NTNU – Trondheim
Norwegian University of
Science and Technology

Dietary effects of different live prey on growth and functional development in ballan wrasse (*Labrus bergylta*) larvae and juveniles

Maria Oknes Sørøy

Marine Coastal Development

Supervisor: Elin Kjørsvik, IBI

Norwegian University of Science and Technology
Department of Biology

Acknowledgments

This master thesis was written at the Department of Biology, at the Norwegian University of Science and Technology (NTNU). The experimental work was carried out at the NTNU Centre of Fisheries and Aquaculture (Sealab) in Trondheim, and in the laboratories of SINTEF Fisheries and Aquaculture Ltd. The experiment was part of the project "Produksjon av berggylte" (900554), and was a research collaboration between SINTEF, Nofima, the Institute of Marine Research, and NTNU. The project was funded by the Norwegian Seafood Research Fund (FHF). The thesis has been written under the guidance of Professor Elin Kjørsvik at the Department of Biology, NTNU, and Research Manager Gunvor Øie at SINTEF Fisheries and Aquaculture.

I would first like to thank Elin Kjørsvik for excellent supervising and informative feedback during the writing of my thesis, and Gunvor Øie for constructive input and discussions, as well as invaluable help with the experiment. I would like to thank Werner Storøy and Marte Schei for great help during the experiment, Marit Holmvaag Hansen for advice and assistance in the behaviour studies and stress tests, Kari Attramadal for help during the experiment and statistical analysis, Tora Bardal for providing excellent guidance and help during the laboratory and analytical work, and Ida Anette Norheim for help during the writing of this thesis. Thanks also to Martin Almli and Martin Berg for cooperation during the experimental work.

Thanks to all the great students at Sealab, for making these two years an enjoyable experience, and a special "thank you" goes to Maren Ranheim Gagnat. Your friendship and support have been greatly appreciated!

Last, but not least, I would like to thank Morten, and the rest of my family, for always supporting me.

Trondheim, May 2012 Maria Oknes Sørøy

Abstract

Ballan wrasse (*Labrus bergylta*) is a new species in aquaculture, the newly initiated intensive production is a response to the increasing demands for wrasse as cleaner fish in salmon and trout farming. The dietary requirements of the ballan wrasse larvae are largely unknown. The nutritional quality of the live feed commonly used in the farming of marine larvae today, rotifers and *Artemia sp.*, may be suboptimal to the dietary need of developing ballan wrasse larvae, and contribute to the problems with growth, survival and skeletal anomalies currently observed in the rearing of this species. Copepods are the natural feed for pelagic marine larvae. To use intensively cultivated copepod nauplii in the farming of marine species, either as a supplement to, or instead of rotifers or *Artemia* sp., have lead to improved growth and normal development, earlier onset of ossification, and less skeletal anomalies.

The aim of this study was to examine the effects of four different feeding regimes from 4 to 30 days post hatch (dph), on larval growth, development and quality. The larvae in the **Copepod** and **Cop7** treatments were fed cultivated copepod nauplii of the species *Acartia tonsa* from 4 to 30 dph, and from 4 to 10 dph, respectively. The larvae in the **RotMG** and **RotChl** treatments were fed enriched and unenriched rotifers (*Brachionus ibericus* Cayman), respectively. All groups were fed *Artemia fransiscana* nauplii from 24 to 51 dph, and formulated feed from 40 to 61 dph. The experiment was terminated on 61 dph. Growth and survival was recorded, and the quality difference between the larvae from the different treatments was assessed through several quality parameters; observations of larval behaviour, response to handling stress, bone ossification, and skeletal anomalies.

The larvae fed copepods instead of rotifers showed better growth and stress tolerance, were more effective predators, showed earlier onset of ossification of the axial and fin ray skeleton, and had significantly less skeletal anomalies per larvae compared to the larvae fed rotifers, when the most common anomaly (twisted arches) was excluded. Larval size was consistently more related to degree of ossification than age. Growth was the parameter most notably affected by the diet, and significant differences in dry weight, standard length and myotome height was found already on 8 dph.

Table of contents

1. Introduction

1.1 Background

The salmon farming in Norway started in the late 1960s, and became a large-scale commercial food producing industry in the 1980s. Today Norway is the biggest producer of farmed Atlantic salmon (*Salmo salar)*, and the salmon is Norway's fourth biggest export commodity (Liu et al., 2011). The infestations of sea lice are a problem for the farmed Atlantic salmon industry, as well as for the wild salmon. To avoid this parasite it is possible to use chemical or biological treatments. The biological treatment utilises wrasse as a cleaner fish, and was first tested and developed at the Institute of Marine Research in Bergen, Norway, at the end of the 1980ies (Bjordal, 1988a ; Bjordal, 1988b ; Bjordal, 1990). The wrasses have been captured from wild populations since 1988. In 2000 the farmers started using a feed containing the chemical emamectin benzoate, and the use of cleaner fish decreased. However, the sea lice developed resistance, and the use of wrasse increased again after 2006 (Espeland et al., 2010). There are four species of wrasse that are commonly used to remove sea lice from salmon in Norway: Goldsinny wrasse (*Ctenolabrus rupestris,* bergnebb), ballan wrasse (*Labrus bergylta,* berggylt), corkwing wrasse (*Symphodus melops,* grønngylt) and rock cook (*Centrolabrus exoletus,* gressgylt). Ballan wrasse is the species that has the biggest potential to remove lice from salmon; it tolerates handling and stress well, with low mortality (Espeland et al., 2010). The reported use of wrasse in salmon and trout aquaculture, was 682,000 in 2006, and increased to 10,976,000 by 2010 (data from the Norwegian Directorate of Fisheries, as of 01.12.11). The need for cleaner fish have recently been estimated to be approximately 15 million individuals year^{-1} (Hamre & Sæle, 2011). These numbers emphasise the need to establish an intensive production of ballan wrasse. Even though there currently are no evidence that the populations of the wrasse species used in aquaculture are declining due to the increase in fisheries, the species can be vulnerable to overexploiting as the populations are small and local (Espeland et al., 2010). It has proven difficult to obtain wild ballan wrasse of the right size at the right time of year, and farming can ensure a stable supply of fish, in the size range demanded. The first experimental production of ballan wrasse in Norway was at the research station Austevoll at the marine institute of research, in cooperation with Villa Miljølaks in 2007. Marine Harvest Labrus

started their production of ballan wrasse in 2009 (Espeland et al., 2010), and has since been followed by several farmers.

The most common live feed used for intensive cultivation of marine fish larvae today are rotifers, mostly *Brachionus plicatilis* (but lately also *B. rotundiformis* for particularly small larvae), often followed by the used of brine shrimp; *Artemia* sp. nauplii (Støttrup & McEvoy, 2003). The same feeding regime is used for rearing of ballan wrasse (Grøntvedt, 2010), but the knowledge of this species' nutritional requirements are limited. The challenges experienced in intensive rearing of ballan wrasse, using rotifers at first-feeding, are low survival, poor growth, and various skeletal anomalies (Grøntvedt, 2010 ; Helland et al., 2012).

Several types of skeletal anomalies commonly occur in intensively reared ballan wrasse. Helland et al. (2012) collected ballan wrasse of different sizes and age groups from several farmers, as well as wild-caught ballan wrasse, and analyzed the occurrence of skeletal anomalies. Examples of anomalies detected were fusions, lordosis, compressions in the vertebrae, and deformities of the lower jaw. The number of fish with skeletal anomalies and the types of anomalies observed varied greatly between the different rearing facilities. In one group, 80-100% of the fish had anomalies in the vertebrae, and 4-24% were diagnosed with lordosis. 3 of a total of 17 wild-caught ballan wrasse was diagnosed with well-organized fusions in the vertebral column.

The natural food for marine fish larvae are copepods and other zooplankton (Hunter, 1981), and it is assumed that copepods cover all the larvae's nutritional needs (Evjemo & Olsen, 1997 ; Evjemo et al., 2003 ; van der Meeren et al., 2008). Marine larvae fed copepods harvested from naturally occurring populations or from intensive cultivation have generally higher growth rates, better survival, increased rates of normal pigmentation (halibut and turbot), better stress resistance, earlier ossification of the axial skeleton, and less skeletal deformities compared to larvae fed rotifers and/or *Artemia* nauplii (e.g. (Kraul et al., 1993 ; McEvoy et al., 1998 ; Shields et al., 1999 ; Sæle et al., 2003 ; Hamre, 2006 ; Rajkumar & Kumaraguru vasagam, 2006 ; Busch et al., 2011 ; Hansen, 2011 ; Zouiten et al., 2011). It is possible that feeding ballan wrasse larvae intensively cultivated zooplankton can alleviate the different problems encountered in the intensive rearing of this species.

1.2 Dietary requirements for growth and development of marine fish larvae

Pelagic marine fish larvae are very small and immature when they hatch, ballan wrasse larvae are only on average 3.6 mm (Skiftesvik & Bjelland, 2003 ; Dunaevskaya, 2010). Many marine larvae, including ballan wrasse and cod, are altricial. The larvae have a rudimentary but functional digestive system, and lack both a morphological and functional stomach when the yolk-sac is exhausted (Govoni et al., 1986). Pelagic marine larvae need easily digestible feed at first feeding, accomplished through supply of live feed (Støttrup & McEvoy, 2003). Live feed have successfully been completely replaced by formulated feed from the onset of first-feeding in a few species, but there are still challenges regarding nutrient leaching from the feed, distribution of feed in the water column, and the ingestion and digestibility of the formulated feed (Yúfera et al., 1999 ; Yúfera et al., 2000 ; Cahu & Zambonino Infante, 2001). This still make live feed the preferred choice. As opposed to cod, and many other marine fish, it has recently been discovered that the ballan wrasse lack a stomach not only in the larval stage, but also in the juvenile and adult stages (Hamre & Sæle, 2011). Further development of feed have to be adapted to the specific challenges this imposes.

Growth in marine fish larvae is mainly due to an increase in body protein, and the specific requirement for proteins in the diet can be very high. Amino acids and proteins are paramount to ensure growth, and proteins are quantitatively more important than lipids (Olsen et al., 2004). The lipids are the main energy source for marine fish larvae (Sargent et al., 2002), as well as being important structural components in membranes (Watanabe & Kiron, 1994). The n-3 highly unsaturated fatty acids (HUFAs) are an essential dietary part for marine fish larvae, because these fatty acids cannot be synthetized in sufficient amounts *de novo* (Bell et al., 2003). Docosahexaenoic acid (22:6n-3, DHA) and eicosapentaenoic acid (20:5n-3, EPA) are both essential fatty acids, and can be supplied as part of the phospholipids (PL), which are the main component in cell membranes, or as neutral lipids (NL). HUFAs incorporated in the dietary polar PL are more available and beneficial for marine fish larvae than HUFAs in the NL, and has given more developed digestive organs, higher dry weight, earlier ossification of the vertebrae, and higher DHA content in the tissues (Izquierdo et al., 2000 ; Gisbert et al., 2005 ; Kjørsvik et al., 2009 ; Wold et al., 2009). Cahu et. al (2003a) found that the proportion of fatty acids incorporated in the NL and PL affected growth and skeletal deformities in seabass (*Dicentrarchus labrax*) larvae more than the total dietary lipid content.

Rotifers and *Artemia* nauplii are naturally nutritionally deficient for the growth and survival of cold-water marine fish larvae, because of the lack of sufficient amounts of n-3 HUFAs, in particular DHA and EPA. The nutritional value of cultivated live feed have to be manipulated through enrichment, and it is suggested that the values of lipids and proteins should resemble that of copepods (Bell et al., 2003 ; Evjemo et al., 2003). The live feed is enriched with marine fish oils, where the EPA and DHA are mainly present in the triacylglycerides (Izquierdo et al., 2000). Through the enrichment of rotifers and *Artemia* nauplii the fatty acid composition can be manipulated (Evjemo & Olsen, 1997), but the lipids will mainly be incorporated in the triacylglycerides, leaving the PL-fraction less affected (Rainuzzo et al., 1994 ; Izquierdo et al., 2000). The predominant lipids in wild-caught copepods (*T. longicornis, C. finmarchicus* and *Eurytemora sp)* are DHA and EPA, and the protein content (percentage of DW) have been found to be higher in copepods (52-57%) than in newly hatched (41%) or enriched (37%) *Artemia* nauplii (Evjemo et al., 2003).

The knowledge of the species-specific requirements for n-3 HUFAs in marine fish larvae is limited, but studies on fish eggs (which are a good indicator of the developing yolk-sac larvae's and the resulting larvae's requirement of lipids until absorption of the yolk sac) have shown that most have a natural ratio of DHA:EPA of ≥ 2 (Tocher & Sargent, 1984). The ratio of DHA:EPA in newly hatched *Acartia tonsa* nauplii, where the eggs were obtained from a culture of adults fed the red algae *Rhodomonas baltica,* was 2.0 (Støttrup et al., 1999), and for several species of wild-caught copepods have been reported to be in the range of 0.84 to 1.86 (Evjemo et al., 2003).

Copepods can either be harvested from naturally occurring populations in fjords or from outdoor ponds, or they can be intensively cultivated. If harvested from nature, the availability of copepods and nauplii will be highly seasonal and variable, disease and parasites can be introduced in the rearing facility, and control of the nutritional value, species and stages of the copepods are limited (van der Meeren & Naas, 1997 ; Støttrup, 2000). Methods for the intensive cultivation of *Acartia tonsa* have been developed, where eggs are harvested and stored so that nauplii can be hatched when necessary (Støttrup et al., 1986). SINTEF Fisheris and Aquaculture Ltd has contributed to the upscaling and further development of the intensive production of copepods, with the goal of making a large scale production viable. Benefits of such a production include control of the supply, size, species, and nutritional value (the

copepods are reared on a mono-algal diet (*Rhodomonas baltica*) (Gunvor Øie, SINTEF, pers. comm.). Intensively cultivated *Acartia tonsa* have successfully been used as a supplement to, or instead of rotifers in the early feeding of cod larvae, leading to larvae of a higher quality (Hansen, 2011).

1.3 Larval quality

Ballan wrasse is a new species in aquaculture, and little is known of its requirements of live feed. It is important to assess the effect different live feed-types and -quality have on the larval quality, as this can be an indicator of the dietary suitability of the live feed.

Important factors to assess larval quality are morphological and physiological characteristics (such as survival and growth) combined with behavior (Fushimi, 2001). Examples of common indicators of differences in larval quality, include growth, survival, tolerance to stress, analysis of skeletal anomalies (deformities), ossification processes, shape variations and behavior (e.g. activity, or ability to catch prey).

1.3.1 Stress tolerance

The number of factors that can cause stress for intensively reared fish are numerous; general handling, tending of the tanks, environmental fluctuations, predation from other larvae, and weaning are some examples. In larger fish, sorting and grading are also a cause of stress (Barton & Iwama, 1991). The effect of stress have been described as "a change in biological condition beyond the normal resting state that challenges homeostasis and, thus, represents a threat to the fish´s health." (Barton & Iwama, 1991). The result of a stress response can be mortality (Barton, 2002).

Differences in live feed quality affect stress tolerance in marine larvae; dietary DHA, and also arachidonic acid (ARA), are beneficial for vitality and tolerance to stressors such as handling, decreased dissolved oxygen and increased water temperature in marine larvae (Watanabe & Kiron, 1994 ; Kanazawa, 1997 ; Tago et al., 1999 ; Koven et al., 2001). Cod larvae fed intensively cultivated copepods (*Acartia* tonsa*)* were found to have better tolerance to air-exposure (handling stress) than larvae fed enriched rotifers (Hansen, 2011).

Common ways to assess the stress tolerance in fish larvae includes altering the temperature or salinity, or exposing the larvae to air, and record the effect of the stressor (e.g. mortality,

cortisol levels) (Barton & Iwama, 1991 ; Kraul et al., 1993 ; Tago et al., 1999 ; Koven et al., 2001 ; Barton, 2002 ; Hansen, 2011).

1.3.2 Larval activity

Cod larvae fed intensively cultivated copepods or rotifers with a high lipid content have been shown to have a higher activity level and to be more effective predators than larvae fed rotifers of a lower quality in regard to lipid content (O'Brien-MacDonald et al., 2006 ; Hansen, 2011). A larva's ability to catch prey will affect its growth, and ultimately its ability to survive, and feeding behavior analysis can be used to assess this ability (von Herbing & Gallager, 2000 ; O'Brien-MacDonald et al., 2006). Measurements of larval quality can hence be based on swimming duration and number of prey caught during a limited time span, as in Puvanendran & Brown (1999), O'Brien-MacDonald et al. (2006), and Hansen (2011).

To my knowledge, no similar behavioral studies on ballan wrasse have been performed, but some general observations of larval feeding behavior was performed on 3 wild-caught larvae (from tow-nets), of which 2 was kept alive until after metamorphosis (Lebour, 1919). Lebour remarked that the ballan wrasse larvae were "the most stupid" (compared to other marine larvae that was observed), with heavy and slow movements. No stalking behavior was observed, rather the larvae would "snap up" copepods in its path, and displayed a "slowness of perception of the food." The larvae would spend most of the time on the bottom except when feeding, and would eat most copepods available in the tank. Only the copepod *Calanus finnmarchicus* was not eaten, possibly being too quick to capture "for such a sluggish creature."

1.3.3 Skeletal development and anomalies

The ossification process is defined by the transformation of cartilage to bone (Harder, 1975). A general developmental pattern of ossification is found in fish; the structures of the feeding apparatus ossify first (mouthparts), followed by the swimming structures (tail fin), cranium, dorsal and anal fins, the axial skeleton and last the pterygiophores (e.g. (Koumoundouros et al., 1999 ; Faustino & Power, 1999 ; Sæle et al., 2004 ; Sfakianakis et al., 2005). The process of ossification is affected by nutritional factors (Cahu et al., 2003b ; Sæle et al., 2003 ; Mazurais et al., 2008 ; Kjørsvik et al., 2009 ; Power, 2009), and supply of DHA mainly through the PL fraction (as opposed to in NL) have been shown to give faster ossification of the vertebral column in cod (Kjørsvik et al., 2009). Also several minerals and vitamins are essential for normal skeletal development and mineralization; such as the levels of calcium, phosphorus and vitamin D (Power, 2009 ; Zambonino Infante et al., 2009a).

Feeding with copepods, either intensively cultivated or from a mesocosm system positively affected ossification in cod (Eidsvik, 2010), sea bass (Zouiten et al., 2011), and halibut larvae (Sæle et al., 2003), when compared to larvae fed rotifers or *Artemia.*

Skeletal anomalies is a problem in intensive cultivation of marine fish such as cod (Bæverfjord et al., 2009b ; Fjelldal et al., 2009), seabream (Boglione et al., 2001) and ballan wrasse (Helland et al., 2012), and in marine fin-fish production in general (Divanach et al., 1996). Boglione et al. (2001) found that 4% of wild-caught fish had deformations of the body, which is substantially lower than what is commonly found in hatchery reared fish (Divanach et al., 1996). Fjelldal et al. (2009) found that 45% of intensively reared cod had vertebral deformities, while the prevalence was 6% in wild-caught cod. A number of different skeletal anomalies have been described in hatchery reared fish such as sea bass, sea bream and milkfish, the majority of which are related to spinal malformations (for review, see Divanach et al., 1996). The causes for skeletal anomalies in fish larvae are numerous, and may be related to diet (Cahu et al., 2003a ; Cahu et al., 2003b ; Lall & Lewis-McCrea, 2007 ; Mazurais et al., 2009 ; Zambonino Infante et al., 2009a ; Hansen, 2011), suboptimal physical or chemical rearing conditions such as too high water current (induces lordosis) (Chatain, 1994), or suboptimal light, salinity or temperature conditions (Bolla & Holmefjord, 1988 ; Ottesen & Bolla, 1998 ; Kurokawa et al., 2008).

No published studies are to date available on the skeletal development of ballan wrasse. The

report from Helland et al. (2012) is the only available description of skeletal anomalies in ballan wrasse, but it describes the occurrences of skeletal anomalies in fish of different size and reared under varying densities and temperatures. There is hence a need for a controlled comparative study, both of the skeletal development, and of the occurrence of skeletal anomalies in larvae of the same age.

Skeletal anomalies can be assessed by different techniques to make the bones visible, such as staining bone and/or cartilage in Alizarin red/Alcian blue (fish <1 g), with mammography (fish <100 g) or with radiography (X-ray, fish >100 g) (Bæverfjord et al., 2009b).

1.4 Aim

The aim of this study was to estimate the effects of different live feed quality on growth and functional development of ballan wrasse larvae. As little is known of the dietary requirements of this new species in aquaculture, the feeding regimes ranged from what is believed to be suboptimal (unenriched rotifers) to what is believed to be the natural, most optimal feed (copepods). It was hypothesised that the larvae fed copepods would be of a higher quality than the larvae fed rotifers. The dietary effects were assessed by examining growth, survival, and larval quality. The quality was measured through observations of stress tolerance, activity and feeding behaviour, bone development and occurrence of skeletal anomalies. These are all parameters known to be affected by the components of the diet, and can hence give a good indication of the suitability of the live feed presented to the ballan wrasse larvae.

2. Materials and methods

The experiment and analytical work took place at Norwegian University of Science and Technology (NTNU), Centre of Fisheries and Aquaculture (Sealab), and in SINTEF Fisheries and Aquaculture's laboratories, in Trondheim. The experiment was performed during the months of June to August 2011.

2.1 Larval rearing

Ballan wrasse larvae, 2 days post hatch (dph), were freighted by air in plastic bags, from Marine Harvest Labrus near Bergen, Norway. Upon arrival the oxygen in the plastic bags were measured. The larvae were acclimatised to the temperature in a large holding tank, before gently releasing the larvae into the water. The holding tank was aerated through a central tube, which ensured that the larvae was evenly distributed. The water temperature was 12°C. The density in the holding tank was estimated by removing several samples of known volume, and counting the removed larvae manually. A measuring jug (1L) was used to transfer 8400 larvae per tank, to 12 cone-bottomed tanks (160 L), with 100 L 34 ppt seawater. The aeration was kept very low, and was gradually increased over several days, to a low/ moderate level. The larvae were kept in the dark for the first 48 hours (until first feeding), after which they had constant light (two fluorescent tubes á 18 W $tank^{-1}$). The rearing water was sand filtered, microbially matured, heated, and filtered through a 1µm mesh (Skjermo et al., 1997). Water-flow was checked every day. The oxygen was always kept above 80%, through adjustment of the water-flow. Temperature was measured daily, with Traceable® VWR® Digital Thermometer (VWR International, USA). Oxygen was measured every second day in the live feed period, and every day in the formulated feed period, with ProODO (YSI, USA). White clay (Vingerling K148, Sibelco, Germany) was added from 4-54 dph, every 15 minutes, with a Storvik feeding robot (Storvik Aqua AS, Norway), up to 54 dph. The clay was used as an alternative to green algae (Attramadal et al., 2012). All tanks contained a central sieve, and surface skimmers were used from 4 dph. The water-flow, temperature, amount of clay added, and mesh size of the central sieve was gradually increased (table 2.1). The bottom of the tanks was cleaned every second day from 13 dph until 49 dph, after which it was cleaned every day. Mortality was registered from 13 dph.

2.2 Feeding regimes

Four different feeding regimes were used from 4 to 30 dph, with 3 replicates per treatment (table 2.1). The larvae in the treatment "Copepod" was fed copepod nauplii (*Acartia tonsa,* clone DFH.AT1), from the onset of exogenous feeding (4 dph) to 30 dph, "Cop7" larvae were fed copepod nauplii from 4 dph to 10 dph (7 days), and then enriched rotifers (*Brachionus ibericus* Cayman, lorica length 180 μm) to 30 dph. Due to production problems, the copepod nauplii were of different ages / stages, ranging from naupliar stage 3 to 5 (NIII-NV) (Nesse, 2010), with the smallest nauplii being fed to the larvae in the first 4-9 days, and the Cop7 treatment was co-fed on 8 and 9 dph (table 2.2). The larvae in the "RotMG" treatment received enriched rotifers, whereas "RotChl" larvae were fed unenriched rotifers. Twelve thousand prey organisms L^{-1} were added manually 3 times day^{-1} till 19 dph, after which the larvae were fed 4 times day^{-1}. The density of feed organisms in the fish tanks was counted several times per day, by using an automatic counter (Alver et al., 2007), to ensure that the water still contained feed organisms before next feeding. From 20 dph the feed density in the tank had to be increased for RotMG and Cop7, to 17500 rotifers L^{-1}.

All larvae were weaned onto *Artemia fransiscana* nauplii from 24 dph, and were exclusively fed enriched *Artemia* from 31 dph. The amount added was adapted to the density of remaining feed in each tank, to maintain a stable supply of prey organisms with a density of 3000 L^{-1}. Both a Storvik feeding robot (used at night in the rotifer and *Artemia* period) and manual feeding was used to administer the *Artemia* and later also the formulated feed. Weaning onto formulated feed started on 40 dph, and the larvae were fed exclusively formulated feed from 51 dph (10 g formulated feed per tank, per day). Feeding from the robot was continuous (every 15 minutes). The formulated feed was an experimental feed developed especially for wrasse, by Nofima, Norway (Appendix 1). The feed had two different particle sizes, 300-600 and 600-800 μm. The smallest particle size was used as the main feed (supplied through the robot), the larger particles were manually distributed to the tanks, from 48 dph, as a supplement to the smaller feed. The experiment was terminated at 61 dph.

