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Effects of feeding with copepod nauplii (Acartia tonsa) compared to rotifers (Brachionus ibericus, Cayman) on quality parameters in Atlantic cod (Gadus morhua) larvae

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Abstract

Good nutritional quality is key for a successful Atlantic cod (*Gadus morhua)* juvenile production. Copepods are the natural prey for marine fish larvae, and their nutritional composition is believed to be optimal for the marine fish larvae. Despite the suitability of copepods for cod larval cultivation, only a few hatcheries have used copepods, and then extensively cultivated. It has several times been documented that nutrition is important for survival, growth and the general quality of cod larvae. Even though these variables are challenges to many farmers of juvenile cod, there has still not been developed any standard evaluation of quality, perhaps with the exception of bone deformation analysis.

Cod larvae were given 4 different treatments from 3 to 28 dph. One was fed the copepod *Acartia tonsa* nauplii (**Copepod**), a second fed enriched rotifers *Brachionus ibericus* (**RotMG**), a third fed unenriched rotifers (**RotChl**) and a forth copepods from 5 to 11 dph and enriched rotifers the rest of the period (**Cop7**). All treatments were fed *Artemia* sp. from 20 to 40 dph and dry feed from 36 to 60 dph. In addition to assessing growth and survival, quality of the fish larvae was assessed by using the following quality parameters: larval feeding activity, response to handling stress, skeletal deformations and shape variation.

Already from 8 dph the larvae fed copepods (Copepod and Cop 7) had a higher dry weight (DW) than larvae fed rotifers. At 60 dph the Copepod had the highest DW and survival rate, followed by the Cop 7 treatment larvae, and with the two rotifer treatments having the lowest DW and survival rate. Larvae from the rotifer treatments swam twice as much per prey caught during analysis of feeding behaviour and had the highest mortality rate after handling by air exposure, compared to the two copepod treatments. The Copepod treatment gave the lowest percentage of deformities, followed by Cop 7, RotMG and RotChl, in that order. The different treatment gave differences in the shape of the larvae. The results from this study show that feeding cod larvae with intensively cultivated copepods nauplii (*Acartia tonsa*) for the first 28 days past hatching results in a better survival, growth and quality of the larvae than feeding with rotifers, and underlines the importance of early larval nutrition.

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1. Introduction

1.1 Background

Commercial aquaculture in Norway began around 1970, and is today one of the major industries in coastal areas. A long, sheltered coastline, together with a reliable and stable water temperature is some of the success factors for Norwegian aquaculture. Farmed salmon is one of the biggest export commodities from Norway (Skonhoft, 2010). Stagnation in the supply of cod catches, together with the success of salmon farming, have led to an increase in the interest of farmed Atlantic cod (*Gadus morhua*) (Rosenlund & Halldorsson, 2007; Nakken, 2008).

In May 1983 the first breakthrough in cod mass cultivation took place: 75 000 cod juveniles were cultivated and collected in a marine pond at the Institute of marine research, Austevoll Biological Station, these cod larvae were cultivated extensively (Øiestad *et al.*, 1985). In extensive cultivation the newly hatched larvae are placed in closed sea basins and natural predators are removed, while natural prey (zooplankton) are added, if not already there (Svåsand *et al.*, 2003). The intensive production, on the other hand, is located on land in a closed and controlled environment, giving the farmers improved control over environmental factors, like: temperature, light, aeration, microbial load etc. It also ensures a stable access to feed in a controlled quantity, and with a more known nutritional and microbial content. The extinctive fish larvae production is more limited compared to the intensive production, especially in terms of unit volume^{-1} and seasonal production (Shields, 2001; Svåsand *et al.*, 2003). These factors give intensive fish cultivation a strong overall incentive in marine fish larvae hatcheries all over Europe (Shields, 2001).

Since copepods are the natural feed for cod larvae and we may assume that their nutritional composition is ideal for cod larvae (Sargent *et al.*, 1999). By intensively cultivating copepods, compared to extensively copepod cultivation, one would be able to gain a continuous year-round supply, in addition to a more controlled nutritional and microbial load (Støttrup, 2000). This would be more suitable for intensive cod larval

production, compared to extensive cod larval production. One of the main differences between extensive and intensive cod larval production is the use of natural zooplankton, like copepods, as live feed in the extensive production (Rosenlund & Halldorsson, 2007). Cod larvae given natural zooplankton instead of rotifers have shown a significantly higher n-3 HUFA level, together with higher growth rates, better survival, less deformities and a generally better quality (Kjørsvik *et al.*, 2009; Busch *et al.*, 2010; Koedijk *et al.*, 2010). These are all variables cod juvenile hatcheries are struggling with, and the use of intensively cultivated copepods has the possibility to be a future solution.

1.2 Larval growth and development

Cod larvae have a great growth potential and are aggressive feeders (Rosenlund & Halldorsson, 2007), and cannibalism has been reported in combination with sub-optimal food availability (Øiestad *et al.*, 1985; Folkvord, 1991). The physical (light, temperature, current, etc.) and chemical environment (salinity, pH and metabolites), biotic factors (viruses, bacteria etc.), nutritional content (live feed) and fish stocking density are all factors important for growth, survival and development of the marine fish larvae (Hamre, 2006; Kjesbu *et al.*, 2006). Cod larvae hatch from small (1.3 mm), pelagic eggs. The small size of the egg limits the amount of yolk (Morrison, 1987). Exogenous food should be given to the larvae prior to yolk-sack absorption, to prevent starvation (Yin & Blaxter, 1986). The gastro-intestinal system of the cod larvae is not fully developed before after metamorphosis, but they will already from hatching posses the functions and structures essential for digestion and absorption of nutrients (Kjørsvik *et al.*, 2004). The start of exogenous feeding is concurrent with the development of organs critical for successful feeding, like vision and muscle development, and optimal conditions are therefore especially important at this stage (Bell *et al.*, 1995; Galloway *et al.*, 1999).

1.3 What is the optimal live feed for cod larvae?

The highest mortality for fish larvae is found during the transition from endogenous (from yolk) to exogenous (from external prey) feeding (Wiborg, 1976; Hewitt *et al.*, 1985). The feed given to the larvae at this stage has been reported vital, as it affects the later performance of the fish larvae (Koven *et al.*, 2001; Hamre, 2006; Rosenlund & Halldorsson, 2007; Kjørsvik *et al.*, 2009).

The growth rates in intensive systems are generally lower than in extensive systems. High mortality, variable quality and poor reproducibility are other key obstacles within intensive production (Hamre, 2006). Normal protocol for intensive cod hatcheries has been the use of enriched rotifers followed by enriched *Artemia* nauplii (Olsen *et al.*, 2004). *Artemia* nauplii have lately gradually being replaced by formulated feed, so-called early weaning, but this has shown to have a negative effect on the growth and survival of the cod larvae (Callan *et al.*, 2003; Hamre, 2006). *Artemia* sp. and rotifers are not a part of the marine fish larva's natural diet, which mainly consists of natural zooplankton, like copepods. The use of natural zooplankton in cod cultivation has shown to have a positive effect in the growth, survival and development of marine fish larvae (Støttrup, 2000; Evjemo *et al.*, 2003; Hamre, 2006). Despite the suitability of copepods as prey for marine fish larvae, there are only a few hatcheries using copepods as live feed, and these are mainly cultivating copepods extensively. Copepods have been tried intensively cultivated, predominantly in small scale and with limited duration (Støttrup, 2000). Støttrup (2000) stated "A basic knowledge of the physiological processes and population dynamics of a species is a prerequisite for the development of rearing techniques". Scaling-up of intensive copepod cultivation has been shown difficult and expensive in terms of commercial production, and the current supply is geographically and qualitatively limited (Støttrup, 2000; Drillet *et al.*, 2011). A possible solution could be a large-scale production of copepod eggs, which could be further distributed to the fish hatcheries. This would be more optimal as distribution of live copepods are more space limited and sensitive to distributions (Drillet *et al.*, 2011). SINTEF have, in cooperation with NTNU, started to intensively cultivate copepods (*Acartia tonsa)* and harvesting eggs for storage at 1.5-4°C (Nesse, 2010). Stored eggs have later been hatched and used to feed cod larvae intensively cultivated. This has resulted in higher

survival and dry weight compared to cod larvae fed rotifers, which has been linked to the superior nutritional content in copepods (Overrein *et al.*, unpublished). For future commercialization in will be essential to develop new technology and automation of the process, gaining a more cost-effective production (Øie, G., SINTEF, pers. com.).

Both *Artemia* sp. and rotifers are easily cultivated, they tolerate high densities and variable salinity (Støttrup, 2003). According to Bush et al., 2010 the growth potential for cod larvae would never be fully exploited when using rotifers instead of natural zooplankton, since their nutritional value don't match the needs of the cod larva. For the nutritional value of the rotifers and *Artemia* sp. to better suit the requirement of the fish larvae they are enriched, mainly with lipids (Evjemo & Olsen, 1997; Støttrup, 2003).

Nutritional imbalance is a well-known factor to affect growth, survival and quality of marine fish larvae, like cod (Cutts *et al.*, 2006; Hamre, 2006). Finn et al. (2002) showed that cod larvae given the right nutritional and environmental condition, could increase their mean weigh 2000 times during the first 50 days of exogenous feeding (Finn *et al.*, 2002). Information about the nutritional requirements of the marine fish larvae has been difficult to achieve, as it is hard to control the live feed nutritional composition precisely (Shields, 2001).

Lipids

The lipid quality and quantity of the feed are central for the viability of the marine fish larvae. Lipids are used either as structural components of the membrane, or for energy production (Watanabe & Kiron, 1994). Several studies have shown that highly unsaturated fatty acids (HUFA) in the n-3 and n-6 series, mainly DHA (docosahexaenoic acid), EPA (eicosapentaenoic acid) and AA (arachidonic acid), are essential for marine fish larvae during start feeding. HUFA deficiency has shown to delay fish growth and development, and induces mortality (Rainuzzo *et al.*, 1992; Watanabe & Kiron, 1994; Izquierdo *et al.*, 2000). In addition to the minimum dietary requirement of n-3 HUFA, the relative proportion is important, and control over both the dietary quantity as well as absolute amounts is necessary (Izquierdo *et al.*, 2000). Evjemo and Olsen (1997) found the following lipid content (% of DW) when analysing different live feed: *Artemia* 21-23%, Rotifers 6-11% and Copepod (*Calanus finnmarchicus, Temora longicoris Acartia* sp.

Eurytemora sp.*)* 12-13% at copepodid I, II and II, and increasing to 24% at copepodid IV, V and adult. Even though *Artemia* sp*.* had higher lipid content compared to copepods, the copepods had a significantly higher level of n-3 HUFA.

It is generally agreed that phospholipids in the diet can improve the performance of marine fish in culture. The greatest advantages are the increase in growth, survival, intestinal maturation and reductions of skeletal deformations (Tocher *et al.*, 2008; Wold *et al.*, 2009). DHA and EPA can be incorporated as phospholipids (PL) or neutral lipids (NL). It is known that when incorporated in the dietary polar PL, the fatty acids are more beneficial to the cod that more efficiently absorb HUFA when incorporated in PL (Cahu *et al.*, 2003a; Gisbert *et al.*, 2005; Kjørsvik *et al.*, 2009; Wold *et al.*, 2009). PL are the main component of cell membranes, and are essential for growth and development of the fish larva, which have a limited ability to synthesise PL *de novo* (Kanazawa *et al.*, 1985; Coutteau *et al.*, 1997). Copepods (*A. tonsa)* has shown to contain a higher percentage of HUFA in their phospholipids, while rotifers and *Artemia* sp. has shown less HUFA and more monounsaturated fatty acids, which has been linked to higher survival and growth rates to cod larvae fed copepods (Overrein, 2010).

Protein

Protein is the most abundant component in marine fish eggs, which could contain as much as 50% of the total amino acid pool as free amino acids (FAA). During the yolk-sac-larvae-stage the FAA pool is depleted (Watanabe & Kiron, 1994; Rønnestad *et al.*, 1999). It is therefore crucial for the fish larvae to get essential FAA trough the first feed. The essential FAA are those that cannot be synthesised or are inadequately synthesised *de novo* by the fish larvae (Li *et al.*, 2009). As commented on earlier, the fish larvae have an great growth potential and this growth is mainly through muscle growth increase by protein synthesis (Rønnestad *et al.*, 1999). The FAA plays an important role in the metabolism and development of the fish, including cell signalling, appetite stimulation, growth, immunity, stress response, metamorphosis, neural development, behaviour, pigmentation and regulation (Li et al., 2009). Skeletal malformations has shown to significantly decrease during optimization of the protein content in fist feed (Cahu *et al.*, 2003b). When enriching *Artemia* sp. and rotifers with lipids the relative protein content will decrease, but the quantitative content will stay fairly unchanged, as the prey is

gaining weight in terms of lipids (Øie & Olsen, 1997; Olsen *et al.*, 2000; Evjemo *et al.*, 2003). Earlier publications have shown the following protein content (% of DW) in live feed: *Artemia* sp. 41% in newly hatched nauplii and 34% after 24-h enrichment and rotifers 24-61% dependent on enrichment, compared to >50% for copepods (*Calanus finnmarchicus, Temora longicoris Acartia* sp. *Eurytemora* sp.*)* at copepodid stages I-IV (Helland *et al.*, 2002; Evjemo *et al.*, 2003; van der Meeren, 2003).

Minerals and vitamins

Minerals, especially calcium (Ca) and phosphorus (P), are essential during larval development, particularly bone formation. Ca can be obtained from the surrounding water, in addition to diet, while P can only be obtained from diet (Power, 2009). Minerals like: manganese, iodine, selenium, copper and zinc, has also been shown essential during skeletal development (Moren, 2009) Research has also shown that Vitamin A is an important nutritional factor during the live feed period of marine fish, especially during bone formation (Moren *et al.*, 2009; Georga *et al.*, 2011). Vitamin A is a fat-soluble vitamin important for gene regulation and sight. It has earlier been shown that too much vitamin A may result in skeletal changes, especially vertebral fusions. Since copepods, *Artemia* sp. and rotifers do not contain any vitamin A, too much is an unlikely problem in aquaculture (Hamre *et al.*, 2008; Moren *et al.*, 2009). On the other hand, both copepod and *Artemia* sp. contains carotenoids, also called pro-vitamin A (the precursor to vitamin A). The level of carotenoids in copepods and *Artemia* sp. is believed to give the larva the right amount of vitamin A needed (Moren *et al.*, 2002; Georga *et al.*, 2011). Rotifers are the live feed firstly given to the cod larvae in traditional intensive production, and the lack of carotenoids in rotifers is believed to possibly affect the skeletal development of the larvae (Moren *et al.*, 2009). Furthermore, vitamin D and C is also regarded as essential during bone formation. Vitamin D is a hormone that maintains calcium and phosphorus haemostasis, which together with vitamin K acts on the bone cells (osteoblasts and osteoclasts) and is therefore indirectly affecting bone development (Hamre *et al.*, 2010). Vitamin C is an antioxidant, essential for collagen synthesis. Deficiency of these vitamins may lead to deformations in the skull and vertebrae of the marine fish larva (Zambonino-Infante *et al.*, 2009). Vitamin C has also shown to increase growth rate and stress response in marine fish larvae (Cahu *et al.*, 2003a).

1.4 Possible parameters for determining cod larvae and juvenile quality

Despite problems with variable quality, there has still not been developed any standard evaluation to determine the cod larval and juvenile quality. The occurrence of deformations has been a common problem for cod hatcheries, and bone deformation analysis are today the most used variable when analysing at marine fish larval quality (Bæverfjord *et al.*, 2009). Morphometics is also a tool used in quality assessment of fish larvae, and the use has been more advanced in recent years (Adams *et al.*, 2004). Other variables like stress responses and feeding behaviour has also been used during studies of marine fish larval and juvenile quality.

The use of these different quality parameters could give a broader picture of the different quality aspects of the cod larvae, and combined provide a good description of cod larval and juvenile quality, and possible predictions on future fish quality. This could be a helpful tool for both farmers and scientists.

Skeletal deformities

One of the most common problems for fish hatcheries is the presence of deformities in the skeleton (Lein *et al.*, 2009). This is mainly a problem for the fish welfare, but also an economical issue for the producers (Boglione *et al.*, 2003; Koumoudouros, 2010). Like all vertebrates, the cod has a highly developed internal skeleton (endoskeleton). Muscles are attached to the vertebra, which bends during normal locomotion. The shape and number of vertebrae varies among different species and different regions of the vertebral column (Harder, 1975). Skeletal deformations in marine fish larvae could be due to dietary factors, e.g. suboptimal lipid (Sargent *et al.*, 1999) and protein content (Cahu *et al.*, 2003b) or lack of vitamins and minerals (Lall & Lewis-McCrea, 2007; Bæverfjord *et al.*, 2009) and abiotic factors, e.g. temperature (Georgakopoulou *et al.*, 2010), or water speed (Helland *et al.*, 2009a).

The vertebral column can be divided into 2 parts; 1) pre-hemal vertebrae (carrying epipleural and pleural ribs, open hemal arch, without hemal spines) and 2) hemal vertebrae (with hemal arches and spines). The unpaired fins are divided in dorsal-, anal-

and caudal fins (Boglione et al., 2003), and the paired are divided in the pectoral- and pelvic fins (Harder, 1975) (figure 1.1).

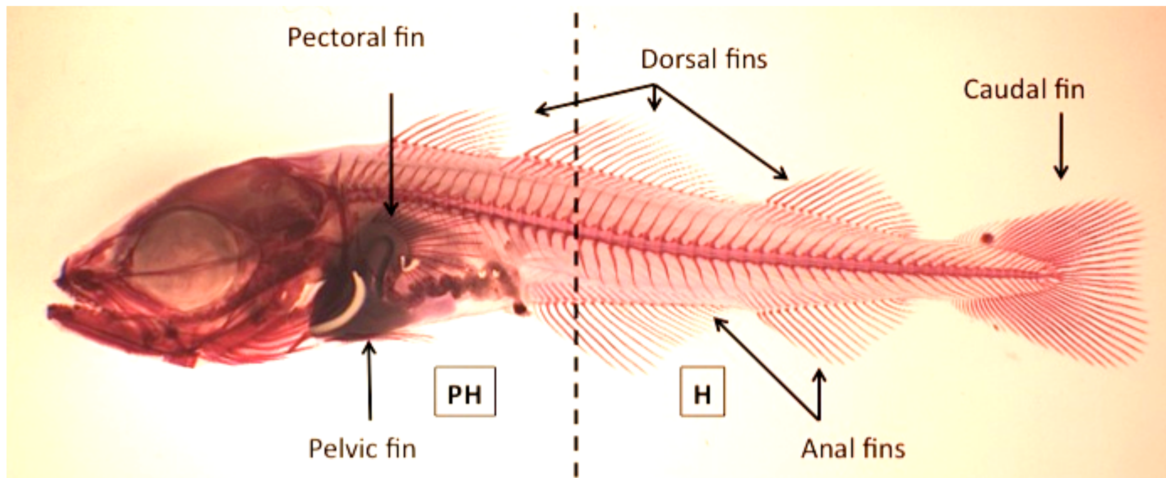

Figure 1.1 *Picture showing the dorsal, anal, caudal, pelvic and pectoral fins on a cod larva stained with Alizarin red at 60 dph,. The different regions throughout the vertebral column are divided in pre-hemal vertebrae (PE) and hemal vertebrae H shown with a stippled line.*

Staining and radiography are well known techniques during malformation diagnostics. Staining with Alizarin Red S is preferred for fish < 1g. Alizarin red only colours bone, not cartilage. Staining at this stage will reveal malformations induced during the whole larval period up to metamorphosis (Bæverfjord *et al.*, 2009).

Ossification is the transformation from cartilage to bone (not membranous bones) (Harder, 1975), and nutrition has earlier been shown to affect this process (Cahu *et al.*, 2003b). Some of the most commonly described deformations in the vertebral column of marine fish, including cod, are lordosis, kyphosis, scoliosis, stargazing and fusion (Boglione *et al.*, 2001; Bæverfjord *et al.*, 2009; Lein *et al.*, 2009). The larval size is important for the frequency and degree of deformities. Lordosis can develop to the better or the worse during larval growth, and low grades of lordisis could even disappear (Hjelde, 2009). Some deformations might not be visible at early stages, e.g. stargazing. Fusion in the vertebrae will never disappear during larval growth, but may get worse. All of these possible changes make further development of deformations in fish larvae unpredictable (Hjelde, 2009).

Shape variation

In addition to deformities, normal phenotypic variation on body shape and meristic characters can also be affected by factors acting during the embryonic and larval stages. The phenotypic plasticity is a genotypes ability to respond to different environmental conditions, as it will maximize its fitness in a fluctuating environment (Pigliucci et al., 1996). This can be seen as an increase in growth of the whole body, a specific morphological structure or both. Variation in shape could be due to both nutritional (Georga *et al.*, 2011) and environmental factors (Koumoundouros *et al.*, 2001; Georga & Koumoundouros, 2010). Cod can grow both isometric, a proportional increase in all morphometric measurements as size increases, and allometric, increase in different parts of the same organism that grow at different rates, affecting the shape of the organism. Most of the allometric growth takes place before and during metamorphosis (Marcil et al., 2006), indicating that the nutrition in the feed prior to metamorphosis is important during the shape development of cod.

