Enrichment of rotifers (*Branchionus plicatilis*), *Artemia* nauplii and artificial dry feed with live yeast (*Debaryomyces hansenii*) for the growth of cultured dusky kob (*Argyrosomus japonicus*) larvae

Meghan Rousseau (RSSMEG004)
Supervisors: Prof John Bolton and Dr Brett Macey

25/10/2013
Department of Biological Sciences
University of Cape Town
Rondebosch
7701

Abstract

Aquaculture has expanded dramatically over the last fifty years and now contributes almost half of the global total fish production. Since fish are a very important source of protein for humans, aquaculture is an important food source and economic practice. It is therefore important to minimise and manage any factors that may negatively impact on aquaculture operations. One of the major problems in marine fish aquaculture is the high rate of larval mortality in captivity. One way of combatting this problem is by improving overall health of larvae. Probiotics, especially yeasts, can be used as immune boosters, disease control agents and a source of additional nutrients to help minimise this problem. These probiotics can be effectively administered to larvae using live feeds, such as rotifers and *Artemia*, and formulated feeds. The dusky kob, *Argryosomus japonicus*, is emerging as a commercially viable marine aquaculture species in South Africa. However, commercial production of this species is being hindered by parasitic disease. Therefore, this study tested the efficacy of (1) incorporating live culturable *Debaryomyces hansenii* (yeast) cells into rotifers (Branchionus plicatilis), Artemia nauplii and a formulated feed (Nutroscience Pre-starter Crumble), and (2) investigated the effects of the probiotic supplemented feeds on growth and survival of dusky kob larvae. We demonstrated that live culturable yeast cells, incubated at a period of 0.5 hours, delivered significantly more yeast cells per rotifer than the longer incubation periods ($p<0.001$). For *Artemia*, the incubation period of 0.5 hours delivered more yeast cells per *Artemia*, but this incubation period was not significantly better than the longer incubation periods ($p=0.204$). The starting yeast concentration of 1×10^{12} yeast cells.ml^{-1} resulted in significantly more yeast cells per rotifer ($p=0.042$) and *Artemia* ($p<0.001$) than the lower starting yeast concentrations. The proliferation of *Debaryomyces hansenii* on the formulated (dry) feed is significantly higher at a storage temperature of 4 °C than it is at a storage temperature of 20 °C ($p<0.001$). This could however, be causing a deterioration in the stability of the feed. The number of yeast cells per g of feed significantly decreases after 14 days, indicating a possible loss of culturability and stability of the yeast in the feed, regardless of the storage temperature ($p<0.001$ for 4 °C; $p<0.05$ for 20 °C). Drying the feed after coating it in yeast also causes a significant reduction in the number of yeast cells per g of feed ($p<0.001$). With more replication and more cautionary experimental design, this experiment could be

repeated in the future. Unfortunately, the mortality rate of the larvae was so high that the results obtained for the larval growth trials could not be statistically analysed, even though rotifer and *Artemia* (live feed) incubations were performed.

Introduction

Fish are an important resource and source of protein and are in great demand. The state of world fisheries is declining and the global capture fisheries production has stabilised at approximately 90 million tonnes per annum (FAO 2012). The world is turning to aquaculture as a solution to sustain the ever-growing human demand for fish and fish products (Macey 2005). Over the past fifty years, aquaculture has expanded dramatically and can now be compared to capture fisheries production in terms of the number of people it feeds, contributing 47% of the total fish production in 2010 (FAO 2012). This suggests that in the near future, aquaculture, the fastest growing food production sector, will become even more important to global food stability. Aquaculture may also be the key to minimising the pressure that anthropogenic activities put on fish stocks (DAFF 2012).

The contribution of South Africa's aquaculture sector to global aquaculture is minimal. In 2010, global aquaculture production totalled 56 million tonnes; whereas, South Africa's aquaculture production totalled 4253 tonnes (DAFF 2012). Marine aquaculture production in South Africa is presently dominated by shellfish, namely abalone (*H. midae*), oysters (*Crassostrea gigas*) and mussels (*Mytilus galoprovincialis*); whereas, the aquaculture of marine fish, primarily dusky kob (*Argyrosomus japonicus*), is an emerging industry that, by the end of 2010, had twenty dusky kob broodstock with approximately 1 million 5 g juveniles in production (DAFF 2012). A pond culture facility, three land-based recirculation facilities and a hatchery producing dusky kob are currently in operation in South Africa (DAFF 2012), and in 2012, 120 tonnes of South African cultivated dusky kob were sold (Roger Krohn, HIK Abalone Farm, *pers. comm*.). In the wild, dusky kob are distributed along the coasts of Australia, Africa, India, Pakistan, China, Korea and Japan (Fielder and Heasman 2011). Once dusky kob are sexually mature, they spawn near the mouths of estuaries (Griffiths 1996; Fielder and Heasman 2011). When the larvae are approximately four weeks old or 20-30 mm in length, they recruit into estuaries (Griffiths 1996). Dusky kob also seem to prefer more turbid areas as nursery grounds (Griffiths 1996).

Even though aquaculture is becoming increasingly important to mankind, often it is economically unsustainable due to disease and changes in environmental conditions

(FAO 2012). Disease and parasitic infection are challenges also associated with dusky kob larval rearing (DAFF 2012). Dusky kob are particularly susceptible to a gill fluke known as *Diplectanum oliveri*, which has caused extensive mortality, particularly among adults, throughout the South African aquaculture systems. This parasite has also been found to infect larvae as young as six weeks old. Bacteria have also proved problematic for the larval stages of this species (Dr Anna Mouton, Amanzi Biosecurity; *pers. comm.*). These negative impacts are exacerbated by the intensive nature of animal production practices and the high rates of larval mortality often encountered in the cultivation of certain species (Macey 2005). These problems can cause bottlenecks that hinder the profitability of the sector (Abidi 2003). Developing methods that will improve larval survival are therefore essential for ensuring the economic viability and survival of aquaculture ventures.

A number of methods exist for the prevention and treatment of diseases that affect the fish and their larvae. Antibiotics have been used extensively by the aquaculture industry to combat and control bacterial disease (Abidi 2003; Abdel-Tawwab et al. 2008). Owing to this intensive antibiotic use, antibiotic-resistant bacteria have evolved and increased in number (Abidi 2003; Abdel-Tawwab et al. 2008; Wang et al. 2008). Antibiotics, such as flumequine and oxolinic acid (Romero et al. 2010) have also been found to leave undesirable residues in the tissues of fish, which could cause trading problems (Abidi 2003). As a consequence, the use of several antibiotics in animal production has been banned by international organisations. Vaccination is another method of disease control, but vaccines for many fish diseases are currently unavailable in certain regions (Abidi 2003) or have not yet been developed for emerging diseases. Also, vaccines are not always effective for juvenile fish (Macey 2005), as it is often difficult to administer vaccines via injection to small juveniles and in addition the immune systems of these juveniles are not yet fully functional. Recently, chemical additive and veterinary medicine use has increased (Wang et al. 2008). This increase in chemical use is associated with environmental problems (Wang et al. 2008). Therefore, studies are currently seeking alternative, environmentally-friendly, disease prevention strategies (Abdel-Tawwab et al. 2008; Wang et al. 2008) and a great deal of emphasis has been placed on the use of non-specific immunostimulants and/or the use of non-pathogenic bacteria as

probiotic control agents (Gram et al. 1999; Gullian et al. 2004; Ringø and Birkbeck 1999; Robertson et al 2000 and Roch 1999).

An aquatic probiotic is defined as "a live microbial adjunct, which has a beneficial effect on the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment" (Verschuere et al 2000). In other words, probiotics will enhance the quality of the host's environment, improve the value of the host's food and ensure the host is able to protect itself from diseases. Often, the benefits of probiotic use are examined, but the modes of action of probiotics are not taken into account (Macey 2005). Probiotics, specifically aquatic probiotics, have a number of different modes of action. Probiotic organisms compete for adhesion to the intestinal tract of the host; produce inhibitory compounds; improve the immune response of the host; improve the environment or water quality for the host; provide a source of macro- and micronutrients; and/or contribute digestive enzymes (Verschuere et al. 2000, Abidi 2003).

Several bacteria, microalgae and yeasts have been evaluated as potential probiotics for feed supplementation and enrichment for use in fish aquaculture (Reyes-Becerril et al. 2008). Yeasts are particularly useful as probiotics as their cell walls are rich in β-glucans and nucleotides (Reyes-Becerril et al. 2008), which are known immunostimulants. Moreover, they are known to have antimicrobial activity. Yeasts produce and secrete polyamines which play a vital role in a number of important biological processes (Tovar-Ramirez et al. 2004) and have been found to increase the rate of maturation of the gut in mammals (Tovar et al. 2002). Yeasts are also able to adhere to the intestinal mucus of fish (Tovar et al. 2002; Tovar-Ramirez et al. 2004), making them ideal probiotic candidates. *Debaryomyces hansenii*, a strain of yeast, has been utilised as a probiotic for abalone *Haliotis midae* (Macey and Coyne 2005) and leopard grouper *Mycteroperca rosacea* (Reyes-Becerril et al. 2008) and is also one of the most halotolerant yeast strains (Reyes-Becerril et al. 2008). This makes *Debaryomyces hansenii* an ideal candidate for an aquatic probiotic that can be administered to dusky kob larvae.

Live feeds make up an essential part of larval rearing (Delbos and Schwarz, 2009) and also provide an effective vehicle for the administration of probiotic cells (Patra and Mohamed 2003). The live feed that has been accepted by most cultivated marine species as a highly favourable first-feed is the rotifer, *Brachionous* species (Delbos and Schwarz 2009). Rotifers have a high enzymatic content which aids in the development of larval digestive systems (Demir and Diken 2011) and has been shown to enhance sea bream and turbot larvae first-feeding performance significantly (Planas and Cunha 1999). Rotifers can be raised in mass cultures and can be distributed evenly in a tank during larval feeding (Delbos and Schwarz 2009; Demir and Diken 2011). The nutrient content of rotifers can also be increased by feeding cells an enrichment diet (Delbos and Schwarz 2009), such as an oil emulsion, microalgal cells (Park et al. 2006) or probiotics (Avella et al. 2010), making rotifers a favourable first live feed option for newly hatched larvae. Brine shrimps or *Artemia* are another highly favoured live feed option for larger larvae that have larger mouths and are able to prey on the *Artemia*, particularly the recently hatched nauplii (Treece 2000). Ballagh et al. (2010) found that larvae weaned directly from rotifers to dry feed grew significantly slower than those that were first fed with *Artemia*, suggesting that *Artemia* are an important part of the larval feeding cycle in cultivation. Larvae require live feeds that are sources of high levels of energy, but *Artemia* and rotifers naturally have low fatty acid contents (Park et al. 2006). Enrichment of the live feeds is therefore necessary before being fed to the larvae. Once larvae have metamorphosed, *Artemia* must be replaced with a formulated dry feed (Fielder and Heasman 2011) which can also be enriched.

