did not touch the sample when dried and we kept an eye on the samples at all time to make sure they did not stay dry too long under the needle. We then added 1 mL of AcOMe/HCl reagent in Toluene; AcOMe adds a Methyl group to the free as well as the bound fatty acids (simultaneously releasing them from e.g. the glycerol backbone of triglycerides), HCl facilitates esterification/transesterification, and Toluene act as a solvent. The reagent is composed by MeOH:Toluene:AcOCl in a volume proportion of 85:66:15. The GC vials were capped and heated in the aluminium block for 2 hours at 95°C in a closed hood. For approximately 15 min before use, the solution of $NaHCO_3$ was bubbled with nitrogen. The vials were cooled down by removing them from the aluminium block. The vials were then de-capped and 500 µL of 5% (W) $NaHCO_3$, which had been bubbled with nitrogen for 15 min,

was added to neutralise the remaining HCl. We then mixed the resulting solution, let it settle and then sucked up the upper phase and transferred it into a clean GC vial. We then extracted again from the first GC vial after adding 500µL Heptane, to retrieve as much of the substrate as possible. We mixed thoroughly, and then sucked up the upper phase and added it to the clean GC vial and did this step once more. We then dried out the content of the new GC vial (the one containing the upper phases) by applying nitrogen gas from a needle in the mouth of the GC vial for about 15 minutes while they were in the 60°C in the aluminium block. Again, the needles were cleaned and did not touch the samples and not kept dried for too long. We finally added exactly 500µL chloroform in the vial, added a cap and stored the sample in a freezer at -20°C until they were ran in Gas Chromatography-Mass Spectrometry (GC-MS) machine (Agilent technologies 5975 Inert Mass Selective Detector and 6890N Network GC System with a 7683B series Injector and a Agilent J&W DB-23 column (length: 60 m, thickness: 250 µm and thickness of inner film: 0.25 µm)), which separates compounds (GC), fragments the compounds, and records the ion fragments (MS). This standard protocol procedure was done to make the samples into Fatty Acid Methyl Esters (FAME), as non-derived fatty acids would get stuck on the column and hinder analysis. The GC-MS was run on Single Ion Monitoring (SIM) procedure with the following ions: m/z = 55, 74, 79, and 81, which are all typical ion fragments of fatty acids. Along the samples, five FAME standard solutions in varying concentrations with a constant concentration of the internal standard (C23:0) were run. From these, calibration curves were created, allowing for accurate determination of the fatty acid contents.

3.5 Statistical analyses:

3.5.1 ANOVA

To analyse our dataset in the density experiment we compare the amount of females with eggs in the different densities.

The ANOVA test compares the means of the several different groups. Here a H_0 hypothesis will state that all the means are the same, i.e. H_0: $\mu1=\mu2=\mu3=$etc., and this hypothesis will then either be proven false or true.
For this dataset, the ANOVA test will be a one-way ANOVA test, as there is only one variable that defines the groups of treatment i.e. number of females with eggs. Calculations for 'ANOVA' were

taken from ‘Statistics for the life Sciences’ by Samuels et al. (2012).

Firstly we find the number of observations per group (n_i), the sum of all observations in each group (sum), the standard deviation (SD), the mean of each group (y_i), the total sum of all observations in all groups (n.), and number of groups (I):

$$n_i = n \; observations \; per \; group$$

$$sum = \sum_{j=1}^{n_i} y_{ij}$$

$$SD = \sqrt{\frac{\sum_{i=1}^{n}(y_i - \bar{\bar{y}})^2}{n_i - 1}}$$

$$y_i = \frac{\sum_{j=1}^{n_i} y_{ij}}{n_i}$$

$$n. = n_1 + n_2 + n_3 + \ldots n_i$$

$$I = n$$

Then we find the total sums of observations, the grand mean ($\bar{\bar{y}}$), and the pooled standard deviation (s^2):

$$Sum \; of \; observations = \sum_{i=1}^{I} \sum_{j=1}^{n_i} y_{i_j}$$

$$\bar{\bar{y}} = \frac{\sum_{i=1}^{I} \sum_{j=1}^{n_i} y_{i_j}}{n. - 1}$$

$$s^2 = \frac{\sum_{i=1}^{I}(n_i - 1)SD_i^2}{\sum_{i=1}^{I}(n_i - 1)}$$

Then we find the sum of squares within (SS (within)), degrees of freedom within groups (Df (within)), and the mean square within groups (MS (within)):

$$SS(within) = \sum_{i=1}^{I}(n_i - 1)SD_i^2$$

$$Df(within) = n. - I$$

$$MS(within) = \frac{SS(within)}{df(within)}$$

Then we find the sum of squares between (SS (between)), degrees of freedom between squares (Df (between)), and the mean square between groups (MS (between)):

$$SS(between) = \sum_{i=1}^{I} n_i(y_i - \bar{\bar{y}})^2$$
$$Df(between) = I - 1$$
$$MS(between) = \frac{SS(between)}{df(between)}$$

Then we find the relationship between sums of squares (SS (total)) and total degrees of freedom (Df (total)):

$$SS(total) = SS(between) + SS(within)$$
$$Df(total) = df(between) + df(within)$$

We then find the F_s and the critical value of the F distribution in a table:

$$F_s = MS(between) + MS(within)$$

Critical values of the F distribution (Table 10): we have a numerator df of 9 and a denominator df of 29, we find that:

$$Table\ 10: F(9{,}29) = 2.22$$
$$0.02 < P > 0.01 \quad : \quad H_0\ is\ false$$

H_0 is false as it does not lie within the 95% confidence interval.

The H_0 hypothesis is refused as the F_s value lies to the right (to the right of the 0.05 tail probability) of the 95% confidence interval of the F_s distribution curve, i.e. not within the 95% interval.

Therefore $H_0: \mu_1 \neq \mu_2 \neq \mu_3$

3.5.2 T-test

To analyse if there is a difference in the CHN and fatty acid profiles between food particles types and copepods fed the two food items, we ran a T-test on the results. The T-test compares the means of two groups. Here a H_0 hypothesis will state that the two means are the same, i.e. H_0: µ1=µ2, and this hypothesis will then either be proven false or true. Calculations for 'T-tests' were taken from 'Statistics for the life Sciences' by Samuels et al. (2012).

As $\overline{y_i} \pm s_i$ is already given to us from our results, we firstly we find the number of observations per group (n), the total sum of all observations in all groups (n.), and degrees of freedom (Df):

$$n_i = n\ observations\ per\ group$$

$$n. = n_1 + n_2 + n_3 + \ldots n_i$$

$$df = n. - 2$$

We can now perform the t-test (t_s):

$$t_s = \frac{(\overline{y_1} - \overline{y_2}) - 0}{SE_{(Y_1 - Y_2)}} = \frac{(\overline{y_1} - \overline{y_2}) - 0}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}}$$

We now look at the table for 'Critical values of the student's t distribution (Table 4) and if our t_s value lies to the right of the 0.025 upper tail probability the H_0 is false. With a df of 4, as that is our degrees of freedom, the numerical value would have to above 2.776, for the H_0 to be false.

3.5.3 Confidence interval for β_1

To analyse if there is a difference between two or more linear regression equation, one can make confidence intervals for both regression equations β_1 value and if an overlap occurs, one can prove that there statically is no difference between the two or more regression equations as a function of their slopes (a). This type of statistical analysis was applied to our logarithmic function of our survival rates, to get a linear function of the survival rate, as a way of determining if the mortality rates of % ovigerous females of the total female population differed when the copepods were fed either algae or yeast. It was also applied to our linear growth rates of the yeast-fed and algae-fed copepods, to estimate if there was a difference in their slopes. Calculations for 'Confidence interval for β_1' were taken from 'Statistics for the life Sciences' by Samuels et al. (2012).

We got Excel to calculate the standard deviations and from there we calculated the variance of the residuals (s_e):

$$s_e = \sum_{i=1}^{I} \sum_{j=1}^{n_i} (y_i - y_m)^2$$

Then we find the standard error of slope (SE_a):

$$SE_a = \frac{s_e}{s_x \sqrt{n-1}}$$

We then find a, which is calculated from the linear logarithmic regression equation (See Appendix F):

$$y = ax + b \ = \ a = \frac{y}{(x+b)}$$

We then find the degrees of freedom (Df):

$$Df = n - 2$$

We then look in the table for 'Critical values of the student's t distribution (Table 4) and find the 0.025 upper tail probability ($t_{0.025}$) from our Df and we find the 95% confidence interval of β_1:

$$\beta_1 = a \pm \ t_{0.025} SE_a$$

These calculations are done for both regression equations and if there occurs an overlap between the 95% confidence interval there is a 95% statistical significance that there is no difference between the two regression equations.

3.5.4 Sigmoidal curve

The fecundity vs. copepod density is modelled by a sigmoidal curve is fitted to the observational data using the software SigmaPlot 13.0, based on the Holling Type III model. This model is adapted from clearance vs. food concentration observations in the copepod *Temora longicornis* as reported in Schultz & Kiørboe (2009). The reason for choosing this model is that the fecundity is following a pattern with a threshold value as often shown for functional response data and fits relatively well with the dataset though it does not give a biological explanation for the results. The below formula is the adapted version used for the present report.

$$G = \left(\frac{ab}{x}\right) e^{1-\frac{a}{x}}$$

Where,

G = female ovigerous rate (%)

x = copepod concentration (n copepods L^{-1})

a = the copepod concentration at which the ovigerous rate is maximum (n copepods L^{-1})

b= coefficient (deduced from the software)

abe^1 equals the maximum ovigerous rate

The purpose of fitting this sigmoidal curve to the experimental data is to be able to estimate the copepod concentration that yields the maximum ovigerous rate, and to be able to estimate the maximum ovigerous rate.

3.6 Methodological considerations - the ‘bottle effect’

When conducting experiments with live organisms that are removed from their natural habitat, in the case of *A. royi* it means that they are removed from their natural habitat in the ocean or in ponds and transferred into smaller and unnatural environments, one should consider the effects this can have on the organism’s performance. This has in some instances been dealt with in terms of supplying the containers that the copepods were in with air, feed, light and dark, changing of the water, salinity, temperature, etc. (See ‘Effect of population density on fecundity’ and ‘Effect of food type on growth’). However, one of the effects that come with moving the copepods into small containers like beakers or cell culture flasks is known as the ‘bottle effect’ (Jungbluth et al., 2017). The bottle effect has been shown to have a negative impact on e.g. ingestion in some organisms and could be explained by the organisms being crowded, having interactions with the container walls, or differing nutrient and light supply (Jungbluth et al., 2017). Therefore, it is suggested that these kinds of experiments, where the organisms are in small containers, are run for the lowest possible time period as results could vary greatly and the lack of ingestion by the organism could potentially lead to a higher mortality rate than observed by the organisms in their natural habitat (Jungbluth et al., 2017). In the present case however, this necessary compromise provides our data with an internal logic-enabling comparison between treatments, so perspectives on the data cannot be extrapolated to natural settings.