Morphometrics is a quantitative way of determining shape variations. Traditional morphometrics has been performed by measuring length, depth and height, this does not provide a sufficient picture of the complex shape of a fish larva (Zelditch, 2004). Geometric morphometrics is a relatively new tool, helping us visualising the shape differences between complex shapes in a more adequate way. When using geometric morphometrics one has to mark a specific numbers of landmarks on the larvae, orientated at homologous anatomical points (Adams *et al.*, 2004). Other factors also important when choosing landmarks are: A) They have to provide adequate coverage of morphology, B) have to be easily found repeatedly and reliably, and C) lie within the same plane. When studying shape variation between different individuals the size of the organisms are unimportant, but they have to be at the same developmental stage. Differences in location and rotation are not shape variations, and are therefore important to remove before analysing shape (Zelditch, 2004).

Behaviour

The larvae's ability to capture prey will have an impact on growth and survival. The larval feeding behaviour could therefore be a good indication on how efficiently the larvae are gaining energy (von Herbing & Gallager, 2000). Atlantic cod larvae are gape-limited predators, meaning that they will not eat the prey if it is too big, even though they may attack it (Puvanendran *et al.*, 2004). The feeding behaviour of Atlantic cod larvae consists of five parts: search, perception, pursuit, attack, and capture, with tree different outcomes: successful attack, aborted attacks, and unsuccessful attacks. During feeding, cod larvae swim by first beating their tails and then gliding, a so-called burst-and-glide-swimming pattern (MacKenzie & Kiørboe, 1995; von Herbing & Gallager, 2000; Peck *et al.*, 2006).

A successful attack will normally have a small attack distance and higher speed, compared to unsuccessful attacks (von Herbing & Gallager, 2000). The duration of the attack will get shorter as the larva grows. As the cod grows, the swimming speed and Reynolds number (ratio of internal forces to viscous forces) increases, all of which affects the attack success-rate positively (Hunter, 1972; von Herbing & Gallager, 2000). The cod is both a pause-travel predator and a cruising predator (von Herbing & Gallager, 2000). A pause-travel predator will stop during prey search and look around for prey in different angles, while a cruising predator is continually moving around, searching for prey in the outer boundary of the search space (O´Brien *et al.*, 1990).

The ratio of successful attacks could depend on the development of the larval eye. Bell and colleagues (Bell et al., 1995) found a linear relationship between dietary DHA and the number of rods in the retina of Herring (*Clupea harengus* L.), and they concluded that dietary deficiency of DHA during first feeding could impair visual performance such that the fish no longer could feed at low light intensities. Also in cod, a large amount of the retina consists of DHA (Bell & Dick, 1991; Sargent *et al.*, 1999). The dietary effect of proteins are important for muscle growth, leading to raised performance during locomotion, as the speed and general activity level of the larvae increases (Müller, 2008).

Tolerance to handling stress

The nutritional content of the live feed has shown to affect the larva's ability to tolerate stress, e.g. handling and weaning, as better nutritional quality results in more hardy larvae (Bell et al., 1985; Castell et al., 1994; Koven et al., 2001). The larval response to stress is important for further development and survival. Stress has been defined as a condition where the dynamic equilibrium of an organism, called homeostasis, is threatened or disturbed as a result of the actions of intrinsic or extrinsic stimuli, commonly defined as stressors (Barton & Iwama, 1991; Chrousos & Gold, 1992; Bonga, 1997; Iversen & Eliassen, 2009). In hatcheries the fish may experience stress during handling. The effects of stress are grouped as: primary (plasma cortisol), secondary (metabolic, cardiovascular, hydromineral balance, respiratory- and immune functions) and tertiary (the whole fish), affecting growth and survival (Barton & Iwama, 1991; Barton, 2002; Braun *et al.*, 2010).

Sudden changes in temperature and salinity, and handling, e.g. air exposure, are all stressors commonly used to display the larval response to stress. Cortisol levels, Na^+, K^+-ATPase activity and mortality are factors than has been monitored during determination of stress effect on fish larvae (Kanazawa, 1997; Koven *et al.*, 2001; Van Anholt *et al.*, 2004). Cortisol level and Na^+, K^+-ATPase activity shows the primary and secondary stress effect, while mortality is a tertiary stress effect (Barton, 2002).

1.5 Study objectives and approach

The aim of this study was to investigate the effect of feeding cod larvae with intensively cultivated copepod nauplii (*Acartia tonsa)* compared to rotifers of different qualities. Also, the effect of feeding with copepods for 28 days compared to 7 days were studied. The 7-day period was at the start of exogenous feeding, which has been characterized as the most crucial period.

The effects were measured as growth, survival and quality. Since there has not been developed any good standardized quality criteria for cod larvae and juveniles, with the exception of bone deformations, the aim of this thesis was also to investigate possible methods of measuring quality in cod larvae and juveniles that could be used by farmers and scientists. The quality parameters tested were based on parameters successfully used in pervious studies, namely skeletal deformities, shape variation, larval feeding activity and response to handling stress.

2. Materials and methods

The experimental and analytical work took place at Norwegian University of Science and Technology (NTNU), Centre of Fisheries and Aquaculture (Sealab) in Trondheim (May 2010 to February 2011). Part of the analytical work was done at the University of Crete, Department of Biology, Heraklio, Greece (December 2010).

2.1 The start feeding experiment

Four different feeding regimes were used during the first feeding experiment (table 2.1). For each treatment there were 3 replicating tanks. The treatment named "Copepod" got the copepod *Acartia tonsa* exclusively as first feed from 3-20 dph. The "Cop 7" treatment was fed copepods exclusively from 5-8 dph, co-fed with copepods and enriched rotifers from 9-11 dph (table 2.2), and enriched rotifers from 3-4 and 12-20 dph. "RotMG" got enriched rotifers exclusively from 3-20 dph, and "RotChl" got unenriched rotifers exclusively from 3-20 dph. 12 000 rotifers/copepods L^{-1} was added to the fish tanks manually 3 times day^{-1}. All treatments were weaned onto *Artemia* sp. 20-28 dph (3000 *Artemia* sp. $feeding^{-1}$ $tank^{-1}$), before being fed *Artemia* sp. exclusively 29-35 dph. After the live feed period there was a short weaning period (35-40 dph) onto dry feed. From 40-60 dph all treatment were fed formulated feed (Gemma micro 300, Skretting, Norway) (10g dry feed $feeding^{-1}$ $tank^{-1}$). *Artemia* sp. and dry feed was fed to the larval tanks with a Storvik feeding robot (Storvik Aqua AS, Norway). The microalgae *Nannochloropsis oculata* (Reed Mariculture Inc., USA) was used during the live feed period, at 2 mg C L^{-1} $feeding^{-1}$ until weaning. The experiment was terminated at 60 dph.

2.2 Larval rearing

The cod eggs came from the Norwegian Cod Breeding Center (Nofima, Tromsø, Norway). The eggs were from a broodstock population with several males and females, spawning together. Directly after arrival the eggs were disinfected with 400 ppm glutaraldehyd for 10 minutes and thoroughly rinsed before transferred to an egg incubator, a 270 l flow through glass fibre tanks, with conical bottoms and 34 ppt seawater. The incubator was tended every day, and temperature (6 ^{0}C) and aeration (moderate) was checked daily. The water flow rate was 3 litres hour^{-1}. Two days prior to hatching the eggs were transferred to 15 flow-through glass fibre tanks, with conical bottoms (100 l) in 34 ppt seawater, with 100 eggs litre^{-1} (10 000 eggs tank^{-1}). Using the mean egg diameter the numbers of eggs ml^{-1} was known using table 7.1 in Holm et al., (1991). The formula $C_1V_1=C_2V_2$ (C=Concentration V=Volume) was used to calculate ml egg tank^{-1}, when the density in each tanks should be 100 eggs litre^{-1}. The hatching rate was >95%.

After hatching the larvae were kept under constant light. The temperature gradually increased from 6 ^{0}C at the start, to 12 ^{0}C at 17 dph. The water exchange rate gradually increased from 2 times day^{-1} to 8 times day^{-1} at 31 dph (table 2.1). The feeding density was not changed according to growth or mortality, and the ratio would therefore go up with increased mortality. Seawater was sand filtered, microbial matured and filtered through a 1µm mesh before use in the fish tanks.

Table 2.1 *The cod start feeding experiment*

Dph	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29-35**	**36**	**37**	**38**	**39**	**40**	**41-60**
Day degrees	6	12	18	24	31	38	46	54	63	72	82	92	103	114	126	138	150	162	174	186	198	210	222	234	246	258	270	282	294-336	348	360	372	384	396	610
Water exchange rate	2	2	2	2	2	2	2	3	3	3	3	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4-8	8	8	8	8	8	8
Temp.	6	6	6	6	7	7	8	8	9	9	10	10	11	11	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
Copepod			**Copepods**																											**Dry feed (Gemma μ)**					
3 tanks																				***Artemia* sp. (Enriched with Multigain)**															
Cop 7					**Copepods**							**Rotifers (enriched with Multigain)**																		**Dry feed (Gemma μ)**					
3 tanks																				***Artemia* sp. (Enriched with Multigain)**															
RotMG			**Rotifers (enriched with Multigain)**																											**Dry feed (Gemma μ)**					
3 tanks																				***Artemia* sp. (Enriched with Multigain)**															
RotChl			**Rotifers (not enriched)**																											**Dry feed (Gemma μ)**					
3 tanks																				***Artemia* sp. (Enriched with Multigain)**															

Table 2.2 *Co-feeding for regime Cop 7*

DPH	5	6	7	8	9	10	11
Copepods	100 %	100 %	100 %	100 %	57 %	40 %	54 %
Rotifers	0 %	0 %	0 %	0 %	43 %	60 %	46 %

2.3 Live feed production

2.3.1 Cultivation and harvesting of microalgae (*Rhodomonas baltica)*

The algae *Rhodomonas baltica* (clone NIVA 5/91 Cryptophycea: Pyrenomonadales) was cultivated in 160 and 200 litres transparent plastic cylinders, and in 300 litre transparent plastic bags, with 34 ppt seawater. Every day 40-50 % of the cultures were harvested, and the volume was refilled with seawater (20 ^{0}C) added Conwy medium (1 – 1.5 ml Conwy L^{-1} added seawater) (Walne, 1974). The seawater was kept in reservoirs where it was chlorinated (100 ml chlorine $litre^{-1}$ seawater), and after a minimum of 5 hours the water was dechlorinated (3 g thiosulphate 100 ml^{-1} chlorine) for a minimum 5 hour under heavy aeration before usage (Hoff & Snell, 1987). At harvest the density of the algae was ~1 million algae ml^{-1}, and the pH was at all times kept between 7.5 – 8.3. CO_2 was added in the aeration, and if the pH was outside the optimal range, more or less CO_2 was added. Around every cylinder and bag there were 3 light sources, each containing 2 X 58 W fluorescent tubes.

The cultures were exchanged every 14th day, and the cylinders were washed, chlorinated and dechlorinated. The bags were changed between every culture. The cultures were started from 20 l bottles of *R. baltica* from intermediate cultures and daily diluted until doubled with seawater and Conwy, before filling 160-200 l in the cylinders and 300 l in the bags. When the density in the cylinders/bags was high enough (1 million algae ml^{-1}) the cultures were harvested as continuous cultures. Seawater was sand filtered and filtered through a 1μm mesh before use.

2.3.2 Cultivation, harvesting and enrichment of rotifers (*Brachionus ibericus*, Cayman)

The rotifer (*B. ibericus)* was cultivated in 270 l flow-through glass fibre tanks, with conical bottoms and 34 ppt seawater. The egg ratio and rotifers ml^{-1} was counted and calculated daily. The rotifers were fed continuously with the algae *Chlorella vulgaris sp.* (Chlorella industry co. Ltd, Japan) (2.4 ml $million^{-1}$ rotifer). For optimal conditions, the temperature (20-25 ^{0}C), oxygen saturation (70-110%) and water exchange rate (100% day^{-1}) were checked daily. The filter in the tanks and the feed containers were washed daily. Once a week the rotifers were transferred to a smaller container and rinsed, and the tanks were thoroughly washed. The density of rotifers in the tank was kept between

300-800 rotifers ml^{-1}. The rotifers were harvested using a siphon, and concentrated using a 60 μm sieve. The rotifers were enriched with Multigain (Biomar, Norway) (0.2 g $million^{-1}$ rotifer) for 2 hours before being washed and fed to the fish larva. The density during the enrichment was never more then 400 rotifers ml^{-1}, due to limited oxygenation. Seawater was sand filtered and filtered through a 1μm mesh before use.

2.3.3 Cultivation of copepods (*Acartia tonsa)*

Production of copepod eggs

The copepods (*A. tonsa)* (clone DFH.AT1) were grown in 1000 and 1600 litre tanks, with 34 ppt seawater, at 20 °C. Algae (*Rhodomonas baltica)* were continuously fed to the Copepods (Nesse, 2010). The algae density in the tanks should be minimum 30 000 cells ml^{-1} (Skogstad, 2010). To ensure this, the density was counted weekly with a cell counter. The eggs were harvested every day. A harvesting arm was used to collect the eggs. The eggs was then washed thoroughly and stored in NUNC EasyFlask™ Nunclon™ (75 cm^3) cell culture bottles at 2 °C. Egg harvesting started 3 month in advance of the experiment, and continued until 23 dph in the experiment. The water in the bottles was exchanged every 14th day. Numbers of eggs in each bottle and the hatching rate were calculated and tested (se appendix 1). Seawater was sand filtered and filtered through a 1μm mesh before use.

Production of copepod nauplii

The copepod eggs were incubated in 100 litres tanks, with a maximum density of 150 eggs ml^{-1}. From 2 dph the nauplii were fed *ad libitum* with *Rhodomonas baltica* (Nesse, 2010*)*. The seawater in the tanks held a temperature for 20-22 °C. The nauplii were fed to the cod larvae at 5 dph. They had then reached copepodid stage IV, and were the same size as the rotifers (*B. ibericus,* Cayman) (180 μm). Before harvesting, the nauplii density in the tanks was counted. A randomly sampled 1-litre sample was counted by using an automatic counter (Alver *et al.*, 2011). Finally the nauplii were harvested with a siphon, and concentrated in a 60μm sieve, before being transferred to the larval tanks. Seawater was sand filtered and filtered through a 1μm mesh before use.

2.3.4 Cultivation of *Artemia* sp.

The decapsulation of the *Artemia* cysts (EG® INEV Aquaculture, Belgium) was done according to Sorgeloos et al. (1977). After decapsulation the *Artemia* cysts were washed in a concentrator to filter out eggshells and cysts. Afterwards they were stored in a fridge for a maximum of one week, before being hatched. After hatching (24 hours) they were short-term enriched with Multigain (Biomar, Norway) (2 X 10 g 60 $litre^{-1}$) for 24 hours. Decapsulation, hatching and enrichment were performed in 60 litre tanks, with conical bottoms and heavy aeration, with seawater (34 ppt). The temperatures were at all-time kept between 25-28 ^{o}C. After enrichment the *Artemia* sp. were again washed in a concentrator, before being fed to the cod larva. Seawater was sand filtered and filtered through a 1µm mesh before use.

2.4 Larval sampling and fixation

All larval sampling was carried out randomly from the tanks. After sampling the larvae were anesthetised in 3-aminobenzoic acid ethyl ester (MS222) and rinsed in freshwater before further treatment.

For dry weight (DW), 10-15 larvae were sampled $tank^{-1}$ at 2, 5, 8, 14, 19, 33, 40 and 60 dph. Larvae from 2-19 dph were analysed for carbon and nitrogen content in an Elemental combustion analyser (Costech Instruments, USA) using acetanilide as standard (analyses preformed by Marthe Schei, SINTEF). Larvae from 33-60 dph were transferred individually to pre-weighted capsules and dried at 60 ^{o}C for minimum 24 hours, before weighed on a microbalance (Mettler-Toledo microgram balance UMX2 automated-s ultra-microbalance, and UM3 precision single-pan balance, Switzerland). The two microbalances used were checked to coincide.

Larvae sampled for morphological examinations were fixated in 4% formalin in phosphate buffered saline (pH 7.4, Apotekproduskjon AS, Norway), and stored at 4°C.

2.5 Growth and survival

Dry weight (DW)

The following formula was used when calculating from µg carbon $larva^{-1}$ to mg dry weight $larvae^{-1}$ (Reitan *et al.*, 1993). It has been tested to also apply for cod (Overrein, I. pers. com).

$$DW = (\mu g \text{ carbon } larva^{-1}) * 2.34$$

Specific growth rate (SGR) and percent daily weight increase (%DWI) was calculated using the following formulas according to Kjørsvik et al., (2004).

$$SGR = \ln(Wt - Wo)/t$$

$$\%DWI = (e^{SGR} - 1) * 100\%$$

Wt is the final weight at time t, and Wo is the start weight. T is the number of days in a measurement period.

Standard length (SL)

The standard length of fixed larva was measured from the tip of the snout to the end of the hypurals (figure 2.1). The measurements were done in a stereomicroscope (Leica MZ7.5), with an ocular micrometer calibrated at 0.63 and 2x.

Myotome height (MH)

The myotome height on fixed larva was measured perpendicular to the axial skeleton right after the anus (see figure 2.1). The measurements were done in a stereomicroscope (Leica MZ7.5), with an ocular micrometer calibrated at 5.0, 2.0 and 1.25x.

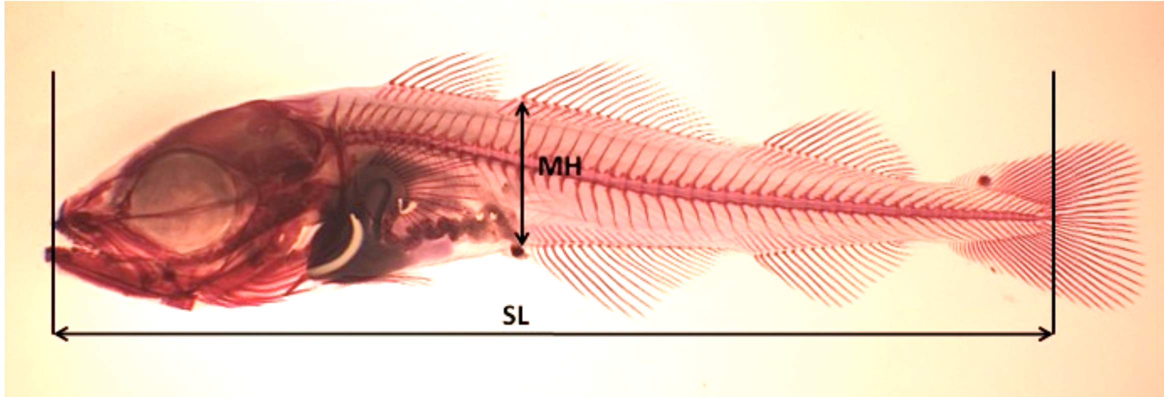

Figure 2.1 *Picture of Alizarin stained cod larva 60 dph, showing standard length (SL) and myotome height (MH).*

Survival

By tending the tanks and counting dead larvae the mortality was estimated. Dead larvae were not counted in the start of the experiment, due to quick resolution. From 38-60 dph the tanks were tended and dead larvae counted daily.

2.6 Larval feeding activity

The observations of larval behaviour were based on previous similar studies (Puvanendran & Brown, 1999; Leirvik, 2007). At 33 dph 15 larvae tank^{-1} were sampled into 3 l plastic aquariums, with seawater (34 ppt), holding the same temperature as the water in the tanks. All larvae were at this point weaned onto *Artemia* sp. The larvae were starved for 3 hours before observation. After 3 hours 750 *Artemia* nauplii litre^{-1} were added to the aquarium, and 10 larvae aquarium^{-1} were observed for 2 minutes each. The sequence of tank observations was according to treatment. The parameters observed were:

-Number of seconds each larva swam.

-Number of times each larva stopped to look around (orientation).

-Number of successful attacks.

Swimming was only measured during movement of caudal area (bursting), since burst is the energy demanding sequence of a burst-and-glide swimming pattern. Number of failed attacks was not counted, due to difficulties in recognizing an attack in advance.

The larvae were removed from the tank after the observation period, fixated and put in a marked container. This was done to avoid observing a larva several times, and in order to measure larval size. For optimal observation conditions the aquariums were placed on a white basis under a lamp with a 60W light bulb. Standard length and myotome height of each larva was measured after fixation.

2.7 Response to handling stress

On 24, 29, 36, 58 and 59 dph the larvae were tested for response to stress by netting and subsequent air exposure. The mortality was used as a measurement for stress response. 15 larvae $tank^{-1}$ were gently sampled and stored in a cup. The tanks firstly sampled were observed first, giving the larvae a recovery of roughly 10 minutes.

- The larvae were transferred in a sieve, which was placed in a small bowl of water.
- The outside of the sieve was then dried on a paper and held in air.
- The larvae were transferred carefully into a small aquarium (3l), filled with seawater, at the same temperature as in the tanks.
- This was repeated for each tank. Larvae from different tanks were transferred to separate aquariums.
- After 1, 5 and 24 hours the dead larvae were taken out, counted and fixated.
- Surviving larvae were registered and fixated after 24 hour.

The specific procedures day^{-1} are summarised in table 2.3. In order to obtain a significant mortality and difference amount the treatments, the procedure was adjusted between the test days. Standard length and myotome height of each larva were measured after fixation. The procedure was modified after Arends et al. (1999) and Van Anholt et al. (2004).