Probiotics, especially yeast, positively impact fish and are administered to fish via live feeds, such as rotifers and *Artemia*, or supplementation of artificial feeds. Many studies have tested the effects of these probiotic-enriched feeds on cultured larvae. Tovar-Ramirez et al. (2004) enriched formulated feed with *Debaryomyces hansenii* and fed this to European sea bass (*Dicentrarchus labrax*) larvae. A 10% increase in survival; decrease in the malformation of larvae; and improvement in pancreas malformation and intestinal development was recorded for the larvae that were fed this probiotic-enriched diet (Tovar-Ramirez et al. 2004). In a more recent study by Tovar-Ramirez et al. (2010), where sea bass larvae were once again fed *Debaryomyces hansenii*-enriched formulated feeds, the larvae that were fed the

probiotic-enriched diet, showed a significant improvement in growth and reduction in oxidative stress. Park et al. (2006) enriched lived feed (rotifers) with probiotics. These probiotics came in the form of four different commercial rotifer enrichments such as oil emulsions and dried microalgae, and contained varying amounts of docosahexaenoic (DHA), an essential fatty acid. These probiotic-enriched rotifers were fed to Atlantic cod (*Gadus Morhua* L.) larvae, and the improved DHA proportions in the rotifers had positive effects on the growth and survival of the larvae (Park et al. 2006). Avella et al. (2010) also enriched live feeds (rotifers and *Artemia*) with probiotics. The probiotic was also used to enrich the rearing water of the false percula clownfish (*Amphiprion ocellaris*) larvae and juveniles. The probiotic used was the bacteria *Lactobacillus rhamnosus*, and the feed and water enriched with this probiotic had a number of positive effects on the larvae and juveniles. A twofold increase in larval body weight; a three day early occurrence of metamorphosis; an increase in growth- and development-associated gene expression; and a reduction in the severity of general stress responses were recorded in larvae and juveniles that were fed the probiotic-enriched feed and reared in the probiotic-enriched water (Avella et al. 2010). A number of studies have therefore shown that probiotic-enriched feeds are beneficial to the aquaculture industry, particularly to emerging aquaculture sectors, such as the cultivation of dusky kob in South Africa.

The aims of the present study are to test the effectiveness of supplementing live and formulated feeds with *Debaryomyces hansenii* in order to test the effects of the supplemented/enriched feeds on larval dusky kob growth and survival by answering the following four questions:

1. Are rotifers (*Branchionus plicatilis*) and *Artemia* nauplii able to accumulate the yeast within their bodies?
2. What is the most efficient incubation period and what is the best starting yeast concentration to use when incubating rotifers (*Branchionus plicatilis*) and *Artemia* nauplii with the yeast?

3. Is *Debaryomyces hansenii* able to coat a formulated dry feed (Nutroscience Pre-starter Crumble) and does the yeast remain culturable and stable when stored at various storage temperatures, namely 4 °C and 20 °C?
4. Do larvae that were fed probiotic-enriched rotifers (*Branchionus plicatilis*) and *Artemia* nauplii have an increased growth rate compared to larvae that were fed normal rotifers and *Artemia*?

Methods

Study site

Microbiological and larval rearing experiments were all conducted at the Department of Agriculture, Forestry and Fisheries (DAFF) Aquaculture Research Facility in Sea Point, Cape Town (33° 55' 14.27" S; 18° 22' 50.75" E). Ethics clearance was obtained (protocol number: 2013/V26/JB) to grow dusky kob (*Agyrosomus japonicus*) larvae in the experimental tanks at this facility. The larval rearing tanks consisted of a heated recirculating seawater system maintained at a salinity of 34-35 ‰ and a temperature of 24 °C. The larvae were kept at a fixed photoperiod of 12 hours light and 12 hours dark. The recirculating seawater system was equipped with a common mechanical-filter, bio-filter and protein skimmer to maintain optimal water quality conditions.The yeast (*Debaryomyces hansenii*), rotifers and *Artemia* were grown separately under the conditions described below.

General Methods

Probiotic Growth

For the preparation of probiotic yeast cells for feed enrichment studies, *Debaromyces hansenii* was inoculated from a 15% glycerol stock solution that had been stored at -80 ºC onto prepared yeast peptone D-glucose (MERCK) agar (YPD agar; 10 g yeast extract, 20 g D-glucose, 20 g peptone, 20 g agar and 1 L distilled water) plates and incubated at 30 °C for approximately 48 hrs. Following incubation, a single colony was transferred from the agar plate into 5 mL of YPD broth which was incubated for a further 24 hours at 30 °C on a vibrating platform set to rotate at 80 rpm. This starter culture was then used to inoculate larger volumes of YPD broth (50 – 100 mL) to achieve a starting concentration of approximately 0.05 – 0.1 at an optical density of 600 nm. The inoculated broths were incubated as described above for approximately 48 hours, at which point the concentration of yeast cells was determined spectrophotometrically and utilised for enrichment studies as described below. This process was repeated a number of times during the experiment in order to keep the yeast colonies in an actively growing state for feed enrichment studies.

Rotifer and Artemia Enrichment Experiments

For the live feed enrichment experiments, the rotifers (*Branchionus plicatilis*) were harvested from the rotifer breeding tanks at the study site, and the *Artemia* were

hatched from cysts in order to obtain *Artemia* nauplii. This was done by weighing out 5 g of *Artemia* cysts and placing them in 1.5 L of seawater acclimated to 28 °C. An airstone and light were added to the system. The cysts were left in the water for two hours to hydrate. After two hours, the *Artemia* cysts were poured into a bucket containing 20 L of 28 °C, aerated seawater. The cysts were incubated for twenty-four hours to hatch.

The concentrations of the rotifers or *Artemia* samples were calculated by counting cells under a light microscope. In order to quantify the number of rotifers or *Artemia* under a dissecting light microscope, a 50 µL aliquot of 10% neutral buffered formalin (NBF) was added to 1 mL of rotifer-containing water and mixed by inversion. Three 200 µl aliquots were taken from the solution and placed in separate grooves of a Bogorov tray. An average number of rotifers or *Artemia* per ml was calculated and if this density was too low, the rotifers or *Artemia* were concentrated further. This was done by gently pouring a large volume of the rotifer- or *Artemia*-containing water through a 40 µm sieve and re-suspending the rotifers or *Artemia* in a smaller volume of seawater that had been pre-acclimated to 28 ºC.

Before incubating the rotifers or *Artemia* with yeast, the concentration of the yeast in the YPD broth, prepared as described above, was quantified using a spectrophotometer (Biochrom Libra S12). The spectrophotometer was set to an

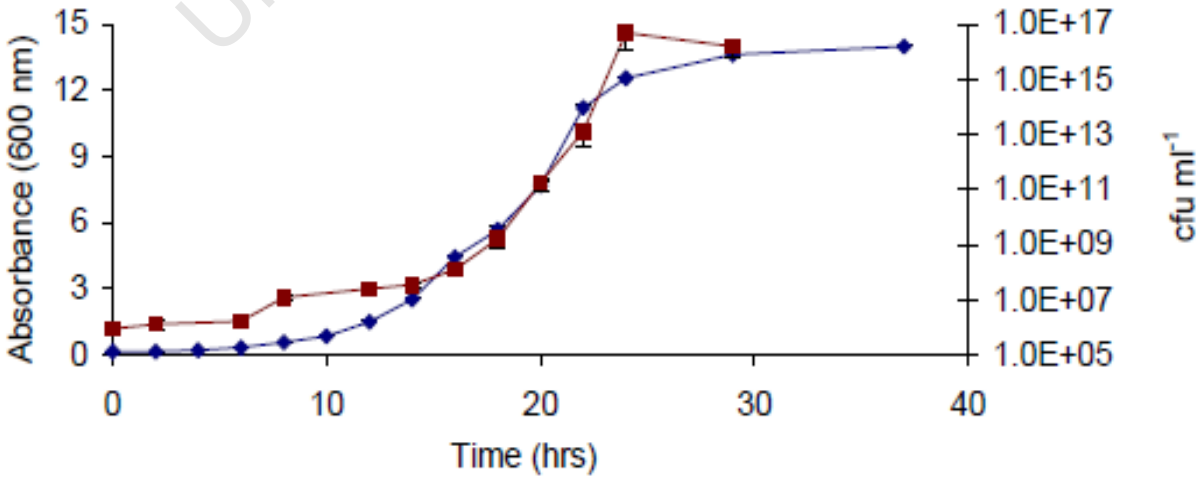

Figure 1: The growth profile of AY1 (*Debaryomyces hansenii*) showing the relationship between absorbance at 600 nm (◆) and the number of colony forming units per ml (■) (Macey 2005)

optical absorption of 600 µm and was blanked with 1 mL of plain YPD broth. The yeast-inoculated broth was diluted into either a 10 fold or 50 fold dilution in sterile YPD broth, depending on the concentration of the yeast. An absorption reading was taken of 1 mL of each dilution. The growth curve of *D. hansenii* (Fig. 1) was used to estimate the number of colony-forming units per ml of broth from the OD units. The volume of yeast-inoculated broth to be added to a set volume (100 mL) of rotifer- or *Artemia*-containing water (C_2) was calculated as follows:

$$C_1V_1 = C_2V_2 \qquad \text{Eq. 1}$$

where C_1 is the concentration of yeast in the yeast-inoculated broth; V_1 is the volume of yeast-inoculated broth to be added; and V_2 is the known volume of rotifer-containing water to which the yeast-inoculated broth must be added.