Table 2.1 *Experimental layout for the start-feeding experiment, with feeding regimes, and regimes for water exchange, clay addition, temperature, mesh size of central sieve, and sampling days.*

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**	**30**	**31**	**32**	**33**	**34-39**	**40**	**41**	**42**	**43**	**44**	**45**	**46**	**47**	**48**	**49**	**50**	**51-60**	**61**
Day degrees	12	24	36	48	60	73	86	99	112	125	138	152	166	180	195	210	225	240	255	270	285	300	316	332	348	364	380	396	412	428	444	460	476	492-572	588	604	620	636	652	668	684	700	716	732	748	764-908	924
Water exchange	2 times day^{-1}														4 times day^{-1}											6 times day^{-1}								8 times day^{-1}													
Temperature (°C)	12						13						14		15											16																							
Clay (g tank^{-1} day^{-1})	0			5			10								13.33				16.7							20			25																		0
Sieve (mesh size)	**200 µm**														**500 µm**																																	**750 µm**
Copepod				**Copepods**																														**Formulated feed (Nofima)**													
3 tanks																								**Artemia (enriched with Multigain)**																								
Cop7				**Copepods**											**Rotifers (enriched with Multigain)**																				**Formulated feed (Nofima)**													
3 tanks																								**Artemia (enriched with Multigain)**																								
RotMG				**Rotifers (enriched with multigain)**																																**Formulated feed (Nofima)**													
3 tanks																								**Artemia (enriched with Multigain)**																								
RotChl				**Rotifers (not enriched)**																																**Formulated feed (Nofima)**													
3 tanks																								**Artemia (enriched with Multigain)**																								
Sampling																																															
CN/Dry weight		x		x				x				x			x						x							x							x							x					x
Mortality														x		x		x		x		x		x		x		x		x				x		x		x		x		x		x	x	x	x
SL / MH				x				x													x							x							x												x	
Bone staining															x						x																											x
Stress																													x					x														
Behaviour																												x						x													x	

Table 2.2 *Feeding regimes for Copepod and Cop7 for the first 4-23 days, with age (days) and stages of copepod nauplii, and the percentage of copepods and rotifers distributed to the larvae per day.*

Cop7 treatment								**Copepod treatment**			
DPH	4-7	8	9	10	11	12-23		4-9	10	11	12-23
Copepod nauplii (%)	100	67	67	100	33	0		100	100	100	100
Rotifers (%)	0	33	33	0	67	100		0	0	0	0
Age of nauplii (days)	3	3	3	3	4	-		3	4	4	5
Stage of nauplii	III-IV					-		III - IV			V (180 µm)

2.3 Live feed production

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

Seawater (34 ppt), sandfiltered, heated to 20 °C and filtered through a 1 µm filter, was kept in two water reservoirs (700 and 1000 litres). Before use the water was chlorinated; 25 ml chlorine 100 L^{-1} seawater in the reservoirs, without aeration for a minimum of 5 hours, and then dechlorinated with sodium thiosulphate pentahydrate (3 g thiosulphate 25 ml^{-1} chlorine) for a minimum of 5 hours, under heavy aeration (Hoff & Snell, 1987).

Rhodomonas baltica (clone NIVA 5/91 Cryptophycea: Pyrenomonadales) was cultivated (Muller-Feuga et al., 2003) in 300 L transparent plastic bags and in 160 and 200 L transparent plastic cylinders, at 20 °C. Each cylinder had heavy aeration from the bottom, with addition of 1-2 % CO_2. The pH was kept between 7.5 and 8.3. There were 3 light sources around each cylinder, with 2x58 W fluorescent tubes in each light source.

The cultures were grown continuously until reaching a density of approximately 1 million algae ml^{-1}, after which 40-50 % of each culture was harvested every day. Water from the reservoirs was used to refill the cultures, and 1 ml of Conwy medium (modified from (Walne, 1974), appendix 2) was added L^{-1} of new seawater.

New cultures were started every 10-14 days. The cylinders were washed, and then filled with filtered seawater, which was chlorinated and dechlorinated while in the cylinder. The plastic bags were changed between every new culture. New cultures were started with 10 or 20 L from intermediate cultures, 150-190 L of seawater and the appropriate amount of Conwy medium. The new culture was spread through splitting the culture in two when the density was high enough.

2.3.2 Cultivation of copepods (*Acartia tonsa*)

Copepods (*A. tonsa*) were grown in two tanks (1000 L and 1600 L), at 20 °C, with 34 ppt seawater (Støttrup et al., 1986 ; Nesse, 2010 ; Eidsvik, 2010 ; Hansen, 2011). All seawater used in the production and storage of copepods and eggs was sand filtered, and filtered through a 1µm mesh. Water-exchange rate was 100% day^{-1}. Oxygen was kept above 60%, by using aeration in the tank coupled with addition of pure oxygen.

The copepods were continuously fed algae; *Rhodomonas baltica* (Støttrup et al., 1986), using one peristaltic pump $tank^{-1}$. The density of algae in the tanks were held at 10-30 000 cells ml^{-1} (Skogstad, 2010), and this was checked by counting samples of the run-out water in a cell counter (Multisizer ™ 3 Coulter counter, Beckman Coulter Inc., USA). A harvesting arm in

the bottom of the tanks was used to collect the eggs, which were siphoned out every day. The siphoned water containing the eggs were filtered through a 120 µm mesh, to remove copepods and shell debris, and then through a 64 µm mesh to retain the eggs. After a thorough wash in seawater the eggs were transferred to NUNC EasyFlask™ Nunclon™ (Nunc A/S, Denmark) cell culture bottles with 75 cm^3 cultivation area. The bottles were filled with chilled (4°C) seawater, 34 ppt, and stored at 2°C. The cultures were run for a maximum of 5 weeks. When the egg-producing culture was 4 weeks old, eggs were hatched to set a new culture one week later. The eggs were incubated in 100 L tanks, for 48 hours, at 20°C. After hatching the nauplii were fed *R. baltica*, 2-3 times per day, to give a density of 10-30 000 cells ml^{-1}. The tanks and equipment was cleaned with 70% ethanol between each culture, and all air tubes and air stones were chlorinated (0.5 ml chlorine L^{-1} seawater). The nauplii were transferred to the large tanks after approximately 7-9 days, when they had reached copepodite stage (determined through visual inspection) and were large enough to be retained by the central sieve.

The eggs harvested from these production cultures were used to produce copepod nauplii, which were used as feed for the fish larvae. The production of eggs started 4-5 months before the first feeding experiment. The hatching percentage and number, of the stored eggs was estimated before the start feeding experiment started (Appendix 3). The copepod eggs were hatched in white, 100 L tanks, for 72 hours. One air stone was used per tank, with very low aeration. Three tanks were used to hatch 1 day's need of nauplii, at a maximum density of 120 nauplii ml^{-1}. After 72 hours, the nauplii were either fed to the fish larvae or transferred to 250 L production tanks, where they were held until they where 96 or 120 hours old, giving them a total age of 3-5 days (table 2.2). At this age, the nauplii will be in nauplii stage 3-5, and the stage 5 nauplii will be of a size ≈ 180 µm (Nesse, 2010), which is a size comparable to that of *Brachionus ibericus* (Ciros-Pérez et al., 2001). During on-growing the nauplii were fed *R. baltica*, *ad libitum*. The nauplii were concentrated before transfer, by removing water through a 64 µm sieve, and the density was estimated after transfer to the reservoirs (Appendix 3).

2.3.3 Cultivation of Rotifers (*Brachionus ibericus*)

Rotifers of the species *Brachionus ibericus* (Cayman) were cultured semi-continously in 270 L glass fibre conical tanks, in 34 ppt seawater. The temperature was 19-25 °C, water exchange rate was 100-150% day^{-1}, and oxygen was held above 80%. The filter in the tanks, and the feed containers were washed every day. The cultures were washed and transferred to a new, clean production tanks once a week. The rotifer cultures were counted manually every day, and density and egg ratio were calculated. The cultures were diluted 25-40% when the density exceeded 750 individuals mL^{-1}. A paste of the green algae *Chlorella* sp. was fed to the rotifers (2.5 ml paste $million^{-1}$ rotifers day^{-1}). The paste was thinned to 10 L by adding filtered (1 μm) seawater, in 10 L plastic buckets. One peristaltic pump per reservoir was used to distribute the feed.

The estimated number of rotifers needed for 24 hours were thoroughly rinsed, transferred to a new reservoir, and enriched with heavy aeration for 2 hours, with Multigain ((BioMar AS, Norway), 0.15 g $million^{-1}$ rotifers) in water heated to 25 °C. The density at enrichment was kept below 400 rotifers mL^{-1}. After enrichment the rotifers were rinsed again, and either manually fed to the larvae immediately, or stored at 8 °C with aeration until feeding (maximum 18 hours). The rotifers which were to be distributed to the larvae during the night, was added to the feeding robot in the evening, after being stored for 6 hours at 8 °C. The feeding robot was positioned in the rearing room, where the temperature was 16 °C.

2.3.4 Cultivation of *Artemia fransiscana*

Artemia fransiscana cysts (EG® INVE Aquaculture, Belgium) were de-capsulated according to (Sorgeloos et al., 1977). Hatching occurred in 60 L conical bottomed cylindrical tanks, in 34 ppt seawater, with aeration. The *Artemia* were rinsed in seawater after hatching, and enriched in 60 L tanks with heavy aeration. The enrichment took place over 24 hours, with 10 g Multigain (BioMar AS, Norway) per 60 L seawater, being supplied twice. Temperature was held at 25-28 °C, and density kept below 300 nauplii ml^{-1}. The *Artemia* was concentrated in a sieve and gently washed after enrichment. The *Artemia* was either manually fed to the larvae immediately after enrichment, or stored at 8°C with aeration. Maximum storage time was 18 hours. The *Artemia* was distributed manually during the 3 daily feeding, and the robot was used to administer the *Artemia* during the nightly feeding. The *Artemia* which were to be

distributed to the larvae during the night, was added to a reservoir connected to the feeding robot, in the evening, after being stored for 6 hours at 8°C. The reservoir was positioned in the rearing room, where the temperature was 16 °C.

2.5 Sampling and analysis

2.5.1 Growth

Dry weight (DW) and daily weight increase (%DWI)

The larvae were sampled for carbon and nitrogen (CN) and dry weight, the sampling days are presented in table 2.1. Twelve (2 and 4 dph), 36 (8, 12, 15, 21 dph), and 72 (27 dph) larvae treatment^{-1} was sampled, and analysed for CN, using an elemental combustion analyser (Costech instruments, USA) with acetanilide as standard. Larvae were sampled from the same tank on 2 and 4 dph (no difference in treatment, as feeding had not yet started). These analysis were performed by Marthe Schei, SINTEF.

To estimate DW, µg carbon larva^{-1} obtained from the CN-analysis was converted to µg dry weight larvae^{-1}, using the following formula obtained from Reitan et al. (1993):

DW= (µg carbon larva-1)*2.34

For measurements of dry weight, 90 (33, 40 and 47 dph) and 150 (61 dph) larvae treatment^{-1} were transferred individually to pre-weighed tin capsules and dried at 60 °C for a minimum of 48 hours, before being weighed on a microbalance (Mettler Toledo microgram balance UMX2, max 2.1 g, *d*=0.1, Switzerland). The daily weight increase (%DWI) for each dry weight-sampling interval was calculated according to (Ricker, 1958):

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

where the growth coefficient *g* is:

$g = (\ln W_2 - \ln W_1) / t_2 - t_1$

where W_2 and W_1 is the weight at time t_2 and t_1, respectively.

Standard length and myotome height

The standard length (SL) and myotome height (MH) was measured on fixated larvae from day 4, 8, 21, 28, 39 and 58. Larvae from 4, 8 and 21 dph were measured from pictures, using the software CAST 2 (Olympus Inc. Denmark). All larvae that had been subject to behavioural tests (28, 39, 58 dph) were measured using a stereomicroscope (Leica M80) with a calibrated ocular micrometer. SL was also measured on all bone stained larvae (see section 2.8 for bone staining procedure). Fixation followed by staining does not alter the size of the larvae, when compared to only fixated specimens (Marit Hansen, pers. comm.).

The standard length was measured from the tip of the mouth to the end of the notochord on larvae where the base of the tail fin was not visible, and to the base of the tail fin on larger larvae. Myotome height was measured perpendicular to the axial skeleton, directly behind the anus (figure 2.1).

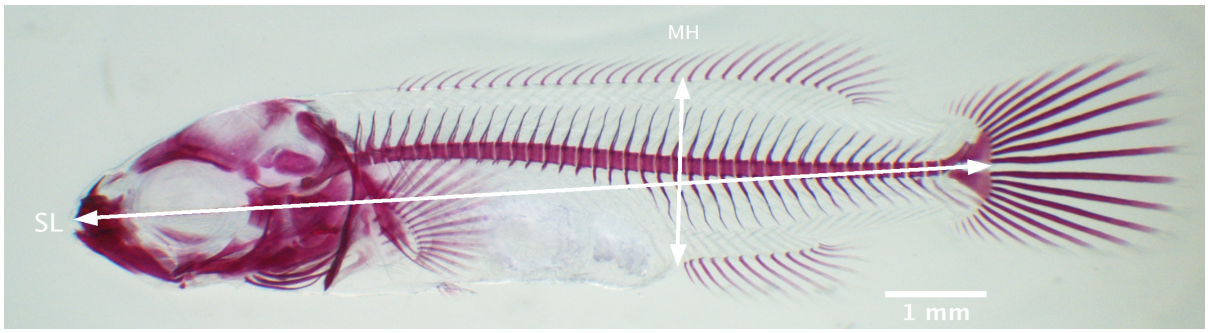

Figure 2.1. *The image shows how SL and MH was measured, on a 33 dph-larva. The larva is stained with Alizarin red S.*

2.5.2 Survival

Survival was calculated based on initial and final number of larvae in each tank, and on regular counting of dead larvae from 13-61 dph. The data was corrected for sampled larvae, and for live larvae accidentally removed during cleaning of the tanks.

2.6. Tolerance to handling stress

The tolerance to handling stress was assessed by a standardised method for netting followed by air-exposure, according to (Hansen, 2011) and Kjørsvik, E. (unpublished). 15 larvae from each tank were sampled with a ladle, and poured into a sieve (a plastic cylinder with a mesh bottom), lying in a bowl with water. The sieve was lifted up from the bowl, and put on a stack of absorbing paper for 15 seconds, to remove most of the water. After 15 seconds it was held in the air for a specific amount of time. The larvae were then transferred from the sieve into a 3L aquarium, having the same water, light condition and temperature as the larvae were accustomed to. No feed was added. Dead fish were removed, counted and fixated 1 and 24 hours after air-exposure, and any remaining live larvae were fixated after 24 hours. SL and MH was measured on all larvae, after fixation in 4% formaldehyde.

The exposure time suitable for the larvae's tolerance limit was found through preliminary tests on larvae from the RotMG treatment. This treatment was chosen as the reference treatment, because it was believed that the larvae would have a tolerance limit between that of the larvae fed copepods and the larvae fed unenriched rotifers. Forty seconds, of which the sieve was held 15 seconds on paper and 25 seconds up in the air, was found to be a suitable exposure time at 29 dph.

A series of preliminary tests was also performed on larvae from the RotMG treatment on 36 dph, to estimate a suitable exposure time for a second stress test (Appendix 7). The larvae were shown to tolerate 4 minutes of air-exposure (zero mortality after 24 hours), and further testing was stopped. Air-exposure was deemed as an unsuitable method to assess the larvae's tolerance to handling stress on 36 dph.

2.7 Larval activity and feeding behaviour

The difference in larval quality was assessed through observations of the larval swimming and feeding behaviour. A focal animal technique was used, where a randomly selected larvae were observed for 2 minutes, and different aspects of the behaviour were recorded (Altmann, 1974). The parameters observed was based on the parameters "Swim" and "Capture" used for cod larvae in (Puvanendran & Brown, 1999, table 1), and "Wriggle" (vrikking) in (Lervik, 2007). The parameter "burst" was chosen after preliminary observations of the larval behaviour. The parameters are described below.

Swimming duration: The time the larvae was swimming. Only the energy demanding swimming, here defined as net movement in the water column caused by the pectoral fins and/or the tail fin was recorded, with a stopwatch.

Prey capture: Successful attacks on prey was counted. Any attack that ended with an *Artemia* entering a larva's mouth was considered a successful attack, also if the *Artemia* was spit out afterwards.

Wriggling*:* A distinct twitching of the body, where the larva twists the body quickly to one side, then straitens (sometimes repeatedly). The number of wriggles were counted.

Burst swimming*:* A larva was said to "burst" when it abruptly started to swim very fast, as opposed to the more common cruising swimming behaviour. The number of burst was counted. The time spent burst-swimming was not measured separately, but was included as swimming-time.

3L transparent plastic aquariums were used for the observations. The aquariums were placed on a white surface, against a white background. The light source (Osram Dulux S, 11 Watt/41, Italy) was positioned so that the light came in at an oblique angle, from above. Fifteen larvae were sampled from each tank, one treatment at a time. They were transferred into separate aquariums (one aquarium treatment^{-1}), and 10 random larvae was observed (30 larvae treatment^{-1}). The water (3L) and the temperature was the same as in the fish tanks. The fish was starved for 3 hours, after which *Artemia fransiscana* nauplii (3000 L^{-1}) was added. Observation of the larvae started 1 minute after feed addition, and lasted for 2 minutes per larva. The larva was removed from the tank immediately after observation, using a wide-

tipped glass pipette, and fixated in 4% formaldehyde. The MH and SL of all observed larva was measured on the fixated samples.

Trial observations of the larval behaviour was also conducted on 39 and 58 dph, but the observation method used at 28 dph was found to be unsuitable at 39 and 58 dph.

2.8 Analysis of ossification and skeletal anomalies

The sampling days are presented in table 2.1. A ladle (or a landing net, from 40 dph) was used to sample larvae randomly from each tank. The larvae were anaesthetised with tricaine methane sulfonate (MS-222, Finquel®, Argent Chemical Laboratories Inc., USA) and rinsed in fresh water, before being fixated in 4% formaldehyde in phosphate buffered saline (pH 7.4, Apotekproduksjon AS, Norway). The samples were stored at 4°C for further analyses.

For analysis of skeletal ossification and skeletal anomalies, 10-15 larvae were sampled from each tank (30-45 per treatment). Only larvae from the Copepod, RotMG and RotChl treatment was analysed. The larvae were stained with alizarin red, adapted from (Kjørsvik et al., 2009) (for detailed procedure see appendix 4). Bone stained larvae were photographed while submerged in 40% glycerol, under a stereomicroscope (Leica MZ7.5, Germany) equipped with a camera (Nikon Digital Sight DS-5M L1, Japan). Analyses of the larvae were done from the pictures and by using a stereomicroscope (Leica M80, Germany). The program Preview for Mac was used to view the images. The software ImageJ v1.45 for Mac OS X, calibrated for 0.63, 1.0, 1.6 and 2.0x was used to measure SL. The larva were studied with emphasis on development of the vertebrae, fin rays and squamation.

2.8.1 Vertebrae

The vertebral bones were classified as fully ossified, compact or transparent, according to (Eidsvik, 2010), based on the extent and saturation of colour. The mean total number of vertebral segments was calculated.

2.8.2 Dorsal fin

Instead of counting the ossified fin rays (not suitable for this species), a method of scoring the ossification of the dorsal fin was developed. The fin was graded from 0 to 3: A score of 0 equalled no ossification, 1= ossification of only the posterior part of the dorsal fin rays, 2= ossification of the anterior and the posterior part of the dorsal fin rays, but not of the

pterygiophores, 3= ossification of entire dorsal fin ray, including the pterygiophores (figure 2.2).

2.8.3 Squamation

The squamation development was assessed by scoring the height of the ossified scale layer with the larva's body height. The scores ranged from 0 to 3; 0= no visible ossification of scales, 1= beginning ossification only along the midline, 2= obvious ossification between the midline and the base of the pterygiophores, 3= ossification of scales dorsal to the base of the pterygiophores (figure 2.3). Squamation was only analysed on the flank.

2.8.4 Ossification of other regions

The preamaxillare, maxillare, dentale and hyoid arch (terminology from Harder (1975)) were collectively referred to as "mouthparts", and characterised as ossified or not, together with the cleithrum, neural and hemal arches, tail fin rays, hypurals, and the urostyle. Any signs of beginning ossification (bone being stained) in any bones of these parts qualified as ossified. The notochord was characterized as having no flexion or flexion. The nomenclature used in to describe the tail region is presented in figure 2.4.

The size intervals (measured as SL) for the ossification of the above-mentioned parts (and for the presence of flexion) was found, to indicate the size above which it was expected that the bone or body part would be ossified. The smallest number in the interval was the size of the smallest larvae observed with the respective part ossified, and the largest number represented the size of the largest larvae that did *not* have this part ossified. A table was constructed to visualize the ossification of the different bones related to larval size, adapted from Sæle et al. (2004).

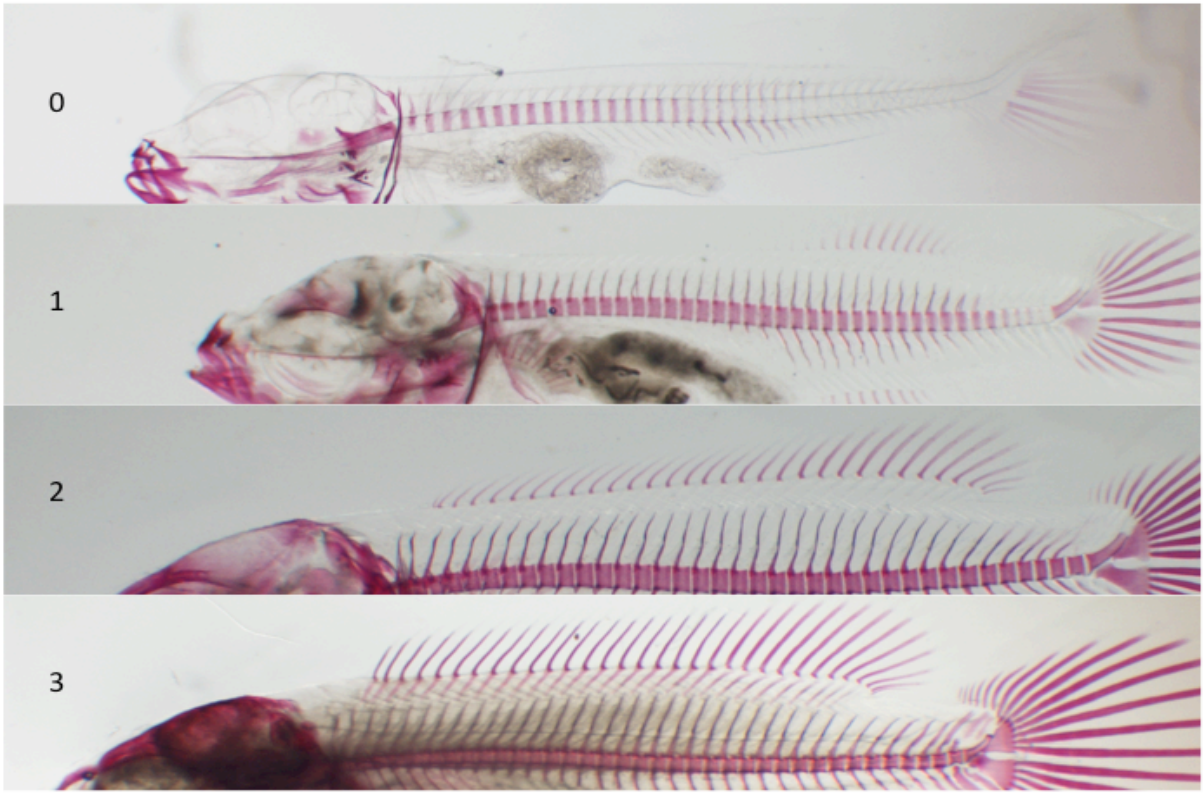

Figure 2.2. *The fin rays were given a score of 0-3, based on the degree of ossification of the dorsal fin rays. The scores are indicated on the left hand side of each image. 0= no ossification, 1= ossification of only the posterior part of the dorsal fin rays, 2= ossification of the anterior and the posterior part of the dorsal fin rays, but not of the pterygiophores, 3= ossification of entire dorsal fin ray, including the pterygiophores.*

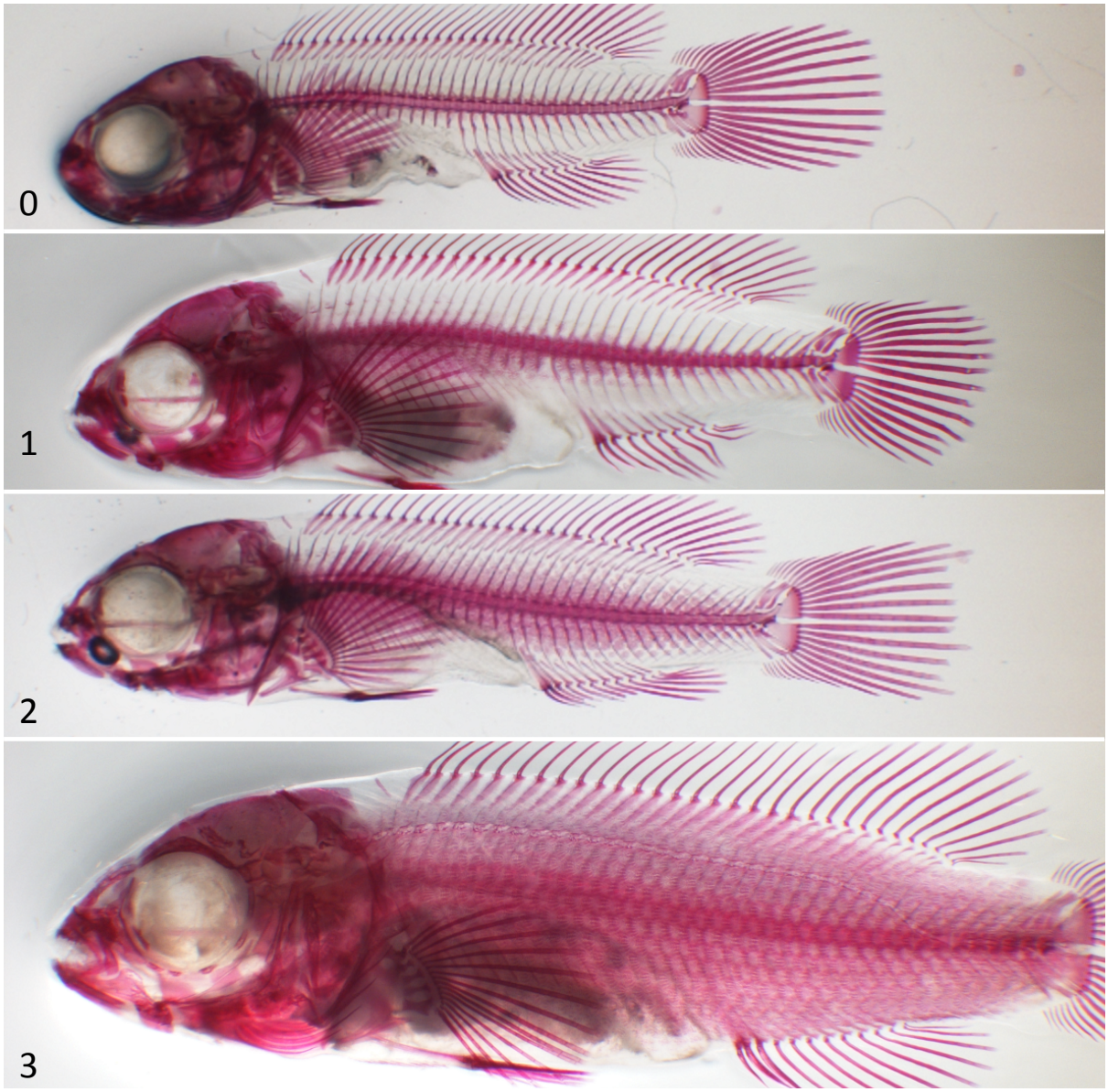

Figure 2.3. *The amount of scales on individual larva is scored, based on the height of the ossified scale layer with the larva's midline and pterygiophores. The scales can be seen as a pink layer, note that this layer is not present in the image marked with "0." The different scores are written in the lower left corner of each of the four images. A score of 0= no visible ossification of scales, 1= beginning ossification only along the midline, 2= obvious ossification between the midline and the base of the pterygiophores, 3= ossification of scales dorsal to the base of the pterygiophores. The images are of the same magnification (0.63x), larval size is comparable.*

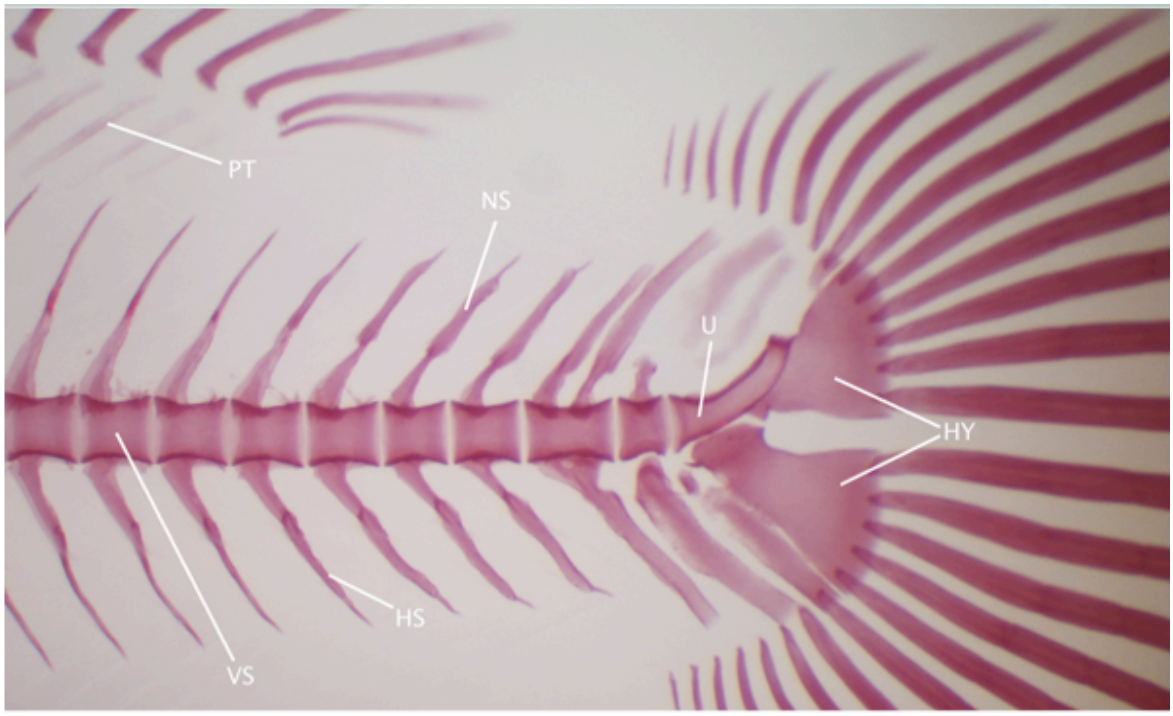

Figure 2.4. *Ossification in the tail region. Nomenclature: Pterygiophores (PT), vertebral segment (VS), neural spine (NS), hemal spine (HS) (the neural arch and hemal arch are positioned medial to the respective spine), urostyle (U) and hypurals (HY). Note skeletal anomaly in the second-to-last vertebral segment, which appears to be longer than the adjoining segments and has two neural spines.*

2.8.5 Skeletal anomalies

All bone stained larvae were checked for skeletal anomalies, using images combined with a stereomicroscope (Leica M80). Calculations were made of the percentage occurrence of the types of anomalies in larvae from the different treatments at 21, 33, 40 and 61 dph, as well as mean percentage of larvae with anomalies and mean number of anomalies per larvae, at 61 dph. An anomaly (also called deformity) is here defined as a "major difference in the shape of body part or organ compared to the average shape of that part" (Bæverfjord et al., 2009a).