Table 2.3 *Procedure during testing of larval response to handling stress.*

Dph	On paper	In air	Total	Repetition
24	-	30	30	-
29	10	20	30	-
37	15	30	45	-
58	15	30	45	After 1 hour
59	20	40	60	After 1 hour

2.8 Bone deformities

In order to visualize bone structures at 60 days post hatching 60 larvae treatment^{-1} (20 larva replica^{-1}) were stained with alizarin red, according to Kjørsvik et al., (2009) (appendix 2). Pictures of the larvae where taken after 2 days in 40% glycerol, with a stereomicroscope (Leica MZ7.5), equipped with a camera (Nikon DS-5M-L1).

The deformed larvae were classifies according to body region (letter) with deformity/abnormality (number) (table 2.4). Regions and deformations are classified according to Boglione and colleagues (2003).

Table 2.4 *Regions along the skeletal axis and types of deformities and abnormalities in the cod larva, with codes.*

Code	Description
A-	Cephalic vertebrae
B-	Pre-hemal vertebrae
C-	Hemal vertebrae
1-	Star gazing – Axial deviation, V-shaped curvature in the neck
2-	Lordosis – Axial deviation, V-shaped bending of the spine
3-	Kyphosis - Axial deviation, A-shaped bending of the spine
4-	Corkscrews – Twisted haemal and neural arches
5-	Fused vertebrae
6-	Not fully ossified

2.9 Shape variation

Shape variation were analysed by geometric morphometrics, carried out at the University of Crete, department of Biology, with the help and assistance of professor Koumoundouros.

45 larvae replicate^{-1} on 60 dph (3 replicates treatment^{-1}-, 4 treatments, in all 540 larva) were bone stained according to Kjørsvik et at. (2009). 20 larvae replica^{-1} were the same as used for skeletal deformities. Only larvae without severe deformities were included in the geometric morphometrics studies after bone staining. The stained cod larvae were photographed with a stereomicroscope (Leica MZ7), equipped with a camera (Nikon DS-5M-L1). Free Tps-softwares were downloaded prior to analyses, from http://life.bio.sunysb.edu/morph/. 16 different landmarks were marked on each picture of the cod larvae, in *X*- and *Y*- co-ordinate at homologous anatomical points, using tpsDig software (v 2.16). The landmarks were placed at the tip of the snout **(1)**, the end of the hypurals **(2)**, and the start and end of the caudal-, dorsal-, and anal fins **(3-15)**, and at the base of the pelvic fin **(16)** (fig. 2.2). A generalized least square method was applied to adjust for centroid size (the minimal sum of square root of the sum of squared distances from the landmarks to the centroid of the larva), and to superimpose all landmark configurations (fig. 2.3 and 2.4). The centroid of the larva, the weight matrix and Relative Warp Scores Matrix were all calculated using the tpsRelw software (V 1.49).

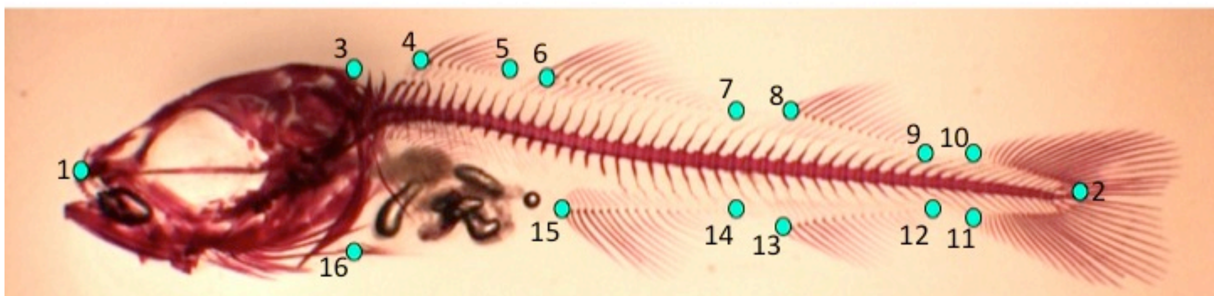

Figure 2.2 *Cod larvae with 16 landmarks at homologous anatomical points.*

The centroid size and the Relative warp scores matrix were plotted in a scatter graph, showing the trendline with R-squared values, to see if size was a significant factor for

shape at this point. The relative warp shows how a object is "warped" or deformed into another, which depicts the overall form of one organism as a distortion in the shape of a reference organism (Adams *et al.*, 2004). A canonical variate analyse was done in PASW statistics v18.0 (SPSS Inc., Chicaco, USA) for Mac on the weight matrix to test the significance of the nutritional differences (fig, 2.5). The latter was estimated by the landmark data set with the thin-plate spline algorithm. The thin-plate spline algorithm is a method to visualize changes between landmarks over the entire form (Zelditch, 2004). The canonical variate analyse allows visualization of the shape variation in the form of deformation grids or vector diagram. TpsRegr (V 1.37) was used to regress the shape components on the canonical variate scores, and vector displacements were obtained.

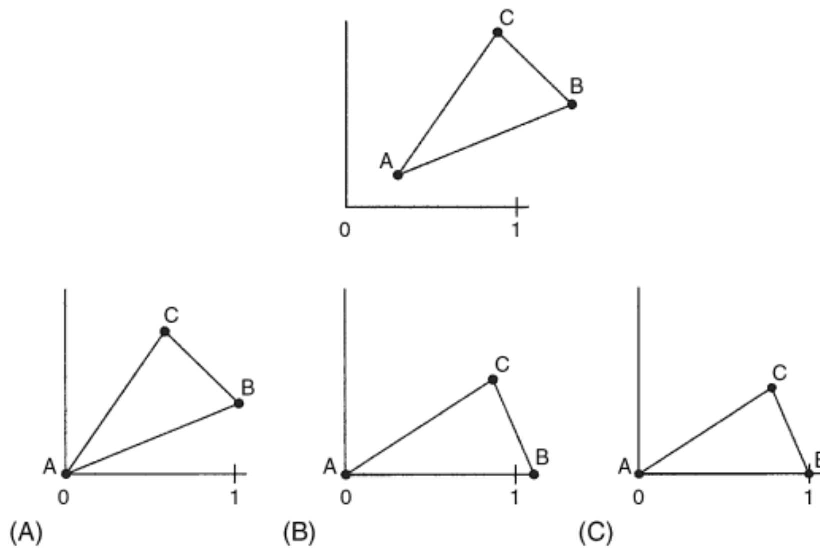

Figure 2.3 *Three operations are here applied to the triangle:* ***A)*** *translation,* ***B)*** *rotation,* ***C)*** *rescaling. This is done to eliminate the effect of location, size and rotation, on shape. Point A and B on the triangle is homologues to landmark 1 and 2 on the cod larvae (fig 2.2), and the rest of the landmarks (3-16) are circled around, like point C in the illustration (Illustration from Zelditch (2004)).*

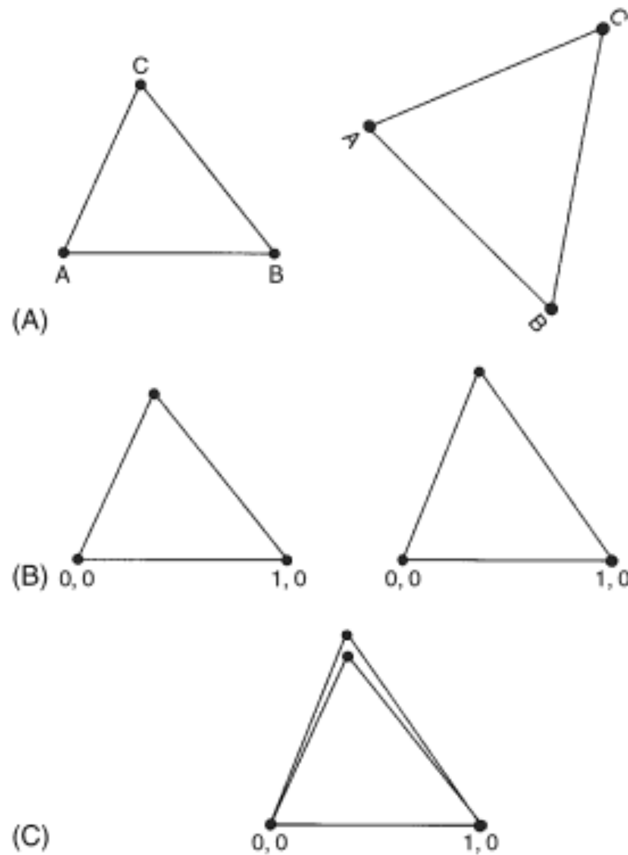

Figure 2.4 *The three steps: A) Translation, B) rotation and C) rescaling applied to two triangles with different shape. Looking at the C illustration the shape variation is easily visualised. Point A and B on the triangle is homologues to landmark 1 and 2 on the cod larvae (fig 2.2), and the rest of the landmarks (3-16) are circled around, like point C in the illustration (Illustration from Zelditch (2004)).*

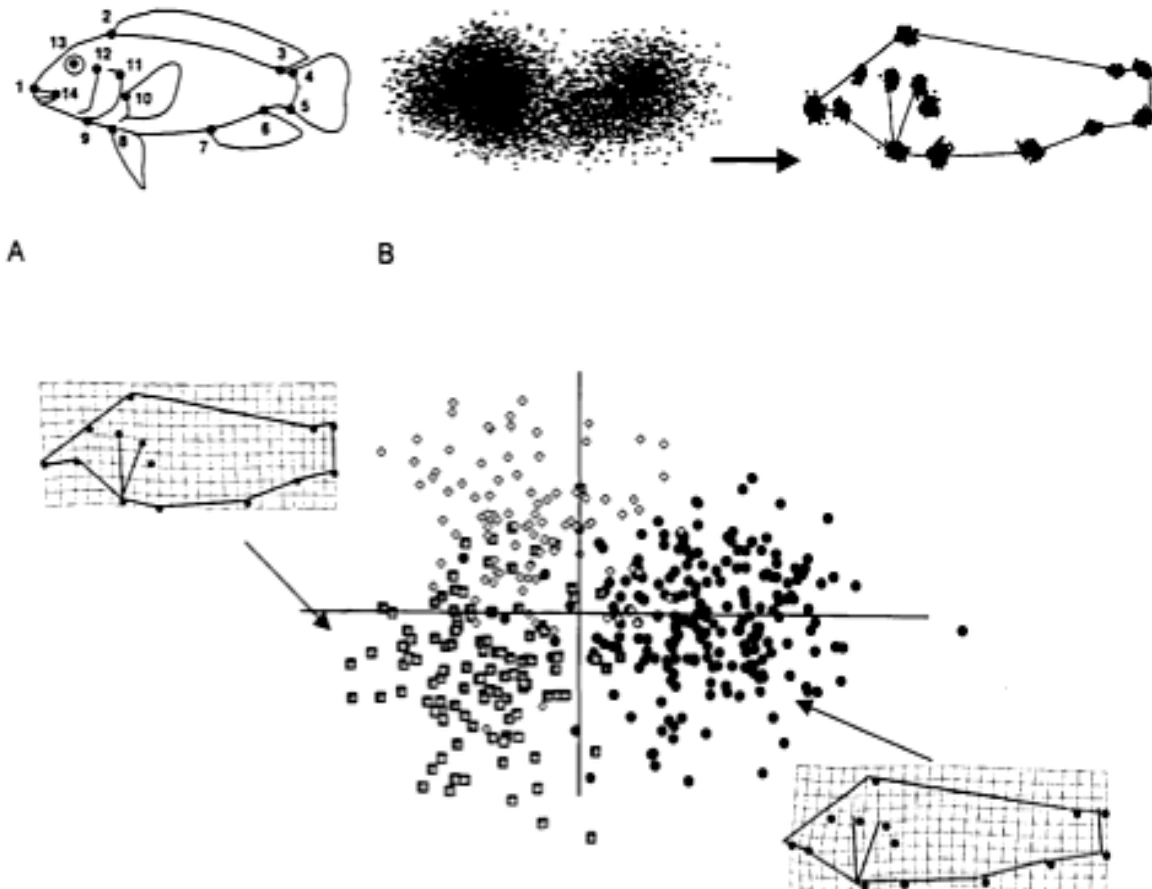

Figure 2.5 A) *14 different landmarks are here marked on the larvae.* ***B)*** *Landmarks of several larvae (412 speciments) put on top of each other first before and than after superimposing (correcting for translocation, scaling and rotation – non shape variations) like seen in figure 2.3 and 2.4.* ***C)*** *Statistical analyse (canonical variance analyse) and graphical presentation of results from the shape analyse. Figure from Adams et al. (2004).*

The procedure was carried out a second time, exclusively with larvae in the size range 16,5-23 mm standard length (n= 81, 102, 86 and 76 for Copepod, Cop 7, RotMG and RotChl respectively. Total of 345 larvae). This was the size range with an average of most larvae tratment^{-1}. By placing number of larvae at different standard length (ranges of $\pm$0.5 mm) in a piviot table in Exel (Microsoft® Exel for Mac, 2008, USA), the size ranges with averagely most larvae treatments^{-1} were selected. This is the result presented in the result section, as the shape is not size dependent in this selection. The procedures were done according to Georga and Koumoundourous 2010 and Kouttouki and colleagues 2006.

2.10 Statistical analysis of data

The data were checked for outliers, and if not due to biological variability outliers were removed. This was decided by the best of one's judgement. Normality was tested using a Shapiro-Wilk´s test (n<100), and a Kolmogorov-Smirnov test (n>100). Data presented in percent were Arcsine transformed before the statistics were run.

Different means were compared using one-way analysis of variance (ANOVA), for normally distributed data. Student-Newman-Keuls Post hoc test was used for homogeneous data, and a Dunnett T3 post hoc test for non-homogeneous data. For non-normal distributed data a Kruskal-Wallis test was used. A significant level of p=0.05 was used.

A Pearson correlation test was used when analysing the correlation of quality to treatment and standard length- The significance level was set at p=0.05. When R-squared values are used for linear regression, the level of correlation is set to $R^2>0.90$.

All graphs, tables, and statistical analyses were made in PASW Statistics v18.0 (SPSS Inc., Chicago, USA) for Mac.

3. Results

3.1 Growth

3.1.1 Dry weight and daily weight increase

The dry weight (DW) of the larvae from the Copepod treatment was significantly higher than DW of larvae from the two rotifer treatments already from 8 dph (Fig 3.1). The DW of Cop 7 treatment larvae were at 60 dph significantly lower than the DW of the Copepod treatment larvae, and higher than the DW of both rotifer treatments, which did not differ significantly from each other (Fig. 3.2) (appendix 3). The general ranking in DW was for the whole period: Copepod treatment larvae having the highest DW, followed by Cop 7, RotMG and RotChl larvae. The DW (mean mg larvae^{-1} ±se) for the larvae in the different treatments was at the end of the experiment (60 dph); Copepod 22.3±1.4, Cop 7 15.2±1.0, RotMG 11.9±0.9, RotChl 12.0±0.7 (appendix 3).

The daily weight increase (%DWI) was generally highest for larvae from the Copepod treatment, and lowest for the RotChl treatment larvae. The growth rate was for all treatments highest during the *Artemia* sp. period (day 19-33), and lowest during the period of weaning onto dry feed (day 33-40), with the Copepod treatment being the only treatment considerably increasing in DW at this stage (fig 3.3) (appendix 4). The %DWI for the whole experiment (2-60 dph) was as follows (mean mg larvae^{-1} ±se): Copepod 10.2±0.2, Cop 7 9.5±0.2, RotMG 9.1±0.2, and RotChl 9.1+0.2.

A pigmentation difference between larvae from different treatments was also observed. The Copepod treatment larvae had a more yellow pigment colour compared to larvae from the other treatments, which were paler and less pigmented. The Copepod treatment larvae were the most pigmented, followed by Cop 7, RotMG and RotChl treatment larvae. This was not quantified in any way, only observed.

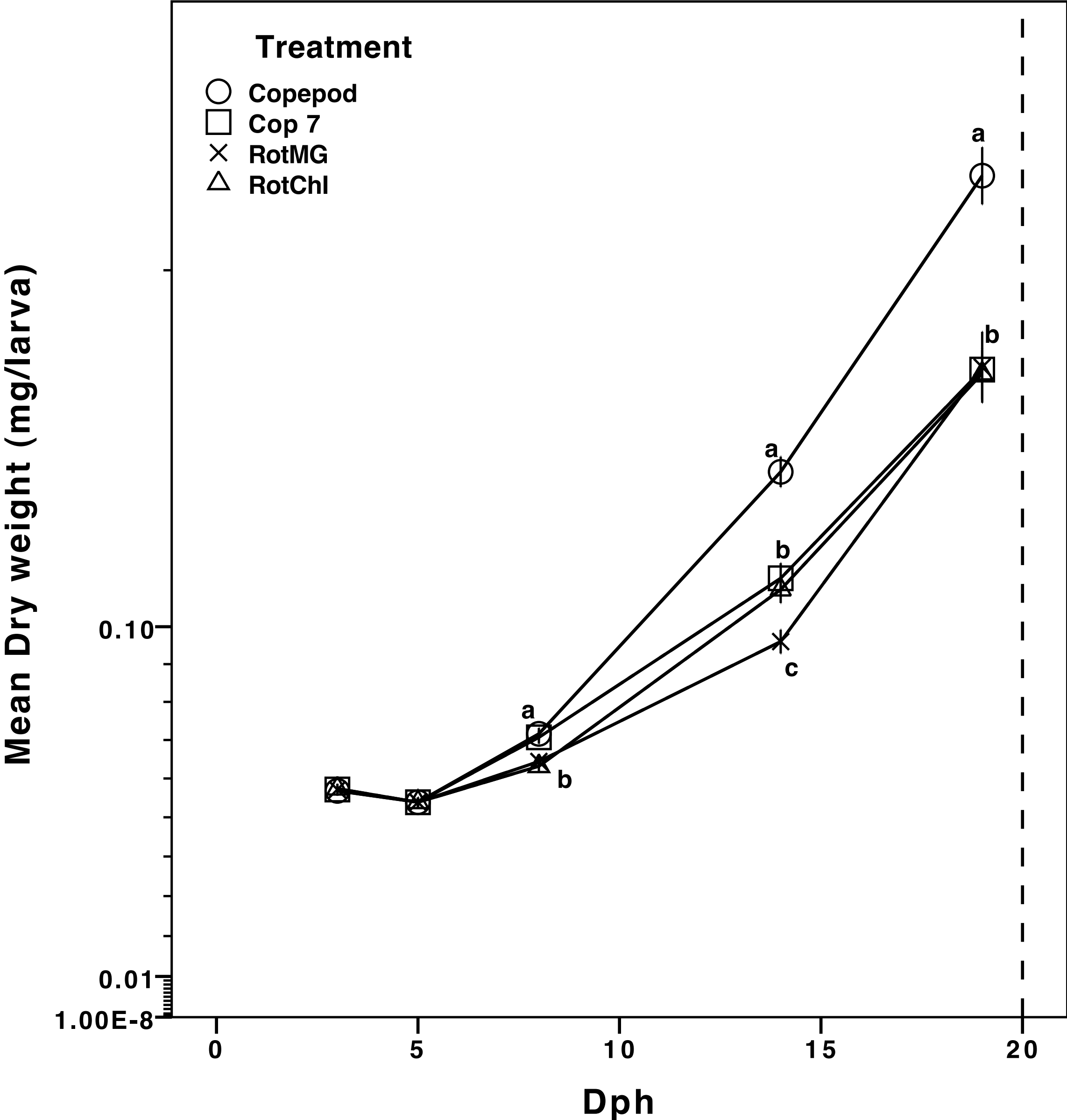

Figure 3.1 *Cod larval dry weight (mg larva^{-1}) from 3-19 dph of the experiment (mean±se), n= 10-50 replica^{-1}. Logarithmic Y-axis. Significant differences could be seen on all sampling days, except on 3 and 5 dph. Dashed line on 20 dph indicates start of weaning onto Artemia sp. Significant differences between treatments are marked with different letters. Error bars indicate ± 1 standard error.*