4. Results

4.1 The effect of population density on copepod fecundity

There does not appear to be an effect on the population composition of the copepod *A. royi* when the population density increases from ~50 to ~4000 ind. L^{-1}. As shown in Figure 1, the nauplii make up approximately 29.4% of the population and the copepodites make up approximately 34.4% of the population. However, there is a considerable amount of variability as evidenced by the high standard deviations from these means (±13.5 and ±10.1 respectively).

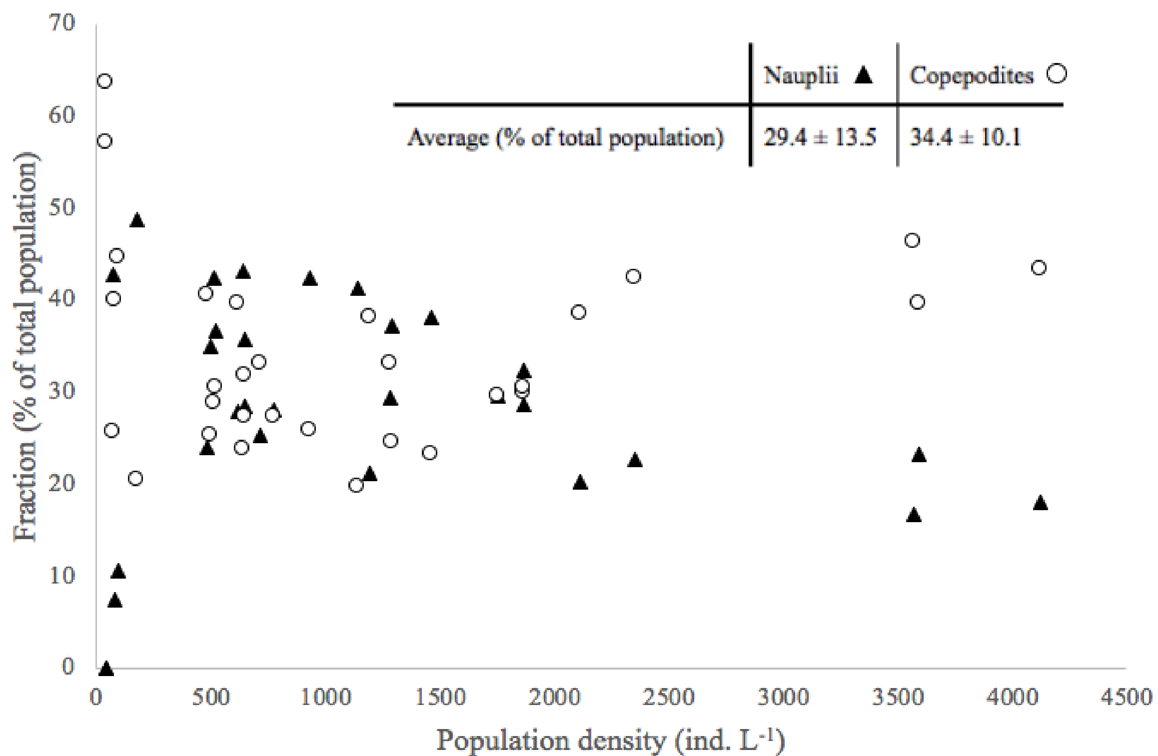


Figure 1: The population composition for the non-reproductive life stages of the copepod *A. royi* (nauplii (black triangle) and copepodites (white circle)) as a fraction (% of total population) of a function of the population density (ind. L^{-1}).

As shown in Figure 2, the males make up approximately 21.6% of the population and the total amount of females make up approximately 14.6% of the population. However, here there is also considerable amount of variability as shown by the high standard deviations from these means (±6.7 and ±4.1 respectively).

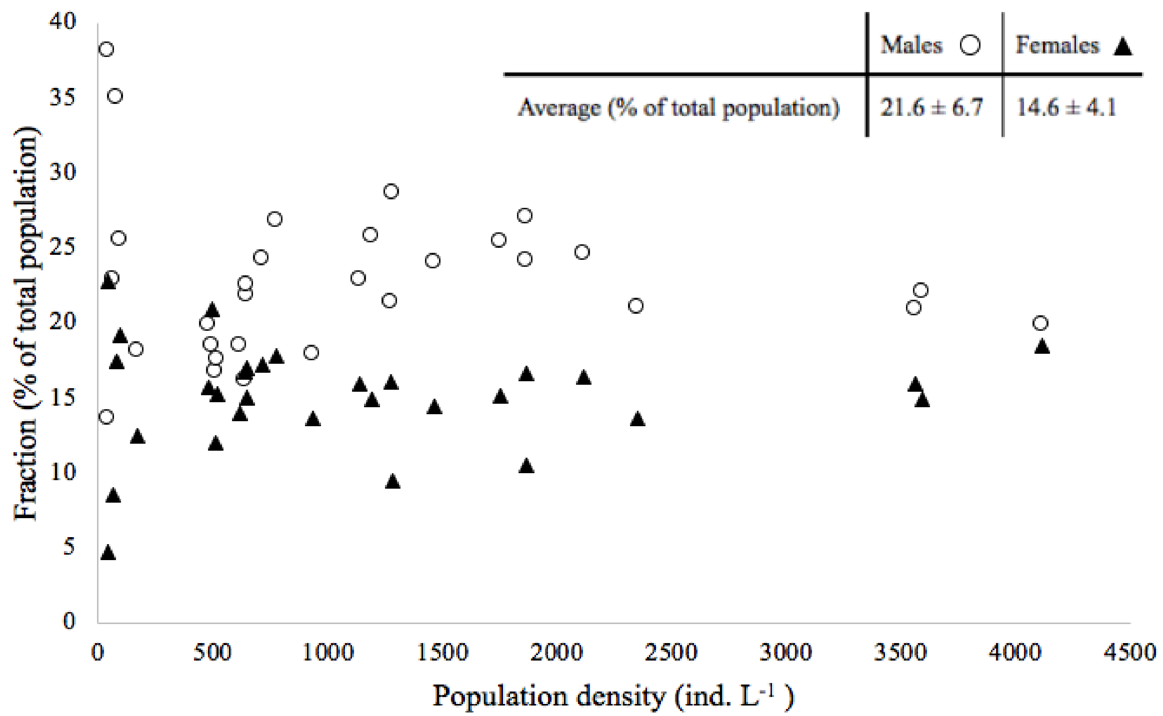


Figure 2: The population composition for the reproductive life stages of the copepod *A. royi* (males (white circle) and females (black triangle)) as a fraction (% of total population) as a function of the population density (ind. L^{-1}).

As shown in Figure 3, the females with egg sacs make up approximately 5.2% of the population and the females without egg sacs make up approximately 9.4% of the population. However, there is yet again a considerable amount of variability as evidenced by the high standard deviations from these means (±2.6 and ±3.8 respectively).

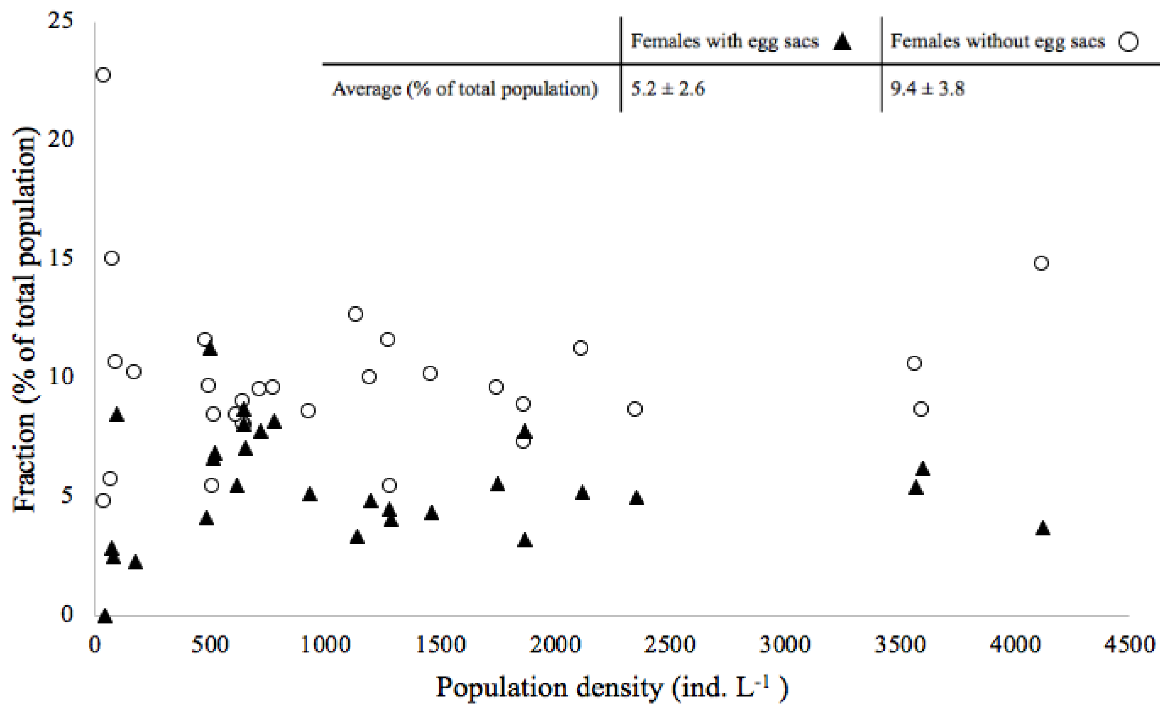


Figure 3: The population composition for the female life stages of the copepod *A. royi* (females with egg sacs (black triangle) and females without egg sacs (white circle)) as a fraction (% of total population) as a function of the population density (ind. L^{-1}).

Though there are differences in the various proportions of the life stages of the copepods, there does not appear to be a trend linked to increasing population density as the means appear to remain relatively stable throughout. However, we are not able to fit a model or line such as a linear regression or exponential curve to this dataset as it does not follow any apparent pattern.

An ANOVA test was carried out, as seen in Table 3, to compare the means of the % of ovigerous females of the total population of females (females with and without egg sacs), which resulted in the $H_0=\mu_1=\mu_2=\mu_3 ...=\mu_n$ being false, i.e. the means are not equal.

Table 3: ANOVA test of the means of the % ovigerous females of the total female population (females with and without egg sacs).