The following terms were used to describe the anomalies that was assessed, adapted from Boglione et al. (2001), and Bæverfjord et al. (2009b).

Abnormal arches: Arches / spines of the vertebral column are bent or otherwise abnormal in shape (but never twisted, see next point).

Twisted arches: Arches / spines are abnormal, forming a spiral at the apex (Synnøve Helland and Elin Kjørsvik, pers. comm.).

Fused vertebrae/arches: Collectively refers to fusion between two or more vertebral segments, and when two vertebral segments share the same neural and/or hemal arch

Deformed jaw: The lower jaw (dentale) is shortened and deformed.

Axis deviations: Weak lordosis, kyphosis or scoliosis.

2.9 Statistical analysis

All statistical analysis, and all graphs were made in IBM SPSS Statistics v.19.0 (SPSS Inc., Chicago, USA) for Mac.

All data was checked for outliers, and extreme values were removed. Data was tested for normality with a Shapiro-Wilk´s test. Percentage data was arcsine transformed before statistics were run.

For normal distributed data, groups were compared using a one-way ANOVA (analysis of variance). Student-Newman-Keuls Post Hoc test was used for data that was homogenous, while a Dunnett T3 Post hoc test was used for non-homogenous data.

Non-normal distributed data was analysed with non-parametric tests. For comparison of two groups, an Independent samples Mann-Whitney U test (2-sided) was applied. For comparison of more than 2 groups, an Independent samples Kruskal-Wallis test (2-sided, asymptotic significances) with inbuilt pairwise comparison and adjustment of the significance value, was applied.

Correlation of activity to SL, and activity to treatment was analysed with a Spearman correlation test, and the correlation coefficient is denoted by r_s in the results. The coefficient of determination, r_s^2, are interpreted as the proportion of variance in *ranks* that two variables share (Field, 2009).

Correlation of stress-tolerance or ossification to SL and MH was analysed with a Pearson correlation test, to find the point-biserial correlation coefficient (r_{pb}) (larvae were characterised as dead or alive / ossified or not ossified). The coefficient of determination, R^2 or r_{pb}^2, are interpreted as the amount of variability that two variables share.

A significance level of p=0.05 was used for all tests. Figures and tables usually presents mean values ± SE.

3. Results

General observations

The larvae fed copepods appeared to have more yellow pigments in the skin compared to the larvae fed rotifers. They were also quicker to react to stimuli than the other larvae, being considerably more challenging to catch when taking samples from the tanks. The RotChl larvae were generally smaller, slower and easier to catch. This trend was notable throughout the duration of the experiment.

One tank from the RotChl was emptied on 40 dph, because there were no larvae left. Larvae from one tank in the Copepod treatment showed very poor growth and survival throughout the experiment, when compared to the other tanks form the Copepod treatment. Late in the experiment the larvae also deviated considerably from the larvae in the other treatments, and had the lowest growth observed in any of the tanks at 61 dph. The results from this tank were omitted from the mean values presented in growth and survival (included in appendix). The tank was, however, included in the results of the behaviour observation, stress test, and the ossification and skeletal anomalies data, as the larvae did not differ from the larvae in the other two Copepod-tanks when regarding these parameters. Due to the high mortality in this tank, there was not enough larvae left at the end of the experiment to use for analysis of skeletal development and anomalies, hence only two Copepod-tanks are included in these results at 61 dph.

3.1 Growth

3.1.1 Dry weight and daily weight increase

The Copepod and Cop7 larval DW was significantly higher than RotMG and RotChl on 8 and 12 dph, and the Copepod larvae had significantly higher DW than all the other treatments on 15-33 dph (figure 3.1 and 3.2, appendix 5). The Cop7 larvae exhibited a decrease in growth rate from 12 to 21 dph, compared to the larvae from the other treatments. The growth of the RotMG and RotChl larvae were similar from 8 to 61 dph. On 40 and 47 dph there were no differences between the larvae in any of the treatments.

At the end of the experiment, at 61 dph, Copepod and Cop7 larval dry weight (DW) was significantly higher than RotChl and RotMG larval DW (figure 3.2). The mean dry weight at

61 dph was 19.6±1.1, 17.1±0.9, 14.0±0.7 and 13.0±0.7 for the Copepod, Cop7, RotMG and RotChl treatment respectively.

The Copepod larvae had the highest %DWI on all sampling days. Significant differences were found between 4-12 and 12-27 dph (figure 3.3, appendix 6). The Copepod and Cop7 larvae had a significantly higher %DWI compared to the larvae from the other treatments on 4-12 dph, and the Copepod larvae had a significantly higher %DWI than all the other treatments on 12-27 dph. The differences between the treatments were especially large from 4-12 dph; the %DWI was 19.3±1.6, 17.9±1.4, 8.5±0.9, and 9.7±0.3 for the Copepod, Cop7, RotMG and RotChl larvae, respectively. The lowest %DWI (ranging from 2.4 % in RotChl to 5.5 % in Copepod) was seen in the interval 47-61 dph. This was the last period of weaning onto formulated feed, and when exclusive feeding of formulated feed occurred. The mean %DWI for 2-61 dph was similar between the treatments; 10.9±0.0, 10.6±0.3, 10.2±0.2, and 10.1±0.3 for the Copepod, Cop7, RotMG, and RotChl larvae respectively. The mean value for all treatments combined was 10.42± 0.1, from 2-61 dph.

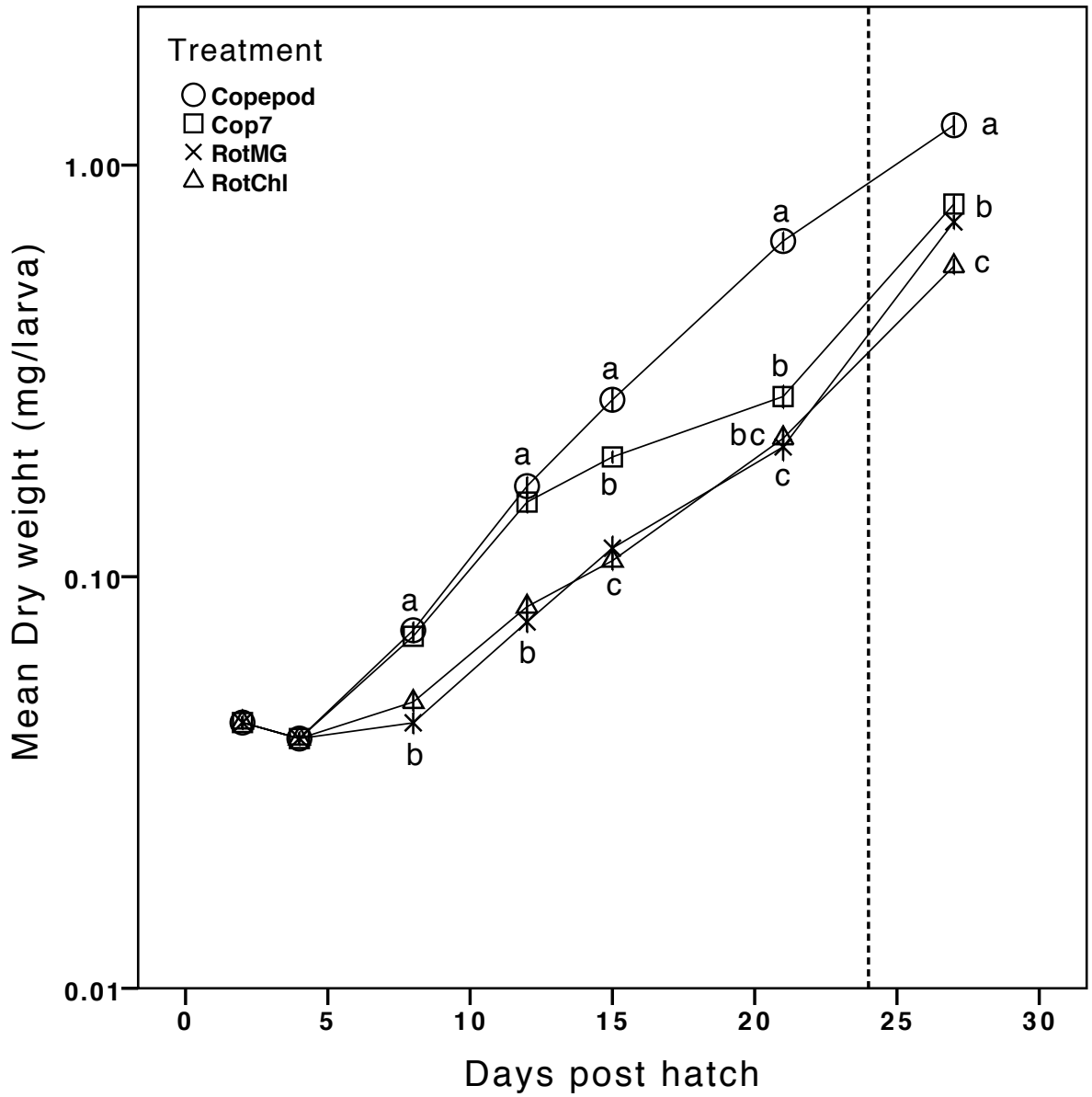

Figure 3.1. *Mean larval dry weight (mg larva^{-1}) from 2-27 dph. n= 12-72 treatment^{-1}. Logarithmic Y-axis. The dotted line on 24 dph marks the transition from only feeding copepod nauplii/rotifers to co-feeding with Artemia. Significant differences between the treatments are indicated by different letters. Error bars indicates ± 1 standard error.*

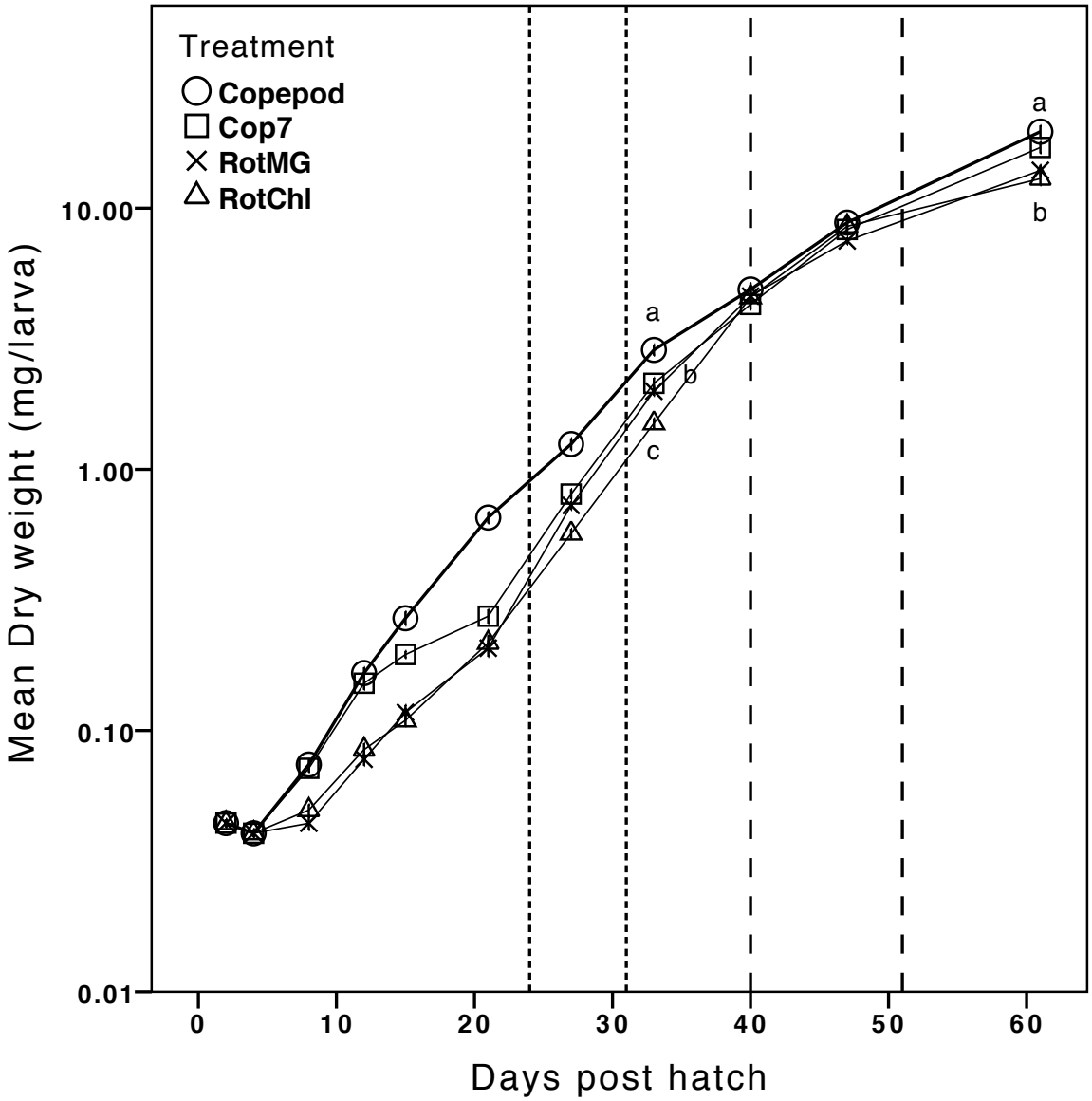

Figure 3.2. *Mean larval dry weight (mg larva^{-1}) from 2-61 dph. n=12-150 treatment^{-1}. Logarithmic Y-axis. The significant differences at 33 and 61 dph are indicated by different letters. No significant differences were found on day 40 and 47. The small dashed lines on 24 and 31 dph is the start and end of co-feeding with rotifers/copepods and Artemia. Between 31-40 dph the larvae was fed only Artemia. The long dashed lines, on 40 and 51 dph, is the start and end of co-feeding with Artemia and formulated feed, before feeding exclusively with formulated feed from 51 dph. Error bars indicates ± 1 standard error.*

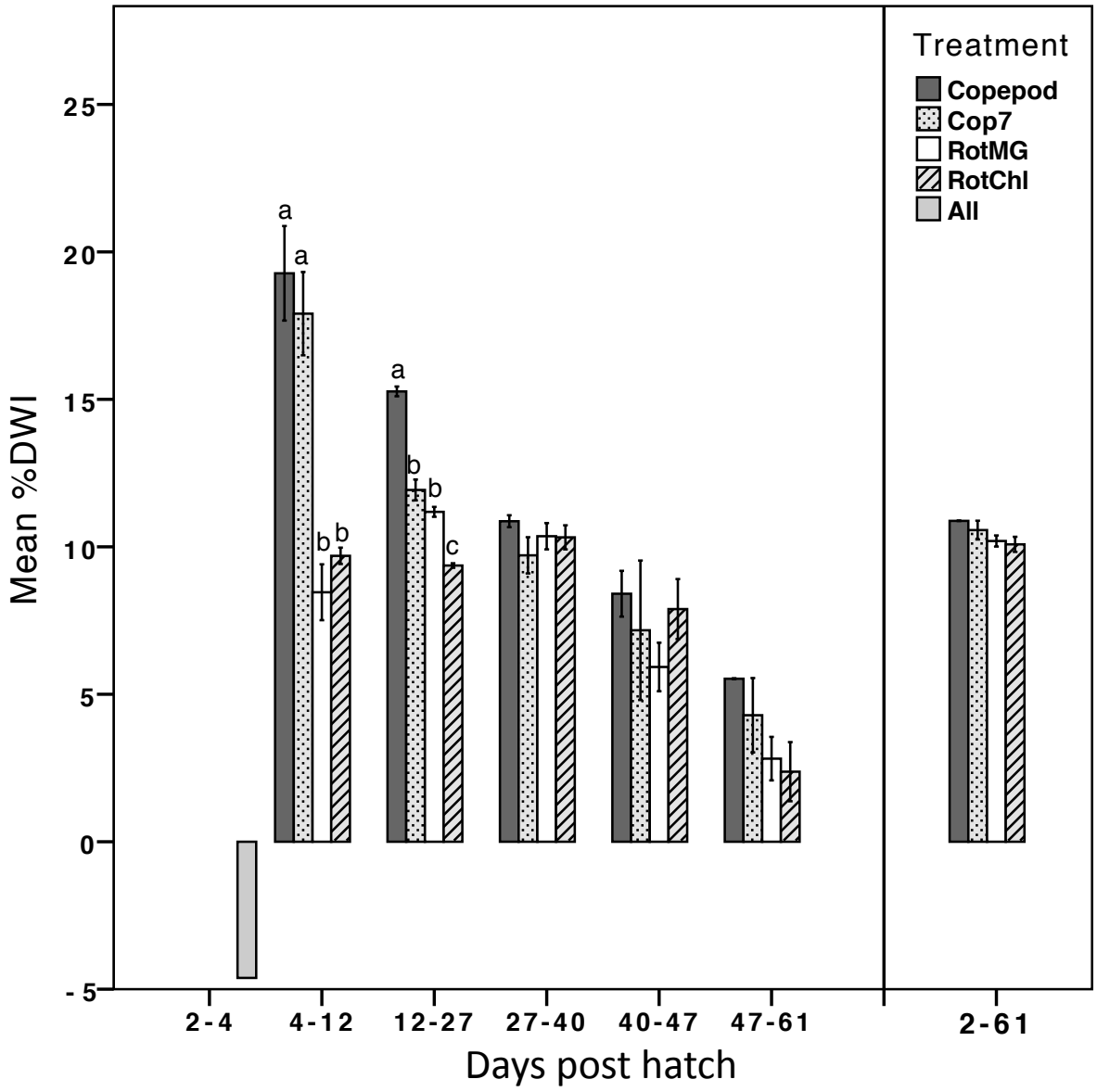

Figure 3.3. *Larval daily weight increase (%DWI) from 2-61 dph. The data are presented in intervals. The DWI-intervals can be said to represents different feeding periods; 2-4 dph is before the larvae had started exogenous feeding, all larvae was sampled from one tank. 4-12 dph is the period where both the Copepod and cop7 treatments are fed copepod nauplii while the RotMG and RotChl treatments are fed rotifers (enriched and unenriched, respectively). From 12-27 dph, all but the Copepod treatment are fed rotifers, and from 27-40 dph weaning onto, and exclusive feeding of Artemia occurs. The treatments are weaned onto formulated feed from 40-47 dph, and are fed mainly formulated feed from 47-61 dph. The interval 2-61 dph represents the %DWI during the entire start feeding experiment. Significant differences on 4-12 and 12-27 dph are indicated by different letters. Error bars indicate ± 1 standard error.*

3.1.2 Standard length and myotome height

The mean SL of Copepod larvae was significantly higher than that of the RotMG larvae on 8 dph, and higher than all other treatments on 21 and 28 dph (figure 3.4). Copepod larvae had a significantly higher SL than RotChl larvae on 39 dph, but no differences was found between Copepod larvae and the larvae of the other treatments on that day. There was no significant difference in mean SL at the end of the experiment, which ranged from 13.1 mm in RotChl, to 13.8 mm in Cop7.

The myotome height followed the same trend as the SL, with larvae from the Copepod and Cop 7 treatment having significantly higher MH than RotMG and RotChl larvae on 8 dph, and the Copepod larvae had significantly higher MH than all other treatments on 21 and 28 dph (figure 3.5). There was no significant difference in mean MH at the end of the experiment, which ranged from 2.7 mm in Copepod to 3.0 mm in RotChl.

The standard length was correlated to the myotome height for all treatments, with a Pearson correlation coefficient of .978, .970, .985 and .976 for the Copepod, Cop7, RotMG and RotChl treatment respectively (Pearson correlation, $p<.01$, 2-tailed).