For the first live feed experiment, a final yeast concentration of 1×10^{12} cfu.mL^{-1} was required to test four different incubation periods: half an hour, one hour, two hours and four hours. The rotifer or *Artemia* concentrations were kept at approximately 200 rotifers.mL^{-1} and 150 *Artemia*.mL^{-1}, respectively, for all tests. The previously calculated volume of yeast-inoculated broth (Eq. 1) was added to three flasks, each containing 100 mL of rotifer- or *Artemia*-containing water. These flasks were incubated at 30 °C on the vibrating platform. When each incubation time was reached, a 6 mL aliquot was removed from each flask. Each aliquot of rotifers or *Artemia* was rinsed using a 40 µm sieve and 28 °C seawater, and re-suspended in 6 mL of the 28 °C seawater. Each rinsed aliquot was divided into two 3 mL aliquots – one was used to determine the rotifer or *Artemia* concentration as previously described and the other was used for homogenisation. Before homogenising the aliquots, 100 µL of seawater (excluding rotifers or *Artemia*) was plated from each aliquot on Potato Glucose Agar (Sigma-Aldrich) plates supplemented with chloramphenicol (39 g potato glucose, 1 L distilled water and 2.5 mL chloramphenicol). These plates were used instead of the YPD agar plates in order to select for yeast cells and retard the growth of opportunistic bacteria. Each homogenisation aliquot was homogenised for 30 s and ten-fold serial dilutions of the homogenised aliquots were plated (100 µL aliquots) on potato glucose agar-

chloramphenicol plates in. All dilutions were plated in triplicate and plates were incubated at 30 °C for 48 hours before the number of yeast colonies on each plate was counted and recorded.

For the second live feed experiment, the pre-determined best incubation time was used to test four different yeast concentrations to determine the optimal starting yeast concentration for maximising the uptake of yeast cells by rotifers and *Artemia*. The four yeast concentrations were 1×10^6 cfu.mL^{-1}, 1×10^8 cfu.mL^{-1}, 1×10^{10} cfu.mL^{-1} and 1×10^{12} cfu.mL^{-1}. Twelve flasks, each containing 100 mL of rotifer- or *Artemia*-containing water with concentrations of 200 rotifers.mL^{-1} and 150 *Artemia*.mL^{-1}, respectively, were subdivided into four groups of three flasks each. Each group of flasks received a different final yeast concentration. All the flasks were incubated for the pre-determined best incubation period. The previously outlined rinsing, counting, homogenising and plating methods were followed.

Dry Feed Enrichment Experiments

A dry feed experiment was also performed in which the formulated dry feed was coated with yeast and the culturability and stability of the yeast on the feed was measured over time. The yeast (170 mL) was prepared as described previously and the concentration determined using the method outlined above. The yeast-inoculated YPD broth was divided into four equal parts in sterile centrifuge tubes. The centrifuge tubes were placed into the centrifuge (Hettich Zentrifugen Rotina 380R) and centrifuged at 8000 rpm for ten minutes at room temperature. The supernatant was carefully removed and the cell pellets from the four tubes were combined and re-suspended in 18 mL sterile YPD broth in order concentrate the yeast cells. The concentrated cells were vortexed (Labnet Vortex Mixer) to re-suspend the cells before transferring 1 mL aliquots to three separate 1.5 mL microcentrifuge tubes. Tenfold serial dilutions (or logarithmic dilutions) were performed and a 100 μL aliquot of each dilution was plated onto Potato Glucose Agar plates supplemented with chloramphenicol in order to determine the number of colony forming units (CFU) in each of the three Eppendorf tubes.

Dry feed (Nutroscience Pre-starter Crumble) was weighed out (150 g) and the remaining 15 mL of concentrated yeast solution (5.27×10^8 Yeast Cells.ml^{-1}) was

used to coat the feed in a beaker covered with parafilm. This was done by vigorously shaking the beaker containing feed and yeast cells for approximately 1 minute. Once the feed was sufficiently coated in yeast, three 0.5 g samples of yeast-coated feed were removed from the beaker and transferred to 10 mL universal bottles containing 5 mL of sterile YPD broth. A sterilised glass rod was used to mash up the yeast-coated feed in the YPD broth, before vortexing the samples for 1 minute each. Samples were serially diluted and aliquots (100 µL) of each dilution were plated on Potato Glucose Agar plates supplemented with chloramphenicol.

The yeast-coated feed was dried for 24 hours by placing the feed on a tray in an oven set to 37 °C. Following drying, the feed was removed from the oven and three 0.5 g samples were removed to determine the number of colony forming units of *D. hansenii* per gram feed, as described above. The remaining 146 g of yeast-coated feed was divided into two equal portions and placed in separate Ziploc bags. One portion was placed in a 4 °C refrigerator and the other portion was placed in a 21 °C incubator to determine the optimal temperature for storage of feed and stability of yeast cells. Every second day, three 0.5 g samples were removed for enumeration of the number of yeast CFUs as described above.

Larval Experiments

Larval rearing experiments were conducted to determine the effects of the probiotic supplemented live feeds on *A. japonicus* larval growth and survival. All experiments were conducted in the recirculating seawater system that was set up at the study site. The system consisted of nine tanks that were cleaned out using chlorine and neutralised using thiosulphate prior to stocking with eggs. Each tank had a volume of 40 l according to which the larvae were fed. System conditions were as described above, under study site, in the materials and methods.

A single dusky kob female spawned naturally at the DAFF Aquaculture Research Facility and fertilised eggs for the experiment were collected from this spawning event. The number of eggs that made up 1 g of eggs was counted and was found to be approximately 1600 eggs. Therefore, 1.5 g of eggs was weighed out and added to each tank in order to obtain an initial stocking density of 60 larvae.L^{-1} as

recommended by industry (Oceanwise, *pers. comm*.). There were approximately 2400 eggs (and therefore larvae) per tank. Since larvae are extremely fragile and sensitive to physical manipulation, regular measurements of larval mortality and survival throughout the study period was not possible. Russell, A. (2013) suggests that very young larvae are fragile and decompose quickly, exacerbating the problem of measuring mortality and survival.

According to Fielder and Heasman (2011), three or four days after hatching, larvae start feeding exogenously. After nine days of feeding on rotifers, *Artemia* was fed to the larvae for the remaining trial time. Since metamorphosis takes place at 23 days post hatch, the trial was run for no longer than 23 days. The live feed concentrations recommended by Fielder and Heasman (2011) and Russell, A. (2013) were adopted for this study. The rotifer concentration in each tank was kept at a minimum of 10 rotifers.mL^{-1} (Russell, A. 2013), and the *Artemia* concentration in each tank was kept at a minimum of 1 *Artemia*.mL^{-1} (Fielder and Heasman 2011). Russell, A. (2013) also suggested that adding *Nannochloropsis oculata* to each tank would contribute to the management of water quality and supplement the live feed. *Nannochloropsis oculata* was added to each tank to achieve and maintain a concentration of 230 000 cells.mL^{-1}. *Nannochloropsis oculata*, rotifers and *Artemia* were added to each tank by hand twice a day, once in the morning and once in the afternoon, as per the protocol described by Russell, A. (2013). The actual volumes added to each tank were calculated daily by determining the concentrations of *Nannochloropsis oculata*, rotifers or *Artemia* on the day of feeding in each 40 L tank and using the following equation:

$$V_{added} = \frac{c_{needed}}{c_{culture}} \qquad \text{Eq. 2}$$

where V_{added} is the volume of *Nannochloropsis oculata*, rotifers or *Artemia* to be added, c_{needed} is the concentration needed in the tank to keep the tank at the abovementioned concentration and $c_{culture}$ is the concentration in the culture tanks at the study site.

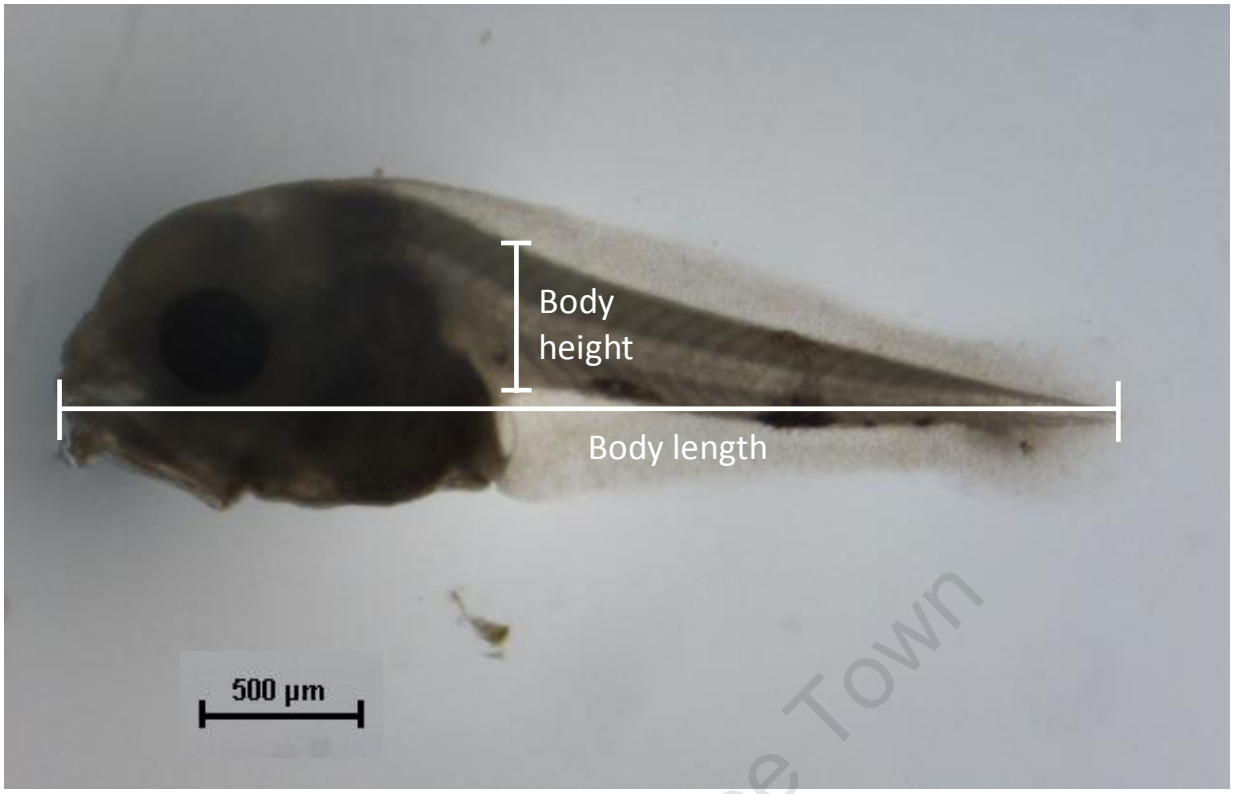

Figure 2: Dusky kob larva on day 7 showing how the different measurements were taken. Body Length: from the snout to the end of the notochord. Body Height: measured vertically upwards from the anus, ignoring the membraneous material (seen as a grey shadow in this picture)

Rotifers and *Artemia* were incubated with yeast for the predetermined best incubation time at the predetermined best yeast concentration. The volume of yeast to be added to the rotifers or *Artemia* was calculated as above (Eq. 1). The enriched rotifers and *Artemia* were fed to five of the nine tanks (probiotic treatment) and were only fed in the morning. In the afternoon, these tanks were fed non-enriched rotifers or *Artemia*. The remaining four tanks were fed non-enriched rotifers or *Artemia* throughout (control tanks).