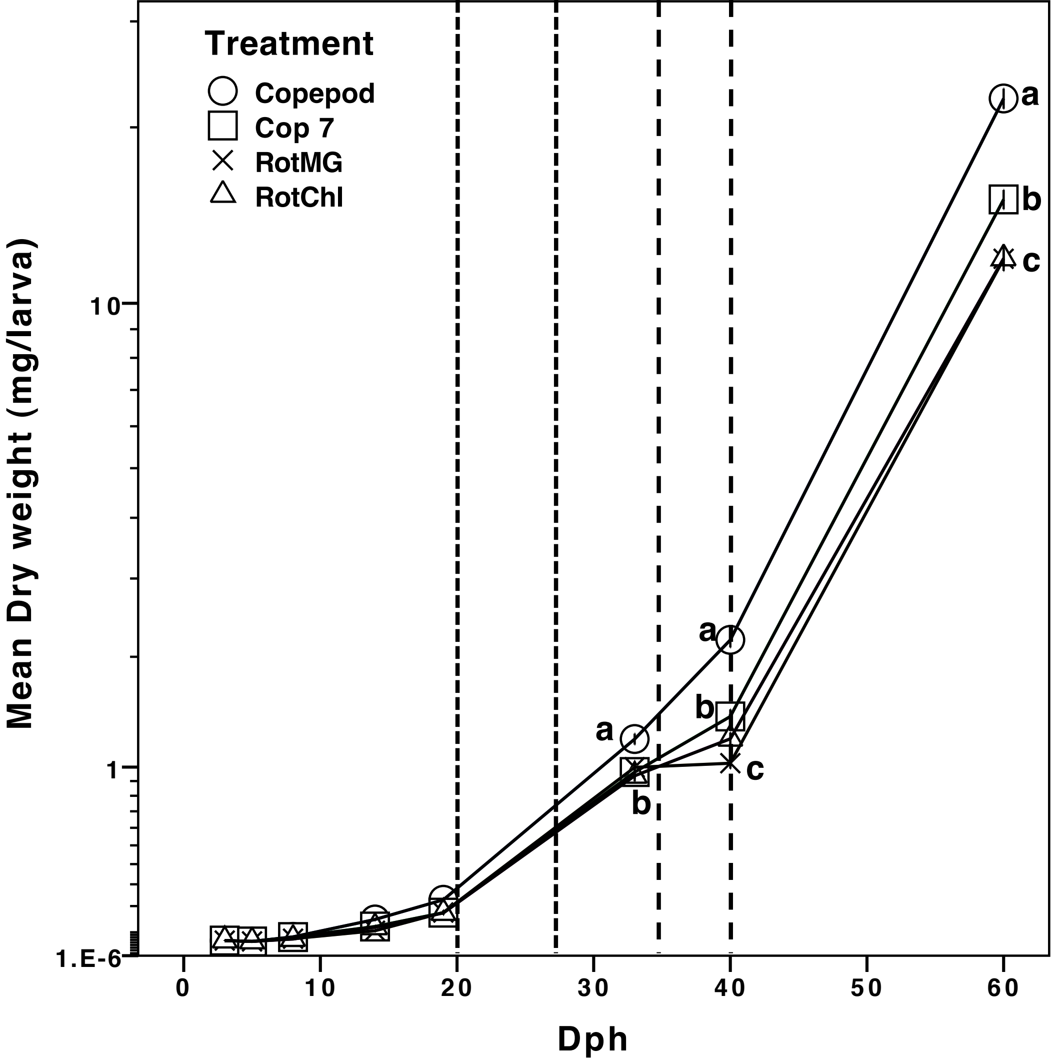

Figure 3.2 *Cod larval dry weight (mg larva^{-1}) from 3-60 dph during the experiment (mean±se), n= 10-50 replica^{-1}. Logarithmic Y-axis. Significant differences at 33, 40 and 60 dph are marked with different letters. The small dashed lines on 20 and 28 dph indicate the co-feeding period with rotifers/copepods and Artemia sp. before feeding only with Artemia sp. from 28 dph. The long dashed lines, 35-40 dph indicate weaning onto dry feed, before feeding only with dry feed from 40 dph. Error bars indicate ± 1 standard error.*

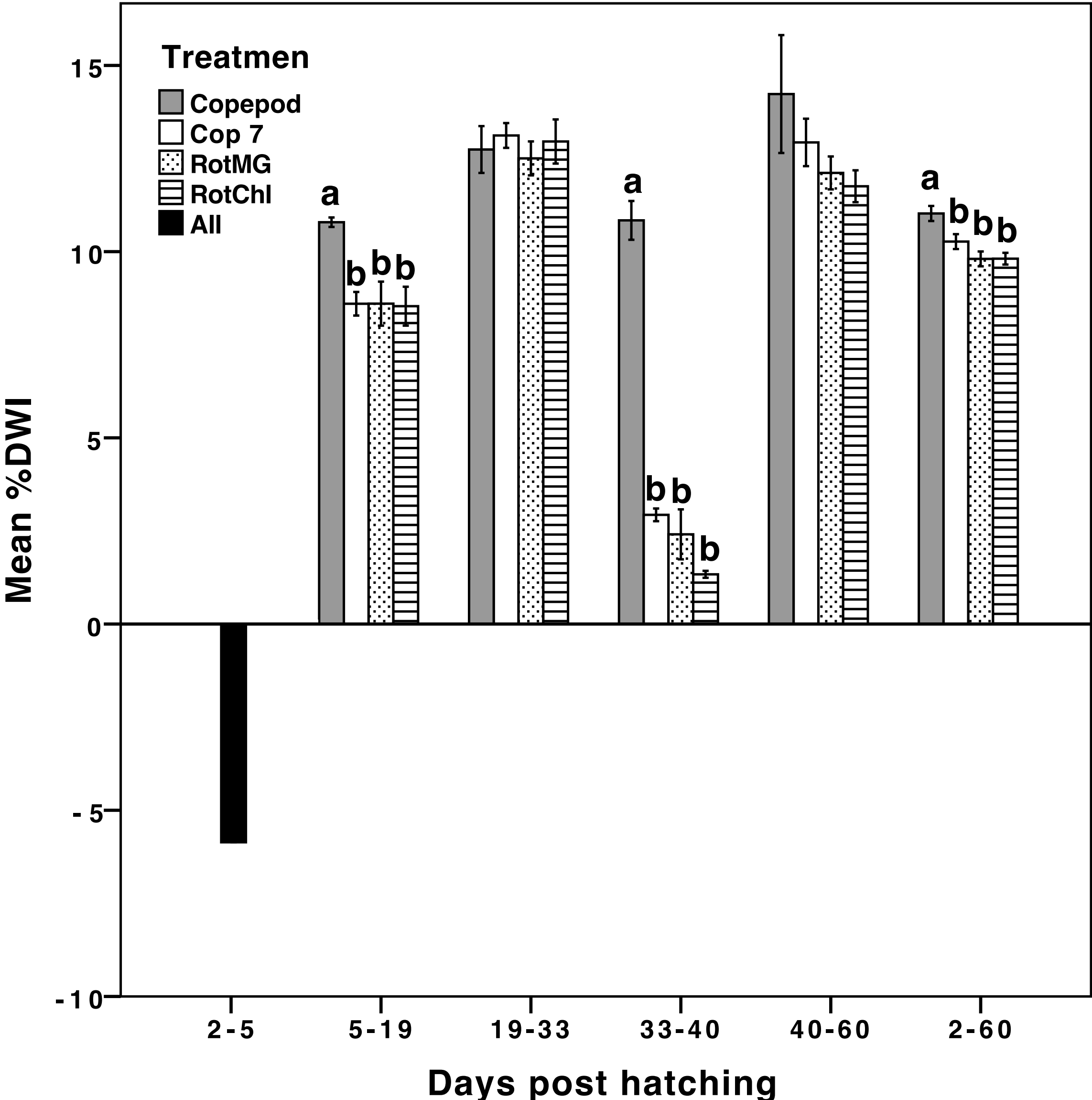

Figure 3.3 *Cod larval daily weight increase (%DWI) during the experiment. n=30-150. Larvae sampled on 2-5 dph were taken from one tank and had not yet started exogenous feeding. 5-19 dph was the first feeding period where the larvae got copepods and rotifers, 19-33 dph the larvae got Artemia nauplii, 33-40 dph was the weaning onto dry feed period and 40-60 dph they got dry feed. 2-60 dph shows %DWI during the whole experiment. Significant differences between treatments are marked with different letters. Error bars indicate ± 1 standard error.*

3.1.2 Standard length and myotome height

From 19 dph the larvae from the Copepod treatment had a significantly higher standard length (SL) and myotome height (MH) compared to larvae from the other treatments (fig. 3.4 and 3.5). At 60 dph larvae from the Cop 7 treatment had a significantly higher SL compared to the rotifer treatments larvae, but a significant lower SL than the Copepod treatment larvae. Like DW, SL and MH were stagnating during the weaning period (35-40 dph). The standard length was for all treatments correlated to myotome height (R^2>0.90), and the Newton quotients were highest for the rotifer treatments and Cop 7 treatment (fig. 3.6), and lowest for the Copepod treatment.

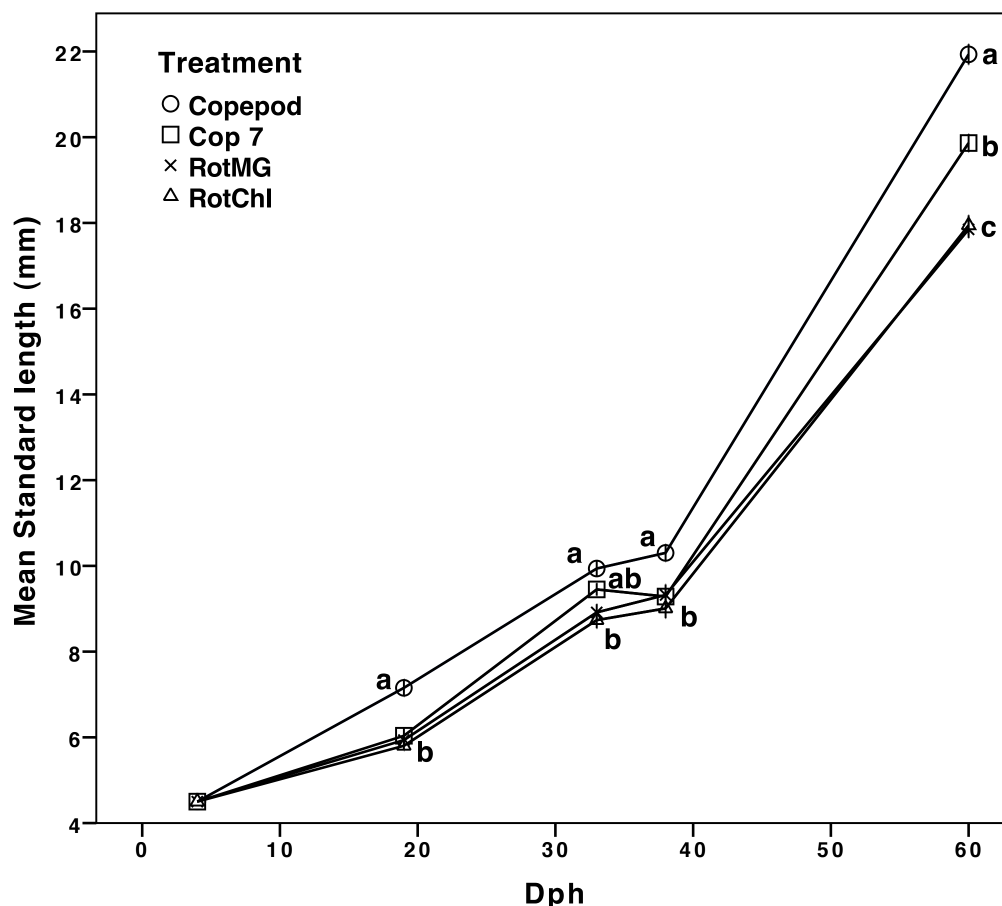

Figure 3.4 *Standard length (mm) from samples taken 4, 19, 33, 38 and 60 dph, n=5, 5, 30, 45 and 45, respectively. On 4 dph there was no difference between the treatments. Where there are significant differences between the treatments these are marked with different letters. Error bars indicate ± 1 standard error.*

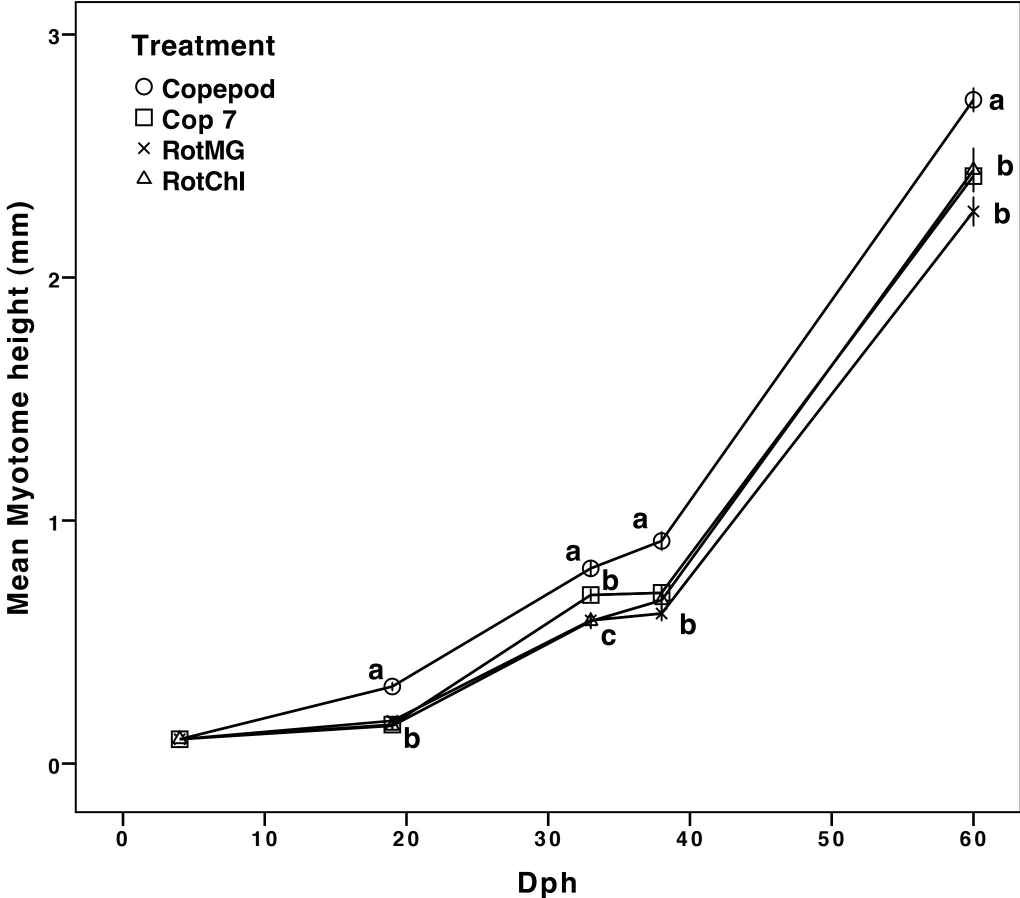

Figure 3.5 *Myotome height (mm) from samples taken 4, 19, 33,38 and 60 dph, n=5, 5, 30, 45, 45, respectively. On 4 dph there was no difference between the treatments. Where there are significant differences between the treatments these are marked with different letters. Error bars indicate ± 1 standard error.*

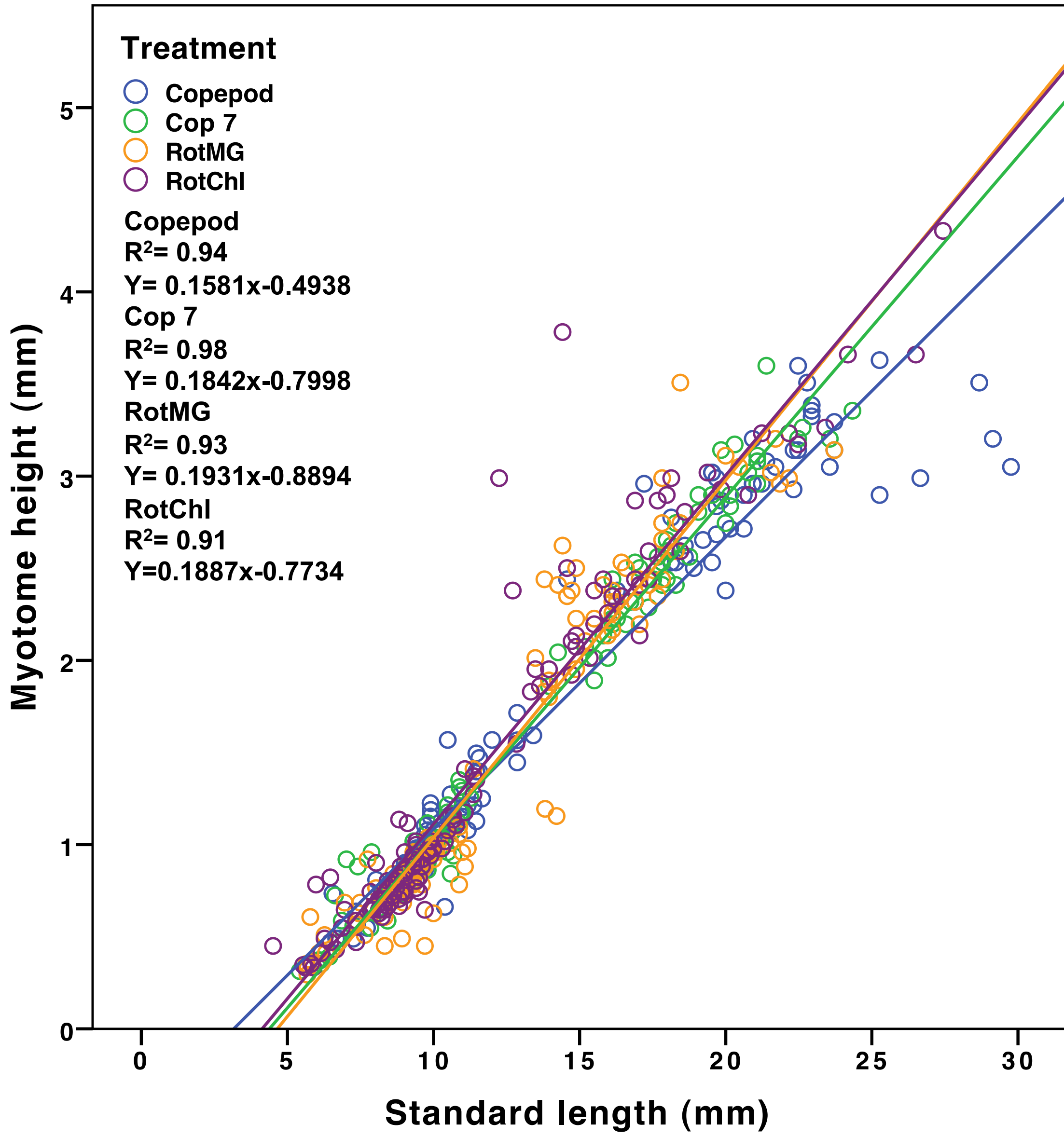

Figure 3.6 *Standard length (SL, mm) plotted against myotome height (MH, mm), showing that SL and MH are correlated (R^2>0.90) for all treatments.*

3.2 Survival

The survival of the larvae from the Copepod treatment was significantly higher than for the other treatments on day 38 and 60 dph, at 45 dph there were no significant differences between the treatments. The survival for all treatments decreased the most during weaning onto dry feed (35-40 dph), but was stabilized from 45 dph until the end of the experiment (60 dph) (table 3.1) (appendix 5). The Cop 7 treatment had the largest difference in survival between the tanks. At 60 dph, the survival rates in the Cop 7 tanks were: tank 1: 12 %, tank 2: 16 % and tank 3: 24 %.

Table 3.1 *Cumulative survival (%) measured daily from 38-60 days post hatching.*

Dph	Treatment	Mean % survival ± SE
38	Copepod	32 ± 1^{A}
	Cop 7	22 ± 4^{B}
	RotMG	16 ± 0^{B}
	RotChl	15 ± 2^{B}
45	Copepod	23 ± 2
	Cop 7	18 ± 4
	RotMG	13 ± 1
	RotChl	13 ± 2
60	Copepod	20 ± 2^{A}
	Cop 7	14 ± 3^{AB}
	RotMG	11 ± 1^{B}
	RotChl	10 ± 2^{B}

3.3 Larval activity

The larvae from the Copepod treatment and the RotChl treatment were the most active swimmers. There were no significant differences in orientation activity between larvae from the different treatments, but there was a clear trend showing that larvae from the Copepod treatment had the highest orientation activity, followed by larvae from the Cop 7 treatment, RotMG treatment and finally larvae from the RotChl treatment with the lowest orientation activity level. The Copepod treatment larvae caught significantly more *Artemia* sp*.* compared to the other treatments (Fig. 3.7) (appendix 6).

How many seconds the larvae were swimming prey^{-1} capture (Fig 3.8) was correlated with both SL and treatment, and larvae from the Copepod and Cop 7 treatments used significantly fewer seconds prey^{-1} capture, compared to the RotMG and RotChl treatments larvae. Mean number of seconds the larvae swam to catch a *Artemia* nauplii was as follows: Copepod 4±8 sec, Cop 7 3.5±6 sec, RotMG 7.6±2.4 sec, RotChl 8.2±1.4 sec.

None of the larvae in this experiment where gape-limited during prey attack. When observing the larvae, those fed copepods seemed to have a clearer predator instinct, and a more 3-dimentional view, compared to the rotifer treatment larvae.