Density (ind. L^{-1})	Average of % ovigerous females of total female population	t_s	$H_0=\mu_1=\mu_2=\mu_3...=\mu_n$
50	11.11 ± 19.25	2.81	False
100	25.64 ± 16.40		
400	42.05 ± 14.49		
600	49.35 ± 3.90		
600	45.92 ± 6.17		
800	34.51 ± 12.41		
1200	30.31 ± 2.22		
1600	36.69 ± 6.01		
2000	38.35 ± 7.73		
4000	31.99 ±10.96		

These results support the observation that fecundity is indeed density-dependent, as there is a statistically significant change in the means across densities with an apparent peak density at 660 ind. L^{-1} (Figure 4). This was calculated by fitting a sigmoidal curve based on the Holling Type III model by Schultz & Kiørboe (2009) to the ovigerous rate of the egg-carrying females as outlined in the Methods: Statistical analyses chapter. While there is still a considerable amount of scatter around the fitted curve, the dataset does appear to follow the general curve.

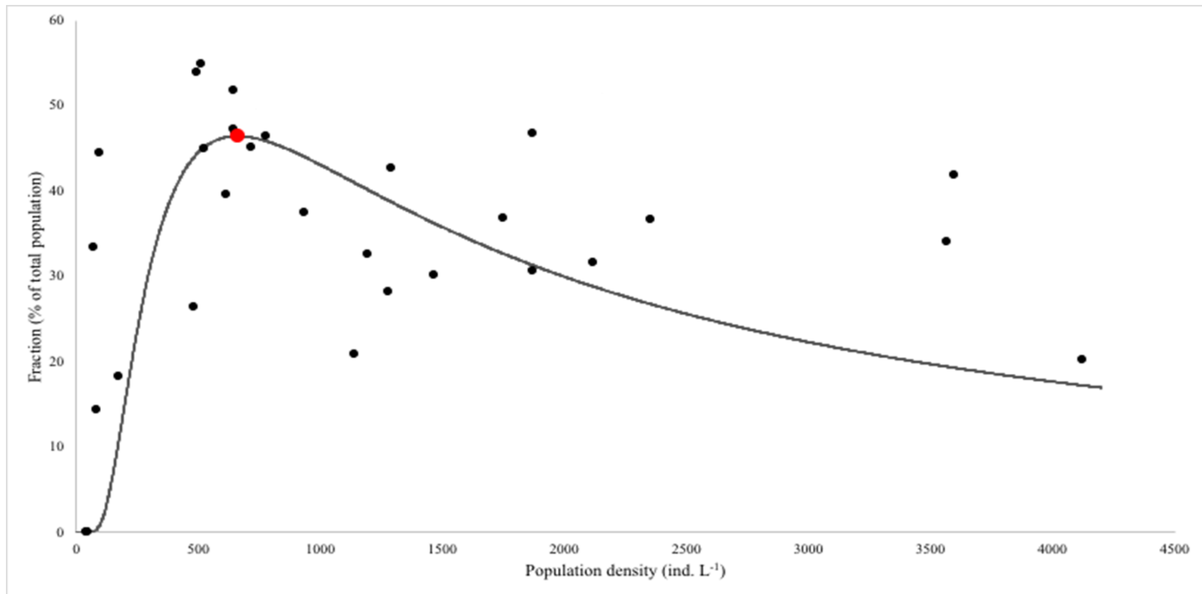


Figure 4: The fraction (% of total female population) of the copepod *A. royi* females with egg sacs (black circles) at the different copepod densities (ind. L^{-1}) as a percentage of the total female population. Fitted with Holling Type III sigmoidal curve (Schultz & Kiørboe, 2009), the red dot marking the estimated population density where the ovigerous rate is at its maximum (660 ind. L^{-1}).

4.2 The effect of food type on copepod growth

A. royi nauplii that were fed a diet of *R. salina* for a period of 144 hours showed faster growth than the nauplii that were fed a diet of *S. cerevisiae* (Figure 5 and 6). The algae-fed nauplii steadily increased in length and started to moult to the copepodite life stage after three days. When the nauplii reached the copepodite life stage, their growth rate increased from 1.4µm h^{-1} to 3.3µm h^{-1}. In contrast, the yeast-fed nauplii increased very slightly in length over the same time period, growing at a rate of 0.25µm h^{-1} and did not moult into the copepodite life stage within the 144 hours. There is a statistically significant difference in the growth rates, as seen from a lack in overlap of the calculated 95% confidence intervals of the growth rates for the faster growing algae-fed nauplii ($0.973 < \beta_{\text{nauplii}} < 1.892$) and copepodites ($2.904 < \beta_{\text{copepodite}} < 3.733$), and the slower-growing yeast-fed nauplii ($-0.043 < \beta_{\text{nauplii}} < 0.546$).

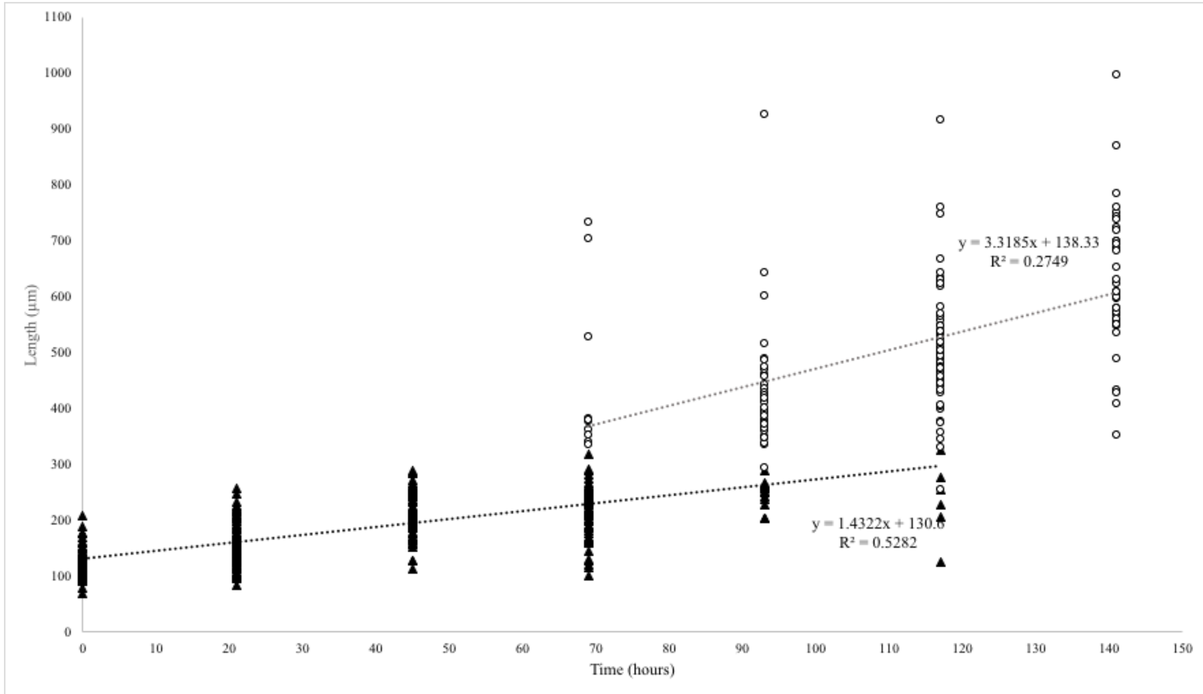


Figure 5: The growth (length (μm) per time unit (hours)) of nauplii (black triangles) and copepodites (white circles) fed on algae for six consecutive days.

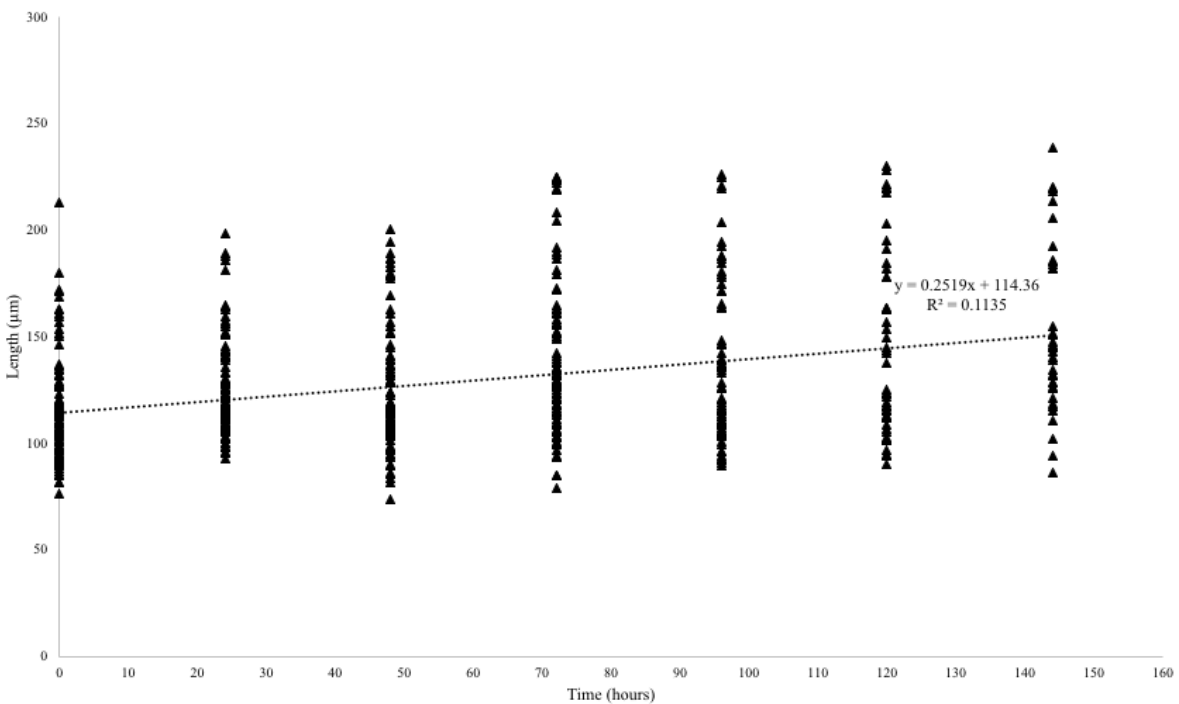


Figure 6: The growth (length (μm) per time unit (hours)) of nauplii (black triangles) fed on yeast for six consecutive days.

The survival rate for the two copepod cultures was measured, with the survival rate of the algae-fed copepods decreasing by 1.7% per hour as compared to a 1.5% decrease per hour for the yeast-fed copepods (Figure 7). However, there is an overlap in the 95% confidence intervals of these rates (Algae-fed copepods: $-1.701 < \beta_{algae} < 1.715$; Yeast-fed copepods: $-2.633 < \beta_{yeast} < 2.646$) when calculated from the linear curve generated by the logarithmic function of the dataset (See Appendix F), so there is no statistically significant difference in the survival rate of copepods when fed either algae or yeast over a period of ~144 hours in *A. royi*.