A beaker and pipette were used to remove 10 larvae from each tank at 8am for the first two days and every second day after that, starting on the day of first feed (3 days post hatch).The sampled larvae were anesthetised using 2-phenoxyethanol at a concentration of 0.5 $ml.L^{-1}$ before being fixed in 5 ml of 10% neutral buffered formalin. Fixed larvae were photographed under a microscope (Nikon SMZ1500 microscope, Nikon D5-Fi2 camera) and were measured using Nikon Imagining Systems (Elements) Basic Research 3.2 software. Measurements of body length, from snout to the end of the notochord, and body height, in a vertical line from the

anus to the top of the body, were taken (Fig. 2) following the measurement methods described by Bjelland and Skiftesvik (2006) and recorded to the nearest 0.01 µm.

Statistical Analyses

SigmaPlot 11 and Statistica 11 were used for the statistical analyses of the enrichment and larval results. The live feed incubation period rotifer data was log transformed to normalise it; whereas, the *Artemia* data was already normal. One-way ANOVA tests and post-hoc Holm-Sidak tests were performed on the transformed rotifer data and the original *Artemia* data to determine the optimal incubation period based on the number of yeast cells per rotifer or *Artemia*. The live feed yeast concentration rotifer data was not normal, even when transformed. A one-way Kruskal-Wallis test and post-hoc Tukey test were performed on the original rotifer data to determine the optimal yeast concentration based on the number of yeast cells per rotifer. The live feed yeast concentration *Artemia* data was normal. A one-way ANOVA and post-hoc Holm-Sidak test was performed to determine the optimal starting yeast concentration. The dry feed data was rank transformed to normalise it. A two-way ANOVA test and a post-hoc Holm-Sidak test were performed on the rank transformed dry feed data to determine the effects of storage temperature and time stored on the number of yeast cells per g of dry feed. A paired t-test was run on the dry feed data that was recorded before the yeast-coated feed was dried and after the yeast-coated feed was dried (day 0). The dry feed drying data were normally distributed. A t-test was performed using the larval body length and larval body height data on Day 0 to determine whether or not the larvae in the different treatment tanks (Probiotic or Control) were significantly different sizes at the beginning of the trial. Regression analyses were performed to calculate the growth rate from the slope for each tank using body length and body height. Not all the data was linear and some of the data had only two sample days. An ANOVA of the growth rates for each tank could therefore not be run.

Results

Rotifer and Artemia Enrichment Experiments

The number of yeast cells per rotifer is highest (169.27±65.15 yeast cells per rotifer) at the shortest incubation period (0.5 hours) and lowest at the longest incubation period of 4 hours (Fig. 3). The number of yeast cells per rotifer differs significantly over the periods of incubation ($F=49.060$, $df_1=3$, $df_2=8$, $p<0.001$). The number of yeast cells per rotifer at an incubation period of 30 minutes is significantly higher than the number of yeast cells per rotifer at the longer incubation periods ($p<0.001$; Fig. 3). The number of yeast cells per rotifer at an incubation period of one hour is not significantly different to the number of yeast cells per rotifer at an incubation period of two hours ($p=0.284$; Fig. 3). The number of yeast cells per rotifer at an incubation period of four hours is significantly lower than at the shorter incubation periods ($p<0.001$; Fig. 3).

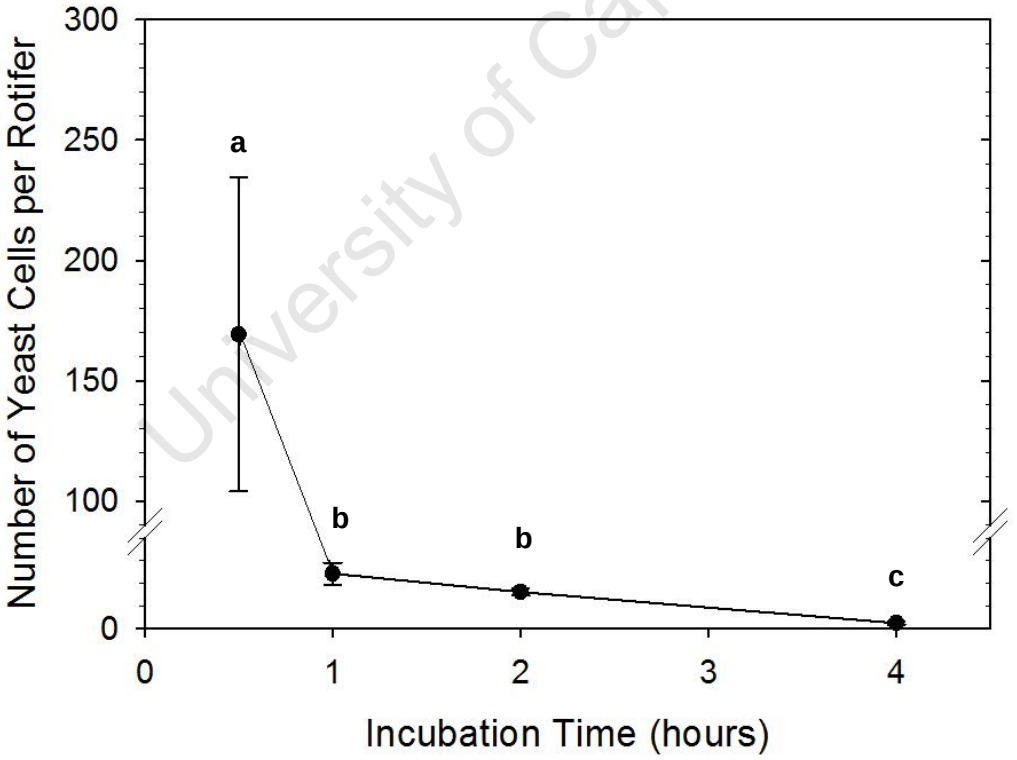

Figure 3: Mean number of yeast cells found per rotifer (±SE) after the rotifers were incubated with yeast of a 1×10^{12} cells.mL^{-1} concentration for varying periods of incubation ("a", "b" and "c" denote significant difference between the various incubation times and the break in the y-axis improves data presentation)

The number of yeast cells per rotifer is highest (18.46±1.82 yeast cells per rotifer) when rotifers are incubated with yeast cells at a concentration of 1×10^{12} yeast cells.mL^{-1} (Fig. 4). Incubation of rotifers with fewer yeast cells (1×10^{10} yeast cells.mL^{-1}, 1×10^{8} yeast cells.mL^{-1} and 1×10^{6} yeast cells.mL^{-1}) resulted in little to no uptake of yeast cells by rotifers over the 30 minute incubation period (Fig. 4). Starting yeast concentration had a significant ($H=8.195$, $df=3$, $p=0.042$) effect on the uptake of yeast cells by rotifers. At a starting yeast concentration of 1×10^{12} yeast cells.mL^{-1}, the number of yeast cells per rotifer is significantly higher compared with the number of yeast cells per rotifer at the other three yeast starting concentrations ($p<0.05$). On the other hand, the numbers of yeast cells per rotifer at the other three yeast starting concentrations are not significantly different from one another ($p>0.05$).

The mean numbers of yeast cells per *Artemia* were between 2003 and 3994 yeast cells per *Artemia* over all the incubation periods (Fig. 5). There were no significant differences between the numbers of yeast cells per *Artemia* at the different

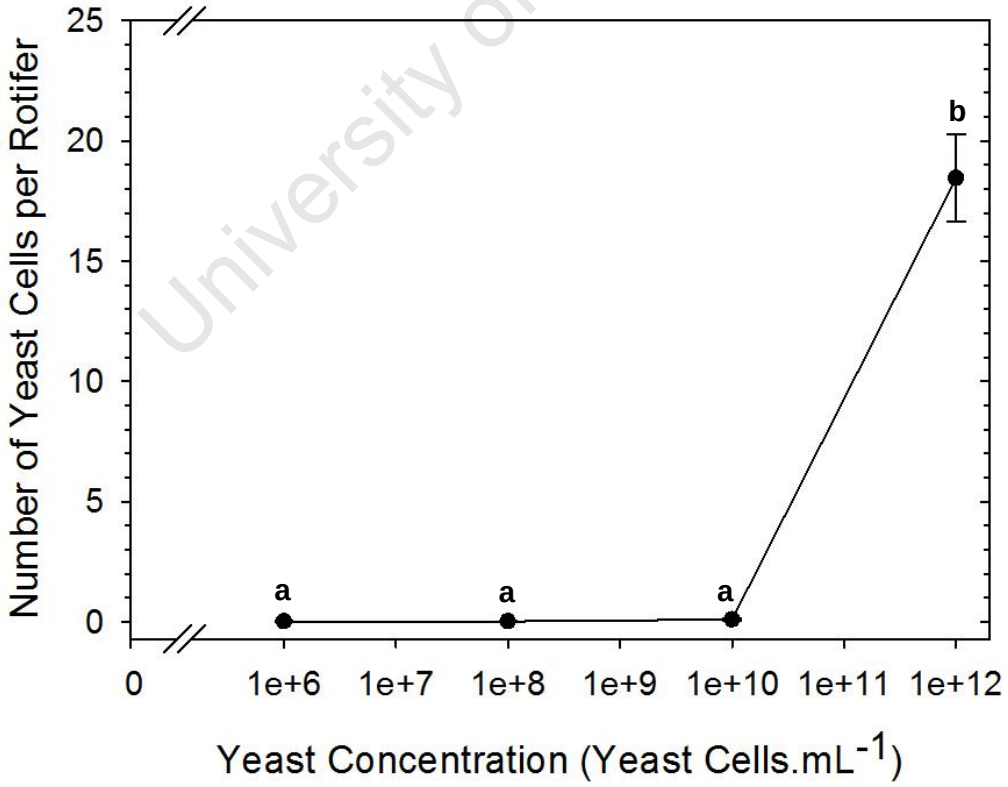

Figure 4: Mean number of yeast cells found per rotifer (±SE) after the rotifers were incubated with yeast of varying concentrations for an incubation period of 0.5 hours ("a" and "b" denote a significant difference between the yeast concentrations)