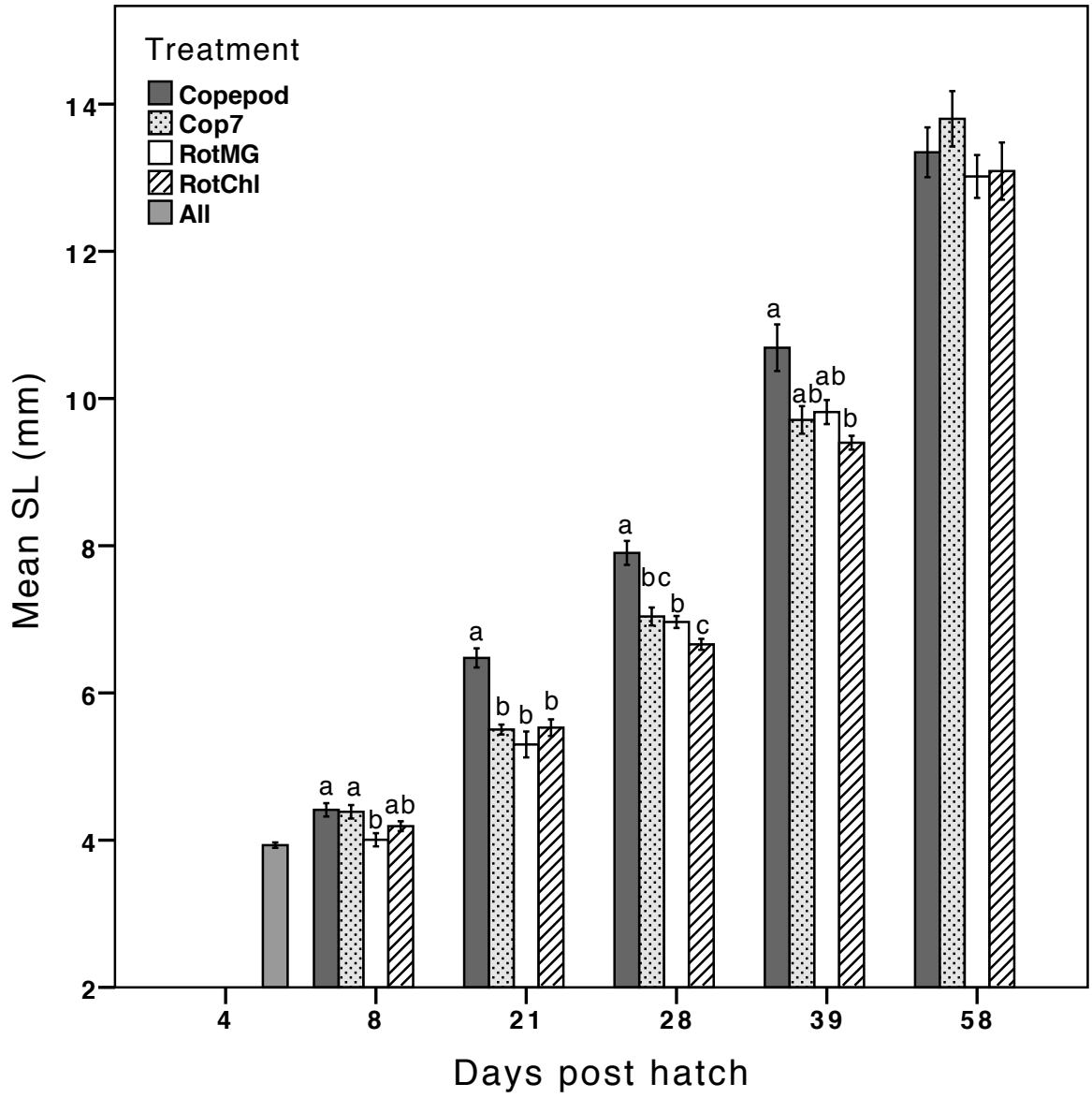

Figure 3.4. *Mean standard length (mm) at 4, 8, 21, 28, 39 and 58 days post hatch. n=12-30 treatment^{-1} on 4-39 dph. On 58 dph, n= 30 for Cop7, and RotMG, and 20 for Copepod and RotChl. Significant differences between the treatments on 8-39 dph are marked by different letters. Larvae were sampled from the same holding tank on 4 dph (exogenous feeding had not yet started). Error bars indicate ± 1 standard error.*

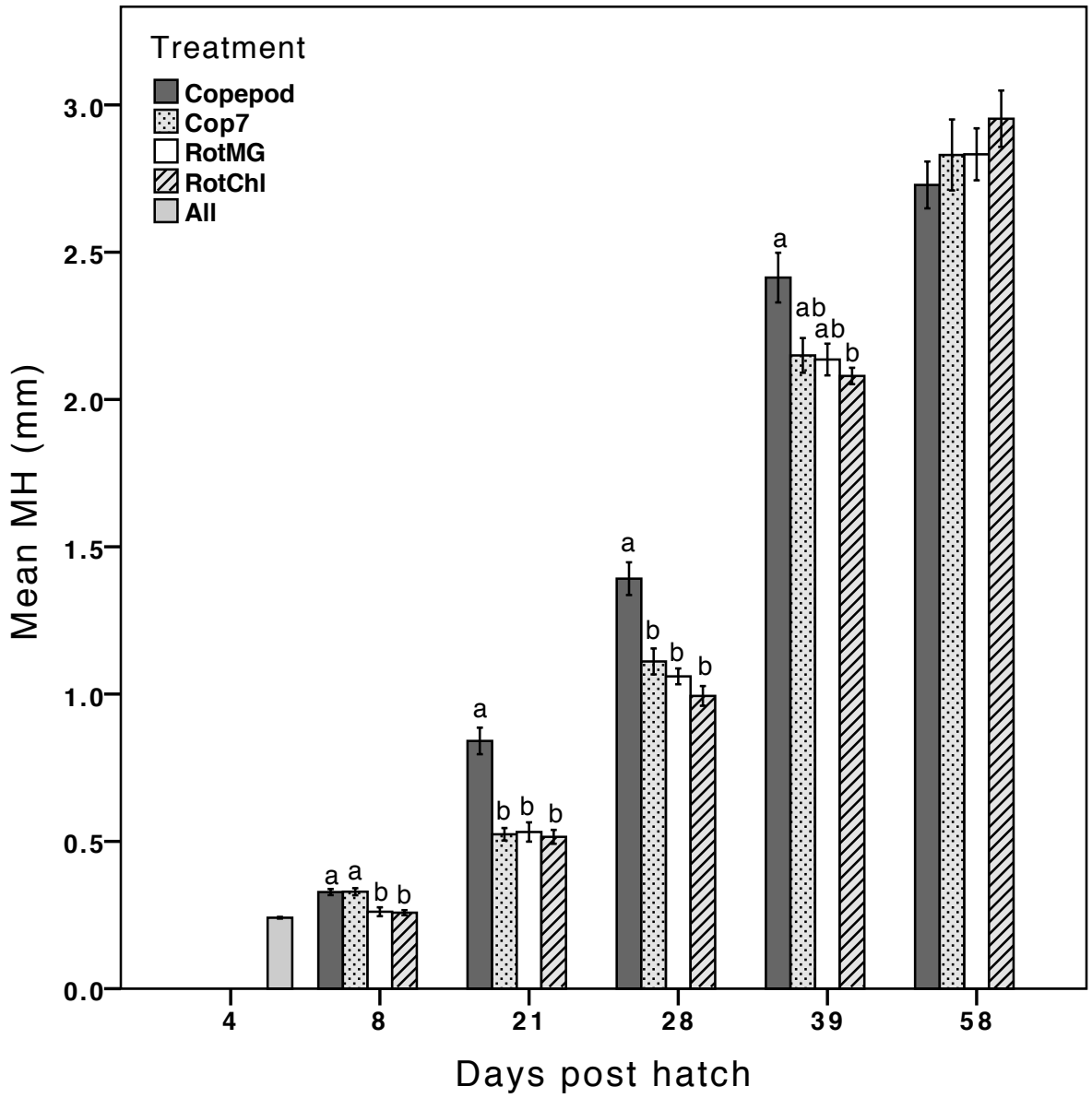

Figure 3.5. *Mean myotome height (mm) at 4, 8, 21, 28, 39 and 58 days post hatch. n=12-30 treatment^{-1} for 4-39 dph. On 28-58 dph, n= 30 for Cop7, and RotMG, and 20 for Copepod and RotChl. Significant differences between the treatments on 8-39 dph are marked by different letters. The larvae was sampled from the same holding tank on day 4 (exogenous feeding had not yet started). Error bars indicate ± 1 standard error.*

3.2 Survival

Most of the mortality occurred before counting of dead larvae started (before 13 dph). Larvae from the Cop7 treatment had significantly higher survival than the RotChl treatment from 24 dph (table 3.1). The survival at 61 dph ranged from 5% in RotChl to 12% in Cop7, and was significantly lower in the RotChl treatment compared to the other treatments.

Table 3.1 *Mean survival (percentage) ± SE per treatment, on a selection of days from 13-61 dph. n=3 tanks treatment-^{1} except for the Copepod treatment, where n=2. Significant differences are indicated by different letters.*

Dph	Copepod	Cop7	RotMG	RotChl
13	16 ± 2.6	24 ± 5.3	17 ± 2.7	12 ± 0.9
24	14 ± 1.5^{ab}	20 ± 4.1^{a}	13 ± 1.6^{ab}	7 ± 1.0^{b}
31	14 ± 1.6^{ab}	18 ± 3.8^{a}	12 ± 1.4^{ab}	6 ± 0.9^{b}
40	13 ± 1.4^{a}	16 ± 3.2^{a}	11 ± 1.5^{ab}	5 ± 0.6^{b}
51	13 ± 1.3^{a}	15 ± 2.8^{a}	10 ± 1.5^{ab}	5 ± 0.6^{b}
61	11 ± 0.5^{a}	12 ± 1.4^{a}	10 ± 1.3^{a}	5 ± 0.6^{b}

3.3 Tolerance to handling stress

The larvae's tolerance to stress was tested on 29 dph (fig 3.6, appendix 7). There was no difference between treatments in mean mortality 1 hour after stress. The Cop treatment had a significantly lower mortality than the RotChl treatment after 24 hours. The mortality after 24 hours ranged from 0% in the Copepod tanks to 25.7 % in the RotChl tanks. There were large differences between the mortality of the larvae from the different tanks, especially in the RotMG and RotChl larvae. The mortality after 24 hours was significantly correlated to treatment (r_s^2 = .74, p<.01), with mortality increasing with lower feed quality, a trend which can be seen in figure 3.6. The Copepod larvae exposed to handling stress had a significantly higher SL and MH than the larvae from the other treatments. The mean SL was 7.5±0.2, 6.8±0.1, 6.8±0.1 and 6.4±0.1 mm for the Copepod, Cop7, RotMG and RotChl treatment, respectively. Whether a larva survived the stress or not was significantly correlated to the larva's SL (r_{pb} = .39, p < .01), and variance in size accounted for 15.2 % of the variance in mortality (r_{pb}^2= .152). The survival after stress was not correlated to MH.

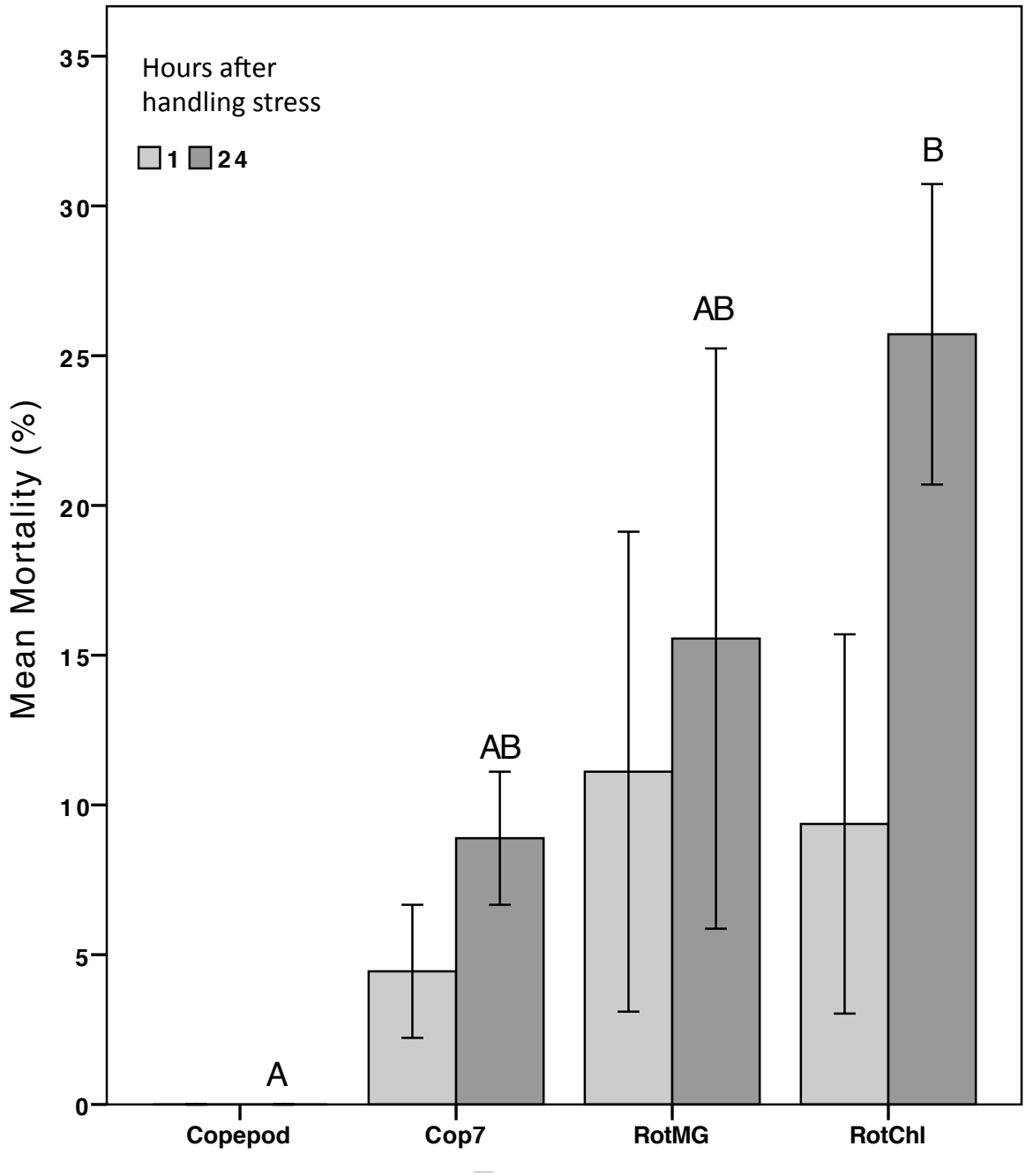

Figure 3.6. *Mean mortality (percentage) 1 and 24 hours after handling stress (netting for 40 seconds), at 29 dph. n=3. Significant differences between the treatments are indicated by different letters. Error bars indicate ± 1 standard error.*

3.4 Larval activity and feeding behaviour

There were significant differences in larval activity between the treatments on 28 dph, presented as mean activity per minute in figure 3.7 (appendix 8). The larvae from the Copepod treatment swam significantly less than the RotMG and RotChl larvae, and the Cop7 larvae swam significantly less than the RotMG larvae. Both the Copepod and Cop7 larvae caught significantly more prey than the RotMG and RotChl larvae. The mean swimming duration per minute was 0.29±0.05, 0.40±0.05, 0.69±0.05 and 0.59±0.03 minutes for the Copepod, Cop7, RotMG and RotChl larvae respectively. The mean number of prey caught per minute was 1±0.14, 0.96±0.16, 0.25±0.08 and 0.57±0.07 for the Copepod, Cop7, RotMG and RotChl larvae respectively.

The larvae swam by beating the tail fin rapidly, and moving the pectoral fins. The tail fin was un-pigmented (transparent) at 28 dph. A change in the swimming pattern was apparent from 28 dph to 39 dph; from swimming mostly with the tail fin and using the pectoral fins as support, the pectoral fins became the main tool of locomotion. Rather than oscillate, the tail fin was often coiled, so the larva's body became U-shaped seen from above. The larva was often observed swimming by means of the pectoral fins, while simultaneously coiling its tail. This coiling of the body was commonly seen in the larvae and juveniles in the rearing tanks, especially when they were resting on the bottom. Generally, the swimming pattern varied from lying still on the bottom for long periods of time, to slowly gliding through the water or darting off in quick bursts. The activity level of different larvae from the same treatment was highly variable, some larvae lay still on the bottom for nearly the entire duration of the observation, while other larvae swam constantly (appendix 8).

The duration of swimming was significantly correlated to both treatment and SL (R_s^2 = .15 and .13, respectively). Also the number of prey caught was correlated to both treatment and SL (R_s^2 = .33 and .26 respectively). There were significant differences between the larvae from the different treatments, in SL and MH at 28 dph (see figure 3.4 and 3.5).

The parameters burst and wriggle was measured, but it was noted that the individual larvae showed very different behaviour when regarding these parameters. The parameter "burst" was especially affected by the movement of other larvae in the aquarium, most larvae would burst-swim if they accidentally came in contact with other larvae, as if becoming startled. The mean number of bursts was 0.27±0.08, per minute for all treatments combined, and there was no

significant difference between the larvae from the different treatments. The number of wriggles in RotMG larvae (1.88±0.40) was significantly higher than in the Copepod treatment (0.68±0.22). Neither the number of bursts nor wriggles was correlated to treatment or SL at 28 dph.

Hunting behavior: At 28 dph a distinct hunting behavior was detected. When localizing a prey, the larva would position itself, by first stopping, focus its eyes directly ahead on the prey, back up and bend its body to an S-shape (seen from above), before darting forward in a quick attack. This behavior was most pronounced in the Copepod and Cop7-treatment, and it was also in these treatments the larvae were most successful at catching prey. Although not quantified in any manner, the larvae from the Copepod and Cop7-treatment were seen to use their eyes more actively; the head region was transparent at 28 dph and the movements of the eyes could easily be followed. Copepod and Cop7 larvae appeared to *look around*, actively searching for prey. The larvae could also move each eyeball separately. The *Artemia* nauplii in the water did not distribute themselves evenly, rather they clustered in the upper and lower corners of the aquarium. The Copepod and Cop7 larvae tended to position themselves inside these clusters, and could lay still on the bottom while eating.

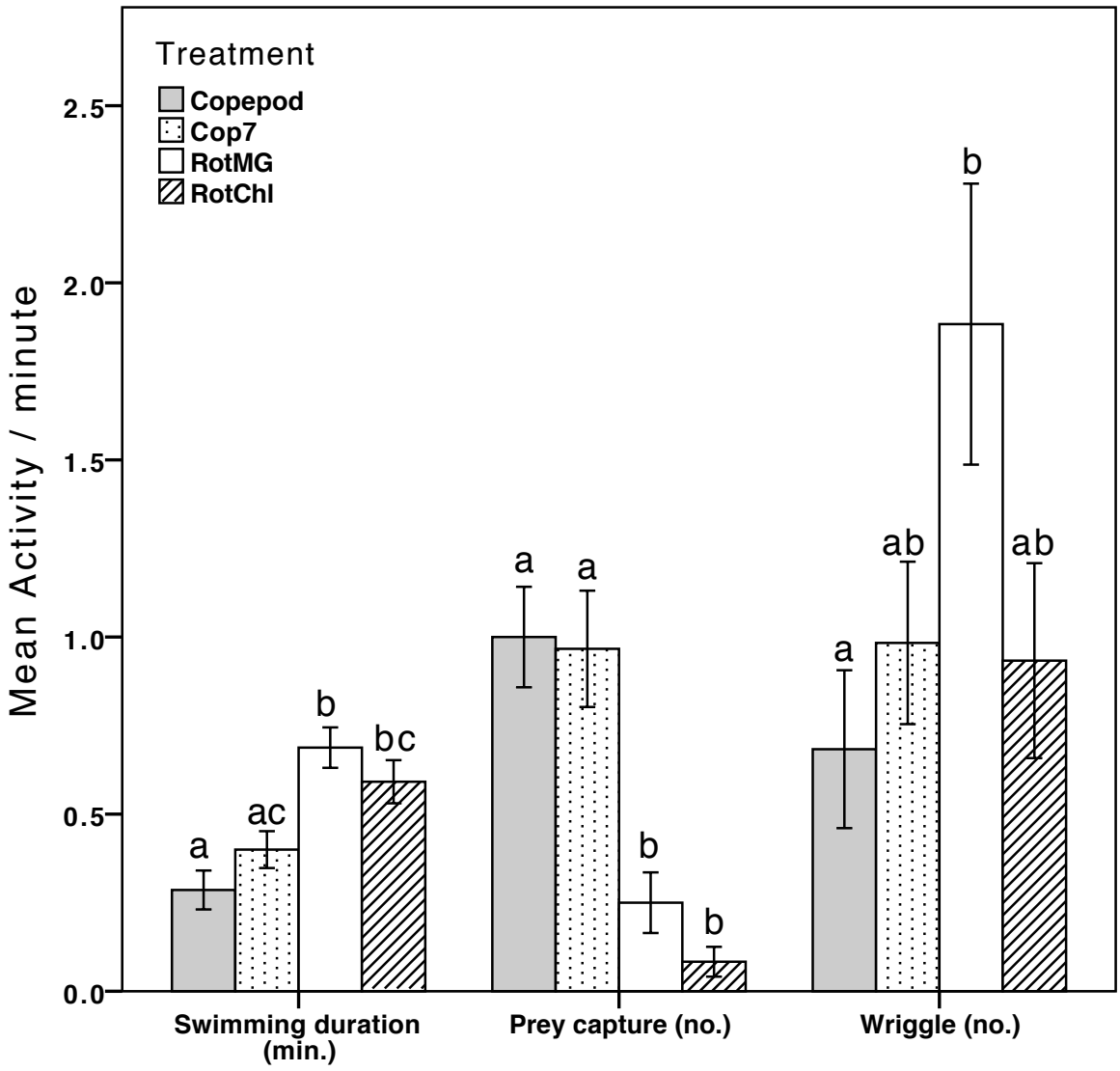

Figure 3.7. *Mean activity observed minute^{-1}, at 28 dph. The presented values are based on 2 minutes observation of 30 larvae per treatment. The activities observed were swimming duration (minutes), number of Artemia captured, and number of wriggles. The Y-axis indicates both minutes, number of prey eaten, and number of wriggles. Significant differences between the treatments are indicated by different letters. Error bars signifies ± 1 Standard error.*

3.5 Analysis of ossification

There were clear differences between the larvae from the different treatments when comparing ossification of the vertebrae, dorsal fin, hemal arches, tail fin rays, urostyle, and presence of flexion, in relation to the age at which this was first observed. The same trend was not found in a size-related comparison of the larvae from the different treatments.

3.5.1 Vertebrae

No vertebral segments were ossified at 15 dph in the Copepod treatment, while a mean number of 26 vertebrae was ossified at 21 dph. Ossified vertebrae was observed at 21 dph for the RotMG larvae (0.6 transparent vertebrae, seen in 3 larvae). No ossified vertebrae were seen in the RotChl larvae at 21 dph. The mean size and the mean number of ossified vertebral segments (transparent, compact, fully and total (summed)) are presented in table 3.2. On 21 and 33 dph the Copepod larvae had a significantly higher number of transparent and compact vertebrae, compared to the other treatments, and on 33 dph, the number of fully ossified vertebrae was significantly higher in the Copepod larvae.

There were no differences between the treatments when comparing the size-related ossification of the vertebral segments (figure 3.8). The size-intervals presented for the ossification of the vertebrae hence refers to all treatments. The first vertebral segments ossified at SL≥5.8-6.8 (transparent), and fully ossified vertebrae was first observed at SL≥ 6.6-8.3. By SL ≥ 8.2-10.6, on 40 dph, the entire vertebral column was fully ossified. When the full ossification of vertebrae is plotted against SL (figure 3.8), it can be seen that the formation of the fully ossified vertebrae occurred between 7-10 mm SL, and that there was no difference between the treatments in at which size the ossification occurred.

The mean number of vertebral segments in larvae at 40 dph were 36.53±0.1 (n=80), and generally ranged from 36 to 37 (35 vertebrae was found in 2 larvae). The mean total number of vertebrae was not calculated for 21 and 33 dph, as not all larvae had ossifications in all vertebrae (RotChl larvae), making a correct count of the total numbers of vertebrae difficult. At 61 dph, most of the larvae were covered with ossified scales, which obstructed the view of the vertebrae, and a count of the total number of vertebrae was not possible.

Table 3.2 *The mean size (SL, mm) ± standard error, number of larvae analysed (N), number of vertebrae which are transparent, compact or fully ossified, and the sum of ossified vertebrae (total) for larvae sampled at 15, 21, 33, 40 and 61 dph. The total number of vertebrae was 36 or 37. Significant differences between the treatments in SL or in number of ossified vertebrae between the treatments on the respective sampling days are indicated by different letters.*

Dph	Treatment	SL (mm)	N	Vertebrae			
				Transparent	Compact	Fully	Total
15	Copepod	5.9±0.1	28	0	0	0	0
21	Copepod	7.6±0.1^{a}	30	10.3±0.86^{a}	15.8±1.5^{a}	0.4±0.43	26.5±1.67^{a}
	RotMG	6.0±0.11^{b}	27	0.6±0.45^{b}	0^{b}	0	0.6±0.45^{b}
	RotChl	5.9±0.1^{b}	29	0^{b}	0^{b}	0	0^{b}
33	Copepod	8.9±0.2^{a}	30	0^{a}	2.9±1.0^{a}	33.7±1.0^{a}	36.6±0.1^{a}
	RotMG	8.2±0.1^{a}	30	1.1±0.5^{a}	9.2±1.9^{b}	25.8±2.3^{b}	36.0±0.2^{ab}
	RotChl	7.5±0.2^{b}	30	2.9±0.7^{b}	14.9±1.5^{c}	14.5±2.2^{c}	32.3±1.4^{b}
40	Copepod	10.2±0.3^{a}	30	0	1.6±0.7	35.1±0.7	36.7±0.1^{a}
	RotMG	11.1±0.2^{b}	30	0.1±0.13	1.5±0.8	34.9±0.9	36.6±0.1^{ab}
	RotChl	11.0±0.2^{ab}	20	0	0	36.3±0.1	36.3±0.1^{b}
61	Copepod	16.1±0.3^{a}	45	0	0	All	All
	RotMG	15.2±0.3^{b}	45	0	0	All	All

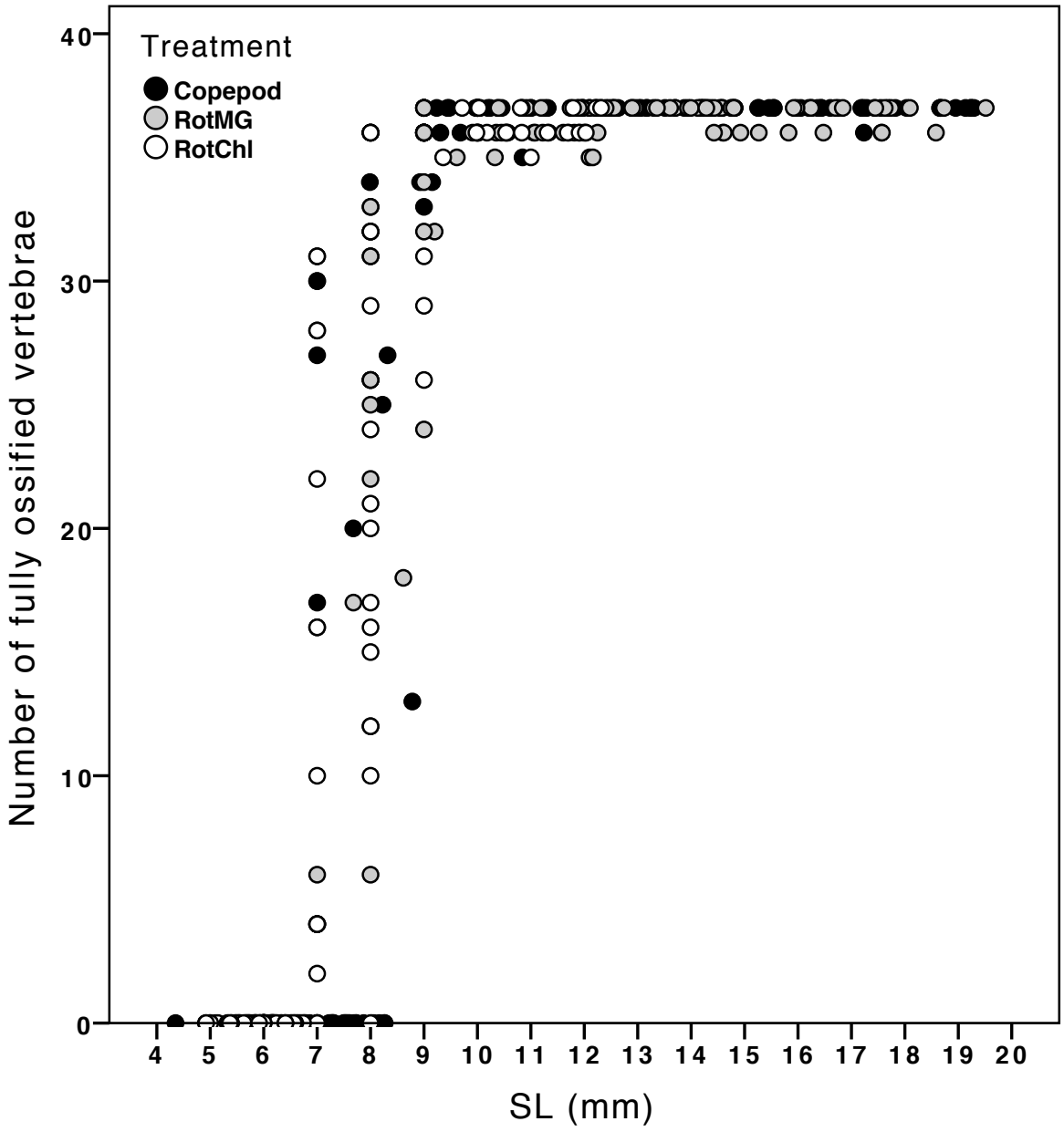

Figure 3.8. *The numbers of vertebrae that are fully ossified in individual larvae are plotted against the larval SL (mm). The total number of vertebrae varied between 36 and 37. The larvae were sampled on 21, 33, 40 and 61 dph. No larvae from the RotChl treatment were sampled on 61 dph. n=30-45 larvae treatment^{-1}, per day^{-1}.*

3.5.2 Dorsal fin

The fin rays initially ossified in the posterior region of the dorsal fin. The ossification process started in the proximal part of the fin rays, and continued towards the distal end. The last parts to ossify of the dorsal fin was the pterygiophores. The degree of ossification of the anal fin was always comparable to that of the posterior part of the dorsal fin.