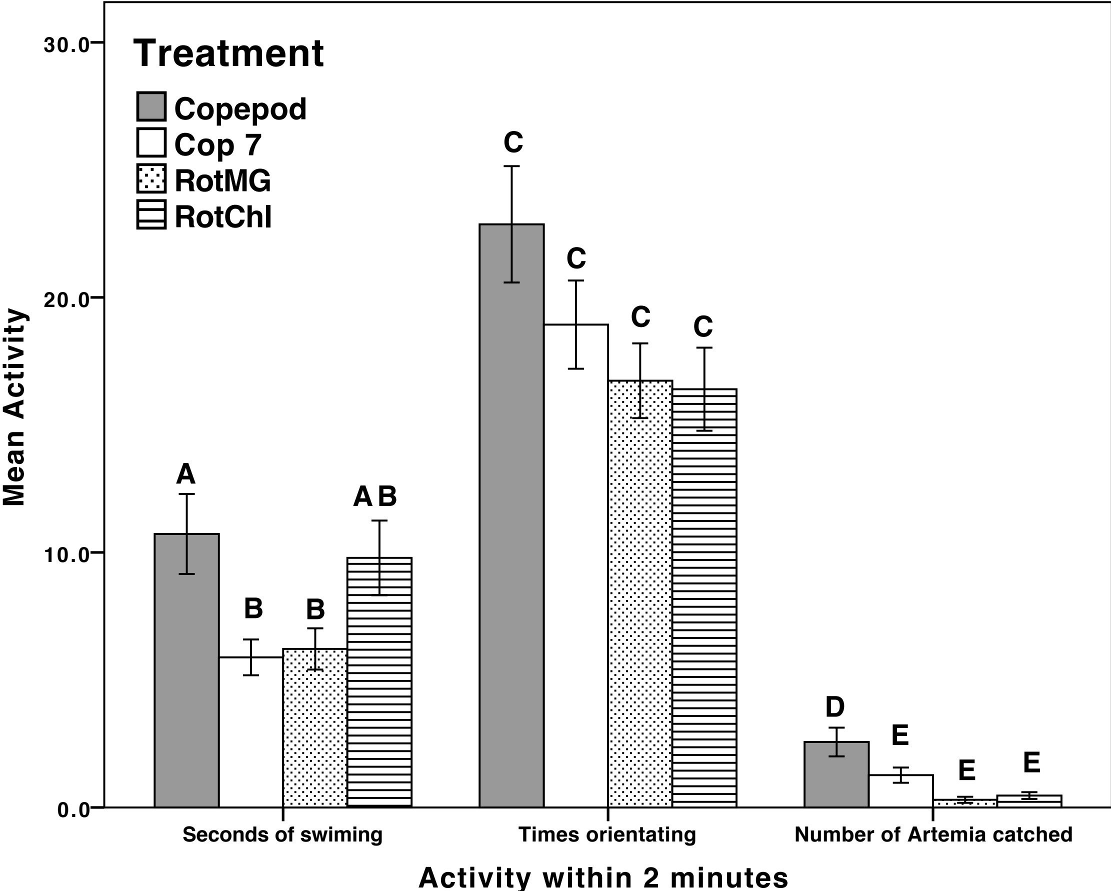

Figure 3.7 *Mean larval activity measured for: seconds swimming, times orientated and Artemia nauplii caught within 2 minutes treatment*$^{-1}$ *at 33 dph (n=30). Significant differences between the treatments are marked with different letters. Error bars indicate* $\pm$ *1 standard error.*

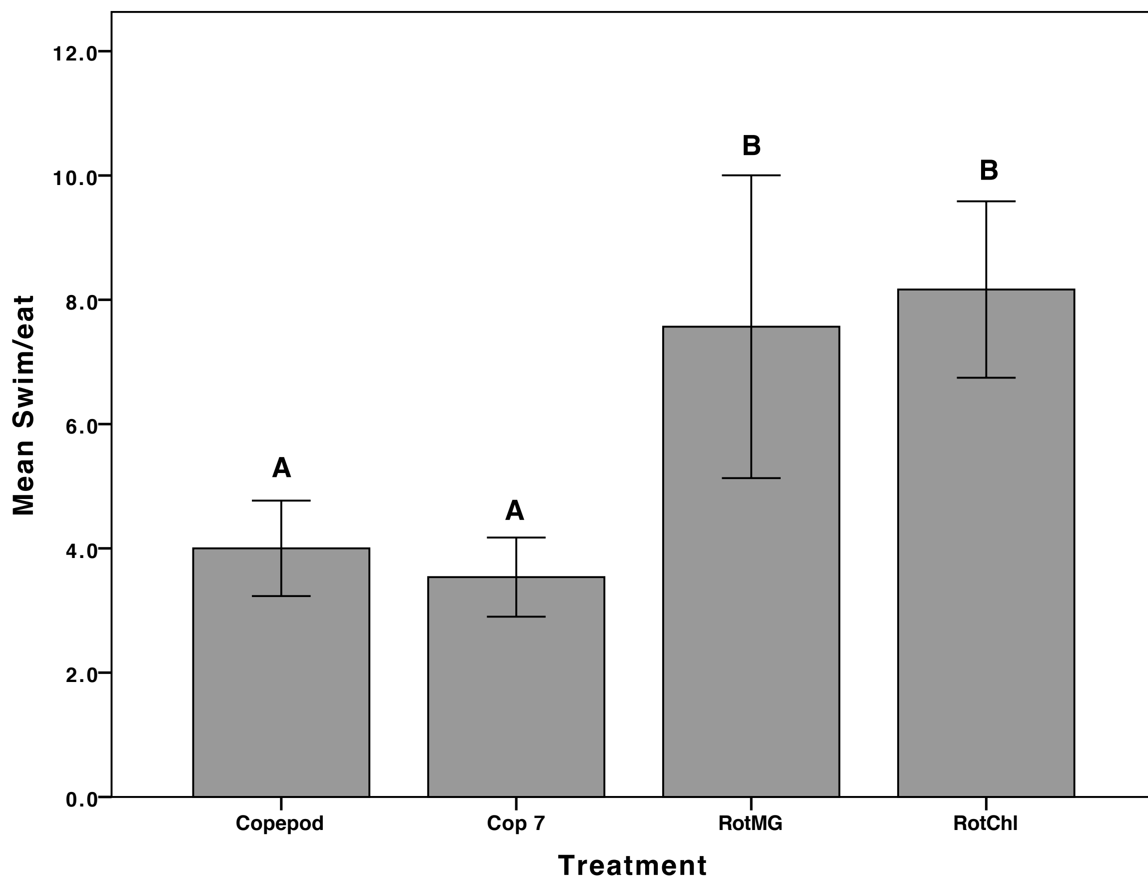

Figure 3.8 *Number of seconds the cod larvae swam prey^{-1} eaten (Swim eat^{-1}), n=30. Significant differences between the treatments are marked with different letters. Error bars indicate $\pm$ 1 standard error.*

3.4 Response to handling stress

Larval response to handling stress was tested by netting followed by air exposure on 24, 29, 37, 58 and 59 dph (appendix 7). The handling stress performed on 37 and 59 dph were the only ones giving significant differences in survival between the treatments. The handling stress tests on the other days generally gave no significant differences between treatments.

At 37 dph there were no significant differences in mortality tank^{-1} between the treatments (n=3) after 1 hour. After 24 hours larvae from the Copepod treatment had a significantly lower mortality rate (mean % ±SE) compared to larvae from the other treatments: Copepods 7±4 %, Cop 7 15±4 %, RotMG 23±1 %, RotChl 39±1 % (Fig. 3.9).

On 59 dph there were no significant differences between treatments 2 hours after the first handling. After 24 hours, the mortality tank^{-1} for the Copepod treatment was significant lower compared to the RotChl treatment, but not compared to the Cop 7 and RotMG treatments: Copepod 10±5 %, Cop 7 6±3 %, RotMG 23±5 %, RotChl 47±7 % (Fig 3.10).

For both 38 and 59 dph a Person correlation analysis was done, asserting that there were no significant correlations between mortality after stress and standard length, confirming the correlation between mortality after stress and treatment.

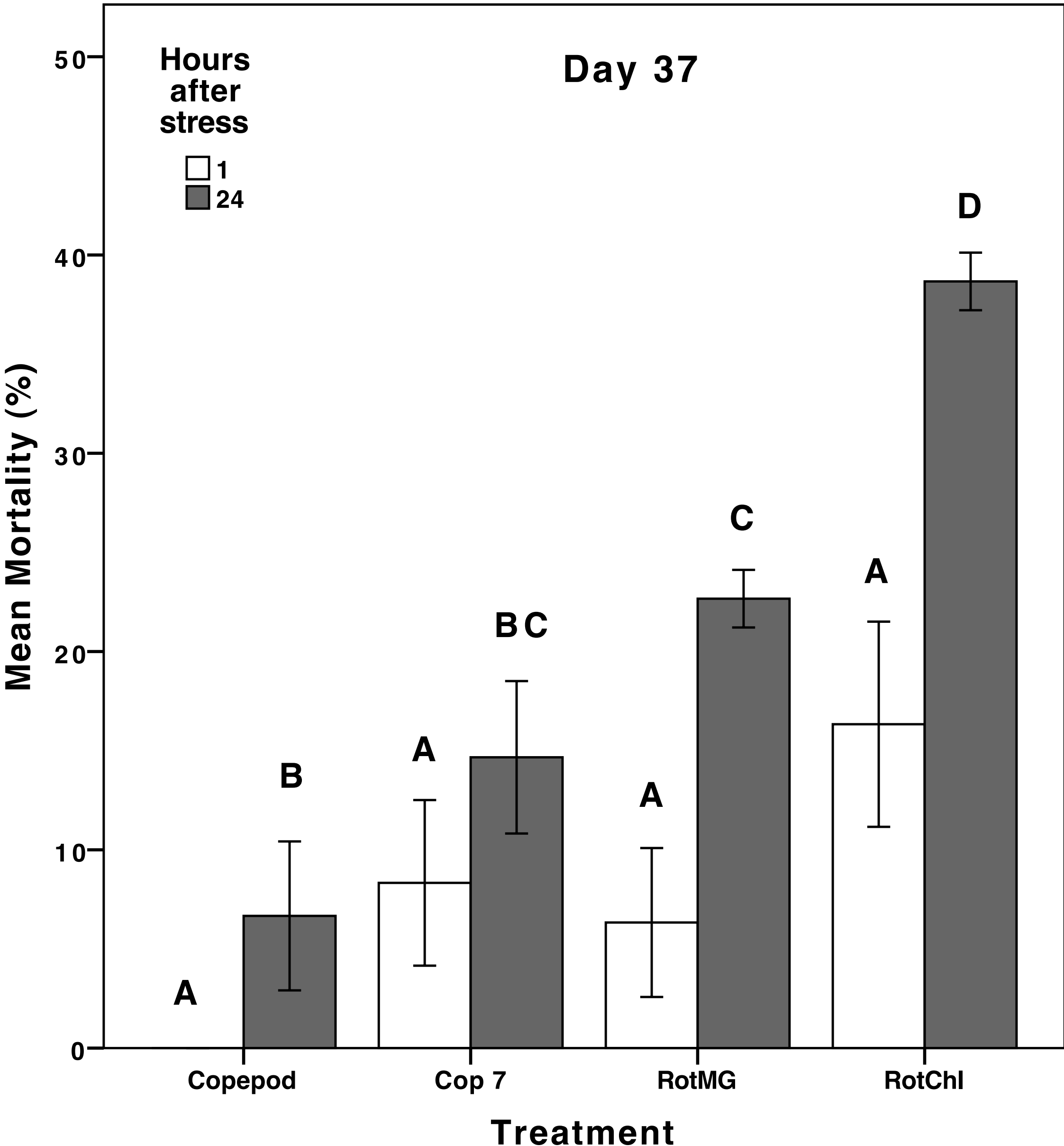

Figure 3.9 *Mean mortality per tank (n=3) for each treatment after the stress test (45 seconds of air exposure) at 37 dph during the experiment. Significant differences between the treatments are marked with different letters. Error bars indicate ± 1 standard error.*

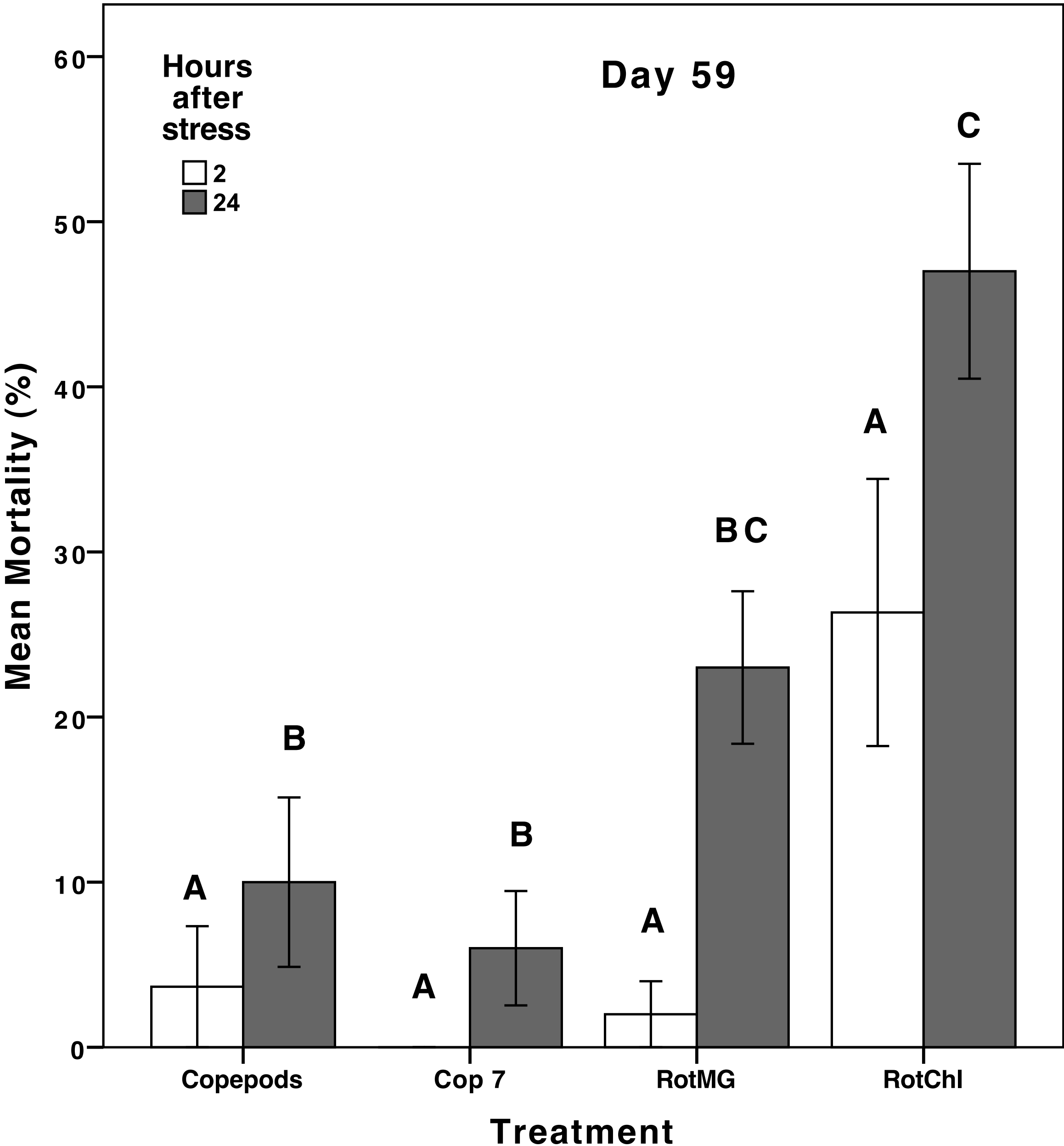

Figure 3.10 *Mean mortality per tank (n=3) for each treatment after stress test (2 x 60 seconds air exposure) at 59 dph during the experiment. Significant differences between the treatments are marked with different letters. Error bars indicate ± 1 standard error.*

3.5 Incidences of malformations and types

Lordosis, kyphosis, “corkscrews” and fused vertebrae were the deformations observed during bone analysis at 60 dph, and some larvae were also showed to have not fully ossified vertebrae (fig 3.11). Lordosis was significantly more common for the Cop 7 treatment compared with the other treatments, while kyphosis was significantly more common in the RotChl treatment compared with the other treatments. Fused vertebra was not a common deformity for any of the treatments, and there were no significant differences in the occurrence between the treatments. Corkscrews and not fully ossified vertebrae were significantly higher for the rotifer groups than for the larvae fed copepods (fig. 3.12).

Mean percentage of deformed larvae tank^{-1} (n=3) according to treatments was highest for the RotChl treatments, both when including and excluding corkscrews (CS) (fig 3.13 and 3.14). When studying the number of deformities larvae^{-1} between the treatments (60 larvae treatment^{-1}), the Copepod treatment larvae had the significantly lowest number of deformities larvae^{-1} and RotChl treatment larvae had the highest amount larvae^{-1} (fig 3.15). When analysing for deformations where corkscrews are excluded there was a general range of the Copepod treatment larvae having the lowest number of deformations larva^{-1}, followed by Cop 7, RotMG and RotChl treatment larvae, but without any significant differences (fig 3.16)

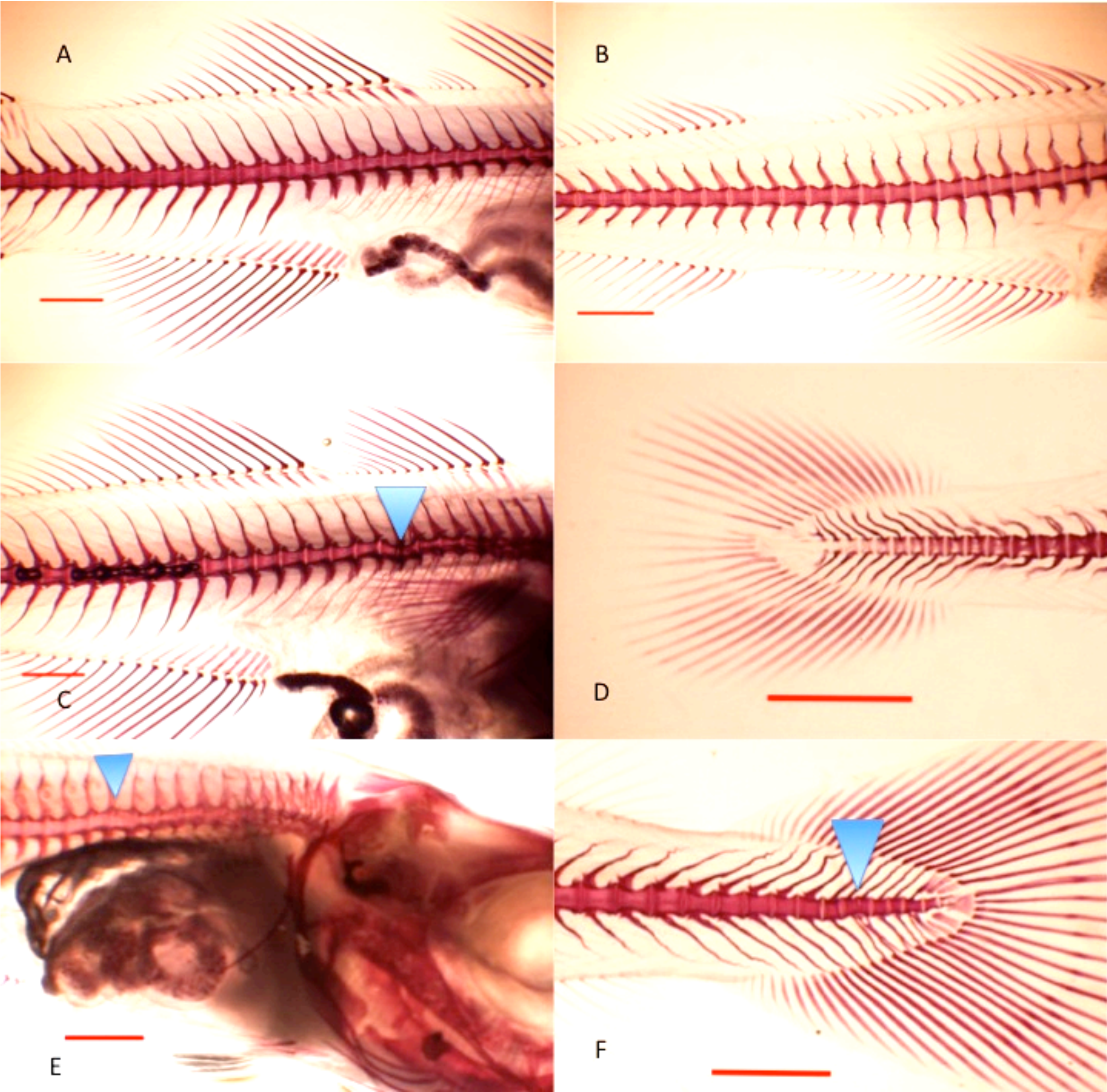

Figure 3.11 *Examples of observed deformities.* ***A)*** *Cod larval vertebral column without any deformations (Copepod).* ***B)*** *Vertebrae with corkscrews (RotChl) – twisted neural and haemal arches. Notice how the neural and hemal arches in picture A appear longer and more solid compared to picture B. The colour of the vertebrae is not as deep as in picture A, indicating that the ossification process in larva B is somewhat delayed compared to larva A.* ***C)*** *Lordosis in the pre-hemal vertebrae, shown with blue arrow (Copepod).* ***D)*** *A not fully ossified vertebra (RotChl).* ***E)*** *Larvae (RotChl) with kyphosis, shown with blue arrow.* ***F)*** *Larvae with fused vertebrae (Cop 7), shown with blue arrow. Red bars equal 1 mm.*