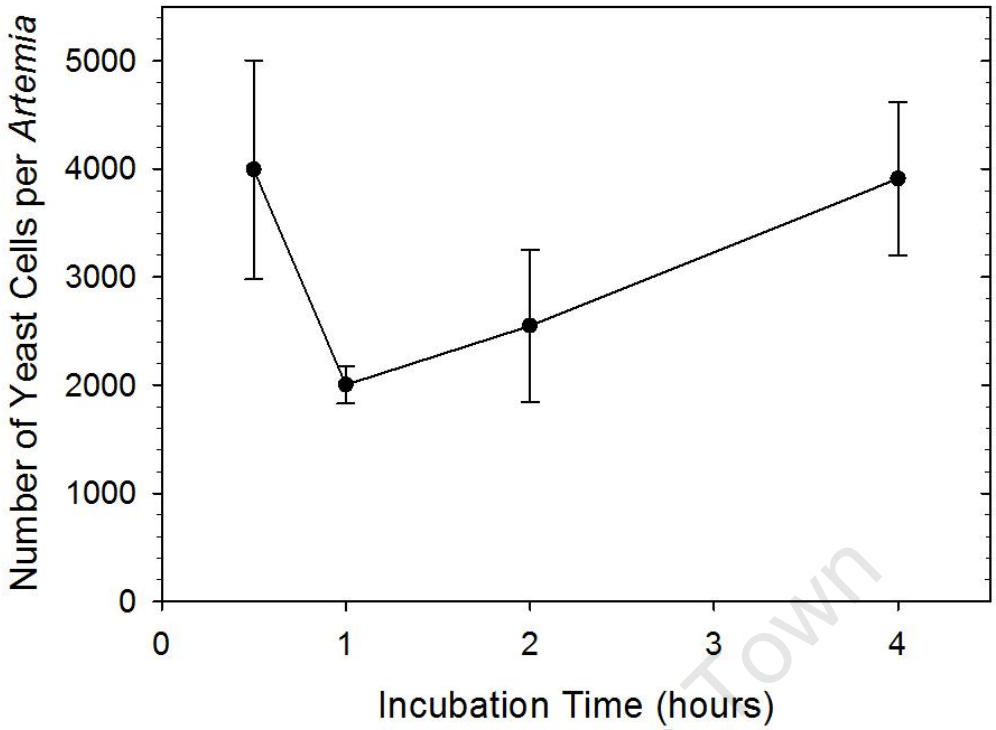

Figure 5: Mean number of yeast cells found per *Artemia* (±SE) after the *Artemia* were incubated with yeast of a 1 × 10^{12} yeast cells.mL^{-1} concentration for varying periods of incubation

incubation periods (F=1.924, df^1=3, df^2=8, p=0.204). The power of this test was only 0.171 which is far lower than the desired power level of 0.800.

Starting yeast concentration had a significant (F=260.504, df_1=3, df_2=8, p<0.001) effect on the uptake of yeast cells by *Artemia*. At a starting yeast concentration of 1 × 10^{12} yeast cells.mL^{-1}, the number of yeast cells per *Artemia* (134.97±7.05) is significantly higher compared with the number of yeast cells per *Artemia* at the other three yeast starting concentrations (p<0.001; Fig. 6). When incubating *Artemia* with yeast cells at a concentration of 1 × 10^{10} yeast cells.mL^{-1}, the number of yeast cells per *Artemia* was significantly lower (13.77±3.95 yeast cells per *Artemia*). Incubation of *Artemia* with fewer yeast cells (1 × 10^8 yeast cells.mL^{-1} and 1 × 10^6 yeast cells.mL^{-1}) resulted in little to no uptake of yeast cells by Artemia over the 30 minute incubation period (Fig. 6). The numbers of yeast cells per *Artemia* at the three lower yeast starting concentrations were not significantly different from one another (p>0.05).

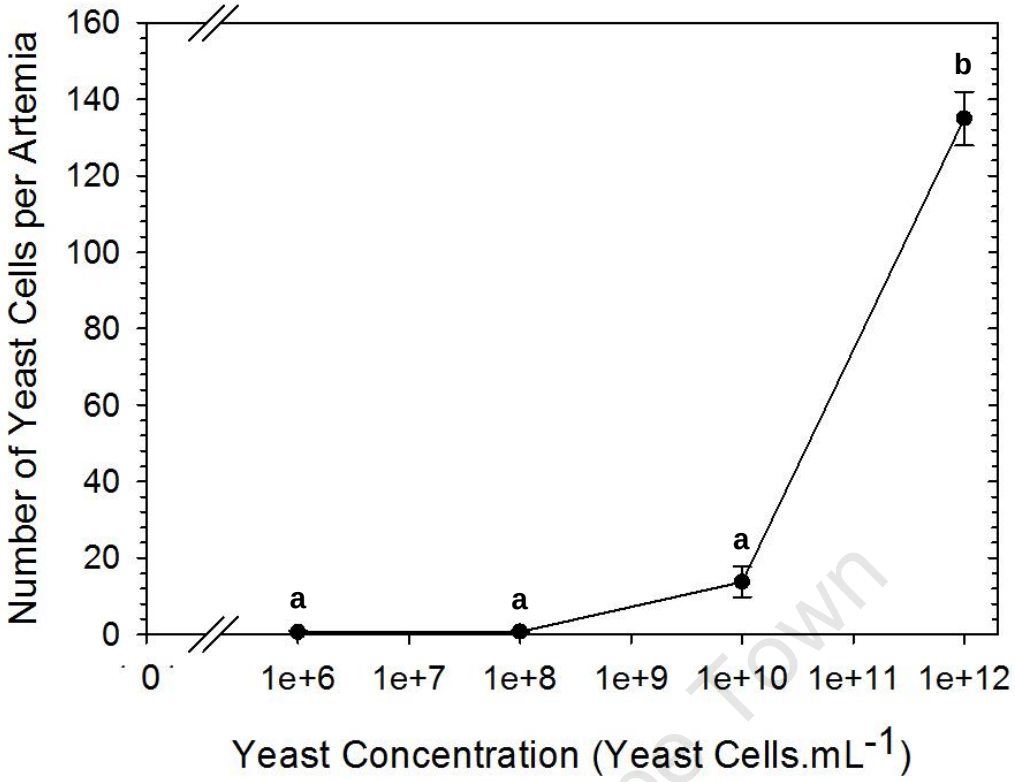

Figure 6: Mean number of yeast cells found per *Artemia* (±SE) after the *Artemia* were incubated with yeast of varying concentrations for an incubation period of 0.5 hours ("a" and "b" denote a significant difference between the yeast concentrations)

Dry Feed Enrichment Experiments

The temperature at which the yeast-coated dry feed was stored had a significant effect on the number of yeast cells per g of feed ($F=45.527$, $df_1=1$, $df_2=24$, $p<0.001$). The time or number of days for which the yeast-coated dry feed was stored also had a significant effect on the number of yeast cells per g of feed ($F=27.291$, $df_1=5$, $df_2=24$, $p<0.001$). Moreover, there was a significant interaction between the two factors, storage temperature and storage time ($F=5.562$, $df_1=5$, $df_2=24$, $p=0.002$).

On days 4, 6 and 9, the number of yeast cells per g of dry feed was significantly lower at the 20 °C storage temperature than at the 4 °C storage temperature ($p<0.001$; Fig. 7). On the other days (days 0, 2 and 14), there was no significant difference between the number of yeast cells per g of feed at these two storage temperatures ($p>0.05$; Fig. 7).

For the 4 °C storage temperature, the number of yeast cells per g of feed on day 2 was significantly lower than the number of yeast cells per g of feed on days 4, 6 and

9 ($p<0.001$; Fig. 7). It was also significantly higher than the number of yeast cells per g of feed on day 14 ($p=0.006$; Fig. 7), but was not significantly different to the number of yeast cells per g of feed on day 0 ($p>0.05$; Fig. 7). The number of yeast cells per g of feed on day 14 was significantly lower than the number of yeast cells per g of feed on any of the previous days, days 0 – 9, for the 4 °C storage temperature ($p<0.001$; Fig. 7). For the 4 °C storage temperature, the number of yeast cells per g of feed on days 0, 4, 6 and 9 were not significantly different from each other ($p>0.05$; Fig. 7).