There were differences between the treatments when comparing ossification in relation to age. Only Copepod larvae had a fin ray score of 1 on 21 dph (table 3.3 and 3.4), the larvae from the other treatments all had a score of 0. The Copepod larvae was also significantly larger compared to the other larvae on 21 dph. The RotChl was the only treatment in which not all larvae obtained a score of ≥ 1 on 33 dph; a significantly larger percentage of the RotChl larvae received a score of 0, compared to the other two treatments (table 3.4). The Copepod larvae were the first to obtain a score of 3 (on 33 dph). The Copepod larvae obtaining a score of 1 on 33 dph and a score of 2 on 40 dph, was significantly smaller than larvae from the other treatments that obtained the same scores (table 3.3). All larvae obtained a fin ray score of 3 at 61 dph (table 3.4). The fin-ray score were positively correlated to SL (r_s= .93, p<.01, 1-tailed), and developed over the size interval SL ≥ 6.6-8.3 (score 1) to SL ≥ 9.5-11.9 (score 3). Figure 3.9 shows the fin ray score of individual larvae plotted against that larva's standard length. There was a large overlap in size between the individual larva obtaining a score of 0-2 and 2-3.

Table 3.3 *Mean Standard length ±SE for the different treatments, on the different sampling days and fin ray scores. Only the scores found on the respective sampling days, and the treatment(s) recorded with these scores, are presented. The score indicates degree of ossification of the dorsal fin; 0=no ossification, 1= ossification of posterior part, 2=ossification of anterior and posterior part, 3=ossification of anterior and posterior part, including the pterygiophores. Significant differences in SL between the treatments with the respective scores on the respective sampling days are indicated by different letters.*

DPH	Fin ray score	Treatment	SL (mm)±SE
21	0	Copepod	7.5±0.1^{a}
		RotMG	6.1±0.1^{b}
		RotChl	5.9±0.1^{b}
	1	Copepod	8.2±0.2
33	0	RotChl	6.4±0.1
	1	Copepod	6.8±0.1^{b}
		RotMG	7.1±0.1^{ab}
		RotChl	7.4±0.1^{a}
	2	Copepod	9.0±0.2^{a}
		RotMG	8.5±0.1^{ab}
		RotChl	8.2±0.1^{b}
	3	Copepod	10.0±0.5
40	1	RotMG	7.67
	2	Copepod	9.4±0.2^{b}
		RotMG	10.6±0.2^{a}
		RotChl	10.6±0.2^{a}
	3	Copepod	11.5±0.3
		RotMG	11.9±0.1
61	3	Copepod	16.0±0.3
		RotMG	15.1±0.3

Table 3.4. *The percentage of larvae per treatment classified with the different fin ray scores, on 21, 33, 40 and 61 dph. n=3 tanks per treatment for the percentage data. N in the table are number of larvae analysed. The score indicates degree of ossification of the dorsal fin; 0=no ossification, 1= ossification of posterior part, 2=ossification of anterior and posterior part, 3=ossification of anterior and posterior part, including the pterygiophores. Mean standard length (SL ± standard error) for the treatments on the different sampling days are included. Significant differences in SL, and in percentage of larvae with the respective fin ray scores are indicated by different letters.*

				Fin ray score			
Dph	**Treatment**	**SL (mm)**	**N**	**0**	**1**	**2**	**3**
21	Copepod	7.6±0.1[a]	30	83	17	-	-
	RotMG	6.1±0.1[b]	30	100	0	-	-
	RotChl	5.9±0.1[b]	30	100	0	-	-
33	Copepod	8.8±0.2[a]	30	0[a]	10	83	7
	RotMG	8.3±0.1[a]	30	0[a]	17	83	0
	RotChl	7.5±0.1[b]	30	27[b]	27	46	0
40	Copepod	10.2±0.2[b]	30	-	0	63	37
	RotMG	11.1±0.2[a]	30	-	3	57	40
	RotChl	11.0±0.2[a]	20	-	0	70	30
61	Copepod	16.0±0.3[a]	45	-	-	-	100
	RotMG	15.6±0.2[b]	45	-	-	-	100

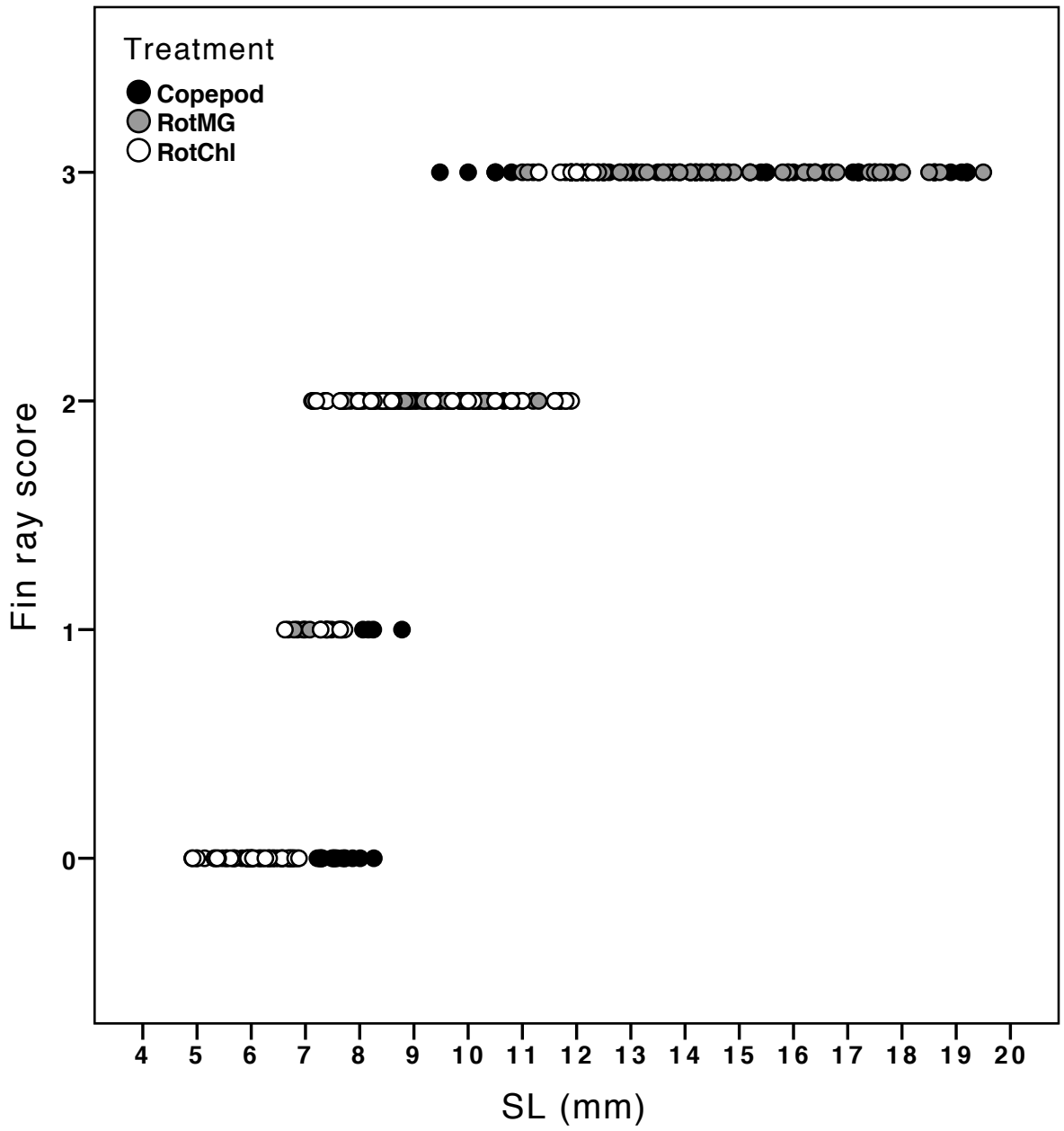

Figure 3.9. *The fin ray score of individual fish larvae from are plotted against standard length (mm). The larvae were sampled on 21, 33, 40 and 61 dph, n=135, 132, and 72 for the Copepod, RotMG and RotChl treatment respectively. No larvae from the RotChl treatment was sampled on 61 dph (empty tanks). The score indicates degree of ossification of the dorsal fin; 0=no ossification, 1= ossification of posterior part, 2=ossification of anterior and posterior part, 3=ossification of anterior and posterior part, including the pterygiophores.*

3.5.3 Squamation

The scales started to ossify along the midline, in a thin line following the vertebral column. An individual scale was triangular in shape, with rounded corners, and the anterior margin of the scale was undulate (figure 3.10). The appearance of the scales was not examined further. Ossified scales were only observed in bone stained larvae at 61 dph, at an SL ≥ 12.1-14.75 (score 1). Score 3 was recorded in the larvae from SL ≥ 15.2-18.7. The squamation score was positively correlated to SL (rs =.76, p< .01, 1-tailed). The Copepod larvae was significantly larger than the RotMG larvae on 61 dph, but obtained the respective squamation scores at a similar size (table 3.5), and there was no differences in the percentage distribution of larvae with the respective scores (table 3.6). At 61 dph, 35.5 and 24.4 % of the Copepod and RotMG larvae, respectively, had a squamation score of 3 (table 3.6).

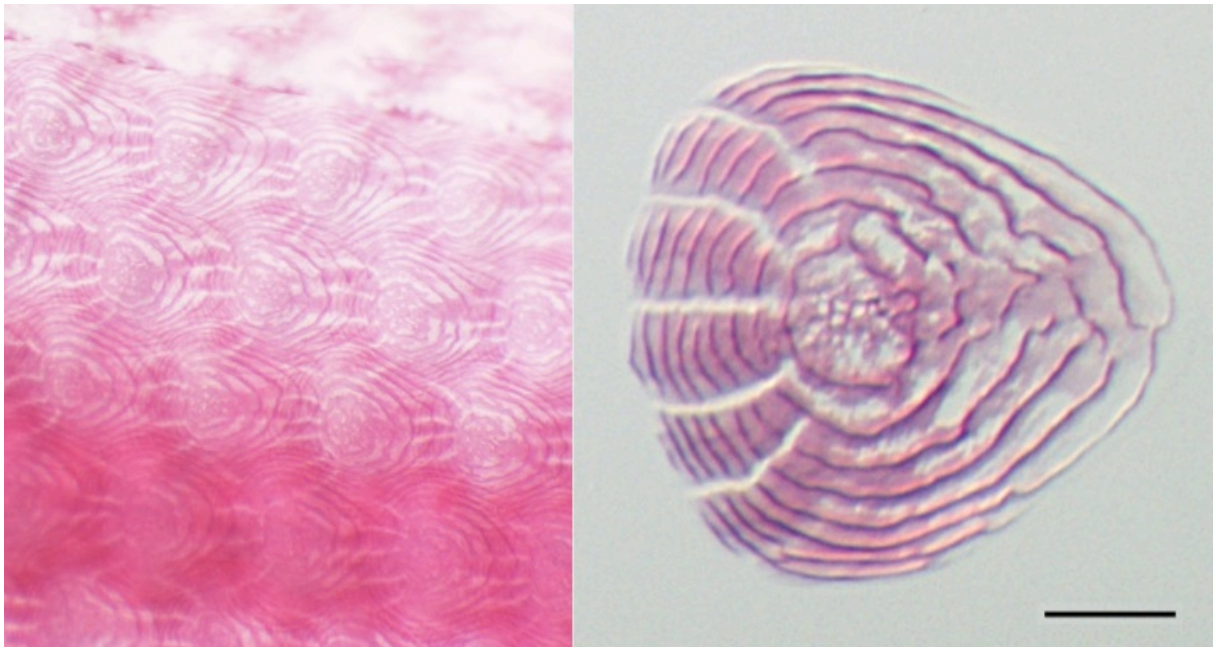

Figure 3.10. *Left: Image shows how the scales are positioned in the skin. The paler line in the upper edge of the image is the sideline, and the darker region in the lower part of the image is the vertebral column, visible through the skin only as a shade. Right: Details of a single scale. The undulating edge is the anterior edge of the scale. The black bar is 100 µm.*

Table 3.5. *Mean SL of larvae obtaining the respective squamation score, on 61 dph. The score indicates degree of squamation of the body. N=45 treatment^{-1}. No larvae from the RotChl treatment were sampled on 61 dph (empty tanks).*

Squamation score	Treatment	Mean±SE	N
0	RotMG	12.2±0.2	4
1	Copepod	13.0±0.6	3
	RotMG	13.2±0.7	3
2	Copepod	15.2±0.3	25
	RotMG	14.8±0.3	27
3	Copepod	17.8±0.3	16
	RotMG	17.3±0.4	11

Table 3.6. *Mean SL and percentage distribution of larvae with the respective squamation score. The score indicates degree of squamation of the body. n=3 tanks for the RotMG treatment and 2 tanks for the Copepod treatment (1 tank was empty). No larvae from the RotChl treatment were sampled on 61 dph (empty tank).*

				Squamation score			
Dph	Treatment	SL (mm)	N	0	1	2	3
61	Copepod	16.0±0.3[a]	45	-	7	58	35
	RotMG	15.6±0.2[b]	45	9	7	60	24

3.5.4 Ossification of other regions

At 15 dph, all the Copepod-treated larvae had ossification of the cleithrum and 75% had ossified mouthparts. Mean SL was 5.9±0.1 mm. The larvae from the RotMG and RotChl treatment was not stained on 15 dph, but 97-100% of the larvae had ossifications of the cleithrum and mouthparts at 21 dph. At 21 dph, the Copepod larvae had a significantly higher mean SL than the other two treatments, and a significantly higher percentage of the Copepod larvae had ossifications in the hemal arches, tail fin rays and the urostyle compared to the other treatments (table 3.7). The r_{pb} values in table 3.7 show that there was a significant correlation between the standard length of the larvae, and whether the larvae had ossifications in the neural arches, hemal arches, tail fin rays, urostyle, and flexion of the notochord. At 33 dph, all larvae had ossifications in the mouthparts, neural arches, hemal arches, tail fin rays and urostyle, and all larvae had flexion.

There was no difference between the treatments in the size-related ossification. The smallest stained larvae measured 4.3 mm (Copepod treatment, 15 dph), and had ossification in the cleithrum but not in the mouthparts. Parts of the splanchocranium; the mouthparts and the pharyngeal teeth, started to ossify at SL ≥ 4.9-5.8, shortly followed by the neural arches (SL ≥ 5.8-6.7) and hemal arches (SL ≥ 5.9-7.3). Ossification of the tail fin rays, and flexion started in the same size-interval, from 5.8-6.9 mm (SL ≥ 5.9-6.8, and 5.8-6.9 for tail fin rays, and flexion, respectively). The urostyle began to ossify in the interval 5.9-7.9 mm.

Table 3.7. *The percentage of larvae with ossifications in mouthparts, cleithrum, neural arches, hemal arches, tail fin rays and the urostyle, and percentage of larvae with flexion of the notochord, at 21 dph. Mean SL (mm ± SE) and the number of larvae analysed are included. The mean percentage values are calculated based on 3 tanks per treatment. Significant differences in ossification (Kruskal-Wallis, $p<0.05$) are indicated by different letters.*
The r_{pb} (point biserial correlation coefficient) and the r_{pb}^2 (coefficient of determination) for the correlation between SL and percentage of larvae with the respective ossified part are also included. Two raised asterisk symbols indicate significant correlation at the p=0.01 level.

Treatment	N	SL (mm)	Mouthparts	Cleithrum	Neural arches	Hemal arches	Tail fin rays	Urostyle	Flexion
Copepod	30	7.6±0.1[a]	100	100	90	83[a]	93[a]	37[a]	90
RotMG	27	6.0±0.1[b]	100	100	11	0[b]	0[b]	0[b]	22
RotChl	29	5.9±0.1[b]	97	100	10	0[b]	0[b]	0[b]	14
r_{pb}			0.12	-	0.80**	0.83**	0.85**	0.63**	0.78**
r_{pb}^2			0.01	-	0.64	0.69	0.72	0.40	0.61

3.5.5 Summary of bone development

As there were no differences between the treatments in at which size the different body parts ossified, a general pattern for the size-related ossification process in ballan wrasse was constructed with the results from the larvae from all treatments (table 3.8). Table 3.8 is hence a summary of the SL at which ossification of different parts occur. The exact size intervals are presented in the sections concerning each part; the sizes where ossification occur are presented to the nearest mm in table 3.8. Figure 3.11 is a visual summary of the skeletal development.

Table 3.8. *The size-related ossification of different parts of the skeleton, and the size (SL, mm) where flexion occurs, are given in the table. The lighter grey represents the size interval wherein the bone was first observed to ossify, and the darker grey represents the size at which all larvae had ossifications in these parts. For the presence of flexion, the lighter grey represents the size interval in which flexion was first observed, and above this size all larvae had flexion. A light grey area followed by a white area indicates that the bone in question was not observed in that state of ossification (e.g. transparent vertebrae) in larvae of a larger size.*

SL (mm)	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
Cleithrum																
Mouthparts																
Neural arches																
Hemal arches																
Tail fin rays																
Urostyle																
Presence of flexion																
Vertebrae																
Transparent																
Compact																
Fully																
Dorsal fin																
score 1																
score 2																
score 3																
Squamation																
score 1																
score 2																
score 3																

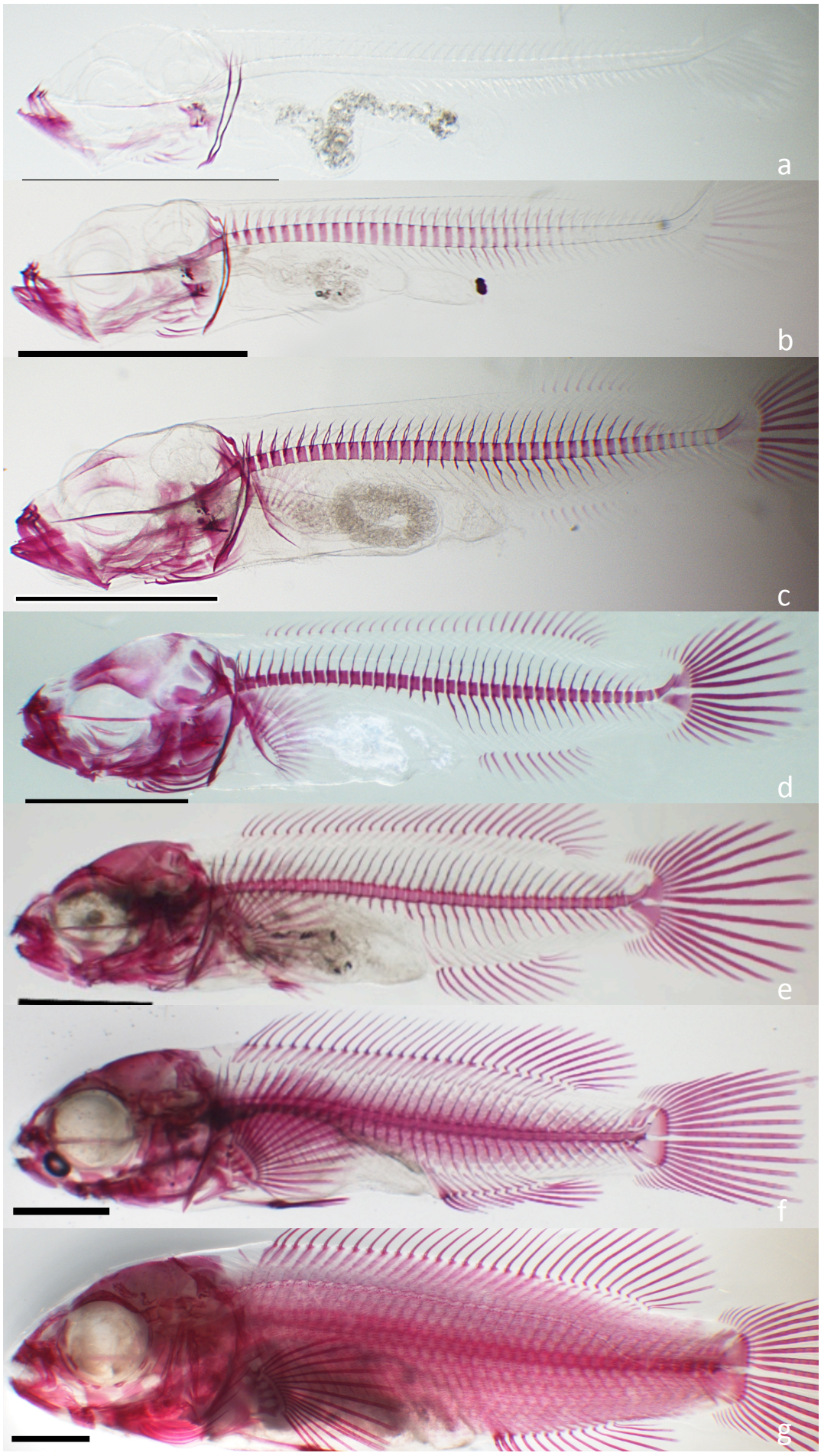
a
b
c
d
e
f
g

Figure 3.11, previous page. *Summary of skeletal development in ballan wrasse larvae, from 15-61 dph. a: SL 5-6 mm, initial ossification of the cleithrum, pharyngeal teeth, hyoid arch, opercular bones and the parasphenoid bone, b: SL 6-7 mm, ossifications in the chordacentra of the vertebral segments, neural and hemal spines, beginning ossification of the tail fin rays, c: SL 7-8 mm, the neurocranium, urostyle, hypurals, pectoral fin rays, posterior part of the dorsal fin and the anal fin is ossified, d: SL 8-9 mm, the ossification of the dorsal fin continues, with also the anterior part ossified, e: SL 10-11 mm, pelvic fin, and full ossification of the dorsal and anal fin, including the pterygiophores, f: SL 12-14 mm, formation of scales along the midline, g: SL > 15 mm, scales are fully formed, and covers the body. The black bars equal 2 mm.*

3.6 Skeletal anomalies

The skeletal anomalies observed are presented in figure 3.12, and 3.13. The percentage occurrences of skeletal anomalies treatment^{-1} at 33, 40 and 61 dph are presented in table 3.9. There were no significant differences between the treatments, in the percentage of larvae afflicted with the different skeletal anomalies, on any of the sampling days. Twisted arches (figure 3.12) was the most common anomaly and were only observed in larvae at 61 dph. A high magnification (5.0x) was necessary to detect the anomaly, as the spiralling at the apex was very weak. Larvae from both treatments had a very high occurrence of twisted arches (55±5 and 49±3% for the Copepod and RotMG treatment, respectively). Only neural arches in the pre-hemal region was found to be twisted.

Fused vertebrae/arches was observed frequently in the tail region, in the second-to-last vertebral segment (figure 3.13 a-c). This type of anomaly was never observed in the pre-hemal region, and always involved only two segments (and a maximum of 2 pairs of neural and hemal arches). Abnormal arches (bent, figure 3.13 d-e) were most common at 33 dph (table 3.9). Weak axis deviations (figure 3.13 f) were found in 47 and 22% of the RotMG larvae on 40 and 61 dph, respectively, compared to 3% and 2% of the Copepod larvae (table 3.9).

Only two types of anomalies were observed on 21 dph, and only in the Copepod larvae; deformed jaw in one larvae, and abnormally shaped vertebral column in two larvae. The larvae from the RotMG and RotChl treatments had only ossifications in the mouthparts at 21 dph, and any skeletal anomalies beside deformed jaw could not be seen. A deformed lower jaw was found in a total of 3 larvae; 2 from the Copepod treatment (21 and 61 dph) and 1 from the RotChl treatment (33 dph).

The percentage of larvae afflicted with one or several skeletal anomalies at 61 dph was 60±5% of the Copepod larvae and 67±7% of the RotMG larvae (n=45 for both). When

excluding twisted arches from the count, 11±6 and 42±2 % of the Copepod and RotMG larvae, respectively, where afflicted with anomalies on 61 dph. The difference was not significant. However, when comparing the mean number of skeletal anomalies per larva on 61 dph (figure 3.14), the Copepod treatment had significantly less skeletal anomalies per larva compared to the RotMG treatment when twisted arches was excluded (0.1±0.1 and 0.5±0.1 anomalies per larva for the Copepod and RotMG treatment, respectively.