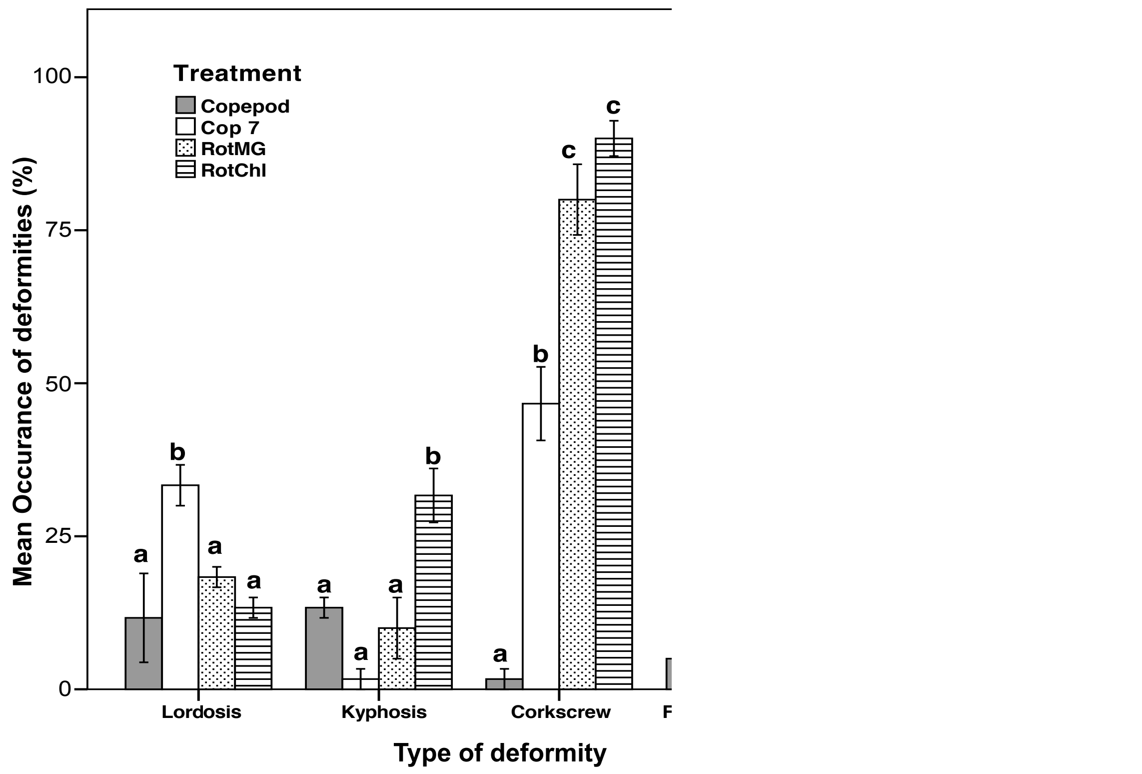

Figure 3.12 *Mean percentage of deformities (lordosis, kyphosis, corkscrew, fused vertebrae) and not fully ossified larvae tank*$^{-1}$ *(n=3) according to the different treatments. 20 larvae tank*$^{-1}$ *were analysed (60 for each treatment). Where there were significant differences between the treatments these are marked with different letters. Error bars indicate ± 1 standard error.*

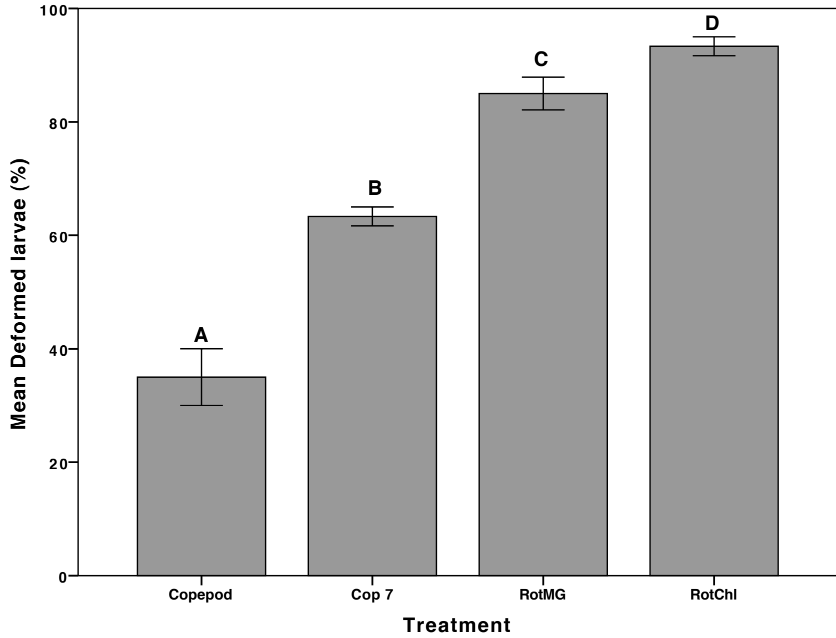

Figure 3.13 *Mean percentage of deformed larvae according to treatments. Numbers of larvae tested from each tank were 20 (60 treatment^{-1}). Significant differences between the treatments are marked with different letters. Error bars indicate* ± *1 standard error.*

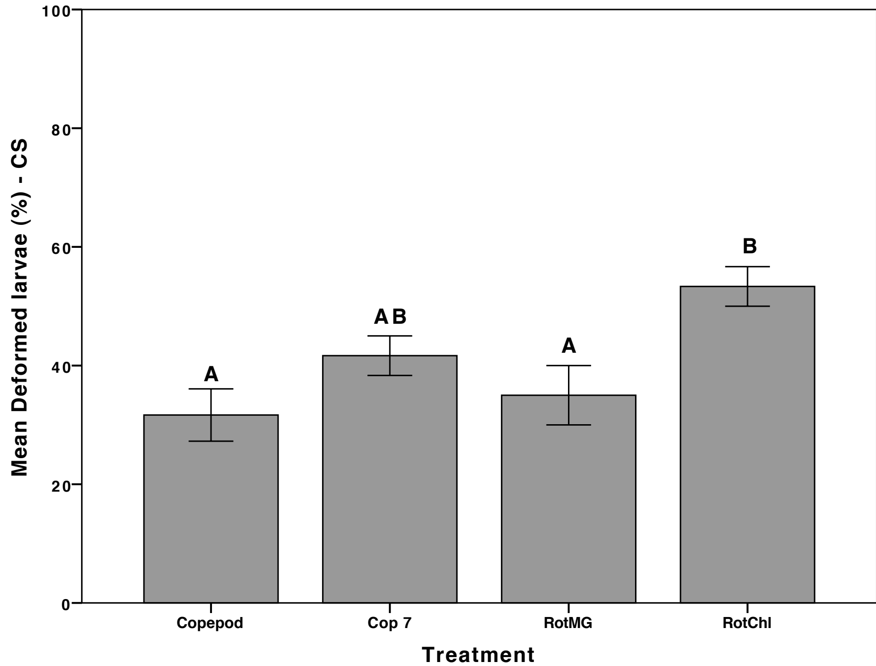

Figure 3.14 *Mean percentage of deformed larvae (excluding corkscrews) according to treatment. Numbers of larvae tested from each tank was 20 (60 treatment^{-1}). Significant differences between the treatments are marked with different letters. Error bars indicate* ± *1 standard error.*

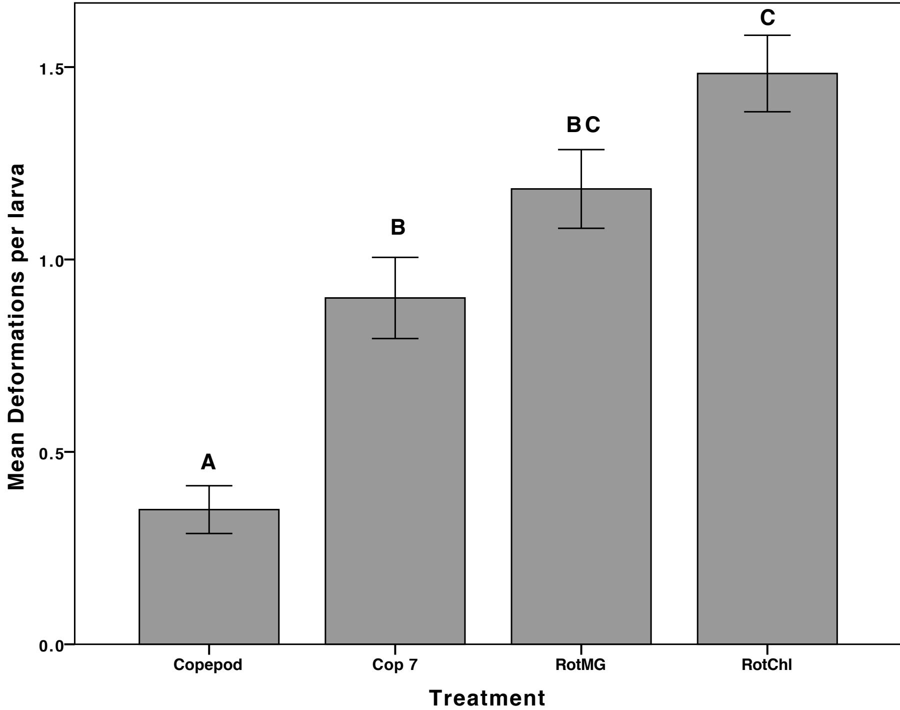

Figure 3.15 *The mean number of deformations larvae^{-1} between the different treatments (n=60). Significant differences between the treatments are marked with different letters. Error bars indicate $\pm$ 1 standard error.*

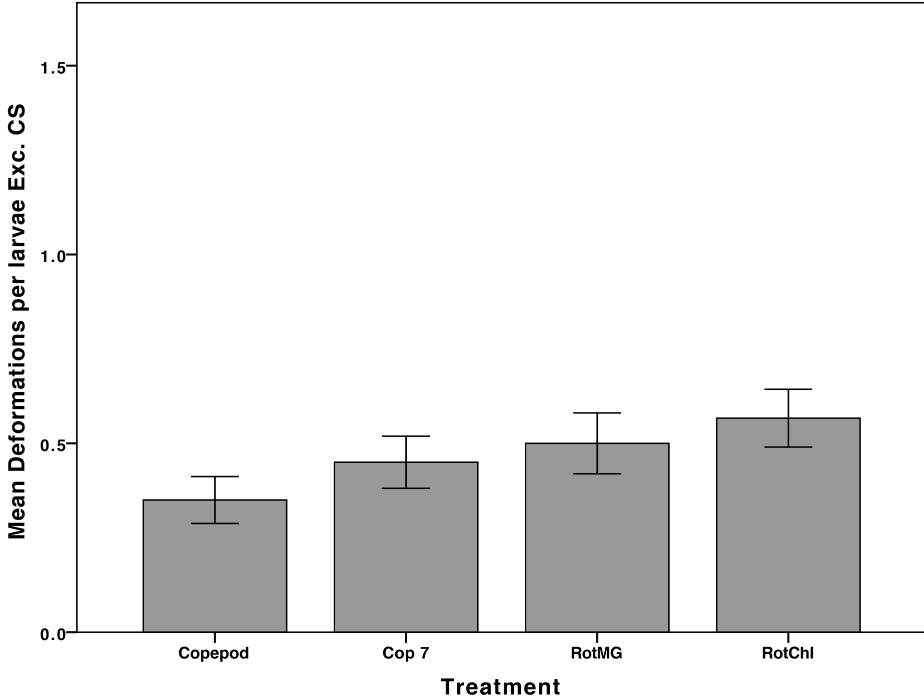

Figure 3.16 *The mean number of deformations larvae^{-1} between the different treatments (n=60), excluding corkscrews. There were no significant differences between the treatments. Error bars indicate $\pm$ 1 standard error.*

3.6 Shape variation

Standard length was correlated to the centroid size (CS) (the minimal sum of square root of the sum of squared distances from the landmarks to the centroid of the larva); as the standard length increased, so did the centroid size ($R^2>0.90$) (fig. 3.17). There was no correlation between the relative warp (RW) (bending energy when warping a shape into the mean shape) and CS (R^2=low) (fig.3.18).

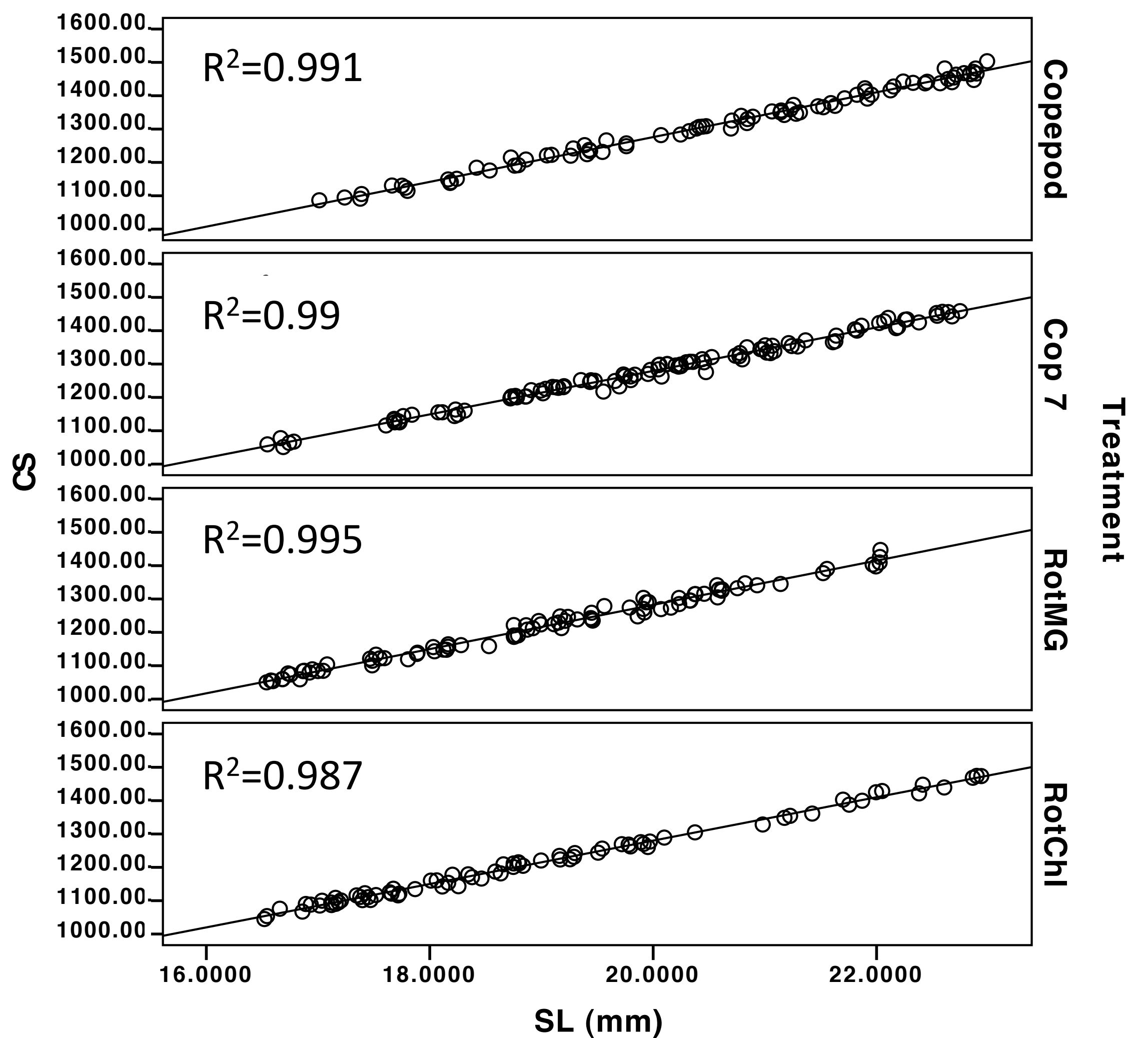

Figure 3.17 *Centroid size (CS) plotted against standard length (SL) for all 4 treatments.*

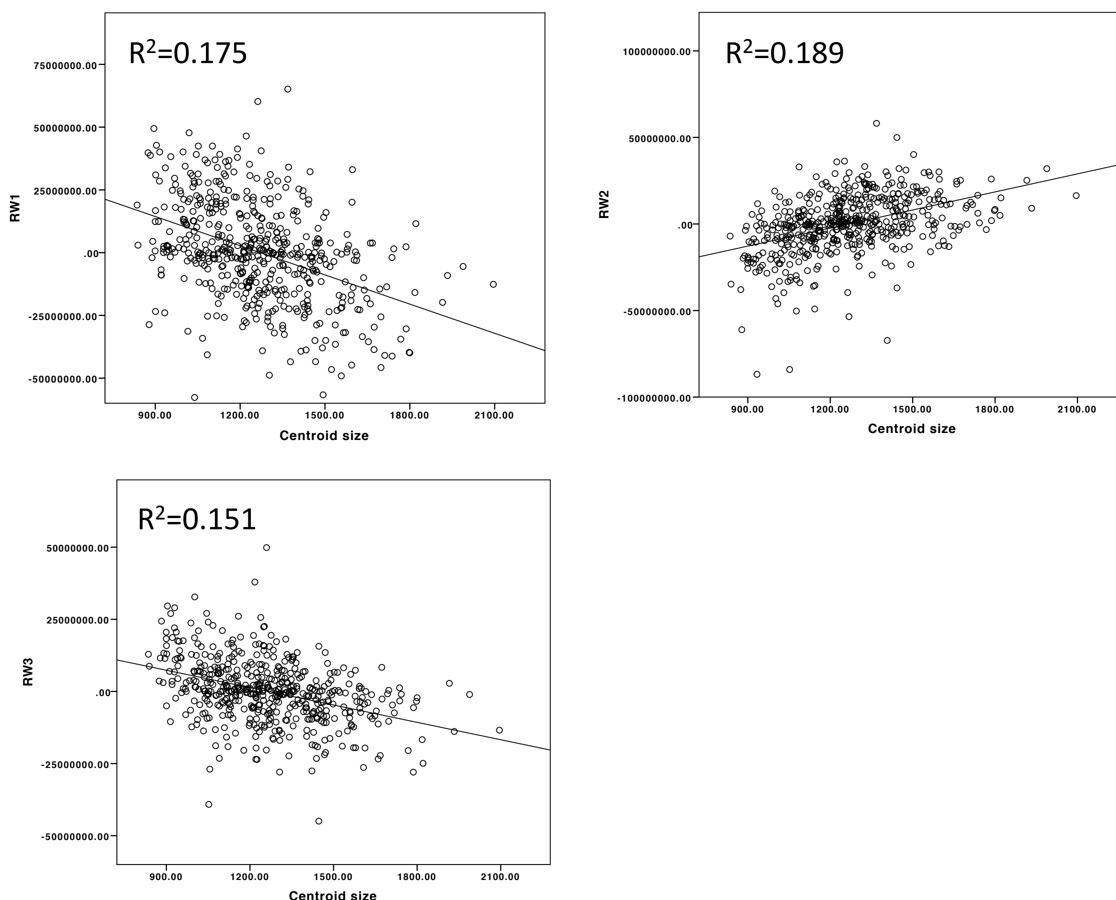

Figure 3.18 *Correlation between RW 1, 2 and 3 and CS*

The larval shape variation was plotted as Canonical variate 1 (CV1) and Canonical variate 2 (CV2) (fig. 3.19). CV 1 and 2 are relative to RW 1 and 2 respectively. The canonical variance visualises shape variation in the form of vector diagrams. CV1 was the one showing the biggest differences according to treatment.

CV 1 and 2 together explained 94.7% of the shape variations in the fish larvae; CV1 explained 78.7 % and CV2 16 %. Figure 3.19 shows how shape is varying according to CV1. The larvae with low CV1 values (Copepod treatment larvae) had a smaller distance between caudal and anal/dorsal fins, were slimmer by the belly and had the nose pointing more downwards compared to larvae from the two rotifer treatments. Cop 7 larvae where in-between. The CV1 variable for the Copepod and Cop 7 treatments were both significantly different from the other treatments. Both rotifer treatments had approximately the same CV1 mean values (fig. 3.20). The CV2 values

for Cop 7 was significantly lower than the CV2 values for the other treatments (fig 3.21).