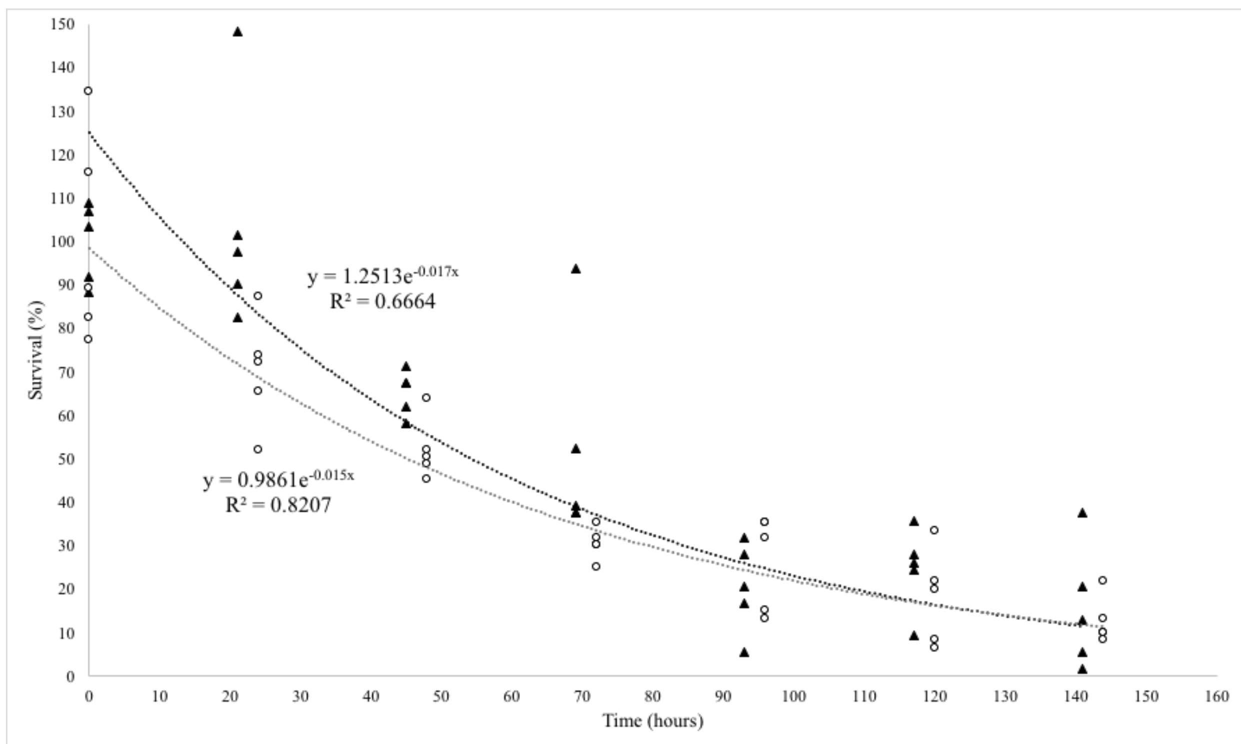


Figure 7: The survival rate of copepods (*A. royi*) fed yeast (*S. cerevisiae* (white circles)) and algae (*R. salina* (black triangles). The survival rate of the algae-fed copepods decreases by 0.017 $hour^{-1}$ (~0.4 day^{-1}), while the survival rate of the yeast-fed copepods decreases by 0.015 $hour^{-1}$ (~0.36 day^{-1}).

4.3 The effect of food type on the nutritional value of copepods

4.3.1 CHN analysis

A CHN analysis was performed on the *R. salina* and *S. cerevisiae* cells, and the copepods fed with these two diets. For the yeast and algae cells, the total amount (mg) of carbon, nitrogen, and hydrogen was calculated per cubic micrometre in order to compare the volume of the two cell types (Table 4). This was necessary as the algae cells were approximately double the size of the yeast cells, making a cell to cell comparison misleading.

Table 4: CHN profile of yeast (*S. cerevisiae*) and algae (*R. salina*).

Carbon	Species	µg C mg^{-1}	cells on filter	pg C cell^{-1}	mg C on filter	mg C / µm^3
	R. salina	373.7	10000000	37.4 ± 1.3	0.37 ± 0.01	0.17 ± 0.01
	S. cerevisiae	436.3	42587141.4	10.2 ± 1.9	0.44 ± 0.08	0.20 ± 004
Nitrogen	Species	µg N mg^{-1}	cells on filter	pg N cell^{-1}	mg N on filter	mg N / µm^3
	R. salina	80.4	10000000	8.04 ± 0.3	0.08 ± 0.003	0.04 ± 0.0012
	S. cerevisiae	67.4	42587141.4	1.58 ± 0.3	0.07 ± 0.01	0.03 ± 0.01
Hydrogen	Species	µg H mg^{-1}	cells on filter	pg H cell^{-1}	mg H on filter	mg H / µm^3
	R. salina	44.1	10000000	4.41 ± 0.2	0.04 ± 0.002	0.02 ± 0.001
	S. cerevisiae	60.4	42587141.4	1.42 ± 0.3	0.06 ± 0.01	0.03 ± 0.01

There was no statistically significant difference found in the CHN contents of the two types of feed (Table 5), so the algae and yeast can be considered equally nutritious to copepods from a standpoint of CHN content when present in equal cell volumes.

Table 5: T-test of CHN profile of yeast (*S. cerevisiae*) and algae (*R. salina*)

	Species	mg / μm^3	t_s	$H_0==\mu_1=\mu_2$
Carbon	*R. salina*	0.17 ± 0.01	1.26	True
	S. cerevisiae	0.2 ± 0.04		
Nitrogen	*R. salina*	0.04 ± 0.001	1.72	True
	S. cerevisiae	0.03 ± 0.01		
Hydrogen	*R. salina*	0.02 ± 0.001	1.72	True
	S. cerevisiae	0.03 ± 0.01		

For the copepods fed with algae and yeast, the total amount (mg) of carbon, nitrogen, and hydrogen was calculated per filter containing the copepods, and in pg copepod^{-1} to allow for easier comparison (Table 6).

Table 6: CHN profile of copepods (*A. royi*) fed yeast (*S. cerevisiae*) and fed algae (*R. salina*).

Carbon	Food Species	µg C mg^{-1}	n copepods on filter	mg C copepod^{-1}	mg C on filter
	R. salina	64.11	43.33	0.0015 ± 0.0002	0.06 ± 0.01
	S. cerevisiae	58.22	45	0.0013 ± 0.0001	0.06 ± 0.005
Nitrogen	Food Species	µg N mg^{-1}	n copepods on filter	mg N copepod^{-1}	mg N on filter
	R. salina	11.75	43.33	0.0003 ± 0.00004	0.01 ± 0.002
	S. cerevisiae	11.64	45	0.0003 ± 0.00002	0.01 ± 0.001
Hydrogen	Food Species	µg H mg^{-1}	n copepods on filter	mg H copepod^{-1}	mg H on filter
	R. salina	7.40	43.33	0.0002 ± 0.00003	0.01 ± 0.001
	S. cerevisiae	8.50	45	0.0002 ± 0.00004	0.01 ± 0.002

There was no statistically significant difference found in the CHN contents of the two types of copepods (Table 7), so the algae- and yeast-fed copepods can be considered equally nutritious to fish larvae from a standpoint of CHN content when present in equal amounts.

Table 7: T-test of CHN profile of copepods fed yeast (*S. cerevisiae*) and fed algae (*R. salina*).

	Food species	mg copepod^{-1}	t_s	$H_0{=}{=}\mu_1{=}\mu_2$
Carbon	*R. salina*	0.0015 ± 0.0002	1.30	True
	S. cerevisiae	0.0013 ± 0.0001		
Nitrogen	*R. salina*	0.0003 ± 0.00004	1.72	True
	S. cerevisiae	0.0003 ± 0.00002		
Hydrogen	*R. salina*	0.0002 ± 0.00003	0.61	True
	S. cerevisiae	0.0002 ± 0.00004		

4.3.2 Lipid analysis:

A fatty acid profile analysis was performed on the *R. salina* and *S. cerevisiae* cells, and the *A. royi* copepods fed with these two diets (Table 8). A set of 34 different fatty acids were included in the analysis, with ALA, ARA, DHA, and EPA being of particular interest due to their role in fish larvae development. The total fatty acid content DHA:EPA ratio was calculated for both the feed (Table 9) and for the two copepod cultures (Table 10). Where no fatty acids were detected in a sample, n.d. (not detected) appears in the table. There was a large amount of variation in the fatty acid levels detected for a number of the samples, leading to some very high standard deviations. This is however just for the rarely occurring fatty acids of minor interest for this project.

Table 8: Fatty acid profile of yeast (*S. cerevisiae*) and algae (*R. salina*), and of copepods (*A. royi*) fed yeast and algae (± SD). Total fatty acid content and DHA:EPA ratio is included (*n.d. = not detected)(** one of the replicates was given double the volume (40µL) of internal standard (C23-methyl tricosanoate dissolved in chloroform (C23:0)) and one replicate was given 0µL of internal standard. As such the replicate with the double volume was divided with 2 and treated as two replicates instead one, to compensate for the replicate that had no internal standard). All values presented for fatty acid results are taken from an average of three replicates.