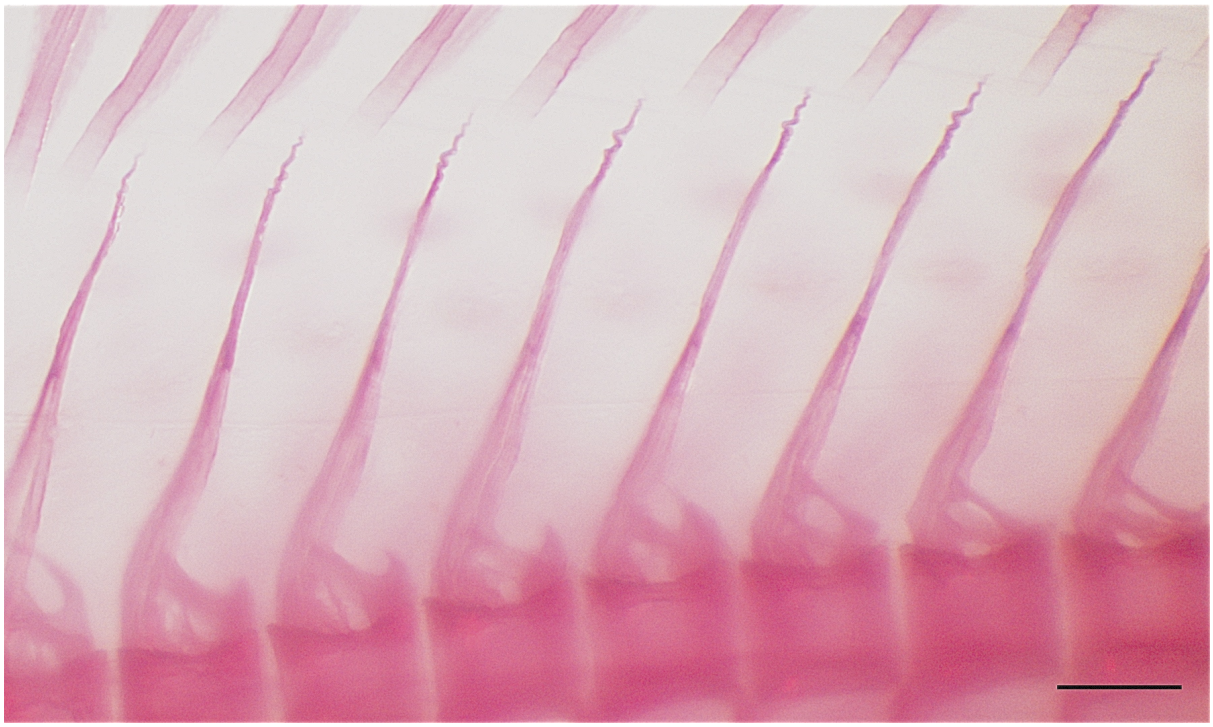

Figure 3.12. *Twisted neural arches in larva at 61 dph. The bones visible above the neural arches are the pterygiophores. The black bar is 200 µm.*

Table 3.9. *Mean percentage ± SE of larvae afflicted with the different skeletal anomalies, on 33, 40 and 61 dph. The percentage values were calculated per tank, n=3 tanks per treatment on all days, except 61 dph, where n=2 tanks for the Copepod treatment (1 empty tank). No larvae from the RotChl treatment was analysed on 61 dph (empty tanks). The data were based on analysis of 30-45 larvae treatment^{-1}, as indicated by the column N.*

			Occurrence of skeletal anomaly (%, ± SE)				
Dph	Treatment	N	Twisted arches	Abnormal arches (bent)	Fused vertebrae/ arches	Axis deviations	Deformed jaw
33	Copepod	30	-	0	17±6	0	0
	RotMG	30	-	30±10	33±15	0	0
	RotChl	30	-	33±18	10±5	7±7	3±3
40	Copepod	30	-	7±7	23±15	3±3	0
	RotMG	30	-	7±3	33±12	47±13	0
	RotChl	20	-	0	55±5	25±2	0
61	Copepod	44	56±5	0	4±4	2±2	2±2
	RotMG	45	49±3	11±2	11±4	22±4	0

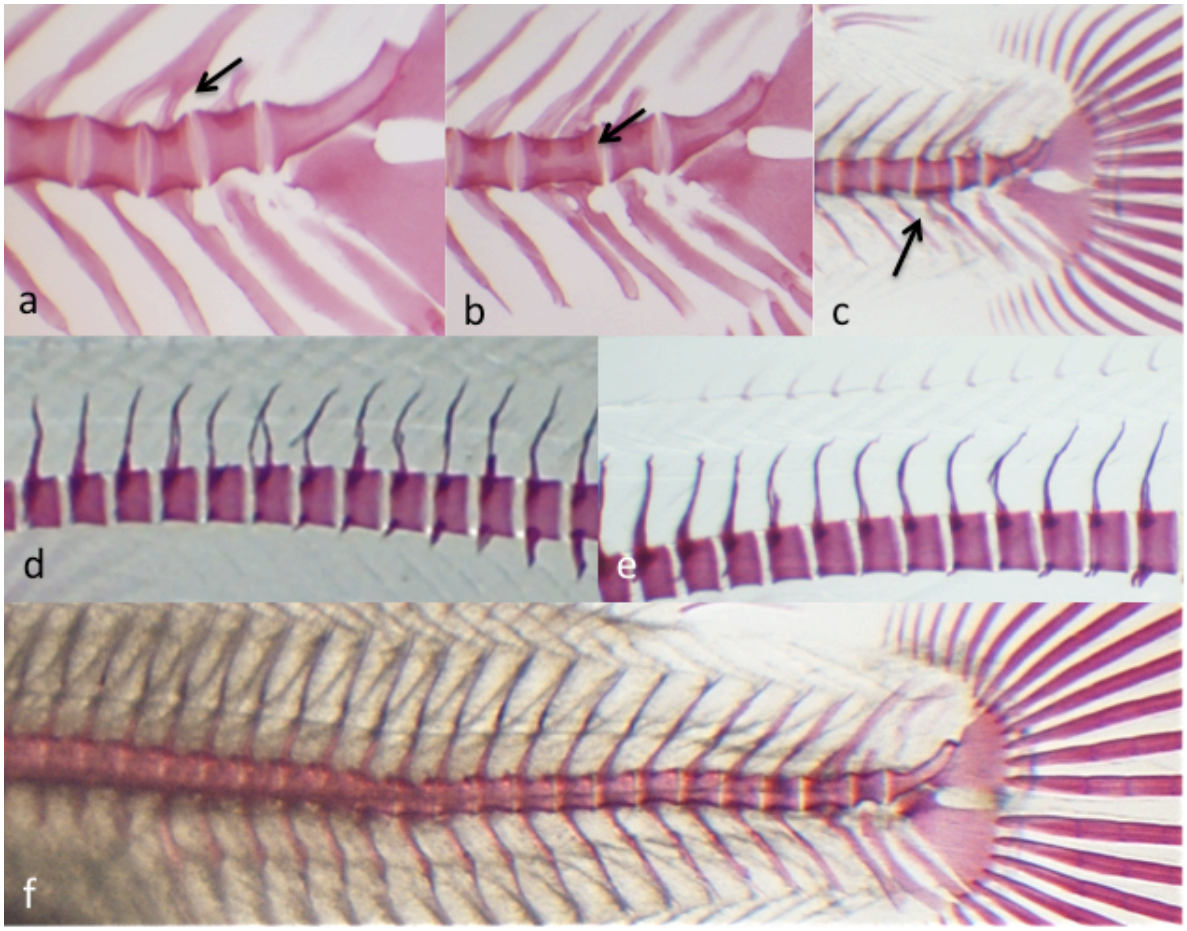

Figure 3.13. *Representative examples of the different skeletal anomalies observed. The larvae is positioned with the head oriented to the left. a-c show fused vertebrae/arches. a: the two vertebral segments indicated by an arrow share the same neural arch. b: the two segments are fused, with two neural and two hemal arches. c: two vertebral segments are incompletely fused. d-e: abnormal neural arches in the pre-hemal region, they are bent and deformed. f: larva with axis deviations, notably in the caudal region. The second-to-last vertebral segments also appears fused, which can be seen by comparing the thickness of the fused segment with the adjoining segments, and both the neural and hemal arch is abnormal.*

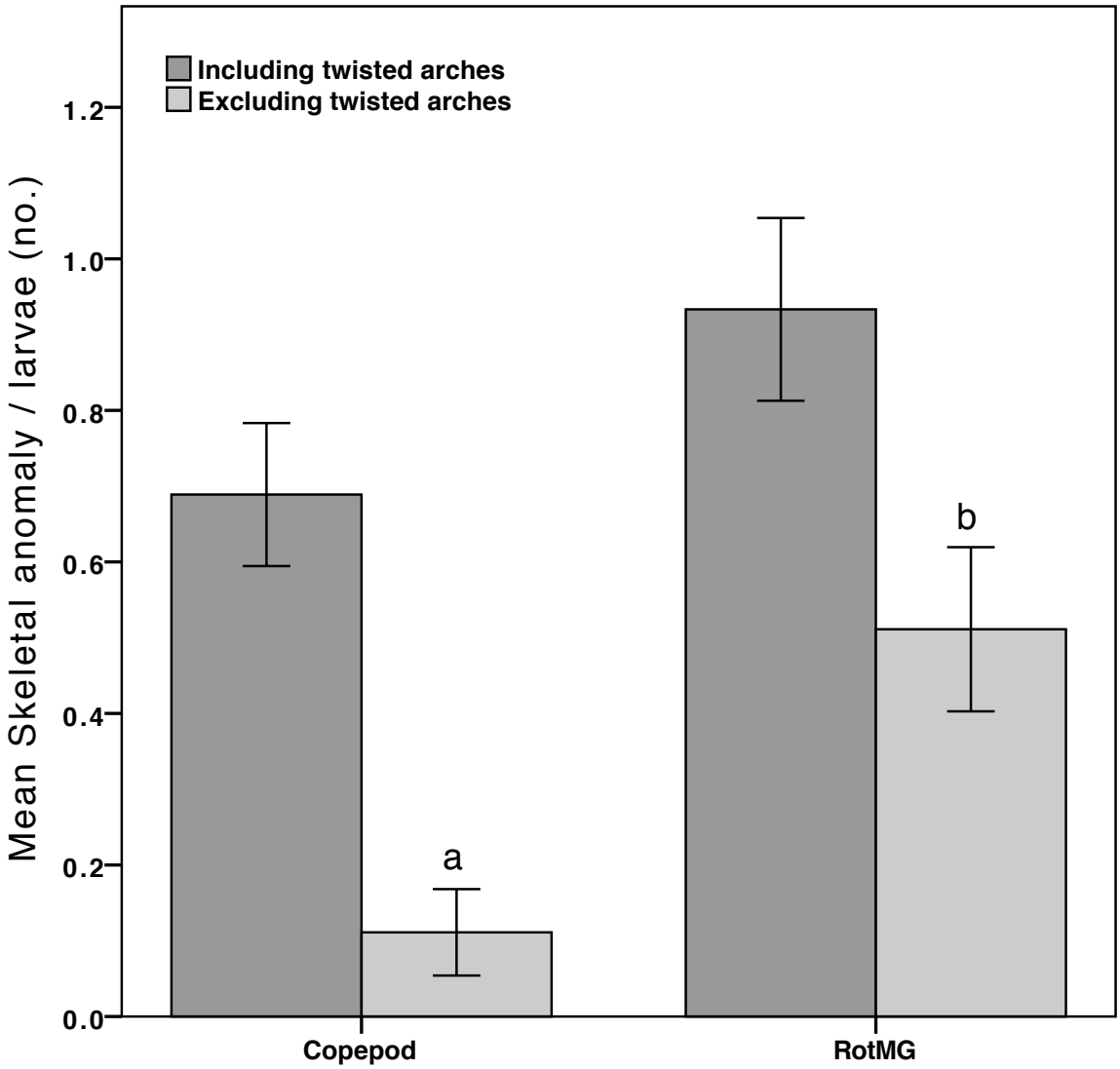

Figure 3.14. *Mean number of skeletal anomalies larva^{-1}, at 61 dph. The bars represents mean number when twisted arches (the most common skeletal anomaly at 61 dph) are included or excluded form the count. n=45 treatment^{-1}. Significant differences (Mann-Whitney) between the treatments are indicated by letters. Error bars signifies ± 1 standard error.*

4. Discussion

Feeding intensively cultivated copepods to ballan wrasse larvae was beneficial for somatic growth, stress tolerance, prey capture efficiency, early ossification of the skeleton and occurrence of anomalies, when compared to larvae fed rotifers. Feeding copepods during the first 7 days was also beneficial for growth, and the larvae tolerated handling stress better than larvae fed rotifers. Feeding enriched rotifers did not improve somatic growth compared to feeding unenriched rotifers, but may have been the cause for the better survival observed. The ossification of the skeleton appeared to be strongest related to larval size (rather than age), and was initiated at an earlier age for the larvae fed copepods due to the larger size, which in turn may be attributed to the high quality diet.

4.1 Dietary effects on growth and survival

Feeding with copepods positively affected somatic growth, resulting in a rapid initial growth during the days the larvae were fed exclusively copepod nauplii. The largest difference in DW was on 8 and 12 dph, the daily weight increase during this first feeding period (4-12 dph) ranged from 8.5 to 19.3 % day^{-1}, with the Copepod and Cop7 larvae having the highest values. This supports observations from other studies on marine larvae, e.g. in cod and sea bass, where larvae fed copepods grow faster than larvae fed enriched live prey (Hamre, 2006 ; Rajkumar & Kumaraguru vasagam, 2006 ; Hansen, 2011). Cod and sea bass larvae fed copepods in the early larval stages have been found to have a consistently higher growth throughout the duration of several start-feeding experiments, and in juvenile stages, when compared to larvae fed rotifers or *Artemia* (Imsland et al., 2006 ; Rajkumar & Kumaraguru vasagam, 2006 ; Hansen, 2011). It has been suggested that there exists a long-term dietary effect on growth, quality and incidences of deformities caused by the diet during the early life stages in cod (Imsland et al., 2006 ; Koedijk et al., 2010), and this should be further investigated in ballan wrasse. The better growth exhibited by the Copepod and Cop7 larvae in the formulated feed period, imply that the early live feed in ballan wrasse larvae affects the later growth, but larvae older than 61 dph should be examined to clarify this.

The larvae fed unenriched and enriched rotifers obtained the same DW in the *Artemia*-period as the larvae fed copepods, which is contradictory to the growth pattern observed in cod and sea bass larvae (Imsland et al., 2006 ; Rajkumar & Kumaraguru vasagam, 2006 ; Hansen,

2011). This may be due to the decrease in growth rate exhibited by the Cop7 and Copepod larvae when exposed to a change in live prey organisms. The Cop7 larvae were clearly negatively affected by the switch of feed on 11 dph, which could be seen as slower growth, compared to larvae from the other treatments. A similar decrease in growth rate was seen in the Copepod larvae, when the feed switched from copepods to *Artemia*. The larvae from the other treatments did not show a decrease in growth rate in these periods, but showed decreased growth rates while being fed formulated feed. A negative effect on growth when switching from zooplankton to rotifers as live feed have also been observed in cod (Koedijk et al., 2010 ; Hansen, 2011). The results in this study indicates that larvae initially fed copepods responds more negatively to a switch in feed organisms than larvae initially fed rotifers, possibly indicating a more pronounced preference for the copepod nauplii over the rotifers and *Artemia*. This may be caused by a difference in several attributes of the live feed, such as palatability or movement. If copepods are used as the first feed, special measures should be taken during the transition to another live feed to maintain good growth. It can for instance be investigated wether a longer co-feeding period will be beneficial. The results also suggest that the switch should not occur early, as the switch experienced by the Cop7 larvae gave a more marked decrease in growth rate compared to that seen in the Copepod larvae when switching to *Artemia*.

At 61 dph the Copepod and Cop7 larvae again had significantly higher DW than the RotMG and RotChl larvae. The behavioral observations revealed that the larvae fed copepods were better at catching prey, and it is possible that this ability was beneficial for the performance also in the formulated-feed period.

The dietary requirements for optimal growth of ballan wrasse larvae was clearly not met by using rotifers enriched with Multigain as live feed, when compared to the growth obtained in larvae fed copepods. The RotMG and RotChl treatments showed similar growth throughout the experiment, and enrichment of rotifers does hence not appear to have a strong impact on the somatic growth in ballan wrasse larvae. The same trend was found in cod larvae in a similar study (Hansen, 2011), while other studies have demonstrated the positive effects on growth of enrichment of rotifers (e.g. Gapasin et al. (1998), Baker et al.(1998), and Copeman et al. (2002)). It is possible that the availability of the fatty acids in the enriched rotifers is insufficient for optimal growth, due to the incorporation in the neutral lipids. The obvious superior effect on growth in larvae fed copepod nauplii may in turn be related to the higher

availability of the fatty acids, being incorporated in the phospholipids. DHA have been found to affect growth and survival to a higher extent than the other essential fatty acids; EPA and arachidonic acid (e.g. (Furuita et al., 1998 ; Gapasin & Duray, 2001 ; Copeman et al., 2002)). Also the higher protein and free amino acid content found in copepods compared to rotifers (van der Meeren et al., 2008 ; Conceicao et al., 2010) may have contributed to the large differences in early growth between the treatments, as proteins and amino acids are an important energy source for marine larvae (Fyhn, 1989). The nutritional value of enriched rotifers was found to be more beneficial for other factors than growth, as the RotMG larvae showed higher survival and stress tolerance than the RotChl larvae. Samples of the live feed used in this experiment have been taken, but the analysis is not completed.

The optimal rearing temperature for ballan wrasse is probably higher than what is commonly used (12-14 degrees) in the commercial rearing of the species today. Skiftesvik & Opstad (2010), found that the highest biomasses was obtained in cultures reared at 16°C, as opposed to 12, 14 and 18°C. In this experiment the larvae were reared with temperatures increasing from 12 °C on 4 dph, to 16 °C from 23 dph and onward, and the temperature was thus probably suitable for optimal growth. Farmers of ballan wrasse have reported a specific growth rate (SGR) of 4% until the larvae reach 1 gram (wet weight) (FHF, 2011). We had similar growth rates in the formulated feed period (47-61 dph, 5.4% DWI), but higher growth rates when measuring growth from 2-61 dph (10.4% DWI, 9.9% when measured as SGR).

The overall survival in this study ranged from 5% in RotChl to 10-12 % in the other treatments. Little data is available on the survival of ballan wrasse larvae, but we had similar survival rates as reported from Ecofish, which was 10% after 3 months (EcoFish, 2010). In a recent experiment at NTNU Sealab, the survival after 40 days was 14-21% in larvae given enriched rotifers (Kari Attramadal, NTNU, pers. comm.). It should be taken into account that the estimated survival numbers are uncertain, as it was difficult to know the exact density of the larvae that was distributed in each tank, at the beginning of the experiment. It was also not possible to start counting dead larvae before 13 dph, as they were very small and disintegrated rapidly.

The mortality in this experiment was highest during the first 2 weeks, and only 12-25 % of the larvae survived past 12 dph. This suggests that the initial mortality is not so much affected

by the quality of live feed, and that the rearing environment during the first days are critical. Possible detrimental microbial conditions during the first days may have caused the high mortality. Marine larvae lack a fully function specific immune system at hatching (Vadstein, 1997), and are vulnerable to infections by opportunistic bacteria (Skjermo et al., 1997 ; Hansen & Olafsen, 1999 ; Skjermo & Vadstein, 1999). Larvae from tank 1 in the Copepod treatment performed particularly poorly compared to the other two tanks; when regarding growth and survival from the start of the experiment, and exhibited a particularly low DW at the end of the experiment (7.7 mg). Of all the other larvae, only those from the RotChl-treatment showed lower survival. Differences in rearing temperature can cause differences in growth, but as the total d° of the larvae from the three copepod tanks only differed by 5-15 d° at 61 dph, this was not the cause. It is possible that the poor growth and survival observed in the larvae from the Copepod tank 1 were due to detrimental bacterial conditions in this tank, and that the bacterial condition differed from that of the other tanks. Microbial samples from the rearing water have been taken, but have yet to be analyzed.

4.2 Dietary effects on tolerance to handling stress

The Copepod treatment had a significantly better stress tolerance compared to the RotChl larvae on 29 dph. The variance in size only accounted for 15.2 % of the variance in mortality, indicating that other factors, such as live feed quality, are more important. The gradient observed in figure 3.6, and the high correlation between treatment and mortality supports this, with the mortality after 24 hours being lowest in the treatment fed the highest quality prey and gradually increasing with lower prey quality. Previous studies have also established the importance of early live feed on the tolerance to handling stress, and especially the fatty acids DHA and ARA, as well as vitamin C have been found to be important (Watanabe & Kiron, 1994 ; Kanazawa, 1997 ; Koven et al., 2001 ; Noori et al., 2011). Copepods have been shown to contain high levels of DHA (Evjemo et al., 2003 ; van der Meeren et al., 2008), in the phospholipids. The levels of fatty acids in *Acartia tonsa* have been found to be affected by the developmental stages and algal enrichment used in the production (Støttrup et al., 1999). Thus, only analyses of the live feed can give a clear picture of the differences in HUFA and vitamin content between the copepods and the rotifers.

The results from the stress test on 28 dph implies that the mortality after a standardised handling stress can be a method to assess general larval quality of ballan wrasse. It has also been found to be suitable for the assessment of quality in the early life stages of cod (Hansen 2011). However, in this study there appeared to be a large difference in stress tolerance of the ballan wrasse larvae from 29 to 36 dph. Although only larvae from the RotMG larvae was exposed to handling stress on 36 dph, this indicates that there was a major general increase in tolerance to air exposure. During metamorphosis the structure of the fish skin thickens, and protects the larvae from different environmental shocks (Le Guellec et al., 2004). An examination of the ossification process revealed that large changes occurred between day 33 and 40, consistent with metamorphosis. It is possible that the increased thickness of the skin makes the larvae less vulnerable to dehydration of the skin during air exposure.

Air exposure was not a suitable method to assess stress tolerance at 36 dph, but can be successfully used in younger larvae. The age of the larvae (preferably d°) when a handling stress is feasible, as well as the duration and method of testing should be standardised. The age at which the larvae tolerate handling stress better should also be determined, as the farmers can adjust the production so that necessary stress (such as transfer and grading) occurs in periods were the larvae are more tolerant. It must be emphasised that air-exposure is a very specific type of stress, and the larvae may not be as tolerant to other stressors.

4.3 Dietary effects on larval activity and feeding behaviour

The Copepod and Cop7 larvae had the lowest activity level, while still catching the most prey at 28 dph. They appeared to master a predatory behaviour with a distinct attack to a larger degree than the RotMG and RotChl larvae. From the observations of prey capture in this study, it can be concluded that the ballan wrasse larvae are visual predators, and better developed vision will thus impact the foraging success. DHA are an important component in neural tissue, mainly in brain and retinal tissue (Neuringer et al., 1988). Feeding with zooplankton as opposed to enriched *Artemia* positively affect eye development in halibut larvae (Shields et al., 1999). O'Brien-MacDonald et al. (2006) found that cod larvae fed high-lipid rotifers exhibited a higher attack rate and activity level than larvae fed low-lipid rotifers, and proposed the cause to be the difference in DHA content of the feed. Copepods are rich in DHA, and it is possible that the larvae fed copepods had better developed vision and neural

system, and hence were more successful predators. This agree with the larval behaviour observed, as the RotChl and RotMG larvae appeared to swim around without detecting that a prey was nearby, as opposed to the larvae from the Copepod and Cop7 treatment. The attack-behaviour observed, with the typical s-shape, was similar to that reported in larval herring (Batty & Morley, 1994) and cod (Munk, 1995).

The density of prey used in the aquariums was clearly too high in the current experiment (3000 prey organisms L^{-1}) compared to that used in Hansen (2011) (750 prey organisms L^{-1}). The density was chosen based on what the larvae would experience in the rearing environment, but as the *Artemia* gathered in clusters, it could preferably have been lower in the aquariums. The Copepod and Cop7 larvae would position themselves inside these clusters, more often than the larvae from the other treatments. This resulted in some larvae exhibiting very short swimming durations, while still capturing several prey organisms, and may have led to the lower activity level when compared to the RotMG and RotChl larvae.

As this was the first standardised behavioural study of ballan wrasse, the results can be said to be preliminary, and further development of the methods used are necessary. The method was based on what have been used during behavioural studies on cod (Lervik, 2007 ; Hansen, 2011), and as the early larval behaviour in ballan wrasse was very different from that of cod larvae, the method was not optimal. Further, the method was even less suitable for observations of older larvae than for younger ones, due to the difficulties involved in catching the very quick larvae at 39 and 58 dph. It was desirable to observe the larvae also at a later stage, to assess if the early feed quality affects later behaviour.

The parameter prey capture revealed a better performance of the larvae fed copepods, which, is consistent with what was observed in relation to growth, skeletal development and tolerance to handling stress. However, neither the number of wriggles nor the numbers of burst were suitable for indications of larval quality. Also the suitability of swimming duration as a quality parameter need to be assessed, as the larvae often appeared to “rest” on the bottom. Based on observation of the larval behaviour in the tanks, this behaviour got more pronounced with increasing age, suggesting that this species is naturally more sedentary in the later stages than for instance cod. Thus, measurements of swimming duration may not be

suitable, as changes in swimming pattern occurs in the transition from larva to juvenile (Koumoundouros et al., 2009).

The behaviour observed on 28 dph was quite different from what is described in Lebour (1919). The larvae observed in this study was very active, and the larvae from the Copepod and Cop7 treatment responded quickly to prey. The differences between these two studies may be attributed to different origin of the larvae (caught in tow-nets versus reared), the conditions during observation, and the large difference in number of larvae observed (3 versus 120), in turn affecting the chances of a representative observation of behaviour.

4.4 Dietary effects on ossification

The general developmental pattern of ossification found in the ballan wrasse larvae was consistent with the general pattern found in other fish species (Kjørsvik et al., 2004). The mouthparts and the cleithrum was the first parts to ossify, implying the importance of feeding and locomotion for larval survival. The cleithrum was the very first bone in which ossification was initiated, as the smallest larvae where found with an ossified cleithrum but not ossified mouthparts. The cleithrum ossifiy together with the first cranial elements also in halibut larvae (Sæle et al., 2004).

The swimming pattern observed in the larvae at 28 dph showed that the pectoral fins was an important source of locomotion, but the tail fin appeared to be the main source. The early ossification of the tail fin rays, urostyle and hypurals represented a separate point of origin of ossification (figure 3.11b and 3.11c), implying the importance of these structures. The pectoral fins became increasingly more important with age, as demonstrated by a marked change in swimming behaviour seen in the trial observations at 39 dph.

The number of vertebrae ranged from 36-37, and this is consistent with what was found in wild-caught ballan wrasse (Helland et al., 2012).

The Copepod larvae was the youngest larvae in which ossification of the vertebrae and fin rays occurred, and they had ossifications in a larger part of the skeleton compared to the other larvae at 21 dph. However, a size related comparison of the ossification of the vertebrae, fin rays and squamation revealed that the Copepod larvae generally did not start the ossification

at a smaller size than the RotMG and RotChl larvae. This suggests that size is more explaining for the differences in ossification and larval development than the age or the dietary treatments in this study. It has been suggested that when comparing the developmental of larvae, size (and not age), should be the common feature (Amara & Lagardere, 1995 ; Wold et al., 2008 ; Sæle & Pittman, 2010). The results obtained when examining allometric growth, organ volume and muscle development in ballan wrasse larvae from this start-feeding experiment, also display large differences between the larvae from the different treatments, when comparing the development using larval age as the common feature. However, it was also found that the larval size (SL), as opposed to age or feeding regime appeared to be the determining variable for the developmental stage, in terms of organ development and growth (Maren Ranheim Gagnet, pers. comm., thesis in prep.), and number and size of muscle fibres (Martin Berg, pers. comm., thesis in prep.).

Only the beginning ossification of the dorsal fin deviated from this highly size-related ossification pattern. The Copepod larvae was significantly *smaller* than the larvae from the RotMG and RotChl treatment when obtaining the fin ray score 1 at 33 dph (only 3 larvae) and the score 2 at 40 dph. It is thus possible that the diet did affect the size related onset of ossification in the ballan wrasse larvae to some extent, leading to onset of ossification at a smaller size.

The first ossified scales appeared on the middle region of the flank, which is one of seven main sites of squamation found in teleosts (Sire & Arnulf, 1990). The morphology of the scales themselves was not studied in detail, and as the shape and appearance of scales can differ depending on which region of the body they are from (Park & Lee, 1987), the scales on different regions of the fish may differ from that seen in figure 3.10. The squamation score was strongly related to size. The ballan wrasse appeared to be fully metamorphosed at 61 dph, with no larval characteristics. The fact that ossified scales was not observed before 61 dph (not seen at 40 dph) is consistent with the conception that the formation of scales occur late in ontogeny, when the fish have lost its larval characters to become a miniature version of the adults (Sire & Akimenko, 2004). When seen in context with the development of the skeleton, scales appeared subsequently to the completion of ossification of the fin rays and vertebrae (table 3.8). The squamation score was strongly related to the size of the larvae, a relation which was also found for the ossification of the other part of the skeleton.