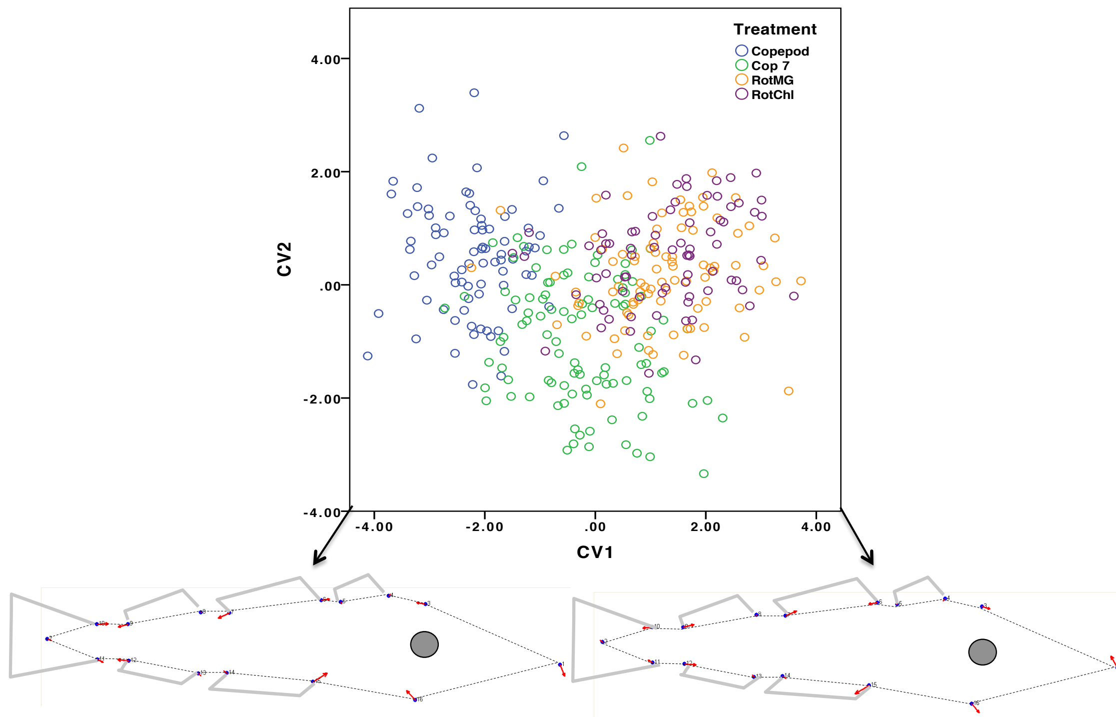

Figure 3.19 *The graph shows the shape variance in the larvae. The vector diagrams at the bottom illustrate how the shape changes from each end of the X-axis (magnified by 3X to emphasize shape differences) (n= 81, 102, 86 and 76 for Copepod, Cop 7, RotMG and RotChl respectively. Total of 345 larvae).*

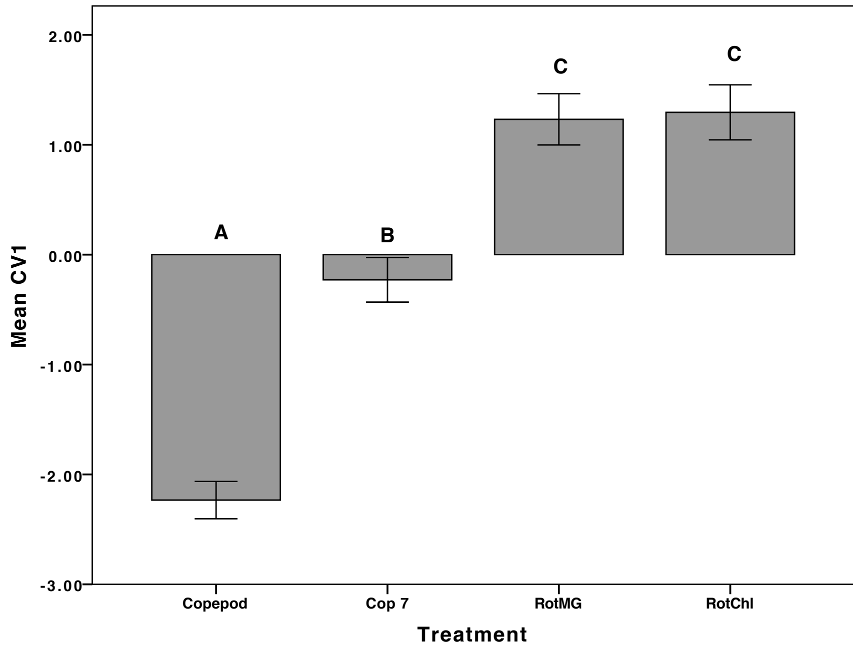

Figure 3.20 *Difference in the CV1 values, which explain 78.7% of the shape variations in the larvae, for the different treatments (n= 81, 102, 86 and 76 for Copepod, Cop 7, RotMG and RotChl respectively. Total of 345 larvae). Significant differences between the treatments are marked with different letters. Error bars indicate* $\pm$ *1 standard error.*

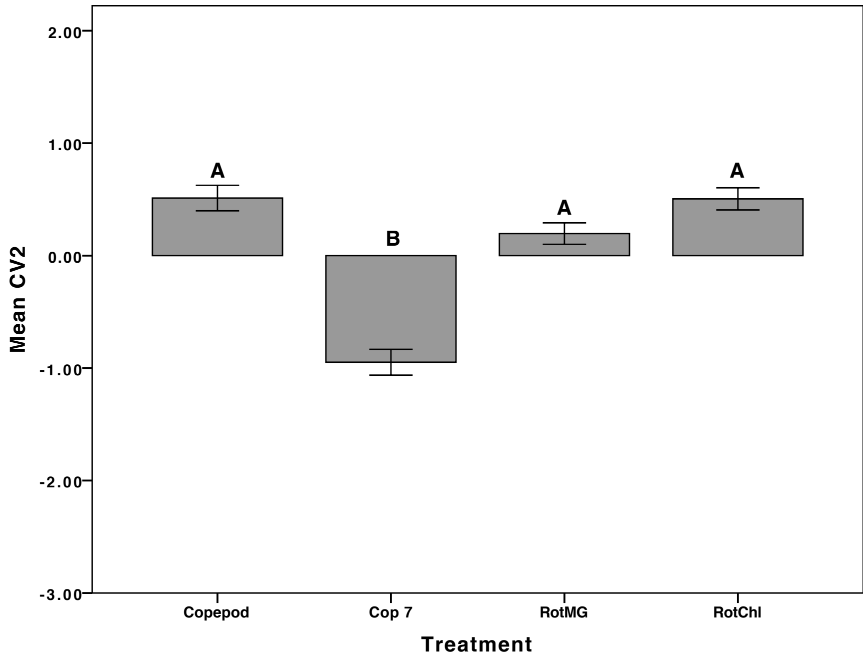

Figure 3.21 *Difference in the CV2 values, which explain 16% of the shape variations in the larva, for the different treatments (n= 81, 102, 86 and 76 for Copepod, Cop 7, RotMG and RotChl respectively. Total of 345 larvae). Significant differences between the treatments are marked with different letters. Error bars indicate* $\pm$ *1 standard error.*

4. Discussion

This study showed that the cod larval rearing success was positively affected by feeding with intensively cultivated copepod nauplii. Growth, survival and quality parameters all showed the same general trend between treatments; the Copepod treatment giving the best results, followed by the Cop 7 treatment, the RotMG treatment and finally the RotChl treatment.

4.1 Growth and survival in relation to live feed

Growth and survival were clearly affected by the different live feed. The treatments fed copepods showed improved growth when compared to those exclusively fed rotifers. The two treatments, Copepod and Cop 7, showed already at 8 dph a significantly higher growth compared to the rotifer treatment. The Copepod treatment larvae had a significantly better growth throughout the experiment, compared to the other treatments, in terms of dry weigh, standard length, myotome height and daily weight increase.

The daily weight increase during first feeding (5-19 dph) in this experiment was 8.5-10.8% day^{-1}, being clearly lower compared to the growth rates achieved by van der Meeren & Næss (1993) at 13.7-21.7% during first feeding (0-16 dph) when feeding with natural zooplankton, extensively cultivated at 11 ^{o}C. In order to make a real comparison of the nutritional effect of copepods with that of rotifers in the present study, the copepods fed to the cod larvae where of the same size as the rotifers in the present study. Also, the Copepod treatment tanks were fed the same amount of prey individuals as the rotifer treatment tanks throughout the experiment, with no feeding to satiation for any of the treatments. However, this may have been a suboptimal feeding regime in terms of growth, since the feed should be in excess and with a higher particle size in order to achieve an optimal growth rate. The particle size of the live feed is a major factor for growth, and the larvae will generally select bigger prey as they grow (Olsen *et al.*, 2000; Kui, 2007). This makes the growth rates in this experiment not directly comparable to other start feeding experiments with natural zooplankton. Feeding to

satiation with intensively cultivated copepods of variable size could probably give a higher growth rate than observed here.

Bush et al. (2010) showed that feeding with rotifers gave a generally lower growth rate compared to feeding with zooplankton. This was also shown in the present study, as growth rate, survival and all quality parameters tested were positively affected by feeding with copepods compared to rotifers, which shows that the nutritional composition of the copepod had a great effect on the success of the cod larvae.

Another master thesis from the same start feeding experiment found mitochondria with interdigitated crista in both the gut and the liver of the RotChl larvae, which indicates a defect in the mitochondrial metabolism (Norheim, 2011). This is a plausible additional reason for the lower growth and survival rates of the larvae fed unenriched rotifers.

In a feeding experiment with harvested copepods for cod larvae, Busch et al. (2010) achieved an approximate 7% survival at 41 dph, compared to 23% survival at 45 dph for the copepod treatment in the present experiment. The shortage of control of live feed and environmental parameters in extensive production compared to intensive production, could possibly be one of many reasons for poor survival rates. Otterlei et al. (1999) also fed with zooplankton extensively sampled, achieving an approximate survival of 5%-45% at 56 dph, indicating that the extensively sampled plankton may be good but not reliable.

During weaning onto dry feed the larvae from the Copepod treatment had a 28% decrease in survival from 38 dph to 45 dph, while RotChl larvae had a 13% decrease in survival during the same period. Also, the Copepod treatment larvae were 56% larger (DW) than the RotChl treatment larvae at 40 dph. This could indicate that the Copepod treatment larvae were in a critical phase of metamorphosis during the 38-45 dph period, and they could be more vulnerable to stress, e.g. weaning. A decrease in growth rate was noticeably seen for the RotChl, RotMG and Cop 7 treatment during weaning, but not for the Copepod treatment. This could be linked to the size difference prior to weaning, as weaning on small larvae has shown to have a negative effect on growth, (Wold *et al.*,

2008) or that the smaller larvae in the Copepod treatment were those dying during the weaning period.

Observations also showed that larvae fed copepods had a more yellow pigmentation compared to larvae fed rotifers, in addition to a higher degree of pigmentation. Chapman (1966) described the pigment *monadoxanthin,* which comprises 15% of the total carotenoids in the microalgae *Rhodomonas*, used in this experiment to feed the copepods. *Monadoxanthin* gives a yellow colour and is therefore a likely cause of the more yellow pigmentation of the larvae fed copepods. Astaxantin is also a pigment found in high concentrations in copepods, having antioxidative properties, preventing the formation of free radicals and protecting against oxidation of lipids and cell membranes. Astaxantin is in comparison found in small amounts in rotifers (van der Meeren, 2003), this is an additional reason for the higher grade of pigmentation in the larvae fed copepods.

4.2 Quality parameters in relation to growth and live feed

All quality parameters, measured from day 33 post hatching onwards, gave the same general gradient as seen for growth and survival, with the Copepod treatment giving the best results, followed by the Cop 7 treatment, the RotMG treatment and finally the RotChl treatment. These findings indicate a correlation between quality of live feed given to the larvae during the first 28 dph and the future performance of the larvae.

Feeding behaviour

The results from the activity observations on 33 dph showed that larvae fed copepods were the most active and that larvae fed rotifer were less active. This indicates that the difference in live feed during the first 28 dph affected the activity level of the cod larvae at 33 dph, and is therefore comparable with previous studies describing the importance of early nutritional quality on behaviour (Gisbert & Williot, 1997). The feeding behaviour in the present study was not only affected by treatment, but also correlated with standard length. Since Reynolds number increases with increase larval size, the water will seem less viscous to a large larvae compared to a smaller larvae, making movement in the water easier for larger larvae(Sakshaug *et al.*, 2009). The general

gradient of activity during feeding, with copepod being the most active, followed by Cop 7, RotMG and RotChl, is the same as the gradient for growth, underlining the importance of size during locomotion in water.

Larvae from the rotifer treatments swam twice as much, compared to larvae from the Copepod and Cop 7 treatments, in order to catch a prey. This may explain the lower growth rates for the rotifer treatments larvae, as they possibly use more energy catching the same amount of food.

Galloway et al. (1999) described the development of axial musculature in first feeding cod larvae, and found higher somatic growth rates in larvae fed DHA-rich diets. High somatic growth rates were associated with an increased contribution of hyperplasia to axial white muscle growth. Copepods have earlier shown to have a higher and qualitatively better HUFA level than rotifers (Evjemo & Olsen, 1997). The higher level of protein in copepods compared to rotifers could possibly lead to a higher muscular growth, since muscle-growth is a product of protein-synthesis (Evjemo *et al.*, 2003; Li *et al.*, 2009). This is consistent with the higher somatic growth rates found for the larvae fed copepods in the present study. Larvae from the Copepod treatment had a significantly greater myotome height from 19 dph, compared to larvae from the other treatments, suggesting a faster growth of axial muscle in larvae fed copepods. A higher degree of contribution of hyperplasia to axial white muscle growth, resulting in more muscle fibres, could possibly affect the muscle power output in the Copepod treatment larvae, which in turn could explain the higher activity level and increase the efficiency of prey capture. In addition another master thesis from the same first feeding experiment, analysing at muscle development, found a higher growth in axial muscle of the Copepod treatment larvae already at 19 dph, compared to the other treatments (Halseth, C. K., NTNU, pers. com. master thesis in prep.).

None of the larvae in this experiment seemed to be gape-limited during prey attack. When observing the larvae, those fed copepods seemed to have a much more clearly predator instinct, and a more 3-dimentional view, compared to the rotifer treatment larvae. This could be seen during prey attacks, where the rotifer treatment larvae seemed to be unintentionally bumping into the prey, not attacking, while the larvae fed

copepods had a more distinct attack. The latter larvae could also make sudden angular turns towards the prey, indicating peripheral vision. This could be seen in the context with previous studies describing the importance of nutrition on larval eye development (DHA content) (Bell *et al.*, 1995) and behaviour (von Herbing & Gallager, 2000).

A camera could have been places over the aquarium, during the behaviour analysis, to better detect differences in activity and behaviour. By using a camera swimming speed, distance and time could have been detected. More parameters could have been analysed for in one study, as a video could have been analysed several times. On the other hand, by not using a camera the procedure is more simple and easier to repeat by others, e.g. farmers.

Larval response to handling stress

The handling stress responses at 37 and 59 dph were affected by treatment, but not correlated to standard length, indicating that the differences seen in these tests were mainly contributed by the differences in treatment the first 28 dph. The larvae from the RotChl treatment had the highest mortality 24 hours after the test, on both 37 and 59 dph. With a clear gradient with copepods being the most hardy, followed by Cop 7, RotMG and finally RotChl, although on day 59 the two treatment fed copepods seemed to be equally hardy. Earlier publications have also shown the importance of early nutrition for the response to stress at later stages (Castell *et al.*, 1994; Koven *et al.*, 2001).

These results have implications in commercial cultivation of cod, by suggesting that early larval nutrition affects the survival of older larvae and juveniles that need to adjust to stress during metamorphosis, weaning, grading and transfer from the hatcheries to sea cages. Larvae stressed according to the same procedure at 37 and 58 dph showed great differences in stress tolerance, suggesting that at a younger stage the larvae are more sensitive to acute stressors, like air exposure. The stress response tested on 24, 29 and 58 dph did not give good results, as the responses were mild. The reason for this is most likely to mild handling. This emphasises the importance of standardizing this method before possible future use as a quality parameter.

The treatments that gave the lowest mortalities after handling stress (Copepod and Cop 7) were also the most successful treatments regarding growth and survival. These results indicate that mortality after stress, e.g. air exposure, could successfully be used as a measure of larval quality. For a better evaluation of stress effects that were not tertiary, one could have studied operculum beats minute^{-1} or cortisol levels prior to stress. These results could have revealed more about the physiological effects of stress. But by making the procedure simple it could more easily be repeated.

Skeletal deformations

Kyphosis, corkscrews and fused vertebras were the deformities mostly affected by the different treatments. The mean number of deformed larvae and deformations larvae^{-1} were also affected by treatment, with the Copepod treatments being superior with the lowest degree of deformities. These results are comparable with earlier studies on other fish species, highlighting the importance of early nutrition on skeletal development. Nutrition has in previous studies shown to particularly affect the skeletal formation of fish larvae, with lipids, proteins, mineral and vitamins all playing essential roles (Cahu *et al.*, 2003b; Lall & Lewis-McCrea, 2007). The overall bone status of larvae from the different treatments showed the same general trend as for growth, survival and other quality parameters, namely that the Copepod treatment gives the best results and the RotChl treatment gives the poorest results. This indicates that bone deformations, as earlier described, are a good indication of the overall success of the fish, in relation to the nutritional quality.

The occurrence of lordosis did not show the same trend as growth, survival and quality. Lordosis appeared to be significantly more common for Cop 7 larvae compared to larvae from the other treatments. Tank 1 in the Cop 7 treatment had both the highest occurrence of lordosis (40%) and the lowest survival rate (12%). This could suggest the occurrence of other suboptimal environmental conditions in one or more of the Cop 7 treatment tanks, affecting survival and development. Previous experiments have also suggested the occurrence of lordosis to be linked with environmental conditions, like water speed and temperature (Helland *et al.*, 2009a; Georgakopoulou *et al.*, 2010). Also for the shape variable CV2 did the Cop 7 larvae significantly different from the other treatments, again suggesting a possible unknown factor affecting this treatment.

About 40% of the larvae from the RotChl treatment and 20% in the RotMG treatment were not fully ossified, while all larvae from the two copepod treatments were fully ossified. This supports earlier studies showing effects of both nutrition and larval size on the ossification process (Cahu *et al.*, 2003b; Kjørsvik *et al.*, 2009; Eidsvik, 2010).

Kjørsvik et al. (2009) studied deformities in cod larvae at 40 dph, and found 11% deformed larvae, while Eidsvik (2010) reported 7.1% deformed larvae from 29-47 dph. The percentage of deformed larvae in this study varied between 35-93% deformed larvae, including so-called corkscrews (twisted arches). Earlier studies have not focused on corkscrews, and the level of deformations when excluding corkscrews was more comparable to former studies, with a range of 32-53% deformed larvae. One reason for the somewhat higher percentage of deformed cod in this study could be the later stage of the larvae when analysed (60 dph), as the percentage of deformed larvae tends to increase with larval size and stage (Grotmol *et al.*, 2005). More recent publications have shown that the use of "Best management practice" significantly reduced the prevalence of deformities, and Helland et al. (2009b) achieved about 3% deformed cod at 20g size when using this practice. The high proportion of deformations in the present experiment is difficult to explain, but since this applies to all treatments and larval sizes it could possibly be related to broodstock quality or rearing practices, since genetics has shown to affect the prevalence of deformations (Kolstad *et al.*, 2006).

Corkscrews were found in almost all larvae from the rotifer treatments (80-90%), and only in ~5% of the larvae from the Copepod treatment. The occurrence of corkscrews in the Cop 7 treatment was somewhat in-between, with approximately 45-50% of the larvae having this deformation. Earlier studies have not focused much upon this deformation, but deformed arches and ribs have previously been linked to both temperature (Sfakianakis *et al.*, 2006) and deficiency of phosphorus and vitamin C (Wimberger, 1993; Lovell, 1998; Halver & Hardy, 2002) in marine fish.

The skeletal deformation results from this experiment clearly show correlation with early larval nutrition. Nutrition has in previous studies shown to particularly affect the skeletal formation of fish larvae, with lipids, proteins, mineral and vitamins all playing

essential roles (Cahu *et al.*, 2003a). The overall bone status of larvae from different treatments show the same general range as for growth, survival and other quality parameters. This indicated that bone deformation, as earlier described, is a good indication of the overall success of the fish, in relation to the nutritional quality of live feed.

When using skeletal deformations as a criterion for larval quality it is important to remember that not all deformities reduce the larval quality. Minor deviations of skeletal elements from the normal, like corkscrews, will not necessarily affect the external appearance and functionality of the fish (Koumoudouros, 2010).

Shape variation

The CV1 shape variation results accounted for 78.7% of the shape variance, and showed that nutritional history significantly affects the body shape of cod larvae. There was a clear shape difference between larvae fed copepods and those fed rotifers, again with the Cop 7 treatment somewhat in-between. All larvae were in the same size range, but the copepod fed larvae were mostly dominating the upper part of this range, and the rotifer fed larvae dominating the lower part of the range, but still there was shown no relation between centroid size and relative warp, indicating that size had no impact on the shape variations in this study.

This study has shown the importance of early nutrition during skeletal development of cod larvae. Skeletal deformities should not contribute to the shape variance observed, as no severely deformed larvae were included in the shape analysis. Whether slight abnormalities that may not yet be detectable could affect shape is uncertain. One of the biggest differences between the larvae from the copepod and rotifer treatments was the higher placement of the snout in the larvae fed rotifers. This may be an indication of a starting stargazing, which is a common deformity in adult cod (Lein *et al.*, 2009). The CV1 shape variance showed a similar trend between treatments as deformations.

Earlier studies have demonstrated that different ways of prey capture and processing of the prey induces morphological differences (Collins & Cheek, 1983; Witte, 1984; Wimberger, 1991), affecting the shape of the fish larvae. The larvae in this study also

showed different feeding behaviours according to treatment, supporting the theory of shape variance due to different prey types.

The distance between the caudal fin and the anal and dorsal fins was much greater for larvae fed rotifers, and may affect the swimming performance of the fish larvae, as it is using the caudal fin during burst swimming. Differences between treatment in swimming activity was not shown in this study, but for further analysis it could be interesting to see whether this shape variance could affect the swimming speed, or perhaps continuous swimming distance and time of the fish larvae/juveniles. The distance between the caudal fin and the anal and dorsal fins has earlier shown to be affected by vitamin C, with larvae fed feed containing no vitamin C having a shorter distance (Wimberger, 1993). This is not a probable reason in the present study, as larvae fed copepods have been shown to have higher contents of vitamin C than larvae fed rotifers (Busch *et al.*, 2010), but it could still be a sign of vitamin malnutrition, most likely for the rotifer treatment larvae, as deficiency of vitamin C also has been linked to stress response and growth, two factors where larvae from the copepod treatment has shown significant better results compared to larvae from the two rotifer treatments.