Fatty acid (%)	**Algae (*R. salina*)**	**Yeast (*S. cerevisiae*)**	**Algae-fed copepods**	**Yeast-fed copepods**
C23:0	n.d.*	n.d.	n.d.	n.d.
C10:0	n.d.	n.d.	n.d.	n.d.
C11:0	n.d.	n.d.	n.d.	n.d.
C12:0	0.01 ± 0.02	0.08 ± 0.07	n.d.	n.d.
C13:0	0.002 ± 0.004	n.d.	n.d.	n.d.
C14:0	3.21 ± 0.73	0.32 ± 0.07	0.26 ± 0.15	0.60 ± 0.26
C14:1	0.16 ± 0.04	n.d.	0.02 ± 0.02	1.15 ± 1.69
C15:0	0.16 ± 0.02	0.12 ± 0.07	0.08 ± 0.02	1.80 ± 1.66
C15:1	0.10 ± 0.01	n.d.	n.d.	1.26 ± 1.25
C16:0	11.69 ± 0.38	7.17 ± 12.42	2.33 ± 0.37	16.35 ± 11.36
C16:1	0.19 ± 0.01	17.85 ± 9.04	0.05 ± 0.02	1.97 ± 3.26
C17:0	0.13 ± 0.002	0.32 ± 0.41	0.37 ± 0.03	5.55 ± 4.91
C17:1	0.05 ± 0.01	4.01 ± 6.53	n.d.	0.39 ± 0.68
C18:0	0.74 ± 0.10	5.40 ± 9.35	1.11 ± 0.75	1.89 ± 3.27
C18:1 trans	0.52 ± 0.02	2.94 ± 3.27	0.05 ± 0.02	0.56 ± 0.78
C18:1 cis	2.52 ± 0.06	16.06 ± 13.05	0.50 ± 0.05	7.35 ± 7.36
C18:2 trans	0.05 ± 0.01	6.76 ± 5.86	n.d.	0.33 ± 0.29

C18:2 cis	6.83 ± 0.06	0.26 ± 0.03	0.62 ± 0.17	1.41 ± 1.37
C18:3 n-6	0.46 ± 0.01	n.d.	0.05 ± 0.01	0.26 ± 0.27
C18:3 n-3 (ALA)	**27.32 ± 0.53**	**0.26 ± 0.03**	**3.15 ± 1.14**	**6.48 ± 8.14**
C18:4	19.31 ± 0.54	n.d.	1.99 ± 0.75	6.98 ± 2.64
C20:0	0.01 ± 0.002	0.66 ± 0.32	0.05 ± 0.03	1.77 ± 1.35
C20:1	0.06 ± 0.01	0.38 ± 0.44	0.05 ± 0.06	0.40 ± 0.53
C20:2	0.02 ± 0.003	n.d.	0.02 ± 0.01	0.30 ± 0.45
C21:0	n.d.	n.d.	0.02 ± 0.01	2.81 ± 3.10
C20:3 n-6	0.02 ± 0.002	n.d.	n.d.	0.02 ± 0.04
C20:4 n-6 (ARA)	**0.39 ± 0.01**	**0.18 ± 0.10**	**0.08 ± 0.02**	**0.22 ± 0.19**
C20:3 n-3	0.07 ± 0.003	n.d.	0.10 ± 0.05	0.28 ± 0.28
C22:0	0.003 ± 0.003	0.06 ± 0.03	0.07 ± 0.02	1.01 ± 1.16
C20:5 n-3 (EPA)	**13.90 ± 0.11**	**2.27 ± 3.93**	**1.80 ± 0.80**	**4.69 ± 4.25**
C22:1	2.05 ± 0.31	26.25 ± 22.73	n.d.	5.30 ± 9.18
C24:0	0.03 ± 0.002	n.d.	0.11 ± 0.01	2.21 ± 2.34
C24:1	0.92 ± 0.80	6.05 ± 10.49	0.03 ± 0.01	2.47 ± 4.10
C22:6 n-3 (DHA)	**9.07 ± 0.47**	**2.61 ± 3.89****	**5.33 ± 1.49**	**24.19 ± 11.63**

Table 9: The total fatty acid content (pg um^3) of the yeast (*S. cerevisiae*) and the algae (*R. salina*) feed, and the DHA:EPA value with standard deviations included for all results. DHA:EPA is the average of the DHA:EPA values for each set of replicates.

	Algae (*R. salina*)	**Yeast (*S. cerevisiae*)**
Total FA (µg / µm^3)	0.09 ± 0.01	0.002 ± 0.0002
DHA:EPA	**0.65 ± 0.03**	**1.15 ± 0.99**

Table 10: The total fatty acid content (mg copepod^{-1}) of the yeast-fed copepods and the algae-fed copepods, and the DHA:EPA value with standard deviations included for all results. DHA:EPA is the average of the DHA:EPA values for each set of replicates.

	Algae-fed copepods	**Yeast-fed copepods**
Total FA (µg copepod^{-1})	469,069.78 ± 97,269.25	242,049.38 ± 305,575.50
DHA:EPA	**2.96 ± 0.70**	**5.16 ± 4.33**

For the *R. salina* and *S. cerevisiae* feed, there was shown to be statistically significantly higher amount of ALA, ARA, EPA, and DHA in the algae than in the yeast. There was no statistically significant difference between them in terms of their DHA:EPA ratio (Table 11).

Table 11: T-test of fatty acid profile of algae (*R. salina*) and yeast (*S. cerevisiae*).

Fatty acid	Species	% of total FA (µg / um^3)	t_s	$H_0 == \mu_1 = \mu_2$
ALA	*R. salina*	27.32 ± 0.53	88.29	False
	S. cerevisiae	026 ± 0.03		
ARA	*R. salina*	0.39 ± 0.01	3.62	False
	S. cerevisiae	0.18 ± 0.1		
DHA	*R. salina*	9.07 ± 0.47	2.86	False
	S. cerevisiae	2.61 ± 3.89		
EPA	*R. salina*	13.9 ± 0.11	5.12	False

	S. cerevisiae	2.27 ± 3.93		
DHA:EPA ratio	*R. salina*	0.65 ± 0.03	0.87	True
	S. cerevisiae	1.15 ± 0.99		

For the *R. salina* and *S. cerevisiae*-fed copepods, there was no significant statistical difference in the amounts of ALA, ARA, and EPA, or in their levels of EPA:DHA. There was statistically significantly more DHA in the yeast-fed copepods than in the algae-fed copepods (Table 12).

Table 12: T-test of fatty acid profile of copepods fed algae (*R. salina*) and copepods fed yeast (*S. cerevisiae*).

Fatty acid	Copepod feed (species)	% of total FA (µg copepod^{-1})	t_s	$H_0=\mu_1=\mu_2$
ALA	*R. salina*	3.15 ± 1.14	0.70	True
	S. cerevisiae	6.48 ± 8.14		
ARA	*R. salina*	0.08 ± 0.2	1.27	True
	S. cerevisiae	0.22 ± 0.19		
DHA	*R. salina*	5.33 ± 1.49	2.79	False
	S. cerevisiae	24.19 ± 11.36		
EPA	*R. salina*	1.8 ± 0.8	1.16	True
	S. cerevisiae	4.69 ± 4.25		
DHA/EPA ratio	*R. salina*	2.96 ± 0.7	0.00002	True
	S. cerevisiae	5.16 ± 4.33		

5. Discussion

The purpose of this report was to further our knowledge about the parameters influencing growth, survival, and reproduction in the copepod *A. royi* in order to optimise its potential for mass cultivation. To this end, we investigated how changes in population density influences the fecundity of *A. royi*, and how food type influences the growth and nutritional value of *A. royi*. Table 13 and 14 provide a brief summary of the results found in this Master's thesis.

5.1 The effect of population density on fecundity:

Table 13: Short overview of density, population composition, and fecundity results.

Population composition	Average (% of total population)
Nauplii	29.4 ± 13.5
Copepodites	34.4 ± 10.1
Males	21.6 ± 6.7
Females	With egg sacs: 5.2 ± 2.6 Without egg sacs: 9.4 ± 3.8 Total: 14.6 ± 4.1
Optimum density for ovigerous females	660 ind. L^{-1}

We found that in our culture of copepods of the species *A. royi*, fecundity is density dependent with an optimum at 660 ind. L^{-1} (Figure 4). Most other copepod studies investigating the effect of population density on fecundity have been with the calanoid copepod *A. tonsa*. Our population density optimum value of 660 ind. L^{-1} is low compared to that found by Vu et al. (2017) for *A. tonsa* Dana. As this species shed their eggs, fecundity is measured by egg harvest instead of the proportion of egg-carrying females. They found that the population density leading to the highest rate of egg harvest was at 5000 ind. L^{-1}, while Drillet et al. (2015) found the optimum egg harvest for *A. tonsa* Dana to be ~2500 ind. L^{-1}. Both results are considerably higher than that for *A. royi*, which could indicate that egg shedding copepods can be kept at higher densities without it affecting the fecundity of the female copepods.

There does not appear to be an effect of density on population composition in this species. All life stages showed a significant amount of scatter around their relative mean with no clear trend, so from this dataset we are drawing the conclusion that increasing density does not change the proportion of life stages present. Some general trends were present: copepodites were present in the highest quantities, followed by nauplii. Males were present in higher numbers than females, possibly due to this adult life stage maturing at a faster rate than that of the female life stage as the males are considerably smaller than full-grown females. This trend of having somewhat similar compositions in all densities is not in line with other findings. Hirst et al., (2010), found that in a laboratory setting i.e. without predation, the copepodite life stage is male-biased and the adult copepod life stage is female-biased, as the female life span is longer than the male life span, for the pelagic species. Furthermore, as no predation occurs in the experiment conducted by Hirst et al., (2010) and in our experiment, a deviance from a 1:1 'Fisherian' male to female sex ratio seen in the copepod populations used for this report would not be due to the presence of any male- or female-biased predation (Gusmão et al., 2013). Furthermore, it has been shown in Voordouw et al. (2005) that there may be a genetic component to the male biased sex ratio as was demonstrated in the harpacticoid copepod *Tigriopus californicus*, which could be an explanation for why we see more males in our *A. royi* population composition.

We do not assume that cannibalism has occurred during the course of the experiments. During the months-long manipulations and microscopic work involved in counting the copepods, we did not observe adults grasping eggs or nauplii, any fractions of copepods, or partial eggs sacs. This would be expected if cannibalism was present. Cannibalism of eggs has been shown to be a significant issue in the calanoid copepod *A. tonsa*, where up to 90% of eggs are consumed as they are shed and sink to the sediment (Vu et al., 2017). This was reduced to negligible levels when water disturbance was decreased so the eggs would sink faster as well as increasing the food concentration. As *A. royi* females carry their eggs, egg predation after the eggs are shed would not be an issue. Furthermore, the microalgae *ad libitum* feeding regime reduced any possible occurrence of cannibalism.