4.5 Dietary effects on the occurrence of skeletal anomalies

The Copepod larvae had a generally lower occurrence of the different skeletal anomalies, fewer larvae with anomalies, and significantly less skeletal anomalies per larvae (when excluding twisted arches), compared to the RotMG and RotChl larvae. The Copepod larvae also showed the best performance when regarding stress tolerance, growth and foraging success. The results in this study thus support the view that the presence of skeletal anomalies can be used to measure the quality of larvae produced in aquaculture (Boglione et al., 2001).
When assessing larval quality based on incidences of skeletal anomalies, it must be emphasized that not all skeletal anomalies necessarily leads to changes in external morphology or larval performance (Loy et al., 1999 ; Loy et al., 2000). Minor deviations from the norm, such as twisted arches, may hence not negatively affect larval and juvenile performance. In this study, the larvae with axial deviations all had only very weak deviations, and the most common anomaly; twisted arches, was of a very low severity. In addition, the deformed lower jaw-anomaly was the only anomaly externally visible, and was only found in 3 out of the 374 larvae analyzed. The degree of severity of the different skeletal anomalies found in this study thus seem low.

On 61 dph, 11% of the Copepod larvae had skeletal anomalies other than twisted arches at 61 dph, compared to 42% of the RotMG larvae, implying the benefits of the high quality diet. As Helland et al. (2012) did not assess twisted arches, the occurrence without twisted arches is what may be comparable to the findings in Helland et al. (2012). There were clear differences between the studies regarding the severity of anomalies observed, implying that the rearing environment and feeding regimes in this study (table 2.1), also for the RotMG treatment, were more optimal than that used by the farmers. However, there were also large differences in age/ size of the fish in this study and in Helland et al. (2012), the fish in this study was considerably younger and smaller. Skeletal anomalies can become more frequent and increasingly severe with increasing larval age, as demonstrated for intensively cultivated halibut (Lewis et al., 2004), so caution should be taken when comparing larvae of different age. The incidences of skeletal anomalies in the commercial rearing of ballan wrasse should be reduced, and a development of a “Best management practice” (BMP) will contribute to this. A BMP has successfully been implement for cod (Helland et al., 2009), seabass, and

gilthead seabream (Zambonino Infante et al., 2009b). The larger research project that this study was a part of (FHF project number 900554), will contribute to the development of a BMP for the rearing of ballan wrasse.

There were no significant differences between the treatments in the percentage occurrence of the respective skeletal anomalies on 61 dph. This may be attributed to the rather large variation between the tanks, and that there was only two parallels from the Copepod treatment on 61 dph. Twisted arches were only observed at 61 dph, suggesting that this was an anomaly developed over time. Hansen (2011) found twisted arches (in her thesis called corkscrews) in significantly more of the cod juveniles initially fed rotifers (80-90%), compared to the juveniles fed copepods (5%), at 60 dph. Such a trend was not found in this study. The anomaly was equally common in the Copepod and RotMG larvae at 61 dph, implying that early nutrition have less effects on this particular anomaly in ballan wrasse, compared to cod. The appearance of this anomaly may be related to suboptimal diet in the later stages; either the nutritional quality of the *Artemia* or the formulated feed, as the feeding regime from 30-61 dph was identical for both treatments. Alternatively, the anomaly may have been induced by abiotic factors in the rearing environment, but suboptimal diet is a more plausible cause based on the results in Hansen (2011). The formulated feed used in this study is recently developed, and the nutritional value has yet to be optimized for ballan wrasse. Older larvae (1 year old in june 2012) from this experiment are being kept in the rearing facilities on Sealab today, and could preferably be analyzed to see if the severity of the twisted arches has increased, as these larvae have been fed the same formulated feed since weaning.

Fusions of the vertebrae have been frequently observed in reared ballan wrasse (Helland et al., 2012), and was found in 3 out of 17 wild caught ballan wrasse. It was also found in this study. However, fusions in other parts than the caudal region was not observed, and the fusions of the vertebrae were always simple fusions (Hjelde et al., 2009), involving only two vertebrae. Fusions in the vertebrae of the tail region occur regularly in many species, and it has been suggested that this may be a normal differentiation of the tail vertebrae in some species (Bæverfjord et al., 2009b). In this study, fusions of the vertebrae/arches were genereally more common in the RotMG and RotChl larvae than in the Copepod larvae. This suggests that the diet may have affected the occurrence of this anomaly, and that it is not a normal differentiation of the tail-associated vertebrae in ballan wrasse. Fusions of the vertebral

segments are often related to spinal deformities, such as lordosis or kyphosis (Divanach et al., 1996). The fusions in the tail region can be an early stage in the development of an axis deviation in the caudal region, and bone analysis of older larvae from this study should be conducted to clarify this.

The presence of scales on the flank at 61 dph made the examination of the vertebral elements difficult, as the scales obstructed the view of the vertebrae below. By using the focus on the microscope, it was possible to "see through" the scales, but not to a satisfactory extent. This may have led to fewer anomalies being detected at 61 dph, and may be the cause for the apparent decrease in the occurrence of vertebral anomalies compared to the other sampling days. As there was no difference in the percentage of larvae with the different squamation scores, the possible diagnostic error will apply equally to the larvae from both treatments. It can thus still be concluded that the RotMG larvae had more skeletal anomalies than the Copepod larvae. A method for removal of ossified scales in small larvae (SL < 20 mm) should be developed before further diagnostics, if bone staining is used as the preferred method to visualize the bones.

4.6 Conclusions and future perspectives

The results in this study showed that feeding with intensively cultivated copepods for the first 30 days after hatching clearly improved the growth and quality in ballan wrasse larvae, when compared to feeding with rotifers. It can hence be concluded that *Acarti tonsa* nauplii are a more optimal feed for ballan wrasse larvae than rotifers, which was expected due to copepods being the natural prey organisms for pelagic marine larvae. The Cop7 larvae generally performed better than the RotMG and RotChl larvae, and it is therefore suggested that feeding with copepods for the first 7 days can be sufficient to positively affect growth and larval quality in ballan wrasse. The enriched rotifers did not have the dietary quality necessary to obtain optimal growth, and alo resulted in a higher occurrence of skeletal anomalies when compared to the larvae fed copepods. The main dietary effects on the ossification process in this study appeared to be related to increased growth rate, resulting in earlier onset of ossification in relation to age.

The quality parameters used in this study all indicated the larval quality, suggesting dietary effects on tolerance to handling stress, foraging success and occurrence of skeletal anomalies. The observations of behavior and the duration and timing of the handling stress procedure should be further optimized for this species.

This study showed that the problems related to growth and skeletal anomalies experienced in the rearing of this species today, can be alleviated by the inclusion of copepod nauplii in the diet. Further scientific effort should be aimed at optimizing the feeding regime, and the rearing environment, to obtain high quality larvae.

If farmers want to implement the use of intensively cultivated copepods as first feed in ballan wrasse larvae, the production of nauplii and harvesting of eggs need to be large-scale, and more cost-effective. Preferably stored eggs could be easily ordered and quickly shipped to the farmers (as with *Artemia* cysts today). The farmers can then hatch the eggs themselves, and feed the copepods a suitable algae paste till required size is achieved. The production need not be any more time consuming or difficult than that of rotifers, but will give larvae of a higher quality.

Bibliography

Altmann J (1974) Observational Study of Behavior: Sampling Methods. *Behaviour,* **49**, 227-266.

Alver MO, Tennøy T, Alfredsen JA, Øie G (2007) Automatic measurement of rotifer Brachionus plicatilis densities in first feeding tanks. *Aquacultural Engineering,* **36**, 115-121.

Amara R, Lagardere F (1995) Size and onset of metamorphosis in sole (*Solea solea* L) of the gulf of Gascogne. *ICES J. Mar. Sci.,* **52**, 247-256.

Attramadal KJK, Tøndel B, Salvesen I, Øie G, Vadstein O, Olsen Y (2012) Ceramic clay reduces the load of organic matter and bacteria in marine fish larval culture tanks. *Aquacultural Engineering*.

Bæverfjord G, Helland S, Hough C (2009a) Control of malformations in fish aquaculture - Science and practice. Federation of European Aquaculture Producers, Liege.

Bæverfjord G, Hjelde K, Helland S, Lein I (2009b) Malformations in farmed fish - Guidelines for classification II Cod (*Gadus morhua*), URL: http://www.aquamedia.org/FileLibrary/28/Cod%20diagnostic%20manual.pdf. In: *Diagnostic manuals*. Finefish.

Baker EP, Alves D, Bengtson DA (1998) Effects of rotifer and Artemia fatty-acid enrichment on survival, growth and pigmentation of summer flounder Paralichthys dentatus larvae. *Journal of the World Aquaculture Society,* **29**, 494-498.

Barton BA (2002) Stress in fishes: A diversity of responses with particular reference to changes in circulating corticosteroids. *Integr. Comp. Biol.,* **42**, 517-525.

Barton BA, Iwama GK (1991) Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. *Annual Review of Fish Diseases,* **1**, 3-26.

Batty RS, Morley SA (1994) Temperature and S-start feeding strikes by larval herring. *J. Fish Biol.,* **45**, 244.

Bell JG, McEvoy LA, Estevez A, Shields RJ, Sargent JR (2003) Optimising lipid nutrition in first-feeding flatfish larvae. *Aquaculture,* **227**, 211-220.

Bjordal Å (1988a) Cleaning symbiosis between wrasses (Labridae) and lice infested salmon (*Salmo salar*) in Mariculture. ICES CM.

Bjordal Å (1988b) Rensefisk mot lakselus. *Norsk fiskeoppdrett,* **37**.

Bjordal Å (1990) Sea lice infestation on farmed salmon: Possible use of cleaner-fish as an alternative method for de-lousing. In: *Canadian Technical Reports of Fisheries and Aquatic Sciences,* pp. 85-89.

Boglione C, Gagliardi F, Scardi M, Cataudella S (2001) Skeletal descriptors and quality assessment in larvae and post-larvae of wild-caught and hatchery-reared gilthead sea bream (Sparus aurata L. 1758). *Aquaculture,* **192**, 1-22.

Bolla S, Holmefjord I (1988) Effect of temperature and light on development of Atlantic halibut larvae. *Aquaculture,* **74**, 355-358.

Busch KET, Peruzzi S, Tonning F, Falk-Petersen IB (2011) Effect of prey type and size on the growth, survival and pigmentation of cod (Gadus morhua L.) larvae. *Aquaculture Nutrition,* **17**, e595-e603.

Cahu C, Zambonino Infante J (2001) Substitution of live food by formulated diets in marine fish larvae. *Aquaculture,* **200**, 161-180.

Cahu C, Zambonino Infante J, Barbosa V (2003a) Effect of dietary phospholipid level and phospholipid:neutral lipid value on the development of sea bass (*Dicentrarchus labrax*) larvae fed a compound diet. *Journal of Nutrition,* **90**, 21-28.

Cahu C, Zambonino Infante J, Takeushi T (2003b) Nutritional components affecting skeletal development in fish larvae. *Aquaculture,* **155**, 245-258.

Chatain B (1994) Abnormal swimbladder development and lordosis in sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus auratus*). *Aquaculture,* **119**, 371-379.

Ciros-Pérez J, Gómez A, Serra M (2001) On the taxonomy of three sympatric sibling species of the Brachionus plicatilis (Rotifera) complex from Spain, with the description of B. ibericus n. sp. *Journal of Plankton Research,* **23**, 1311-1328.

Conceicao LEC, Yufera M, Makridis P, Morais S, Dinis MT (2010) Live feeds for early stages of fish rearing. *Aquac. Res.,* **41**, 613-640.

Copeman LA, Parrish CC, Brown JA, Harel M (2002) Effects of docosahexaenoic, eicosapentaenoic, and arachidonic acids on the early growth, survival, lipid composition and pigmentation of yellowtail flounder (Limanda ferruginea): a live food enrichment experiment. *Aquaculture,* **210**, 285-304.

Divanach P, Boglione C, Menu B, Koumoundouros G, Kentouri M, Cataudella S (1996) Abnormalities in finfish mariculture: an overview of the problem, causes and solutions. In: *Seabass and seabream culture: problems and prospects: handbook of contributions and short communications presented at the International Workshop on "Seabass and seabream culture: problems and prospects"* (ed by Chatain B*ea*). European Aquaculture Society, Oostende*,* pp. 45-66.

Dunaevskaya E (2010) Histological investigations of organs and tissues development of ballan wrasse larvae during ontogenesis. In: *Faculty of Biosciences and Aquaculture*. Bodø University College, Bodø.

EcoFish (2010) Summary of preliminary results. In: *URL: http://www.eco-fish.org/modules/news/article.php?storyid=4, accessed 25.04.12, last updated 15.04.10*.

Eidsvik E (2010) Co-feeding Atlantic cod larvae (*Gadus morhua)* with copepod nauplii (*Acartia tonsa)* and rotifers *(Brachionus plicatilis):* -effect on growth and osteological development. In: *Institute of Biology*. NTNU, Trondheim.

Espeland SH, Nedreaas K, Mortensen S, Agnalt A-L, Skiftesvik AB, Harkestad LS, Karlsbakk E, Knutsen H, Thangstad T, Jørstad K, Bjordal Å, Gjøsæter J (2010) Kunnskapsstatus leppefisk

Utfordringer i et økende fiskeri (foreløpig utgave pr 15.11.10). In: *Fisken og havet*. Havforskningsinstituttet, Bergen*,* pp. 36.

Evjemo JO, Olsen Y (1997) Lipid and fatty acid content in cultivated live feed organisms compared to marine copepods. *Hydrobiologia,* **358**, 159-162.

Evjemo JO, Reitan KI, Olsen Y (2003) Copepods as live food organisms in the larval rearing of halibut larvae (*Hippoglossus hippoglossus* L.) with special emphasis on the nutritional value.

Faustino M, Power DM (1999) Development of the pectoral, pelvic, dorsal and anal fins in cultured sea bream. *J. Fish Biol.,* **54**, 1094-1110.

FHF (2011) FHF-Prosjektet "Produksjon av berggylt" Nyhetsbrev 3. 1. Desember 2011, URL: http://www.fiskerifond.no/files/news/attach/nyhetsbrev_nr_3_desember_2011.pdf , accessed 30.04.12. Fiskeri- og havruksnæringens forskningsfond.

Field A (2009) *Discovering statistics using SPSS,* SAGE Publications Ltd, London.

Fjelldal PG, van der Meeren T, Jørstad KE, Hansen TJ (2009) A radiological study on vertebral deformities in cultured and wild Atlantic cod (Gadus morhua, L.). *Aquaculture,* **289**, 6-12.

Furuita H, Takeuchi T, Uematsu K (1998) Effects of eicosapentaenoic and docosahexaenoic acids on growth, survival and brain development of larval Japanese flounder (Paralichthys olivaceus). *Aquaculture,* **161**, 269-279.

Fushimi H (2001) Production of juvenile marine finfish for stock enhancement in Japan. *Aquaculture,* **200**.

Fyhn HJ (1989) First feeding of marine fish larvae: Are free amino acids the source of energy? *Aquaculture,* **80**, 111-120.

Gapasin RSJ, Bombeo R, Lavens P, Sorgeloos P, Nelis H (1998) Enrichment of live food with essential fatty acids and vitamin C: effects on milkfish (Chanos chanos) larval performance. *Aquaculture,* **162**, 269-286.

Gapasin RSJ, Duray MN (2001) Effects of DHA-enriched live food on growth, survival and incidence of opercular deformities in milkfish (Chanos chanos). *Aquaculture,* **193**, 49-63.

Gisbert E, Villeneuve L, Zambonino-Infante J, Quazuguel P, Cahu C (2005) Dietary phospholipids are more efficient than neutral lipids for long-chain polyunsaturated fatty acid supply in European sea bass (*Dicentrarchus labrax*) larval development. *Lipids,* **40**, 609-618.

Govoni J, Boehlert G, Watanabe Y (1986) The physiology of digestion in fish larvae. *Environmental Biology of Fishes,* **16**, 59-77.

Grøntvedt RN (2010) Oppdrett av berggylt - muligheter og utfordringer. *Norsk fiskeoppdrett,* **5**, 56-57.

Hamre K (2006) Nutrition in cod (Gadus morhua) larvae and juveniles. *ICES J. Mar. Sci.,* **63**, 267-274.

Hamre K, Sæle Ø (2011) Oppdrett av leppefisk til lakselusbekjempelse: Hva står på menyen? *Norsk fiskeoppdrett,* **9**.

Hansen GH, Olafsen JA (1999) Bacterial interactions in early life stages of marine cold water fish. *Microb. Ecol.,* **38**, 1-26.

Hansen MH (2011) Effects of feeding with copepod nauplii (*Acartia tonsa*) compared to rotifers (*Brachionus ibericus,* Cayman) on quality parameters in Atlantic cod (*Gadus morhua*) larvae. In: *Institute of Biology*. NTNU, Trondheim.

Harder W (1975) *Anatomy of fishes,* Schweizerbart'sche Verlagsbuchhandlung, Stuttgart.

Helland S, Bæverfjord G, Lein I (2012) Produksjon av berggylt (*Ballan wrasse*) - Statusrapport fra Sunndalsøra. In: *Leppeprod Dialogmøte*. Nofima, Stord.

Helland S, Lein I, Barr Y, Terjesen BF, Bæverfjord G (2009) Best management practice - Cod (*Gadus morhua* L.). In: *Control of malformations in fish aquaculture - Science and practice* (ed. by Bæverfjord G, Helland S, Hough C). Federation of European Aquaculture Producers, Liege, pp. 119-127.

Hjelde K, Helland S, Lein I, Bæverfjord G (2009) Guidelines for classification A. salmon, rainbow trout and cod, available from http://www.aquamedia.org/FileLibrary/24/Hjelde_FF.pdf , accessed 11.05.12. FineFish, Ghent.

Hoff FH, Snell TW (1987) *Plankton culture manual,* Florida Aqua Farms Inc., USA.

Hunter JR (1981) *Feeding ecology and predation of marine fish larvae,* Washington Sea Grant Program.

Imsland AK, Foss A, Koedijk R, Folkvord A, Stefansson SO, Jonassen TM (2006) Short- and long-term differences in growth, feed conversion efficiency and deformities in juvenile Atlantic cod (Gadus morhua) startfed on rotifers or zooplankton. *Aquac. Res.,* **37**, 1015-1027.

Izquierdo MS, Socorro J, Arantzamendi L, Hernandez-Crus CM (2000) Recent advances in lipid nutrition in fish larvae. *Fish Biochem. Physiol.,* **22**, 91-107.

Kanazawa A (1997) Effects of docosahexaenoic acid and phospholipids on stress tolerance of fish. *Aquaculture,* **155**, 129-134.

Kjørsvik E, Olsen C, Wold P-A, Hohne-Reitan K, Cahu CL, Rainuzzo J, Olsen AI, Øie G, Olsen Y (2009) Comparison of the dietary phospholipids and natural lipids on skeletal development and fatty acid composition in Atlantic cod (*Gadus morhua*). *Aquaculture,* **294**, 246-255.

Kjørsvik E, Pittman K, Pavlov D (2004) From fertilisation to the end of metamorphosis - Functional development. In: *Culture of Cold-Water Marine Fish* (ed. by Moksness E, Kjørsvik E, Olsen Y). Blackwell Publishing Ltd, Oxford.

Koedijk RM, Folkvord A, Foss A, Pittman K, Stefansson SO, Handeland S, Imsland AK (2010) The influence of first-feeding diet on the Atlantic cod Gadus morhua phenotype: survival, development and long-term consequences for growth. *J. Fish Biol.,* **77**, 1-19.

Koumoundouros G, Ashton C, Xenikoudakis G, Giopanou I, Georgakopoulou E, Stickland N (2009) Ontogenetic differentiation of swimming performance in Gilthead seabream (Sparus aurata, Linnaeus 1758) during metamorphosis. *J. Exp. Mar. Biol. Ecol.,* **370**, 75-81.

Koumoundouros G, Divanach P, Kentouri M (1999) Osteological development of the vertebral column and of the caudal complex in Dentex dentex. *J. Fish Biol.,* **54**, 424-436.

Koven W, Barr Y, Lutzky S, Ben-Atia I, Weiss R, Harel M, Behrens P, Tandler A (2001) The effect of dietary arachidonic acid (20 : 4n-6) on growth, survival and resistance to handling stress in gilthead seabream (Sparus aurata) larvae. *Aquaculture,* **193**, 107-122.

Kraul S, Brittain K, Cantrell R, Nagao T, Ogasawara A, Ako H, Kitagawa H (1993) Nutritional Factors Affecting Stress Resistance in the Larval Mahimahi Coryphaena hippurus. *Journal of the World Aquaculture Society,* **24**, 186-193.

Kurokawa T, Okamoto T, Gen K, Uji S, Murashita K, Unuma T, Nomura K, Matsubara H, Kim S-K, Ohta H, Tanaka H (2008) Influence of Water Temperature on Morphological Deformities in Cultured Larvae of Japanese Eel, Anguilla japonica, at Completion of Yolk Resorption. *Journal of the World Aquaculture Society,* **39**, 726-735.

Lall SP, Lewis-McCrea LM (2007) Role of nutrients in skeletal metabolism and pathology in fish - An overview. *Aquaculture,* **267**, 3-19.

Le Guellec D, Morvan-Dubois G, Sire JY (2004) Skin development in bony fish with particular emphasis on collagen deposition in the dermis of the zebrafish (Danio rerio). *Int. J. Dev. Biol.,* **48**, 217-231.

Lebour MV (1919) Feeding habits of some young fish. *J. Mar. Biol. Assoc. U.K.,* **12**, 9.

Lervik M (2007) Variasjon i egg og larvekvalitet i forhold til larveatferd for en blandet stamfiskgruppe av torsk (*Gadus morhus*). In: *Institute for Biology*. NTNU, Trondheim.

Lewis LM, Lall SP, Eckhard Witten P (2004) Morphological descriptions of the early stages of spine and vertebral development in hatchery-reared larval and juvenile Atlantic halibut (Hippoglossus hippoglossus). *Aquaculture,* **241**, 47-59.

Liu Y, Olaf Olaussen J, Skonhoft A (2011) Wild and farmed salmon in Norway--A review. *Marine Policy,* **35**, 413-418.

Loy A, Boglione C, Gagliardi F, Ferrucci L, Cataudella S (2000) Geometric morphometrics and internal anatomy in sea bass shape analysis (Dicentrarchus labrax L., Moronidae). *Aquaculture,* **186**, 33-44.

Loy BA, Boglione C, Cataudella S (1999) Geometric morphometrics and morpho-anatomy: a combined tool in the study of sea bream (Sparus aurata, sparidae) shape. *J. Appl. Ichthyol.,* **15**, 104-110.

Mazurais D, Darias MJ, Gouillou-Coustans MF, Le Gall MM, Huelvan C, Desbruyeres E, Quazuguel P, Cahu C, Zambonino-Infante JL (2008) Dietary vitamin mix levels influence the ossification process in European sea bass (Dicentrarchus labrax) larvae. *Am. J. Physiol.-Regul. Integr. Comp. Physiol.,* **294**, R520-R527.

Mazurais D, Glynatsi N, Darias MJ, Christodoulopoulou S, Cahu CL, Zambonino-Infante JL, Koumoundouros G (2009) Optimal levels of dietary vitamin A for reduced deformity incidence during development of European sea bass larvae (Dicentrarchus labrax) depend on malformation type. *Aquaculture,* **294**, 262-270.

McEvoy L, Næss T, Bell JG, Lie O (1998) Lipid and fatty acid composition of normal and malpigmented Atlantic halibut (*Hippoglossus hippoglossus)* fed enriched *Artemia:* a comparison with fry fed wild copepods. *Aquaculture,* **163**, 235-248.

Muller-Feuga A, Moal J, Kaas R (2003) The Microalgae of Aquaculture. In: *Live feeds in marine aquaculture* (ed. by Støttrup JG, McEvoy LA). Blackwell Science Ltd, Oxford.

Munk P (1995) Foraging behavior of larval cod (*Gadus morhua*), influenced by prey density and hunger. *Marine biology,* **122**, 205-212.

Nesse SI (2010) Kultivering av *Acartia tonsa*- nauplier til bruk i startfôring av marine fiskelarver. In: *Institutt for biologi*. NTNU, Trondheim.

Neuringer M, Anderson GJ, Connor WE (1988) The Essentiality of N-3 Fatty Acids for the Development and Function of the Retina and Brain. *Annual Review of Nutrition,* **8**, 517-541.

Noori F, Takami GA, Van Speybroeck M, Van Stappen G, Sorgeloos P (2011) Feeding Acipenser persicus and Huso huso (Acipenseriformes) larvae with Artemia urmiana nauplii enriched with HUFA and vitamin C: II. Effect on tolerance to shock exposure of environmental factors. *J. Appl. Ichthyol.,* **27**, 787-795.

O'Brien-MacDonald K, Brown JA, Parrish CC (2006) Growth, behaviour, and digestive enzyme activity in larval Atlantic cod (Gadus morhua) in relation to rotifer lipid. *ICES J. Mar. Sci.,* **63**, 275-284.

Olsen Y, van der Meeren T, Reitan KI (2004) First Feeding Technology. In: *Culture of Cold-Water Marine Fish* (ed. by Moksness E, Kjørsvik E, Olsen y). Blackwell Publishing Ltd, Oxford*,* pp. 279-336.

Ottesen OH, Bolla S (1998) Combined effects of temperature and salinity on development and survival of Atlantic halibut larvae. *Aquaculture International,* **6**, 103-120.

Park E-H, Lee S-H (1987) Scale growth and squamation chronology for the laboratory-reared hermaphroditic fish *Rivulus marmoratus* (Cyprinodontidae). *Japanse Journal of Ichtyology,* **34**, 476-482.

Power D (2009) The impact of dietary minerals on rainbow trout development. In: *Control of malformations in fish aquaculture* (ed. by Bæverfjord G, Helland S, Hough C). Federation of European Aquaculture Producers, Liege.

Puvanendran V, Brown JA (1999) Foraging, growth and survival of Atlantic cod larvae reared in different prey concentrations. *Aquaculture,* **175**, 77-92.

Rainuzzo JR, Reitan KI, J√∏rgensen L, Olsen Y (1994) Lipid composition in turbot larvae fed live feed cultured by emulsions of different lipid classes. *Comparative Biochemistry and Physiology Part A: Physiology,* **107**, 699-710.

Rajkumar M, Kumaraguru vasagam KP (2006) Suitability of the copepod, Acartia clausi as a live feed for Seabass larvae (Lates calcarifer Bloch): Compared to traditional live-food organisms with special emphasis on the nutritional value. *Aquaculture,* **261**, 649-658.