The myotome height (MH) of larvae from the rotifer treatments was greater than that of the copepod treatment larvae. This was also seen when correlating MH and standard length (SL), as Newton quotient for the two rotifer treatments followed by Cop 7, and finally with the Copepod treatment having the lowest Newton quotient (fig, 3.6). This may be due to different developmental stages of the larvae, with the Copepod treatment larvae probably being at a more advanced developmental stage. When studying the correlation between MH and SL, a flattening in the growth curve of the MH in Copepod treatment larvae was observed at 25-30 mm SL, which could indicate a change in shape due to metamorphosis. However, it may also be linked to starvation, if the largest larvae did not get enough feed at the end of the experiment, since there was no feeding to satiation. This change could be linked to the observed thicker belly of the RotChl and RotMG during shape analysis.

When analysing shape variance as a quality parameter it is important to know if the shape variance has an effect on the success of the larvae. The treatments that gave low

CV1 levels had a clearly better success in terms of growth and survival in this experiment, but it is unclear if these shape differences are related to these success differences.

4.3 Conclusions and future perspectives

The results from this study show that feeding cod larvae with intensively cultivated copepods (*Acartia tonsa*) for the first 28 days post hatching results in a better survival, growth and quality of the larvae than feeding with rotifers, and underlines the importance of early larval nutrition Feeding with copepods for only a short time (Cop 7) also gave larger and more viable larvae than feeding with only rotifers.

All quality parameters tested in this study showed clear differences between the treatments, and could successfully be used for quality assessment of cod larvae and juveniles. Combined they showed the same general range of effects from the treatments, enhancing the conclusion that intensively cultivated copepods successfully could be used as first feed for cod larvae.

Future perspectives

The cod larval feeding regime with intensively cultivated copepods nauplii as prey should be optimized for the larvae to get the best possible effect of the copepods. The length and timing of the shorter copepod-feeding period should be studied further, for optimal output. The production should also be made more cost-effective.

All quality parameters, perhaps with the exception of deformities, need standardization before being used as quality parameter by farmers and scientists working with cod larvae. These parameters could in addition be tested and perhaps standardized for other species where quality variations are a problem.

The use of geometric morphometrics related to skeletal deformation studies could make a clearer definition of when a skeletal deviation is a deformity, in addition to detect deformities not visible externally at an earlier stage. Geometric morphometrics could also be used for further analysis of cod larval allometry.

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APPENDIX 1

Egg counting and hatching test.

The NUNC EasyFlask™ Nunclon™ cell culture bottles containing *A. tonsa* eggs was shaken so the eggs were evenly distributed in the bottle. 50µl was taken out of the bottle with a pipette and diluted with seawater until 1ml. From this; 50 µl was taken out and placed as a droplet in a petri dish. The eggs in the droplet were then counted trough a microscope. The bottle was then weighed. The volume (V) was calculated by taking the weight of the bottle with eggs (B), minus an empty bottle (B_0), multiplied with the seawater density (1,028):

$$V=(B-B_0)*1,028$$

Number of eggs (N) is then calculated by taking number of eggs per ml (X), and multiplying it with the volume

$$N=X*V$$

The petri dish was added 10 ml destilled water, closed with parafilm and placed in room temperature with continues illumination. After 48 hours the parafilm was removed and the sample was fixated with phytofix. Number of nauplii in sample was counted in a microscope, using a peristaltic pump. The hatching rate (HR) was calculated divining the number of nauplii (N) with number of eggs (E), multiplied by 100.

$$HR=N/E*100$$

APPENDIX 2

Procedure for bone staining with Alizarin Red A,

The procedure is according to Kjørsvik et al. (2009).

Larva size	**10-20 mm**	**> 20 mm**
Step 1 - Fixation		
• Fix in 4% natural formalin		
• Rinse in distilled water	2 x 10 min	2 x 20 min
Step 2 – Rehydration and bleaching		
• 95% ethanol	2 x 1 hour	2 x 2 hours
• 75% ethanol	1 hour	2 hours
• 40% ethanol	1 hour	2 hours
• 15% ethanol	1 hour	2 hours
• Distilled water	1,5 hours	Over night
Bleach in 1:9 3% H_2O_2 : 1% KOH	4 hours	6 hours
Clear in trypsin buffer	20 hours	24-48 hours
Step 3 – Staining		
• Cover samples with 1% KOH, and add drops of Alizarin solution until the solution turns purple	20 hours	48 hours
Step 4 – Preservation		
• Rinse in distilled water	5 minutes	5 minutes
• Rinse in 1% KOH	20 minutes	20 minutes
• 40% Glycerol	1 day	1 day
• 70% Glycerol	1 day	1 day
• Store in 100% Glycerol		

Pictures where taken when the larva has been in 40% glycerol for 2 days.

APPENDIX 3

Dry weight

			Dry weight (mg/larva)		
Dph	Treatment	Tank	Mean	Standard Error of Mean	Total N
3	Copepod		.	.	
		1	,0558	,0020	12
		2	,0571	,0023	12
		3	,0575	,0025	11
	Cop 7		.	.	
		1	,0579	,0020	9
		2	,0571	,0023	12
		3	,0567	,0023	15
	RotMG		.	.	
		1	,0571	,0023	12
		2	,0571	,0023	12
		3	,0571	,0023	12
	RotChl		.	.	
		1	,0578	,0022	12
		2	,0571	,0023	12
		3	,0571	,0023	12
5	Copepod		.	.	
		1	,0544	,0022	12
		2	,0539	,0021	12
		3	,0533	,0023	11
	Cop 7		.	.	
		1	,0533	,0025	10
		2	,0539	,0021	12
		3	,0538	,0020	13
	RotMG		.	.	
		1	,0539	,0021	12
		2	,0539	,0021	12
		3	,0539	,0021	12
	RotChl		.	.	
		1	,0539	,0021	12
		2	,0539	,0021	12
		3	,0539	,0021	12
8	Copepod		.	.	
		1	,0742	,0028	12
		2	,0705	,0024	12
		3	,0700	,0029	11
	Cop 7		.	.	
		1	,0639	,0022	12
		2	,0754	,0029	12
		3	,0729	,0024	11

	RotMG		.	.	
		1	,0654	,0015	12
		2	,0578	,0040	12
		3	,0700	,0022	12
	RotChl		.	.	
		1	,0660	,0024	12
		2	,0613	,0023	12
		3	,0622	,0026	12
14	Copepod		.	.	
		1	,1369	,0061	12
		2	,1397	,0088	12
		3	,1512	,0068	11
	Cop 7		.	.	
		1	,1218	,0058	12
		2	,1122	,0062	11
		3	,1052	,0086	12
	RotMG		.	.	
		1	,0884	,0046	12
		2	,1082	,0038	12
		3	,0915	,0063	12
	RotChl		.	.	
		1	,1078	,0055	12
		2	,1095	,0084	12
		3	,1127	,0049	12
19	Copepod		.	.	
		1	,2217	,0122	12
		2	,2276	,0164	12
		3	,2355	,0169	11
	Cop 7		.	.	
		1	,1793	,0177	12
		2	,1592	,0104	12
		3	,1753	,0172	12
	RotMG		.	.	
		1	,1836	,0081	12
		2	,1506	,0199	12
		3	,1816	,0215	12
	RotChl		.	.	
		1	,1872	,0041	12
		2	,1728	,0113	12
		3	,1486	,0180	11
33	Copepod		.	.	
		1	1,0881	,0840	20
		2	1,3692	,0707	20
		3	1,1937	,0649	19
	Cop 7		.	.	
		1	1,0593	,0744	20
		2	,8534	,0886	20
		3	,9796	,0853	20

	RotMG		.	.	
		1	,9007	,0735	20
		2	1,1003	,0924	20
		3	,9950	,0562	20
	RotChl		.	.	
		1	1,0603	,0603	20
		2	,8359	,0585	20
		3	,9160	,0769	20
40	Copepod		.	.	
		1	1,9436	,1417	40
		2	2,5393	,1493	40
		3	2,0920	,1529	39
	Cop 7		.	.	
		1	1,3485	,0977	40
		2	1,5911	,1345	40
		3	1,2880	,0911	40
	RotMG		,6315	.	
		1	1,0163	,0655	40
		2	,9846	,0711	40
		3	1,0975	,0752	39
	RotChl		.	.	
		1	1,4450	,1115	40
		2	1,0187	,0691	39
		3	1,1900	,0715	39
60	Copepod		18,3543	.	
		1	23,0411	1,4971	48
		2	18,1025	1,2519	46
		3	25,6804	1,5650	50
	Cop 7		.	.	
		1	16,6316	1,0472	49
		2	16,9039	,9974	50
		3	12,1357	,8001	55
	RotMG		.	.	
		1	10,9420	,7068	50
		2	10,3994	,8053	49
		3	14,4905	1,2500	50
	RotChl		.	.	
		1	12,2723	,7200	48
		2	10,1739	,5445	50
		3	13,4890	,7593	50

APPENDIX 4

% Daily Weight Increase

Dph	Treatment	Mean %DWI	±SE
2-5	Copepod	.	.
	Cop 7	.	.
	RotMG	.	.
	RotChl	.	.
	All	-5.9	.0
5-19	Copepod	10.8	.1
	Cop 7	8.6	.3
	RotMG	8.6	.6
	RotChl	8.5	.5
		.	.
19-33	Copepod	12.7	.6
	Cop 7	13.1	.3
	RotMG	12.5	.5
	RotChl	13.0	.6
	All	.	.
33-40	Copepod	10.8	.5
	Cop 7	2.9	.2
	RotMG	2.4	.7
	RotChl	1.3	.1
		.	.
40-60	Copepod	14.2	1.6
	Cop 7	12.9	.6
	RotMG	12.1	.4
	RotChl	11.8	.4
		.	.
3-60	Copepod	11.0	.2
	Cop 7	10.3	.2
	RotMG	9.8	.2
	RotChl	9.8	.2
		.	.

APPENDIX 5

Survival

				Survival (%)	
				Mean	+SE
Dph	38	Treatment	Copepod	31,81	1,34
			Cop 7	21,84	3,79
			RotMG	15,60	,34
			RotChl	15,15	2,29
	39	Treatment	Copepod	31,02	1,07
			Cop 7	21,66	3,72
			RotMG	15,47	,32
			RotChl	15,09	2,31
	40	Treatment	Copepod	29,70	1,93
			Cop 7	21,48	3,70
			RotMG	15,40	,32
			RotChl	14,98	2,32
	41	Treatment	Copepod	29,40	1,99
			Cop 7	21,13	3,71
			RotMG	14,96	,64
			RotChl	14,84	2,35
	42	Treatment	Copepod	26,51	2,08
			Cop 7	19,92	3,59
			RotMG	14,52	,55
			RotChl	14,57	2,35
	43	Treatment	Copepod	24,32	2,22
			Cop 7	18,82	3,56
			RotMG	13,94	,48
			RotChl	14,14	2,27
	44	Treatment	Copepod	23,55	2,14
			Cop 7	18,20	3,61
			RotMG	13,52	,56
			RotChl	13,62	2,29
	45	Treatment	Copepod	22,80	2,03
			Cop 7	17,62	3,65
			RotMG	12,96	,55
			RotChl	12,79	2,14
	46	Treatment	Copepod	22,45	1,97
			Cop 7	17,22	3,73
			RotMG	12,51	,49
			RotChl	12,37	2,06
	47	Treatment	Copepod	22,26	1,96
			Cop 7	16,92	3,69
			RotMG	12,13	,41
			RotChl	12,00	2,02
	48	Treatment	Copepod	22,08	1,98
			Cop 7	16,71	3,63

			RotMG	11,91	,38
			RotChl	11,73	2,02
	49	Treatment	Copepod	21,82	1,97
			Cop 7	16,50	3,51
			RotMG	11,72	,42
			RotChl	11,48	1,91
	50	Treatment	Copepod	21,51	2,01
			Cop 7	16,28	3,53
			RotMG	11,55	,42
			RotChl	11,35	1,88
	51	Treatment	Copepod	21,31	2,03
			Cop 7	16,09	3,42
			RotMG	11,39	,42
			RotChl	11,18	1,80
	52	Treatment	Copepod	21,24	2,03
			Cop 7	16,03	3,41
			RotMG	11,21	,51
			RotChl	11,09	1,76
	53	Treatment	Copepod	21,20	2,01
			Cop 7	15,95	3,41
			RotMG	11,11	,54
			RotChl	10,98	1,70
	54	Treatment	Copepod	21,17	2,00
			Cop 7	15,81	3,39
			RotMG	11,03	,56
			RotChl	10,91	1,68
	55	Treatment	Copepod	21,03	2,01
			Cop 7	15,66	3,42
			RotMG	10,95	,58
			RotChl	10,71	1,75
	56	Treatment	Copepod	20,77	1,99
			Cop 7	15,38	3,45
			RotMG	10,85	,58
			RotChl	10,48	1,81
	57	Treatment	Copepod	20,53	2,02
			Cop 7	15,18	3,45
			RotMG	10,76	,58
			RotChl	10,40	1,81
	58	Treatment	Copepod	20,38	2,02
			Cop 7	15,02	3,42
			RotMG	10,70	,60
			RotChl	10,28	1,78
	59	Treatment	Copepod	19,98	1,99
			Cop 7	14,60	3,29
			RotMG	10,61	,60
			RotChl	10,16	1,80
	60	Treatment	Copepod	19,53	2,04
			Cop 7	14,28	3,31
			RotMG	10,52	,61
			RotChl	9,95	1,73

APPENDIX 6

Behaviour analysis

Treatment	Tank	Larva	Swimming (sec.)	Orientation (No.)	Eat (No.)
RotMG	1	1	18,5	21	1
RotMG	1	2	8,6	16	0
RotMG	1	3	2,7	13	0
RotMG	1	4	10,0	28	1
RotMG	1	5	6,4	24	0
RotMG	1	6	11,7	27	0
RotMG	1	7	3,9	8	0
RotMG	1	8	10,0	11	0
RotMG	1	9	3,2	7	3
RotMG	1	10	7,2	17	1
RotMG	2	1	4,0	9	0
RotMG	2	2	15,6	10	0
RotMG	2	3	10,6	9	0
RotMG	2	4	12,1	10	1
RotMG	2	5	2,9	19	0
RotMG	2	6	2,4	19	0
RotMG	2	7	0,0	1	0
RotMG	2	8	3,0	21	0
RotMG	2	9	1,5	15	0
RotMG	2	10	7,3	16	0
RotMG	3	1	2,0	14	1
RotMG	3	2	4,8	27	0
RotMG	3	3	1,2	22	0
RotMG	3	4	4,6	16	0
RotMG	3	5	6,0	34	0
RotMG	3	6	2,1	3	1
RotMG	3	7	2,9	23	0
RotMG	3	8	8,7	12	0
RotMG	3	9	6,8	29	0
RotMG	3	10	5,8	21	0
Average			*6,2*	*16,7*	*0,3*
Copepod	1	1	8,2	29	0
Copepod	1	2	7,5	33	2
Copepod	1	3	8,3	58	0
Copepod	1	4	5,2	23	1
Copepod	1	5	13,9	26	1
Copepod	1	6	0,9	14	1
Copepod	1	7	38,2	1	0
Copepod	1	8	0,4	13	0
Copepod	1	9	0,2	22	0

Copepod	1	10	19,1	53	0
Copepod	2	1	6,4	29	4
Copepod	2	2	1,9	18	0
Copepod	2	3	7,0	16	10
Copepod	2	4	12,3	21	8
Copepod	2	5	7,4	31	2
Copepod	2	6	10,0	39	1
Copepod	2	7	2,7	25	1
Copepod	2	8	1,2	13	0
Copepod	2	9	4,6	29	0
Copepod	2	10	13,3	33	2
Copepod	3	1	20,3	19	5
Copepod	3	2	13,6	18	8
Copepod	3	3	15,3	20	5
Copepod	3	4	14,8	17	9
Copepod	3	5	12,0	21	4
Copepod	3	6	13,6	19	2
Copepod	3	7	30,3	2	0
Copepod	3	8	17,8	16	6
Copepod	3	9	10,7	23	5
Copepod	3	10	4,6	5	0
Average			*10,7*	*22,87*	*2,57*

Cop 7	1	1	0,0	10	0
Cop 7	1	2	5,5	4	4
Cop 7	1	3	5,4	14	2
Cop 7	1	4	7,6	46	0
Cop 7	1	5	2,2	14	2
Cop 7	1	6	14,9	15	0
Cop 7	1	7	4,4	13	0
Cop 7	1	8	2,8	19	0
Cop 7	1	9	3,1	17	0
Cop 7	1	10	0,0	18	0
Cop 7	2	1	1,5	44	1
Cop 7	2	2	6,3	20	7
Cop 7	2	3	10,2	13	3
Cop 7	2	4	5,4	22	1
Cop 7	2	5	13,6	28	0
Cop 7	2	6	11,1	25	1
Cop 7	2	7	1,5	17	0
Cop 7	2	8	5,5	13	0
Cop 7	2	9	11,2	22	2
Cop 7	2	10	7,6	28	0
Cop 7	3	1	1,7	20	2
Cop 7	3	2	4,6	14	1
Cop 7	3	3	7,8	3	0
Cop 7	3	4	6,0	10	0
Cop 7	3	5	5,5	21	4
Cop 7	3	6	4,3	28	1

Cop 7	3	7	10,2	24	2
Cop 7	3	8	8,8	20	3
Cop 7	3	9	2,2	13	1
Cop 7	3	10	5,7	13	1
Average			*5,9*	*18,9*	*1,3*

RotChl	1	1	8,8	6	0
RotChl	1	2	21,7	14	0
RotChl	1	3	31,5	11	0
RotChl	1	4	5,7	16	2
RotChl	1	5	8,5	17	0
RotChl	1	6	4,9	22	0
RotChl	1	7	5,9	10	0
RotChl	1	8	2,5	18	0
RotChl	1	9	3,0	19	0
RotChl	1	10	7,8	23	2
RotChl	2	1	4,5	30	2
RotChl	2	2	1,5	40	0
RotChl	2	3	0,8	36	0
RotChl	2	4	1,6	7	0
RotChl	2	5	9,1	14	2
RotChl	2	6	8,4	22	1
RotChl	2	7	13,8	25	0
RotChl	2	8	4,7	12	0
RotChl	2	9	0,7	17	0
RotChl	2	10	13,4	13	1
RotChl	3	1	26,2	5	0
RotChl	3	2	12,0	16	1
RotChl	3	3	4,5	9	0
RotChl	3	4	23,5	11	0
RotChl	3	5	22,5	6	0
RotChl	3	6	5,3	9	0
RotChl	3	7	6,5	29	0
RotChl	3	8	8,6	7	1
RotChl	3	9	13,4	9	1
RotChl	3	10	12,3	19	1
Average			*9,8*	*16,4*	*0,5*

APPENDIX 7

Stress tests

DAY 24

Treatment/ tank	Dead after1 h	Dead after 5 h	Dead after 24 h	Survivals
Cop -1	0	1	1	12
Cop -2	2	1	3	8
Cop -3	3	0	0	12
Cop 7-1	0	0	0	11
Cop 7-2	2	0	1	11
Cop 7-3	1	0	2	14
RotMG -1	1	0	1	11
RotMG -2	1	1	0	11
RotMG -3	1	2	1	10
RotChl -1	1	0	0	9
RotChl -2	0	1	0	9
RotChl -3	2	0	1	11

Day 29

Treatment/ tank	Dead after1 h	Dead after 5 h	Dead after 24 h	Survivals
Cop -1			2	15
Cop -2	1		1	13
Cop -3		1		13
Cop 7-1	1	1	2	16
Cop 7-2	1		2	12
Cop 7-3				15
RotMG -1			2	15
RotMG -2	1			13
RotMG -3	1			13
RotChl -1	1			15
RotChl -2	2	1		13
RotChl -3			2	11

Day 37

Treatment/ tank	Dead after1 h	Dead after 5 h	Dead after 24 h	Survivals
Cop -1	0	1	1	14
Cop -2	0	1	0	14
Cop -3	0	0	0	15
Cop 7-1	2	0	1	14
Cop 7-2	0	0	1	14
Cop 7-3	2	0	1	13
RotMG -1	2	0	1	12
RotMG -2	1	1	2	12
RotMG -3	0	1	2	10
RotChl -1	3	0	2	9
RotChl -2	1	2	4	10
RotChl -3	4	1	2	11

Day 58

Treatment/ tank	Dead after1 h	Dead after 5 h	Dead after 24 h	Survivals
Cop -1	0	0	1	14
Cop -2	0	0	0	15
Cop -3	0	2	1	12
Cop 7-1	0	3	1	11
Cop 7-2	0	0	0	15
Cop 7-3	0	1	1	13
RotMG -1	0	0	2	13
RotMG -2	0	0	0	15
RotMG -3	0	2	4	9
RotChl -1	0	0	1	14
RotChl -2	1	1	0	13
RotChl -3	0	1	1	13

Day 59

Treatment/ tank	Dead after1 h	Dead after 5 h	Dead after 24 h	Survivals
Cop -1	0	2	0	14
Cop -2	2	1	0	15
Cop -3	0	0	0	13
Cop 7-1	0	1	1	15
Cop 7-2	0	0	0	14
Cop 7-3	0	0	1	16
RotMG -1	0	2	0	11
RotMG -2	1	2	2	11
RotMG -3	0	3	0	10
RotChl -1	2	5	0	10
RotChl -2	4	5	0	6
RotChl -3	1	2	3	9