For the 20 °C storage temperature, the number of yeast cells per g of feed on day 14 was significantly lower than the number of yeast cells per g of feed on days 0, 4, 6 and 9 ($p<0.05$; Fig. 7), but was not significantly different to the number of yeast cells per g of feed on day 2 ($p>0.05$; Fig. 7). The number of yeast cells per g of feed on day 0 was significantly higher than the number of yeast cells per g of feed on days 2, 4, 9 and 14 ($p<0.05$; Fig. 7), but was not significantly different to the number of yeast

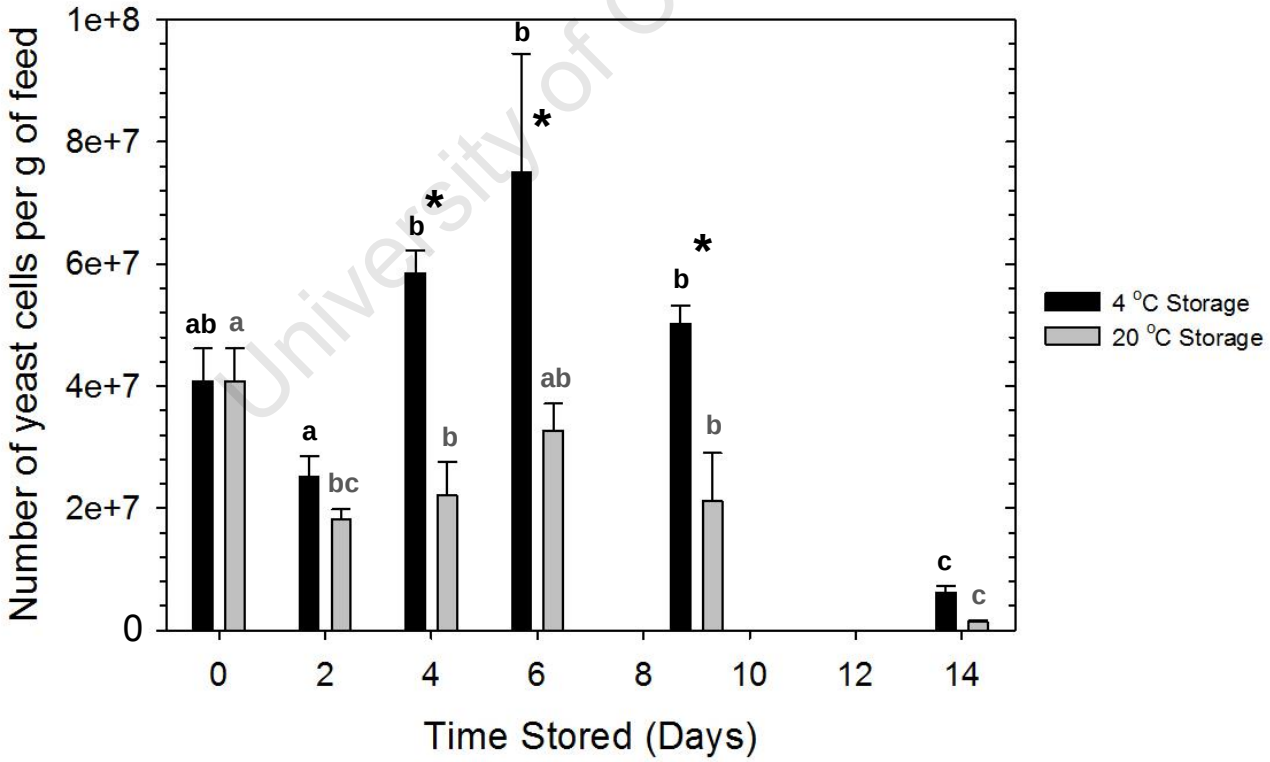

Figure 7: Mean number of yeast cells found in 1 g of dry feed (±SE) after the feed was coated with the yeast and stored at two different temperatures (4 °C and 20 °C) for 14 days ("a", "b" and "c" denote a significant difference in yeast cells among the number of days stored within each storage temperature, and a * denotes a significant difference in yeast cells between the storage temperatures on a specific day)

cells per g of feed on day 6 for the 20 °C storage temperature ($p>0.05$; Fig. 7). For the 20 °C storage temperature, the number of yeast cells per g of feed on days 2, 4, 6 and 9 were not significantly different from each other ($p>0.05$; Fig. 7).

The drying of the feed after it had been coated with the yeast also had a significant effect on the number of yeast cells per g of feed. Just after the feed had been coated with the yeast, approximately 419 644 444 yeast cells per g of feed were recorded.

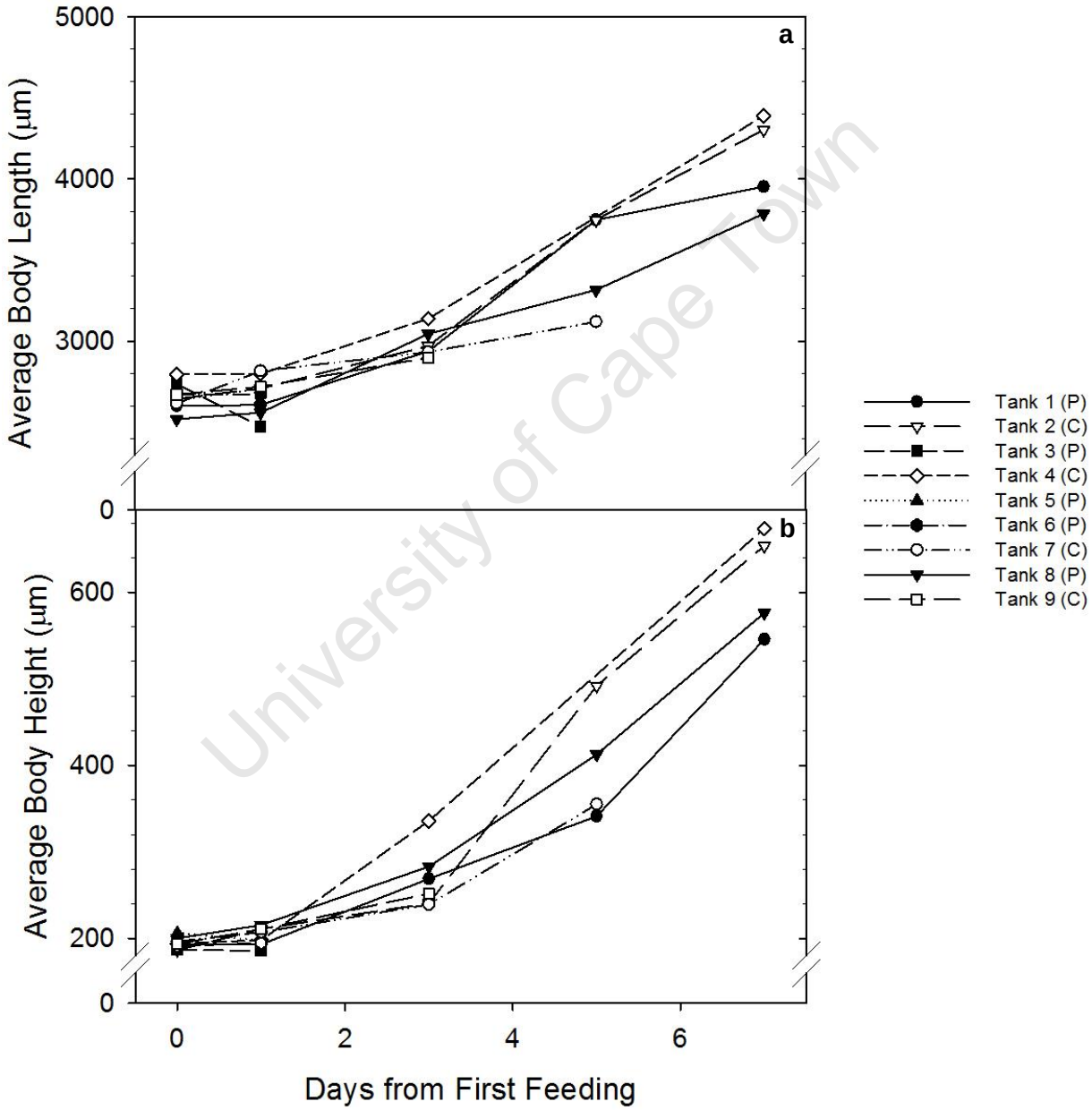

Figure 8: Mean body length (a), measured from the snout to the end of the notochord, and mean body height (b), measured vertically upwards from the anus, for samples of dusky kob (*Agyrosomus japonicus*) larvae from nine tanks, five of which were probiotic-enriched (filled symbols) and four of which were controls (hollow symbols) over a number of days starting from the day of first feed

Whereas, just after the yeast-coated feed had been dried for 24 hours, approximately 40 826 667 yeast cells per g of feed were recorded. Drying the yeast-coated feed for 24 hours therefore resulted in a significant reduction in the number of yeast cells per g of feed ($t=22.081$, $df=4$, $p<0.001$).

Larval Experiments

The mean body length and body height of the larvae increased with time in all treatment groups from the first feed (Fig. 8a&b). On Day 0, the mean body length of the control (C) larvae (2681.44 µm) is slightly greater than the mean body length of the probiotic-enriched (P) larvae (2632.28 µm). There was however, no significant difference between the mean body length of the larvae in these two treatment groups on Day 0 ($t=-0.917$, $df=7$, $p=0.390$). The same non-significant difference between treatments was recorded for the mean body height on Day 0 ($t=0.623$, $df=7$, $p=0.553$). Owing to the high mortality rate, only four tanks still had larvae on Day 7 and only one tank (Tank 8) had surviving larvae after Day 7. On this day, the water from the other three tanks was siphoned out and any larvae remaining in these three tanks were collected for measurements. Two of these tanks (replicates) were probiotic-treatment tanks and the other two were control tanks (replicates). There were therefore only two replicates per treatment on Day 7. Tank 8 was the only tank that had 10 larvae samples throughout the experiment (until Day 7). The three tanks from which the water was siphoned only had between one and three larval samples by Day 7. This reduced the robustness of any statistical analyses and made testing statistical significance impossible. The experiment could also not run to completion (Day 23).

Even though statistical tests cannot be performed, it can be seen that after 7 days of feeding, the surviving larvae had grown. This growth was seen regardless of treatment.