5.2 The effect of food type on growth:

Table 14: Short overview of growth and nutrition results (*Indicates there was a statistical difference found between *R. salina* and *S. cerevisiae*)

	Growth rate	Life stages reached	Survival rate	C:H:N	Fatty acids
R. salina (feed)	-	-	-	187 : 22 : 40	*ALA, *ARA, *DHA, and *EPA (all higher)
S. cerevisiae (feed)	-	-	-	436 : 67 : 60	-
R. salina (copepods)	1.4µm h^{-1} (nauplii) 3.3µm h^{-1} (copepodite)	Nauplii + copepodite	-1.7% h^{-1}	64 : 12 : 7	-
S. cerevisiae (copepods)	0.25µm h^{-1} (nauplii)	Nauplii	-1.5% h^{-1}	58 : 12 : 9	* DHA (higher)

In our results we found the yeast-fed copepods grow considerably slower than the algae-fed copepods, and they did not reach the copepodite life stage over the course of the six days, while the algae-fed nauplii reached the copepodite life stage after three days. Also, the yeast-fed nauplii grew at a significantly slower rate than both the algae-fed nauplii and the algae-fed copepodites (Table 5 and 6). This could be due to the significantly lower amounts of ALA, ARA, DHA, and EPA in the yeast feed, which means that actual concentrations of the fatty acids DHA and EPA, which are involved in growth, was at an insufficient amount for the copepods to grow at the same rate as the algae-fed copepods. This halted growth of the yeast-fed copepods could however have a positive aspect as it has been shown that nauplii of approximately 80-100µm is the preferred size of the feed for numerous species of fish larvae (Højgaard, 2018). Furthermore, as the *A. royi* copepods fed either yeast or algae do not statistically differ in their ALA, ARA, or EPA content, but do differ in DHA, with the yeast fed having statistically more DHA, the yeast-fed copepods are both nutritionally and size-wise preferable as live feed for certain sizes of fish larvae. However, as the yeast-fed copepods' growth is halted it also prevents them from growing as fast as the algae-fed copepods, thereby reaching maturity later, which means that their capability of producing new generations of nauplii is reduced. Vice versa the algae-fed copepods reach maturity earlier and are

thereby capable of producing new generations of nauplii quicker, which means that there is a quicker turnover of new nauplii.

We also found that there is no statistical difference in the decline in survival rate between copepods fed the two items, meaning that although yeast is statistically a less nutritional food source for the copepods, it does not affect the overall survival of the copepods compared to the copepods fed algae for the first six days. The survival rate of the algae-fed copepods decreased by 0.017 $hour^{-1}$ (~0.4 day^{-1}), while the survival rate of the yeast-fed copepods decreased by 0.015 $hour^{-1}$ (~0.36 day^{-1}). We were unable to elucidate why the copepods died at a considerably higher rate than otherwise seen in the literature. Bonnet and Carlotti (2001) had mortality rates in the copepod species *Centropages typicus* of 0.01 ind. day^{-1} for nauplii and 0.09 - 0.17 ind. day^{-1} for copepodites fed various algae and diatom diets. *Acartia tranteri* was shown to have mortality rates of 0.16 ind. day^{-1} for the early nauplii stages and 0.02 ind. day^{-1} for the later nauplii stages (Kimmerer & McKinnon, 1987), so the mortality rates seen in the *A. royi* nauplii were far higher than expected. In the density experiment we kept the copepods in densities as high as 4000 ind. L^{-1} in 1L beakers compared to the 1000 ind. L^{-1} in 77 mL cell culture flasks in the growth experiment, where we saw no discernible increases in mortality during the experiment. This high mortality in the growth experiment could therefore be due to the 'bottle effect' (See 'Methodological considerations-the 'bottle effect''), which could have affected the copepods in such a negative way that their overall survival was affected.

The two continuous copepod stock cultures that were maintained for months in the climate-controlled rooms produced sufficient numbers to run both the density, the growth, and the nutrition experiments. However, while the growth experiment produced a very low growth rate for the yeast-fed copepods, we did see copepodites and adult life stages in our continuous yeast culture, indicating that the yeast-fed copepods in fact are able to reach the copepodite and adult life stages. Therefore, while the conclusion that algae-fed copepods grow faster than the yeast-fed copepods can be considered reliable, the result could vary if the experiment was repeated in larger volumes of water and for a longer period of time, as was the case in the continuous culture buckets. A certain 'bottle effect' may therefore also have had an impact on the growth results in the small incubation volumes used, as we suspect it had on the survival rate.

When cultivating copepods as live feed for fish larvae, size and nutritional value is of great importance, but a third thing that is also important is the visibility of the copepods as the fish larvae's raptorial instinct is triggered by both the copepods movement, by the prey being alive, but also by being visible (Højgaard, 2018). Here we would argue that the algae-fed copepods would be superior to the yeast-fed copepods as the gut content of the algae-fed copepods is more visible than that of the yeast-fed. It would therefore be easier for the fish larvae to detect the algae-fed nauplii and thereby ensuring that the fish larvae feed and get their required nutrients.

5.3 The effect of food type on nutritional value:

We found that neither yeast vs. algae nor yeast-fed copepods vs. algae-fed copepods differed statistically in their CHN content. This means that from an elemental standpoint both the feed and the copepods fed either algae or yeast are equally favourable.

We did however find that although algae contained a statistically higher amount of ALA, ARA, DHA, and EPA, the nutrition experiment resulted in no statistically significant difference in the amounts of ALA, ARA, and EPA in the yeast-fed and algae-fed copepods. This means that although yeast is clearly a lesser nutritional source for the copepods it apparently does not affect the overall nutritional value of the copepods themselves in terms of ALA, ARA, and EPA content. However, the yeast-fed copepods do supersede the algae-fed copepods in terms of DHA content. As shown in Table 9, it appears that the yeast's low total fatty acid content, and lower amounts of ALA, ARA, EPA, and DHA, has an effect on copepod growth. This may be because the yeast-fed copepods are using more resources producing ALA, ARA, DHA and EPA in order to acquire the building blocks necessary to grow. ALA is a precursor for the more complex essential fatty acids ARA, DHA, and EPA, and so the copepod is investing its resources in upgrading its short chain fatty acids rather than into growth. The algae-fed copepods would have had sufficient amounts of the essential fatty acids in their diet, allowing them to grow faster as they did not need to divert resources in the same manner.

The finding that despite the large discrepancy in fatty acid quantity in the feed (algae=0.09$\mu g/\mu m^3$ and yeast=0.002$\mu g/\mu m^3$), both the yeast-fed copepods and the algae-fed copepods did not significantly differ in their nutritional value with the exception of DHA. This suggests that *A. royi* is capable of trophic upgrading their pool of fatty acids. Though the exact mechanism behind this

process is not made clear, it opens up some interesting possibilities in replacing or supplementing copepod feed with less nutritious and cheaper feed. The benefits of using yeast as a replacement for algae are primarily the elimination of the laborious work needed to cultivate live algae. This includes not regularly making growth media for the algae, not diluting the algae to avoid population crashes, and eliminating the water quantity necessary to produce the algae, because one can simply buy a 50g packet of yeast for 1DKK in most shopping centres. This must however be balanced with the trade-off of a markedly decreased growth rate for the yeast-fed copepods.

A similar trend is seen when *A. tonsa* copepods are fed an algal paste made of *Isochrysis galbana*. The algal paste had a superior fatty acid profile in total content of fatty acids (pg cell^{-1}) and in the four essential fatty acids that are of interest to this report, as compared to the live algae (Rayner & Hansen, in press). The paste-fed *A. tonsa* were hence able to maintain a favourable ratio of DHA to EPA like the yeast-fed *A. royi*. However, this also resulted in slower growth in the copepods as similarly seen in the *A. royi* yeast-fed copepods.

5.4 Implications for aquaculture use

When applying our result to the aquaculture industry we see both advantages and disadvantages. As yeast-fed copepods are found to be equally nutritious to algae-fed copepods, and even superior in terms of DHA content than the algae-fed copepods, it is worth considering if the yeast diet should be implemented in the aquaculture industry. To support this claim even further, yeast is as mentioned a cheaper type of feed in terms of time, labour, and overall costs compared to live microalgae. We would therefore suggest that yeast-fed copepods could be used as live feed for first feeding fish larvae for the first few days. This would be favourable as the yeast-fed copepods are of the right size during this period and provides the fish with the superior nutritional value that copepods possess, as opposed to rotifers and *Artemia* (Abate et al., 2015; Højgaard, 2018). This is further substantiated by DiMaggio et al. (2017), who found that the regal blue tang (*Paracanthurus hepatus*) could be successfully cultivated when the fish larvae were fed copepods for the first three days. Previous attempts at cultivating this species had been unsuccessful and therefore speak to the value of copepods as live feed. However, due to the low growth rate of copepods when fed yeast, we would then suggest that the live feed for the fish larvae is predominantly switched to the traditional feed of *Artemia*, while keeping the remaining copepods as a supplement.

In terms of copepods being able to compete with *Artemia* and rotifers, there are a number of factors to consider. We believe that the replacement of rotifers with copepod nauplii is economically feasible and worth the effort for fish farmers as stated by Abate et al. (2015). Copepod nauplii are of the ideal size for first-feeding fish larvae, and result in at least 15% higher survival and pigmentation as compared with alternate live feeds such as *Artemia* and rotifers (Abate et al. 2016). While copepod densities do not reach comparable densities to rotifers when looking at counts of individuals, according to Schipp et al. (2006) it is more accurate to compare copepod densities of one copepod per millilitre as equal to 20 rotifers per millilitre instead when used as feed. This means in comparisons of densities, all copepod values should be multiplied by 20 to take into account their superior nutritional value and the difference in biomass.

As it was indicated by the sigmoidal curve for the population density effect on fecundity, higher densities will result in lower fecundity for *A. royi*. However, as we did not see a total population crash of the copepods even when densities reached 4000 ind. L^{-1}, we can assume that the maximum possible population density was far from reached. This means that the total nauplii production by females at higher densities (> 660 ind. L^{-1}) will result in higher actual numbers of nauplii than at the fecundity optimum density of 660 ind. L^{-1}.

6. Conclusion

In conclusion we found that the fecundity at the different population densities was significantly different and that the fecundity of *A. royi* therefore is density dependent. The optimum was found to be at 660 ind. L^{-1}, and the population composition did not appear to be affected by the population density i.e. proportions of males, females without egg sacs, copepodites, and nauplii. Moreover, a promising fraction of females were fecund even at 4000 ind. L^{-1}, indicating that largescale production is realistic at several thousand individuals per L.
We found that food type had a significant effect on growth but not on survival rate, with the growth rate of the algae-fed copepods being significantly higher than that of the yeast-fed copepods. There was no statistically significant difference in the CHN content of algae or yeast cells, and neither in the algae-fed nor the yeast-fed copepods. There was however a statistically significant difference in the fatty acids profile of the algae and yeast, with algae being superior to yeast, with no statistically significant difference in their DHA:EPA ratio to yeast. In addition, algae also have a higher total content of fatty acids than yeast. However, this does not translate into a difference in the fatty acid profile of algae-fed copepods compared to yeast-fed copepods. There was no statistically significant difference in the yeast-fed copepods and algae-fed copepods in terms of ALA, ARA, and EPA, but yeast-fed copepods have a higher % of DHA.

Since yeast-fed copepods remain at a size fraction that is ideal for first-feeding fish larvae for longer than algae-fed copepods, and since it would be considerably cheaper to feed copepods with yeast instead of live algae, we conclude that it is highly promising to seriously investigate the perspective of replacing rotifers with yeast-fed nauplii in fish aquaculture. Since copepods are as nutritious to fish larvae in terms of CHN and fatty acid content when fed yeast as compared to algae, we firmly believe that *A. royi* has the potential to be mass cultivated for aquaculture purposes.

7. Perspective

There are numerous studies on copepods and their potential for use in aquaculture, but they are not yet widely utilised commercially. Abate et al. (2016) states that this is likely due to a lack of communication between copepod researchers and the fish farmers that would be utilising them, in addition to the technical challenges still involved in mass cultivating copepods. As such, further studies into reducing or overcoming these challenges are still necessary.

Building on the experimental results from this project, it would be of interest to find out if the optimum feeding quantity of yeast cells is different from that of algae due to their differing fatty acid profiles. The maximum possible population density for this species is not yet known, as well as how important the bottle effect could be. These could be relatively easily studied, by further increasing the population densities, and conducting experiments with constant population density and different volumes of experimental containers respectively. The mechanism behind the trophic upgrade of short chain fatty acids to PUFAs is also still unknown.

For the rotifer *Brachionus plicatilis*, it was found that feeding with marine yeast (*Candida* sp.) instead of baker's yeast, in addition to the chlorophyte *Chlorella*, significantly increased the population density at which the rotifers could be cultivated as well as their production rates (James et al., 1987). It might be of interest to repeat the nutrition experiment from this report with this marine yeast as it has a higher fatty acid content than baker's yeast. However, based on the results seen when feeding copepods algal paste compared to live algae, despite a high fatty acid profile the copepods still had a low growth rate. In this case, the experiment may not produce favourable results. This may simply be because the copepods are too selective in their food choice.

Another interesting avenue of investigation would be to cultivate *A. royi* at increasingly high densities to see if it is possible through long-term selective pressure to breed a more stress-resistant copepod that can survive and reproduce at higher densities. This would improve their chances of sustainably replacing *Artemia* as feed for fish larvae. This is especially true as the market price of *Artemia* cysts can fluctuate dramatically over time. This is due to the cysts being sourced from hypersaline environments, primarily the Great Salt Lakes in Utah, USA, where production can vary seasonally and annually. These changes will presumably be exasperated with the increasing effect from global climate changes and further jeopardise stable availability for the fish hatcheries. A

steady supply of cultured copepods will in this case become an even more attractive solution for fish farmers.

It should however be noted that although aquaculture has a positive impact on human health and in restoring wild fish population sizes and therefore reducing the impact on ecosystems, there is much to be done in terms of making aquaculture more environmentally friendly. Insufficient management practices can lead to nutrient pollution of local ecosystems, and excessive harvesting of *Artemia* and the like can have unforeseen consequences (FAO, accessed 2018). While a great deal of progress has been made in aquaculture already, there are still a range of challenges that have to be met, investigated, and overcome.

8. References

Abate, T.G., Nielsen, R., Nielsen, M., Jepsen, P.M. and Hansen, B.W., 2016: A cost-effectiveness analysis of live feeds in juvenile turbot *Scophthalmus maximus* (Linnaeus, 1758) farming: copepods versus Artemia. *Aquaculture nutrition*, *22*(4), pp.899-910.

Abate, T.G., Nielsen, R., Nielsen, M., Drillet, G., Jepsen, P.M. and Hansen, B.W., 2015: Economic feasibility of copepod production for commercial use: result from a prototype production facility. *Aquaculture*, *436*, pp.72-79.

Azam, F., Fenchel, T., Field, J.G., Gray, J.S., Meyer-Reil, L.A. and Thingstad, F., 1983: The ecological role of water-column microbes in the sea. *Marine ecology progress series*, pp.257-263.

Balian, E.V., Segers, H., Lévêque, C. and Martens, K. 2008: The freshwater animal diversity assessment: an overview of the results. *Hydrobiologia*, *595*(1), pp.627-637.

Berggreen, U., Hansen, B. and Kiørboe, T., 1988: Food size spectra, ingestion and growth of the copepod *Acartia tonsa* during development: Implications for determination of copepod production. *Marine biology*, *99*(3), pp.341-352.

BiologyDiscussion: *Saccharomyces*: Reproduction and Life Cycle - Vegetative Body of *Saccharomyces*.
URL:http://www.biologydiscussion.com/fungi/saccharomyces-reproduction-and-life-cycle-eumycota/46654 [Accessed 27 Nov. 2018].

Blanda, E., Drillet, G., Huang, C.C., Hwang, J.S., Jakobsen, H.H., Rayner, T.A., Su, H.M., Wu, C.H. and Hansen, B.W., 2015: Trophic interactions and productivity of copepods as live feed from tropical Taiwanese outdoor aquaculture ponds. *Aquaculture*, *445*, pp.11-21.

Bonnet, D. and Carlotti, F., 2001: Development and egg production in Centropages typicus (Copepoda: Calanoida) fed different food types: a laboratory study. *Marine Ecology Progress Series*, *224*, pp.133-148.

Boyd, C.E. and Tucker, C.S., 1998: Ecology of aquaculture ponds. In *Pond Aquaculture Water Quality Management* (pp. 8-86). Springer, Boston, MA.

Brown, M.R., 2002: Nutritional value and use of microalgae in aquaculture. *Avances en Nutrición Acuícola VI. Memorias del VI Simposium Internacional de Nutrición Acuícola*, *3*, pp.281-292.

Chang, W.B., Lei, C.H., 1993: Development and energy content of a brackish-water copepod, *Apocyclops royi* (Lindberg) reared in a laboratory. *Bull. Inst. Zool. Acad. Sin*, *32*, pp.62-81.

Daan, R., Gonzalez, S.R. and Klein Breteler, W.C.M., 1988: Cannibalism in omnivorous calanoid copepods. *Marine ecology progress series. Oldendorf*, *47*(1), pp.45-54..

Dalsgaard, J., John, M.S., Kattner, G., Müller-Navarra, D. and Hagen, W., 2003: Fatty acid trophic markers in the pelagic marine environment. *Advances in Marine Biology*, *46*, pp.225-340.

Dhanker, R., Hwang, J.S., 2013: Predation by *Apocyclops royi* (Cyclopoid: copepod) on ciliates and rotifers. *Journal of Marine Science and Technology*, *21*, pp.246-251.

Dhont, J., Dierckens, K., Støttrup, J., Van Stappen, G., Wille, M. and Sorgeloos, P., 2013: Rotifers, *Artemia* and copepods as live feeds for fish larvae in aquaculture. In *Advances in aquaculture hatchery technology* (pp. 157-202).

DiMaggio, M.A., Cassiano, E.J., Barden, K.P., Ramee, S.W., Ohs, C.L. and Watson, C.A., 2017. First record of captive larval culture and metamorphosis of the Pacific Blue Tang, Paracanthurus hepatus. Journal of the world Aquaculture Society, 48(3), pp.393-401.

Drillet, G., 2018: Live feed for aquaculture - Developing plankton production systems for optimized larval rearing.
URL:https://www.dhigroup.com/-/media/shared%20content/dhi/flyers%20and%20pdf/solution%20flyers/live%20feed%20for%20aquaculture%20-%20dhi%20solutions_v2,-d-,1.pdf

Drillet, G., Frouël, S., Sichlau, M.H., Jepsen, P.M., Højgaard, J.K., Joarder, A.K. and Hansen, B.W., 2011: Status and recommendations on marine copepod cultivation for use as live feed. *Aquaculture*, *315*(3-4), pp.155-166.

Drillet, G., Iversen, M.H., Sørensen, T.F., Ramløv, H., Lund, T. and Hansen, B.W., 2006: Effect of cold storage upon eggs of a calanoid copepod, *Acartia tonsa* (Dana) and their offspring. *Aquaculture*, *254*(1-4), pp.714-729.

Drillet, G., Jepsen, P.M., Højgaard, J.K., Jørgensen, N.O. and Hansen, B.W., 2008: Strain-specific vital rates in four *Acartia tonsa* cultures II: life history traits and biochemical contents of eggs and adults. *Aquaculture*, *279*(1), pp.47-54.

Drillet, G., Rais, M., Novac, A., Jepsen, P.M., Mahjoub, M.S. and Hansen, B.W., 2015. Total egg harvest by the calanoid copepod A cartia tonsa (Dana) in intensive culture–effects of high stocking densities on daily egg harvest and egg quality. *Aquaculture research*, *46*(12), pp.3028-3039.

FAO, 2018: Aquaculture - FAO‘s role in aquaculture - Aquaculture development.
URL: http://www.fao.org/aquaculture/en/ [Accessed 23rd Nov. 2018].

Gusmão, L. F. M., McKinnon, A. D., & Richardson, A. J., 2013: No evidence of predation causing female-biased sex ratios in marine pelagic copepods. *Marine Ecology Progress Series*, *482*, pp.279-298.

Hammer, A., Schumann, R. and Schubert, H., 2002: Light and temperature acclimation of *Rhodomonas salina* (Cryptophyceae): photosynthetic performance. *Aquatic Microbial Ecology*, *29*(3), pp.287-296.

Hansen, B.W., Blanda, E., Drillet, G., Højgaard, J.K., Mahjoub, M.S. and Rayner, T.A., 2016: Outdoor rearing facilities of free spawning calanoid copepods for turbot larva can host a bank of resting eggs in the sediment. *Aquaculture international*, *24*(4), pp.949-964.

Hansen, B.W., Buttino, I., Cunha, M.E. and Drillet, G., 2016a: Embryonic cold storage capability from seven strains of *Acartia* spp. isolated in different geographical areas. *Aquaculture*, *457*, pp.131-139.

Hansen, B.W., Drillet, G., Kozmér, A., Madsen, K.V., Pedersen, M.F. and Sørensen, T.F., 2009: Temperature effects on copepod egg hatching: does acclimatization matter? *Journal of Plankton Research*, *32*(3), pp.305-315.

Hoef-Emden, K. and Archibald, J.M., 2017: Cryptophyta (cryptomonads). *Handbook of the Protists*, pp.851-891.

Højgaard, J. K., 2018: Size of Copepods - Their Relevance and use in Fish Larvae Culture. PhD, Department of Science and Environment, RUC, Denmark. Includes unpublished manuscripts 1-4.

Izquierdo, M. and Fernandez-Palacios, H, 1997: Nutritional requirements of marine fish larvae and broodstock. *CIHEAM, Options Méditerranéennes*, pp.243-264.

Izquierdo, M, 2005: Essential fatty acid requirements in Mediterranean fish species. *Cahiers Options Méditerranéennes*, *63*, pp.91-102.

James, C.M., Dias, P. and Salman, A.E., 1987: The use of marine yeast (*Candida* sp.) and bakers' yeast (*Saccharomyces cerevisiae*) in combination with Chlorella sp. for mass culture of the rotifer *Brachionus plicatilis*. *Hydrobiologia*, *147*(1), pp.263-268.