Discussion

This experiment successfully highlights the best conditions (incubation period and starting yeast concentration) for incubating two live feeds, rotifers (*Branchionus plicatilis*) and *Artemia* nauplii, with the probiotic yeast, *Debaryomyces hansenii*. Even though the 0.5 hour incubation period is not significantly different in comparison to the longer incubation periods when incubating *Artemia* with the yeast, the number of yeast cells per *Artemia* is still at a maximum (approximately 3994 yeast cells per *Artemia*) at this point, indicating that a short incubation period of 30 minutes is sufficient for uptake of yeast cells by *Artemia*. It is worth noting that the power of the ANOVA test that resulted in the aforementioned insignificant result (17.1%) was much lower than the desired power of 80%. This means that the probability of the test rejecting the null hypothesis when the alternative hypothesis is true, is only 17.1%. This also suggests that the sample size of the experiment was too small. A 30 minute incubation period was found to be optimal for uptake of yeast cells by rotifers and resulted in the uptake of approximately 169 yeast cells per rotifer. Even though the uptake of yeast cells by the rotifers and *Artemia* are highest at an incubation period of 30 minutes and a starting concentration of 1×10^{12} yeast cells.mL^{-1}, the yeast cells per rotifer and *Artemia* might not be at a suitable level for the optimisation of growth and survival of the larvae. The number of yeast cells taken up by the larvae depends on the number of rotifers or Artemia eaten by the larvae per g of their own body weight to satisfy their energy requirements. Dusky kob larvae have a better feed conversion ratio when fed a restricted food ration of 3.41% or 3.6% of their own body weight per day (Collett 2008). The yeast uptake that optimises larval growth and survival is species dependent and can be attributed to the differences in the amounts of polyamines secreted in the gut lumen of larvae of different species (Tovar-Ramirez et al. 2004). For instance, sea bass have an optimal growth rate when *Debaryomyces hansenii* has been taken up to a concentration of 1×10^{6} cfu.g^{-1} (Tovar-Ramirez et al. 2004). It is therefore important to determine the suitable level of probiotics that optimises the growth and survival of the dusky kob in future studies. It is also important to remember that in large-scale commercial live feed production and enrichment where time is of the essence, the shorter incubation periods would be advantageous.

The results of incubating rotifers with the yeast show a reduction in the number of yeast cells per rotifer as the incubation period increases. This suggests that the yeast is either losing its culturability over time or is possibly being used as a source of nutrients and digested by the rotifers. This was however not investigated in this study. Other studies have shown that culturing live feeds, such as cladocerans (*Moina macrocopa*) with *Debaryomyces hansenii* resulted in an increase in the essential amino acid content in the live feed (Kang et al. 2006), suggesting that the yeast cells were utilised as a source of nutrients. It might therefore be valuable to investigate culturing rotifers with live yeast and testing whether or not this increases the nutrient content of the rotifer and the rotifer yield. This is especially true for this study, as the mass cultures of rotifers were not always at sufficient concentrations for enrichment and larval feeding.

Since studies have shown that probiotics need to be at high concentration levels for the probiotic to be effective in the host (Wang et al. 2008), the experiment testing the starting concentration of the yeast is valuable. We showed that rotifers and *Artemia* are able to accumulate significantly higher numbers of yeast cells within their bodies at a higher starting yeast concentration of 1×10^{12} yeast cells.mL^{-1} when incubated with the yeast for 30 minutes. It is important to test the effectiveness of different starting yeast concentrations and assess the effects of the inclusion levels of probiotics in the feed and ultimately in the larvae. Certain concentration levels will be more effective for the optimisation of the growth and survival of certain species than others. These enrichment experiments involving rotifers and *Artemia* are also beneficial to the aquaculture industry, as these commercially used live feeds do not always fulfil the nutrient requirements of larvae as well as their wild feed, copepods, does (Penglase et al. 2011). Rotifers and *Artemia* therefore need to be enriched with unicellular algae, yeast or fish oil to bolster their nutrient contents (Kang et al. 2006).

At an age of 23 days post hatch, dusky kob larvae metamorphose and must be weaned onto formulated, dry feed (Fielder and Heasman 2011). It is therefore important to investigate the ability of live yeast to adhere to dry feeds and determine the stability and culturability of the adhered yeast cells when stored at different temperatures. We found that from days 4 to 9, the number of yeast cells per g of feed in the feed stored at 20 °C, was significantly lower than the number of yeast

cells per g of feed in the feed stored at 4 °C. We also found a pattern of decrease in the number of yeast cells per g of feed from days 0 to 2, followed by an increase in the number of yeast cells per g of feed from days 2 to 6, and then another decrease in the number of yeast cells per g of feed from days 6 to 14. This pattern was apparent in the two varying storage temperatures. Moreover, the reduction in the number of yeast cells from days 9 to 14 was significant for both storage temperatures, yet the increase in the number of yeast cells per g of feed from days 2 to 6 was significant only for the feed stored at 4 °C and not for the feed stored at 20 °C. On the other hand, the reduction in the number of yeast cells per g of feed from days 0 to 2 was significant for the feed stored at 20 °C and not for the feed stored at 4 °C. It is also valuable to note that during the mashing of the feed in YPD broth, we found that the feed stored at 20 °C was much harder to mash than the feed stored at 4 °C. The softening of the feed stored at 4 °C, as well as the significant increase in the number of yeast cells per g of feed from days 2 to 6, suggests that the yeast is proliferating on the dry feed and could be utilising the binders within the feed. Since this means that the yeast could be breaking down the feed in order to grow, the stability of the feed could be deteriorating. This deterioration of stability will be a problem when the feed is placed in water to be fed to larvae, as the feed might disintegrate before the larvae are able to feed on it. Future studies could therefore incorporate feed in water stability tests. The significant difference in the number of yeast cells per g of feed between the feed stored at 4 °C and the feed stored at 20 °C, suggests that *Debaryomyces hansenii* does not proliferate as well on this feed at 20 °C as it does at 4 °C. The higher storage temperature might be making it more difficult for the yeast to utilise the binders in the feed and break down the feed in order to grow as well as it is able to at the lower storage temperature. The significant reduction in the number of yeast cells per g of feed from days 9 to 14 suggest that the yeast is only able to grow on the feed for a short period of time. After this period, the culturability of the yeast might be reduced. Further studies could continue this experiment for a longer period of time to determine if this reduction is a permanent reduction or if it is part of the cycle of the yeast.

Studies suggest that coating dry feed in yeast after the feed extrusion process can have negative impacts on the growth rate of larvae because the buoyancy of the

feed is reduced by the yeast (Tovar et al. 2002). Further tests need to be conducted on the feeds developed in this study to determine whether or not *Debaryomyces hansenii* will have an effect on the buoyancy of the feed. Drying the feed after coating it in yeast also significantly reduces the number of yeast cells per g of feed. Further studies could investigate different drying temperatures and perhaps even other methods of drying the feed. As suggested by Tovar et al. 2002, the trade-offs of incorporating yeast into dry feed prior to extrusion and drying must be evaluated, as the preparation and storage of the enriched feed can become a major bottleneck when producing feed on a commercial scale (Wang et al. 2008).

Feeds enriched with probiotics are of value to industry, as these supplemented feeds can pass their potential immune boosting and improved nutritional qualities on to the larvae. Unfortunately, the mortality rate of the dusky kob larvae was very high in this study and a sufficient number of replicate tank samples could not be maintained for the duration of the trial. It is, however, important to note that the few larvae that did survive actually did grow, with or without probiotic enrichment.

Even though the probiotic yeast was incubated with live and formulated feeds in this experiment, the yeast-enriched live feed and dry feed could not be incorporated in a larval rearing experiment because of the high larval mortality rate. However, other studies have evaluated the effects of yeast supplemented live and formulated feeds on larval growth, development and survival (such as in Tovar-Ramirez et al. 2004; Park et al. 2006; Avella et al. 2010; and Tovar-Ramirez et al. 2010). Overall, these studies have demonstrated that a probiotic-enriched diet enhances the growth and improves the survival of the larvae that are fed these diets when compared with lavae fed non-probiotic supplemented feeds. In contrast, Tovar et al. (2002) found the growth rate of sea bass (*Dicentrarchus labrax*) fed diets enriched with *Debaryomyces hansenii* was less than that of the control sea bass fed non-supplemented diets, but the survival rate of larvae fed the enriched diets was higher. More recently, Tovar-Ramirez et al. (2010) recorded a positive growth result for sea bass fed diets enriched with *Debaryomyces hansenii*, and subsequently testing for the effect of the yeast on the enzymatic antioxidative status of the sea bass found that sea bass fed diets enriched with *Debaryomyces hansenii* had reduced oxidative stress. Since studies have shown varying results and no statistical analyses could be

performed, it is impossible to draw any conclusion from the larval length data presented in this study.

Owing to the high larval mortality rate, the growth trial could not be completed (did not run to Day 23) and too few replicate tank samples were obtained for statistical purposes. A possible limitation of the study that could have contributed to the high larval mortality rate is the number of times the larvae were fed per day. In this study, the larvae were fed twice per day – once in the morning and once in the afternoon. In a study conducted by Avella et al. (2010), the larvae were fed four times per day. Another limitation that could have been a contributing factor to the high larval mortality rate is the concentration of *Nannochloropsis oculata* maintained in the tanks. *Nannochloropsis oculata* is a type of microalgae (phytoplankton) that can be added to closed-water systems to form pseudo-green water. Pseudo-green water is formed by adding phytoplankton cells under light intensities that will not allow further proliferation of these cells (Hellenic Centre for Marine Research (HCMR) 2011). Even though pseudo-green water and green water are essential for aquaculture, Neori (2011) mentions that neither of these larval rearing methods have been quantified nor reported. For this reason, larval rearing studies tend to use varying concentrations of phytoplankton for pseudo-green or green water. In this study the *Nannochloropsis oculata* concentration was maintained at 230 000 cells.mL^{-1}; whereas, in a study performed by Papandroulakis et al. (2002), a *Nannochloropsis oculata* concentration of 750000±200000 cells.mL^{-1} was maintained. Since phytoplankton cells in pseudo-green water have been shown to act as a protective agent against pathogenic bacteria (Papandroulakis et al. 2002), increasing the *Nannochloropsis oculata* concentration could be beneficial to the larvae. Fielder and Heasman (2002) also worked with dusky kob and suggested that the optimal salinity for larviculture is 5-10 ‰ and that this salinity level is important for larval survival. This is far lower than the salinity of seawater (34-35 ‰) which was used in this study, even though dusky kob larvae are reared successfully at these salinities at several production facilities within South Africa. Moreover, dusky kob in the wild spawn near the mouths of estuaries and the larvae later recruit into these estuaries that are at salinities lower than that of seawater. It is possible that an increase in larval feeding intensity and *Nannochloropsis oculata* concentration and a decrease in salinity could have a positive effect on kob larval survival and should therefore be considered for

future investigations. The number of replicates should also be increased to ensure that all results are statistically viable.