Jungbluth, M.J., Selph, K.E., Lenz, P.H. and Goetze, E., 2017: Incubation duration effects on copepod naupliar grazing estimates. *Journal of Experimental Marine Biology and Ecology*, *494*, pp.54-62.

Kainz, M.J., Hager, H.H., Rasconi, S., Kahilainen, K.K., Amundsen, P.A. and Hayden, B., 2017: Polyunsaturated fatty acids in fishes increase with total lipids irrespective of feeding sources and trophic position. *Ecosphere*, *8*(4).

Karlsen, Ø., van der Meeren, T., Rønnestad, I., Mangor-Jensen, A., Galloway, T.F., Kjørsvik, E. and Hamre, K., 2015: Copepods enhance nutritional status, growth and development in Atlantic cod (*Gadus morhua* L.) larvae—can we identify the underlying factors? *PeerJ*, *3*, p.e902.

Khan, H., Parks, N., Kozera, C., Curtis, B.A., Parsons, B.J., Bowman, S. and Archibald, J.M., 2007: Plastid genome sequence of the cryptophyte alga *Rhodomonas salina* CCMP1319: lateral transfer of putative DNA replication machinery and a test of chromist plastid phylogeny. *Molecular biology and evolution*, *24*(8), pp.1832-1842.

Kimmerer, W.J. and McKinnon, A.D., 1987: Growth, mortality, and secondary production of the copepod Acartia tranteri in Westemport Bay, Australia 1. *Limnology and Oceanography*, *32*(1), pp.14-28.

Lavens, P. and Sorgeloos, P., 1991: Production of *Artemia* in culture tanks. *Artemia Biology*, pp.317-350.

Lee, K.W., Kwon, O.N. and Park, H.G., 2005: Effects of Temperature, Salinity and Diet on the Productivity of the Cyclopoid Copepod, *Apocyclops royi*. *Journal of Aquaculture*, *18*(1), pp.52-59.

Lee, K.W., Dahms, H.U., Park, H.G. and Kang, J.H., 2013: Population growth and productivity of the cyclopoid copepods *Paracyclopina nana*, *Apocyclops royi* and the harpacticoid copepod *Tigriopus japonicus* in mono and polyculture conditions: a laboratory study. *Aquaculture Research*, *44*(5), pp.836-840.

Lee, C.S., O'Bryen, P.J. and Marcus, N.H. eds., 2008: *Copepods in aquaculture*. John Wiley & Sons.

Live Copepods and Phytoplankton - AlgaeBarn. 2018: *Feeding the Mandarin Fish with a Mix of Live and Prepared Foods*. [online]
URL: https://www.algaebarn.com/feeding-the-mandarin-fish/ [Accessed 5 Jan. 2018]

Mapelli, V., 2016. *Yeast metabolic engineering - Methods and protocols*. Humana Press.

Marinespecies.org. 2018: *WoRMS - World Register of Marine Species - Apocyclops royi (Lindberg, 1940).*[online]
URL: http://www.marinespecies.org/aphia.php?p=taxdetails&id=357094 [Accessed 21 Aug. 2018].

Miller, D.D. and Marcus, N.H., 1994: The effects of salinity and temperature on the density and sinking velocity of eggs of the calanoid copepod *Acartia tonsa* Dana. *Journal of Experimental Marine Biology and Ecology*, *179*(2), pp.235-252.

Nemlig, 2018: Gær fra Malteserkorsgær. Image.
URL: https://www.nemlig.com/gaer-711001 [Accessed 27 Nov. 2018].

Nielsen, B. L. H. and Gøtterup, L., 2017: Trophic upgrade of long-chain polyunsaturated fatty acids by cyclopoid copepod *Apocyclops royi*. Masters thesis, RUC.

Nielsen, R., Nielsen, M., Abate, T.G., Hansen, B.W., Jepsen, P.M., Nielsen, S.L., Støttrup, J.G. and Buchmann, K., 2017: The importance of live-feed traps–farming marine fish species. *Aquaculture Research*, *48*(6), pp.2623-2641.

Nilsson, B., Jakobsen, H.H., Stief, P., Drillet, G. and Hansen, B.W., 2017: Copepod swimming behavior, respiration, and expression of stress-related genes in response to high stocking densities. *Aquaculture Reports*, *6*, pp.35-42.

NOAA, 2018: What is aquaculture?
URL: https://oceanservice.noaa.gov/facts/aquaculture.html [Accessed 23 Nov. 2018].

NOAA, 2018a: Aquaculture - Feeds for Aquaculture
URL: https://www.fisheries.noaa.gov/insight/feeds-aquaculture [Accessed 27 Nov. 2018].

Onofre, S.B., Bertoldo, I.C., Abatti, D. and Refosco, D., Chemical Composition of the Biomass of *Saccharomyces cerevisiae* (Meyen ex EC Hansen, 1883), 2017: Yeast obtained from the Beer Manufacturing Process. *International Journal of Environment, Agriculture and Biotechnology*, *2*(2).

Pan, Y.J., Souissi, A., Sadovskaya, I., Hansen, B.W., Hwang, J.S. and Souissi, S., 2017: Effects of cold selective breeding on the body length, fatty acid content, and productivity of the tropical copepod *Apocyclops royi* (Cyclopoida, Copepoda). *Journal of Plankton Research*, *39*(6), pp.994-1003.

Pan, Y.J., Souissi, A., Souissi, S. and Hwang, J.S., 2016: Effects of salinity on the reproductive performance of *Apocyclops royi* (Copepoda, Cyclopoida). *Journal of Experimental Marine Biology and Ecology*, *475*, pp.108-113.

Payne, M.F. and Rippingale, R.J., 2001: Effects of salinity, cold storage and enrichment on the calanoid copepod *Gladioferens imparipes*. *Aquaculture*, *201*(3), pp.251-262.

Penglase, S., Hamre, K., Sweetman, J.W. and Nordgreen, A., 2011: A new method to increase and maintain the concentration of selenium in rotifers (*Brachionus* spp.). *Aquaculture*, *315*(1-2), pp.144-153.

Rainuzzo, J.R., Reitan, K.I. and Olsen, Y., 1997: The significance of lipids at early stages of marine fish: a review. *Aquaculture*, *155*(1-4), pp.103-115.

Rayner, T.A., 2017: *Copepods as live feed nutrition for fish larvae: a natural choice for aquaculture?* (Doctoral dissertation, Roskilde Universitet).

Rayner, T.A. and Hansen, B.W., in press: Applying algal paste as food for copepod live feed - a growth study on *Acartia tonsa* nauplii using the microalga *Isochrysis galbana*. DOI: 10.1111/are.13948

Rayner, T.A., Højgaard, J.K., Hansen, B.W. and Hwang, J.S., 2016: Density effect on the ovigerous rate of the calanoid copepod *Pseudodiaptomus annandalei* (Sewell 1919): implications for aquaculture. *Aquaculture Research*, *48*(8), pp.4573-4577.

Samuels, M.L., Witmer, J.A. and Schaffner, A.A., 2003: *Statistics for the life sciences* (Vol. 4). Upper Saddle River: Prentice Hall.

Schipp, G., 2006: The use of calanoid copepods in semi-intensive, tropical marine fish larviculture. *Advances en Nutrición Acuíscola VIII. VIII Simposium Internacional de Nutrición Acuicola*, pp.15-17.

Schultz, M. and Kiørboe, T., 2009: Active prey selection in two pelagic copepods feeding on potentially toxic and non-toxic dinoflagellates. *Journal of plankton research*, 31(5), pp.553-561.

Sorgeloos, P., Dhert, P. and Candreva, P., 2001: Use of the brine shrimp, *Artemia* spp., in marine fish larviculture. *Aquaculture*, *200*(1-2), pp.147-159.

Støttrup, J.G. and Jensen, J., 1990: Influence of algal diet on feeding and egg-production of the calanoid copepod *Acartia tonsa* Dana. *Journal of Experimental Marine Biology and Ecology*, *141*(2-3), pp.87-105.

Støttrup, J.G. and Norsker, N.H., 1997: Production and use of copepods in marine fish larviculture. *Aquaculture*, *155*(1-4), pp.231-247.

Støttrup, J.G., 2003: Production and nutritional value of copepods. *Live feeds in marine aquaculture*, pp.145-205.

Støttrup, J.G., Shields, R., Gillespie, M., Gara, M.B., Sargent, J.R., Bell, J.G., Henderson, R.J., Tocher, D.R., Sutherland, R., Næss, T. and Mangor-Jensen, A., 1998: The production and use of copepods in larval rearing of halibut, turbot and cod. *Bulletin-Aquaculture Association Of Canada*, pp.41-45.

Su, H.M., Su, M.S. and Liao, I.C., 1997: Collection and culture of live foods for aquaculture in Taiwan. *Hydrobiologia*,*358*(1-3), pp.37-40.

The Spruce, 2018: *Saltwater Copepods and Amphipods: the Little White Bugs in Your Tank*. [online]
URL: https://www.thespruce.com/those-little-white-bugs-in-your-tank-2921281 [Accessed 7 Jan. 2018].

Toledo, J.D., Golez, M.S., Doi, M. and Ohno, A., 1999: Use of copepod nauplii during early feeding stage of grouper *Epinephelus coioides*. *Fisheries Science*, *65*(3), pp.390-397.

Voordouw, M.J., Robinson, H.E. and Anholt, B.R., 2005: Paternal inheritance of the primary sex ratio in a copepod. *Journal of evolutionary biology*, *18*(5), pp.1304-1314.

Vu, M.T., Hansen, B.W. and Kiørboe, T., 2017: The constraints of high density production of the calanoid copepod *Acartia tonsa* Dana. *Journal of Plankton Research*, *39*(6), pp.1028-1039.

Vu, M. T. T., Douëtte, C., Rayner, T. A., Thoisen, C., Nielsen, S. L. and Hansen, B. W., 2016: Optimization of photosynthesis, growth, and biochemical composition of the microalga *Rhodomonas salina*—an established diet for live feed copepods in aquaculture. *Journal of applied phycology*, *28*(3), pp.1485-1500.

Walter, T.C. and Boxshall, G., 2018: World of Copepods database.
URL: http://www.marinespecies.org/copepoda on 2018-01-05

Watanabe, T., Kitajima, C. and Fujita, S., 1983: Nutritional values of live organisms used in Japan for mass propagation of fish: a review. *Aquaculture*, *34*(1-2), pp.115-143.

WWF, 2015: "Living Blue Planet Report - Species, habitats and human well-being". ISBN: 978-2-940529-24-7

Yoshimatsu, T. and Hossain, M.A., 2014: Recent advances in the high-density rotifer culture in Japan. *Aquaculture international*, *22*(5), pp.1587-1603.