In summary, three out of the four objectives of this study have been addressed. These three questions all pertain to the development of suitable methods for enrichment of live and formulated feeds with the probiotic yeast *D. hansenii*. We showed that rotifers and *Artemia* are able to accumulate high numbers of yeast cells within their bodies at an efficient incubation time of 0.5 hours and a starting yeast concentration of 1×10^{12} yeast cells.mL^{-1}. *Debaryomyces hansenii* is able to proliferate on the formulated feed significantly more when the yeast-coated feed is stored at 4 °C than when it is stored at 20 °C. The yeast could, however, be deteriorating the stability of the feed by utilising the binders within the feed for growth. At both storage temperatures, the number of yeast cells per g of feed significantly decreases after 14 days, which could indicate a loss of culturability and reduction in stability of the yeast in the feed after this period of time. Drying the feed after coating it in yeast also causes a significant reduction in the number of yeast cells per g of feed. Even though statistically analysed results for the growth of dusky kob (*Argyrosomus japonicus*) larvae being fed these enriched feeds could not be produced due to heavy larval mortality, the degree of sensitivity of the larvae was highlighted. Even in a controlled environment, larvae are sensitive and highly susceptible to many diseases and environmental changes. Future studies must therefore recognise the value of the live feed and dry feed experiments performed in this study and the importance of keeping larvae alive, against so many odds.

Acknowledgements

I gratefully acknowledge Dr Brett Macey of the Department of Agriculture, Forestry and Fisheries (DAFF) for allowing me to set up a hatchery at the research facility in Sea Point, providing me with the thesis idea and tools to complete the thesis and his constructive comments on earlier drafts of the thesis; Mark Goodman of DAFF for his help in setting up the hatchery, hatching the *Artemia* and keeping the rotifer and microalgae cultures going; Esau Mobokane of DAFF for his help in feeding the larvae; Dr Kevin Christison of DAFF for his help with the Nikon Software and with measuring the larval samples; Jared du Plessis for his help with counting the many yeast plates and rotifer samples; Oceanwise for their help with stocking densities and larval rearing suggestions; and Prof John Bolton for his constructive comments on earlier drafts of this thesis.

References

Abdel-Tawwab, M., Abdel-Rahman, A. M. and Ismael, N. E. M. 2008. Evaluation of commercial live baker's yeast, *Saccharomyces cerevisiae* as a growth and immunity promoter for Fry Nile Tilapia, *Oreochromis niloticus* (L.) challenged *in situ* with *Aeromonas hydrophila*. *Aquaculture* **280**: 185-189.

Abidi, R. 2003. Use of probiotics in larval rearing of new candidate species. *Aquaculture Asia* **8(2)**: 15-16.

Russell, A. 2013. Evaluation of the biological feasibility of white stumpnose, Rhabdosargus globiceps, as a potential aquaculture candidate in South Africa. *M.Sc. Thesis*, University of Cape Town.

Avella, M. A., Olivotto, I., Silvi, S., Place, A. R. and Carnevali, O. 2010. Effect of dietary probiotics on clownfish: a molecular approach to define how lactic acid bacteria modulate development in a marine fish. *American Journal of Physiology – Regulatory, Integrative and Comparative Physiology* **298**: R359-R371.

Ballagh, D. A., Fielder, D. S. and Pankhurst, P. M. 2010. Weaning requirements of larval mulloway, *Argyrosomus japonicus*. *Aquaculture Research* **41**: e493-e504.

Bjelland, R. M. and Skiftesvik, A. B. 2006. Larval development in European hake (*Merluccius merluccius* L.) reared in a semi-intensive culture system. *Aquaculture Research* **37**: 1117-1129.

Collett, P. D. 2008. Toward the development of a rearing protocol for juvenile dusky kob*, Argyrosomus japonicus* (Pisces: Sciaenidae). *MSc thesis*, Rhodes University.

DAFF. 2012. *South Africa's Aquaculture Annual Report 2011*. ISBN: 978-1-86871-369-1.

Delbos, B. and Schwarz, M. H. 2009. Rotifer production (as a first feed item) for intensive finfish larviculture. *Virginia Cooperative Extension Publication* **600 (105)**: 1-3.

Demir, O. and Diken, G. 2011. Effects of commercial enrichment products on fatty acid components of rotifer, *Brachionus plicatilis*. *African Journal of Biotechnology* **10(66)**: 15065-15071.

FAO. 2012. *The State of World Fisheries and Aquaculture*. FAO Fisheries and Aquaculture Department, Rome. 209 pp.

Fielder, D. S. and Heasman, M. P. 2011. Hatchery Manual for the production of Australian Bass, Mulloway and Yellowtail Kingfish. Industry and Investment NSW, New South Wales. 170 pp.

Gram, L., Melchiorsen, J., Spanggaard, B., Huber, I. and Nielsen, T. F. 1999. Inhibition of *Vibrio anguillarum* by *Pseudomonas fluorescens* AH2, a possible probiotic treatment for fish. *Applied and Environmental Microbiology* **65**: 969-973.

Griffiths, M. H. 1996. Life history of the dusky kob *Argyrosomus japonicus* (Sciaenidae) off the east coast of South Africa. *South African Journal of Marine Science* **17 (1)**: 135-154.

Gullian, M., Thompson, F. and Rodriguez, J. 2004. Selection of probiotic bacteria and study of their immunostimulatory effect in *Penaeus vannamei*. *Aquaculture* **233**: 1-14.

Hellenic Centre for Marine Research (HCMR). 2011. "The institute aquaculture - Hatchery technologies and larviculture", viewed on 24 October 2013, from http://www.imbc.gr/institute/aqua/hatchery.html.

Kang, C-K., Park, H. Y., Kim, M-C. and Lee, W. J. 2006. Use of marine yeasts as an available diet for mass cultures of *Moina macrocopa*. *Aquaculture Research* **37**: 1227-1237.

Macey, B. M. 2005. Probiotic effect of *Vibrio midae SY9, Cryptococcus sp. SS1* and *Debaryomyces hansenii AY1* on the growth and disease resistance of farmed *Haliotis midae*. *PhD thesis,* University of Cape Town.

Macey, B. M. and Coyne, V. E. 2005. Improved growth rate and disease resistance in farmed *Haliotis midae* through probiotic treatment. *Aquaculture* **245**: 249-261.

Neori, A. 2011. “Green water” microalgae: the leading sector in world aquaculture. *Journal of Applied Phycology* **23**: 143-149.

Papandroulakis, N., Divanach, P. and Kentouri, M. 2002. Enhanced biological performance of intensive sea bream (Sparus aurata) larviculture in the presence of phytoplankton with long photophase. *Aquaculture* **204**: 45-63.

Park, H. G., Puvanendran, V., Kellett, A., Parrish, C. C. and Brown, J. A. 2006. Effect of enriched rotifers on growth, survival and composition of larval Atlantic cod (*Gadus morhua*). *ICES Journal of Marine Science* **63**: 285-295.

Patra, S. K. and Mohamed, K. S. 2003. Enrichment of *Artemia* nauplii with the probiotic yeast *Saccharomyces boulardii* and its resistance against a pathogenic *Vibrio*. *Aquaculture International* **11**: 505-514.

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**: 144-153.

Planas, M. and Cunha, I. 1999. Larviculture of marine fish: problems and perspectives. *Aquaculture* **177**: 171-190.

Reyes-Becerril, M., Tovar-Ramirez, D., Ascencio-Valle, F., Civera-Cerecedo, R., Gracia-Lopez, V. and Barbosa-Solomieu, V. 2008 Effects of dietary live yeast *Debaryomyces hansenii* on the immune and antioxidant system in juvenile leopard grouper *Mycteroperca rosacea* exposed to stress. *Aquaculture* **280**: 39-44.

Ringø, E. and Birkbeck, T. H. 1999. Intestinal microflora of fish larvae and fry. Aquaculture research 30: 73-93.

Robertson, P. A. W., O'Dowd, C., Burrels, C., Williams, P. and Austin, B. 2000. Use of *Carnobacterium* sp. as a probiotic for atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss*, Walbaum). *Aquaculture* **185**: 235-243.

Roch, P. 1999. Defence mechanisms and disease prevention in farmed marine invertebrates. *Aquaculture* **172**: 125-145.

Romero, G. R., Fernandez, R. F., Martinez, V. J. L., Sanchez, M. M. J. and Garrido, F. A. 2010. Depletion of veterinary drugs used in aquaculture after administration in feed to gilthead seabream (*Sparus aurata*). *Journal of Food Protection* **73 (9)**: 1664-1670.

Tovar, D., Zambonino, J., Cahu, C., Gatesoupe, F. J., Vazquez-Juarez, R. and Lesel, R. 2002. Effect of live yeast incorporation in compound diet on digestive enzyme activity in sea bass (*Dicentrarchus labrax*) larvae. *Aquaculture* **204**: 113-123.

Tovar-Ramirez, D., Mazurais, D., Gatesoupe, J. F., Quazuguel, P., Cahu, C. L. and Zambonino-Infante, J. L. 2010. Dietary probiotic live yeast modulates antioxidant enzyme activities and gene expression of sea bass (*Dicentrarchus labrax*) larvae. *Aquaculture* **300(1-4)**: 142-147.

Tovar-Ramirez, D., Zambonino Infante, J., Cahu, C. Gatesoupe, F. J. and Vazquez-Juarez. 2004. Influence of dietary live yeast on European sea bass (*Dicentrarcchus labrax*) larval development. *Aquaculture* **234**: 415-427.

Treece. G. D. 2000. *Artemia* Production for Marine Larval Fish Culture. *Southern Regional Aquaculture Center* **702**: 1-7.

Verschuere, L., Rombaut, G., Sorgeloos, P. and Verstraete, W. 2000. Probiotic bacteria as biological control agents in aquaculture. *Microbiology and Molecular Biology Reviews* **64(4)**: 655-671.

Wang, Y. B., Li, J. R. and Lin, J. 2008. Probiotics in aquaculture: Challenges and outlook. *Aquaculture* **281**: 1-4.