Reitan KI, Rainuzzo JR, Øie G, Olsen Y (1993) Nutritional effects of algal addition in 1st feeding of Turbot (*Scopthalmus maximus* L) larvae. *Aquaculture,* **118**, 257-275.

Ricker WE (1958) Handbook of computations for biological statistics of fish populations. *Bulletin of the Fisheries Research Board of Canada,* **119**, 1-30.

Sæle Ø, Pittman KA (2010) Looking closer at the determining of a phenotype? Compare by stages or size, not age. *J. Appl. Ichthyol.,* **26**, 294-297.

Sæle Ø, Solbakken JS, Watanabe K, Hamre K, Pittman K (2003) The effect of diet on ossification and eye migration in Atlantic halibut larvae (Hippoglossus hippoglossus L.). *Aquaculture,* **220**, 683-696.

Sæle Ø, Solbakken JS, Watanabe K, Hamre K, Power D, Pittman K (2004) Staging of Atlantic halibut (Hippoglossus hippoglossus L.) from first feeding through metamorphosis, including cranial ossification independent of eye migration. *Aquaculture,* **239**, 445-465.

Sargent JR, Tocher DR, Bell JG (2002) The lipids. In: *Fish nutrition* (ed. by Halver JE, Hardy RW). Academic press, California, USA, pp. 181-257.

Sfakianakis DG, Doxa CK, Kouttouki S, Koumoundouros G, Maingot E, Divanach P, Kentouri M (2005) Osteological development of the vertebral column and of the fins in Diplodus puntazzo (Cetti, 1777). *Aquaculture,* **250**, 36-46.

Shields RJ, Bell JG, Luizi FS, Gara B, Bromage NR, Sargent JR (1999) Natural copepods are superior to enriched Artemia nauplii as feed for halibut larvae (Hippoglossus hippoglossus) in terms of survival, pigmentation and retinal morphology: Relation to dietary essential fatty acids. *Journal of Nutrition,* **129**, 1186-1194.

Sire JY, Akimenko MA (2004) Scale development in fish: a review, with description of sonic hedgehog (shh) expression in the zebrafish (Danio rerio). *Int. J. Dev. Biol.,* **48**, 233-247.

Sire JY, Arnulf I (1990) The development of squamation in four teleostean fishes with a survey of the literature. *Japanse Journal of Ichtyology,* **37**, 133-143.

Skiftesvik AB, Bjelland RM (2003) Oppdrett av berggylt (*Labrus bergylta*). *Norsk fiskeoppdrett,* **28**, 5.

Skiftesvik AB, Opstad I (2010) Berggylt, en ny oppdrettsart? presentation in "Arbeidsseminar og artikkel om oppdrett av berggylt", URL http://www.fiskerifond.no/files/projects/attach/havforskningsinstituttet_ab_skiftesvik_og_i_opstad-08022010.pdf , accessed 04.05.12. Institute of Marine Research, Bergen.

Skjermo J, Salvesen I, Øie G, Olsen Y, Vadstein O (1997) Microbially matured water: a technique for selection of a non-opportunistic bacterial flora in water that may improve performance of marine larvae. *Aquaculture International,* **5**, 13-28.

Skjermo J, Vadstein O (1999) Techniques for microbial control in the intensive rearing of marine larvae. *Aquaculture,* **177**, 333-343.

Skogstad ME (2010) Effect of food concentration on growth, egg production and hatching success in *Acartia tonsa* (Copepoda: Calanoida) feeding on *Rhodomonas baltica*. In: *Institute of Biology*. NTNU, Trondheim.

Støttrup JG (2000) The elusive copepods: their production and suitability in marine aquaculture. *Aquac. Res.,* **31**, 703-711.

Støttrup JG, Bell JG, Sargent JR (1999) The fate of lipids during development and cold-storage of eggs in the laboratory-reared calanoid copepod, Acartia tonsa Dana, and in response to different algal diets. *Aquaculture,* **176**, 257-269.
Støttrup JG, McEvoy LA (2003) Live feeds in marine aquaculture. Blackwell Publishing, Oxford.
Støttrup JG, Richardson K, Kirkegaard E, Pihl NJ (1986) The cultivation of *Acarti tonsa* Dana for use as live feed for marine fish larvae. *Aquaculture,* **52**, 87-96.
Tago A, Yamamoto Y, Teshima S-i, Kanazawa A (1999) Effects of 1,2-di-20:5 phosphatidylcholine (PC) and 1,2-di-22:6 PC on growth and stress tolerance of Japanese flounder (Paralichthys olivaceus) larvae. *Aquaculture,* **179**, 231-239.
Tocher D, Sargent J (1984) Analyses of lipids and fatty acids in ripe roes of some Northwest European marine fish. *Lipids,* **19**, 492-499.
Vadstein O (1997) The use of immunostimulation in marine larviculture: possibilities and challenges. *Aquaculture,* **155**, 401-417.
van der Meeren T, Naas KE (1997) Development of rearing techniques using large enclosed ecosystems in the mass production of marine fish fry. *Reviews in Fisheries Science,* **5**, 367-390.
van der Meeren T, Olsen RE, Hamre K, Fyhn HJ (2008) Biochemical composition of copepods for evaluation of feed quality in production of juvenile marine fish. *Aquaculture,* **274**, 375-397.
von Herbing IH, Gallager SM (2000) Foraging behavior in early Atlantic cod larvae (Gadus morhua) feeding on a protozoan (Balanion sp.) and a copepod nauplius (Pseudodiaptomus sp.). *Marine biology,* **136**, 591-602.
Walne PR (1974) *Culture of bivalve molluscs: 50 years' experience at Conwy,* Fishing news (Books) Ltd., West Byfleet, Surrey.
Watanabe T, Kiron V (1994) Prospects in larval fish dietetics. *Aquaculture,* **124**, 223-251.
Wold PA, Hoehne-Reitan K, Cahu CL, Infante JZ, Rainuzzo J, KjØRsvik E (2009) Comparison of dietary phospholipids and neutral lipids: effects on gut, liver and pancreas histology in Atlantic cod (Gadus morha L.) larvae. *Aquaculture Nutrition,* **15**, 73-84.
Wold PA, Hoehne-Reitan K, Rainuzzo J, Kjorsvik E (2008) Allometric growth and functional development of the gut in developing cod Gadus morhua L. larvae. *J. Fish Biol.,* **72**, 1637-1658.
Yúfera, Fernández D, Pascual, Sarasquete, Moyano, Díaz, Alarcón, García G, Parra (2000) Towards an inert diet for first-feeding gilthead seabream Sparus aurata L. larvae. *Aquaculture Nutrition,* **6**, 143-152.
Yúfera M, Pascual E, Fernández-Díaz C (1999) A highly efficient microencapsulated food for rearing early larvae of marine fish. *Aquaculture,* **177**, 249-256.
Zambonino Infante J, Koumoundouros G, Tandler A (2009a) The influence of nutrition at the larval stages in marine fish. In: *Control of malformations in fish aquaculture - Science and practice* (ed. by Bæverfjord G, Helland S, Hough C). Federation of European Aquaculture Producers, Liege.
Zambonino Infante J, Pantelis K, Koumoundouros G, Tandler A (2009b) Recommendations on prevention of malformations in seabass and seabream. In: *Control of malformations in fish aquaculture Science and practice* (ed. by Bæverfjord G, Helland S, Hough C). Federation of European Aquaculture Producers (FineFish), Liege.
Zouiten D, Ben Khemis I, Slaheddin Masmoudi A, Huelvan C, Cahu C (2011) Comparison of growth, digestive system maturation and skeletal development

in sea bass larvae reared in an intensive or a mesocosm system. *Aquac. Res.,* **42**, 1723-1736.

APPENDIX 1

Recipe for formulated feed

Feed production and feed recipe, from Andres Nordgreen, Nofima

"The feed was produced at Nofima, Bergen, Norway. The ingredients were homogeneous mixed (Bjørn mixer) for a minimum of 20 minutes. The mixed ingredients were sieved through a sieve with a mesh opening of 0.6 mm (Allgaier 1200mm). The fraction with a particle size bigger than 0.6 mm was milled with a Retsch mill and thereby homogeneous mixed with the ingredients. The ingredient mixture was added 25 mg^{-kg} etoxiquin (0,05 g FEQ 500 per kg ingredient mixture). Etoxiquin was dissolved in 96 % ethanol and sprayed onto the mixture during continuous mixing. The feed was produced with a pilot scale scale twin-screw, co-rotating extruder (Wenger. The nozzle opening was 1.5 mm. After extrusion the diet was directly dried for 50-55minutes in a carousel dryer (GMBH) at 60 °C. Water content during drying was measured (HG 53 Halogen Moisture AnalyzerMettler Toledo). The feed was left overnight at ambient room temperature for cooling, before the feed was crushed/granulated on a Retsch mill and sieved (Allgaier) to the wanted particle sizes. The feed was packed in plastic bags and were stored at room temperature until transport."

Recipe	
	% (ww)
Fish meal LT [a]	47,662
Shrimp meal[b]	26
Wheat[c]	17,8
Soy lecithin[d]	3
Fish Oil[e]	2
Betafin[f]	1,5
Vitamin mix[g]	0,31
Mineral mix[h]	0,52
Monosodiumphosphate (24% P)i	2
Carop. Pink (10%)[j]	0,03
Taurine[k]	0,2

[a]LT-Fishmeal, Karmsund Fiskemel AS, Norway
[b]Shrimp powder (7411), Seagarden AS, Avaldsnes Norway
[c]Wheat grain (510130), Norgesmøllene AS, Nesttun Norway.
[d]Soylecithin GMO powder (20022), Agrosom, Mölln Germany
[e]NorSalmoil (Batch: 11304), Egersund Sildeoljefabrikk, Egersund Norway.
[f]Betafin S1, Danisco Animal Nutrition, Helsinki Finland.
[g] D3 3000 IE^{-kg}, E 160 mg^{-kg}, K3 20 mg^{-kg}, C 500 mg^{-kg}, B1 20 mg^{-kg}, B2 30 mg^{-kg}, B6 25 mg^{-kg}, B12 5 μg^{-kg}, B5 60 mg^{-kg}, Folic acid 10 mg^{-kg}, Niacin 200 mg^{-kg}, Biotin 1 mg^{-kg},
[h]Mn 30 mg^{-kg},, Mg 750 mg^{-kg}, Fe 60 mg^{-kg}, Zn mg^{-kg}, 120 mg^{-kg}, Cu 6 mg^{-kg}, K 800 mg^{-kg}, Se 0,3 mg^{-kg}.
[i]BOLIFOR® MSP, Yara AS, Norway
[j]Carophyll Pink (10 %),DSM, Basel Switzerland.
[k]Taurine, Sigma Aldrich

APPENDIX 2

Conwy Medium
Modified from (Walne, 1974).

$NaNO_3$ (Sodium Nitrate)	100.0 g
Na-EDTA (EDTA disodium salt)	45.0 g
H_3BO_3 (Boric Acid)	33.6 g
$NaH_2PO_4 \bullet 2H_2O$ (Sodium Phosphate, monobasic)	20.0 g
$FeCl_3 \bullet 6H_2O$ (Ferric Chloride, 6–hydrate)	1.3 g
$MnCl_2 \bullet 4H_2O$ (Manganous Chloride, 4–hydrate)	0.136 g
Vitamin B_1 (Thiamin HCl)	0.1 g
Vitamin B_{12} (Cyanocobalamin)	0.05 g
Trace Metal Solution *	1.0 ml
Distilled water (to make)	1 000.0 ml.

(Note: use 1 ml Conwy medium/litre of seawater)

Trace Metal Stock Solution *	
$ZnCl_2$ Zinc Chloride	2.1 g
$CoCl_2 \bullet 6H_2O$ (Cobalt Chloride, 6–hydrate)	2.1 g
$(NH_4)_6Mo_7O_{24} \bullet 6H_2O$ (Ammonium Molybdate, 4–hydrate)	2.1 g
$CuSO_4 \bullet 5H_2O$ (Copper Sulphate)	2.0 g
Distilled water	100.0 ml

(Note: acidify with 1 M HCl until solution is clear)

APPENDIX 3

Estimation of hatching percentage and density

Estimation of hatching percentage of *Acartia tonsa* eggs

The culture bottles containing the eggs was shaken, and 50 µl was removed with a pipette. This was diluted with seawater, to 1 ml. From this, another 50 µl was removed, and placed in a Petri dish as a droplet. A picture of the droplet was taken through a microscope, and the eggs were counted. This number was multiplied with the dilution factor, to give eggs ml^{-1} (X).

To estimate the volume (V), the bottle was weighed with eggs (B) and the weight of an empty bottle (B_0) was subtracted. This number was then divided by the seawater density (1.028):

$V= (B-B_0) / 1.028$

The number of eggs (N) in the bottle was then calculated using the following formula:

$N= X*V$

Where X is the number of egg ml^{-1}.

The Petri dish with the eggs was added 10 ml of distilled water, covered with parafilm and hatched under constant light for 48 hours. After hatching the nauplii was fixated with phytofix and counted under a microscope, using a pump. The hatching proportion (H) was estimated by dividing the number of hatched eggs (N) with the number of eggs in the sample. The resulting value was then multiplied with the total number of eggs in the bottle, to give an estimate of how many nauplii we could expect from each bottle.

Estimation of density of Copepod nauplii

A 10 L bucket was used to stir the water in the reservoir, so that the copepods would be evenly distributed in the water column. A small sample was poured into a cup. From this sample, 1 ml was removed, into a Petri dish. Three drops of phytofix and approximately 5 ml of fresh water was added. The sample was counted under a stereomicroscope, by removing nauplii with a pipette connected to a pump, and counting them simultaneously. This was done on 3 samples.
The density L^{-1}, as well as the total numbers of copepods in the reservoir was estimated. The density in the reservoirs varied from 40,000 to 120,000 L^{-1}, depending on hatching success.

APPENDIX 4

Bone staining

Procedures for bone staining ballan wrasse with Alizarin Red A, modified from (Kjørsvik et al., 2009) and (Hansen, 2011).

Larval size	**<10 mm**	**10-20mm**
Step 1 - Fixation		
• Fix in 10 % neutral formalin		
• Rinse in distilled water	2 x 5 min	2 x 10 min
Step 2 - Rehydration and bleaching		
• 95 % ethanol	2 x 30 min	2 x 1 hour
• 50 % ethanol	30 min	1 hour
• 15 % ethanol	30 min	1 hour
• Distilled water	30 min	1 hour
• Sodium borate buffer (working solution): Skip or store over night for both sizes.		

Bleach in 1:9 3% H_2O_2: 1 % KOH under strong light, until eyes become light brown and no pigments can be seen in the skin. This can take 30 minutes-6 hours. Watch every sample carefully; stop immediately if bubbles start forming in the bones in the vertebra or tail region.

Clear in trypsin buffer, to almost transparent	1-2 days	3-7 days
If the samples are submersed for more than 2 days, change buffer at least once.		
Step 3 – Staining		
• Cover specimen in 1% KOH, add drops of Alizarin solution until the solution turns purple.	20 hours	2 days
Step 4 – Second clearing		
A second clearing can be performed, if necessary, at this stage in the process.		
Step 5 – Preservation		
• Rinse in distilled water	5 min	10 min
• Rinse in 10% KOH until surplus colour is gone	2 x	2 x
• 40% glycerol in 1% KOH	2 days	2 days
• (Take pictures)		
• 70% glycerol in 1% KOH	1 day	1 day
• Store in 100% glycerol.For long term storage, add a few crystals of thymol.		

APPENDIX 5

Dry weight

The Copepod tank 1 is in bold letters, and are not included in the mean values for growth presented in the results.

DPH	Treatment	Tank	Mean DW (mg)
2	All	-	0.044
4	All	-	0.040
8	**Copepod**	**1**	**0.073**
	Copepod	2	0.072
	Copepod	3	0.076
	Cop7	1	0.080
	Cop7	2	0.064
	Cop7	3	0.071
	RotMG	1	0.051
	RotMG	2	0.039
	RotMG	4	0.042
	RotChl	1	0.043
	RotChl	2	0.053
	RotChl	3	0.052
12	**Copepod**	**1**	**0.139**
	Copepod	2	0.148
	Copepod	3	0.184
	Cop7	1	0.178
	Cop7	2	0.149
	Cop7	3	0.128
	RotMG	1	0.067
	RotMG	2	0.082
	RotMG	4	0.083
	RotChl	1	0.085
	RotChl	2	0.087
	RotChl	3	0.081
15	**Copepod**	**1**	**0.218**
	Copepod	2	0.255
	Copepod	3	0.284
	Cop7	1	0.220
	Cop7	2	0.178
	Cop7	3	0.188
	RotMG	1	0.133
	RotMG	2	0.119
	RotMG	4	0.101
	RotChl	1	0.124
	RotChl	2	0.112
	RotChl	3	0.093
21	**Copepod**	**1**	**0.442**
	Copepod	2	0.625
	Copepod	3	0.683
	Cop7	1	0.347
	Cop7	2	0.244
	Cop7	3	0.231
	RotMG	1	0.263
	RotMG	2	0.171
	RotMG	4	0.186
	RotChl	1	0.236
	RotChl	2	0.221
	RotChl	3	0.195
27	**Copepod**	**1**	**1.010**
	Copepod	2	1.277
	Copepod	3	1.223
	Cop7	1	0.882
	Cop7	2	0.777
	Cop7	3	0.757
	RotMG	1	0.712
	RotMG	2	0.761
	RotMG	4	0.710
	RotChl	1	0.556
	RotChl	2	0.575
	RotChl	3	0.572
33	**Copepod**	**1**	**2.494**
	Copepod	2	3.018
	Copepod	3	2.713
	Cop7	1	3.045
	Cop7	2	1.595
	Cop7	3	1.766
	RotMG	1	1.901
	RotMG	2	2.147
	RotMG	4	1.927
	RotChl	1	1.410
	RotChl	2	1.533
	RotChl	3	1.560
40	**Copepod**	**1**	**3.879**
	Copepod	2	4.998
	Copepod	3	4.767
	Cop7	1	4.906
	Cop7	2	3.858
	Cop7	3	4.077
	RotMG	1	4.179
	RotMG	2	4.640
	RotMG	4	5.010
	RotChl	1	4.762
	RotChl	2	4.158
	RotChl	3	4.842
47	**Copepod**	**1**	**7.351**
	Copepod	2	9.246
	Copepod	3	8.364
	Cop7	1	10.965
	Cop7	2	7.128
	Cop7	3	6.771
	RotMG	1	7.125
	RotMG	2	8.329
	RotMG	4	7.039
	RotChl	1	7.959
	RotChl	2	9.082
61	**Copepod**	**1**	**7.754**
	Copepod	2	19.594
	Copepod	3	19.678
	Cop7	1	21.520
	Cop7	2	12.064
	Cop7	3	17.662
	RotMG	1	16.063
	RotMG	2	11.353
	RotMG	4	13.892
	RotChl	1	14.722
	RotChl	2	11.190

APPENDIX 6

Daily weight increase
The Copepod tank 1 is in bold letters, and are not included in the mean values for growth presented in the results.

DPH	Treatment	Tank	%DWI
2-4	All	-	-4.62
4-12	**Copepod**	**1**	**16.77**
	Copepod	2	17.67
	Copepod	3	20.88
	Cop7	1	20.43
	Cop7	2	17.75
	Cop7	3	15.54
	RotMG	1	6.57
	RotMG	2	9.32
	RotMG	4	9.50
	RotChl	1	9.78
	RotChl	2	10.13
	RotChl	3	9.18
12-27	**Copepod**	**1**	**14.11**
	Copepod	2	15.91
	Copepod	3	15.57
	Cop7	1	13.09
	Cop7	2	12.13
	Cop7	3	11.94
	RotMG	1	11.48
	RotMG	2	11.98
	RotMG	4	11.46
	RotChl	1	9.66
	RotChl	2	9.91
	RotChl	3	9.87
27-40	**Copepod**	**1**	**10.90**
	Copepod	2	13.09
	Copepod	3	12.68
	Cop7	1	12.93
	Cop7	2	10.86
	Cop7	3	11.33
	RotMG	1	11.54
	RotMG	2	12.44
	RotMG	4	13.11
	RotChl	1	12.67
	RotChl	2	11.50
	RotChl	3	12.81
40-47	**Copepod**	**1**	**9.56**
	Copepod	2	13.21
	Copepod	3	11.60
	Cop7	1	16.00
	Cop7	2	9.08
	Cop7	3	8.28
	RotMG	1	9.07
	RotMG	2	11.53
	RotMG	4	8.88
	RotChl	1	10.81
	RotChl	2	12.92
47-61	**Copepod**	**1**	**0.38**
	Copepod	2	7.25
	Copepod	3	7.29
	Cop7	1	7.97
	Cop7	2	3.60
	Cop7	3	6.46
	RotMG	1	5.74
	RotMG	2	3.15
	RotMG	4	4.65
	RotChl	1	5.09
	RotChl	2	3.05
2-61	**Copepod**	**1**	**9.15**
	Copepod	2	10.88
	Copepod	3	10.88
	Cop7	1	11.05
	Cop7	2	9.97
	Cop7	3	10.68
	RotMG	1	10.50
	RotMG	2	9.86
	RotMG	3	10.23
	RotChl	1	10.34
	RotChl	2	9.83

APPENDIX 7

Results of handling stress on 29 and 36 dph

Stress test on 29 dph

Treatment (tank)	Dead after 1 hour	Dead after 24 hours	Number of larvae	Survivours
Copepod (1)	0	0	15	15
Copepod (2)	0	0	15	15
Copepod (3)	0	0	15	15
Cop7 (1)	1	1	15	13
Cop7 (2)	1	0	15	14
Cop7 (3)	0	1	15	14
RotMG (1)	0	0	16	16
RotMG (2)	4	1	15	10
RotMG (4)	1	1	15	13
RotChl (1)	3	2	14	9
RotChl (2)	0	3	14	11
RotChl (3)	1	2	15	12

Preliminary stress test on 36 dph, on RotMG larvae. Table includes seconds on paper and in air, and total duration of handling stress.

On paper	In air	Total (min.sec)	Number of larvae	Dead after 24 hours
15	0.35	0.50	15	0
15	0.45	1.00	15	0
15	0.55	1.10	15	0
15	1.15	1.30	15	0
15	1.25	1.40	15	0
15	1.45	2.00	15	0
15	2.00	2.15	15	0
15	2.15	2.30	15	0
15	2.25	2.40	15	0
30	2.30	3.00	10	0
30	3.00	3.30	10	1
30	3.30	4.00	10	0

APPENDIX 8

Behaviour analysis on 28 dph

Treatment	Tank	Larva	Swimming duration (min.)	Prey capture (no.)	Wriggle (no.)	Burst (no.)
Copepod	1	1	1.64	3	0	0
Copepod	1	2	0.09	1	0	0
Copepod	1	3	1.97	0	1	0
Copepod	1	4	1.94	0	2	0
Copepod	1	5	1.15	6	4	0
Copepod	1	6	0.09	0	0	0
Copepod	1	7	0.19	1	2	0
Copepod	1	8	1.58	1	3	1
Copepod	1	9	0.64	2	4	0
Copepod	1	10	0.17	5	0	0
Copepod	2	1	1.40	3	0	0
Copepod	2	2	0.42	5	1	3
Copepod	2	3	0.27	2	0	0
Copepod	2	4	0.04	1	2	0
Copepod	2	5	0.21	1	0	0
Copepod	2	6	0.11	2	0	0
Copepod	2	7	0.01	0	0	1
Copepod	2	8	0.09	2	0	0
Copepod	2	9	0.45	3	0	0
Copepod	2	10	0.39	3	3	0
Copepod	3	1	0.21	2	1	0
Copepod	3	2	0.92	1	0	0
Copepod	3	3	1.19	3	10	0
Copepod	3	4	0.37	3	0	0
Copepod	3	5	0.39	3	0	0
Copepod	3	6	0.30	1	0	0
Copepod	3	7	0.10	0	0	0
Copepod	3	8	0.11	3	0	0
Copepod	3	9	0.57	2	8	1
Copepod	3	10	0.17	1	0	0
Average			0.57	2.00	1.37	0.20
Cop7	1	1	1.33	3	2	2
Cop7	1	2	1.33	4	0	0
Cop7	1	3	1.15	3	4	0
Cop7	1	4	0.09	0	0	0
Cop7	1	5	1.03	2	0	10
Cop7	1	6	1.66	0	0	0
Cop7	1	7	0.81	5	7	0
Cop7	1	8	0.60	1	6	2
Cop7	1	9	1.12	0	0	0
Cop7	1	10	0.11	4	3	0
Cop7	2	1	0.43	0	2	1
Cop7	2	2	1.05	4	6	0
Cop7	2	3	0.54	6	3	0
Cop7	2	4	1.59	0	1	0
Cop7	2	5	1.05	0	10	0
Cop7	2	6	1.09	1	1	0
Cop7	2	7	0.74	0	0	0
Cop7	2	8	0.05	4	1	0
Cop7	2	9	0.19	2	0	0
Cop7	2	10	0.14	2	1	0
Cop7	3	1	0.74	2	2	0
Cop7	3	2	0.41	3	1	0
Cop7	3	3	1.33	1	0	0
Cop7	3	4	1.96	1	4	2
Cop7	3	5	0.67	0	0	0
Cop7	3	6	1.90	5	1	0
Cop7	3	7	0.30	1	1	0
Cop7	3	8	0.43	2	3	0
Cop7	3	9	0.07	0	0	0
Cop7	3	10	0.10	2	0	0
Average			0.80	1.93	1.97	0.57

RotMG	1	1	0.63	0	3	0
RotMG	1	2	2.00	0	1	0
RotMG	1	3	1.11	0	5	0
RotMG	1	4	1.04	0	2	0
RotMG	1	5	1.67	0	3	0
RotMG	1	6	0.08	0	0	0
RotMG	1	7	0.38	0	1	0
RotMG	1	8	1.87	0	1	0
RotMG	1	9	1.38	1	2	0
RotMG	1	10	1.92	0	2	0
RotMG	2	1	1.75	1	5	0
RotMG	2	2	1.89	0	0	0
RotMG	2	3	2.00	0	6	5
RotMG	2	4	0.65	0	0	0
RotMG	2	5	2.00	1	1	1
RotMG	2	6	2.00	0	7	0
RotMG	2	7	0.91	0	0	0
RotMG	2	8	2.00	4	13	12
RotMG	2	9	1.78	2	10	8
RotMG	2	10	1.96	0	7	0
RotMG	4	1	1.07	2	12	4
RotMG	4	2	1.44	0	5	0
RotMG	4	3	1.68	0	0	0
RotMG	4	4	0.16	0	3	0
RotMG	4	5	0.35	0	4	0
RotMG	4	6	1.06	0	1	0
RotMG	4	7	1.65	1	1	0
RotMG	4	8	2.00	2	0	1
RotMG	4	9	1.85	1	17	2
RotMG	4	10	0.99	0	1	0
Average			1.38	0.50	3.77	1.10

RotChl	1	1	0.42	0	1	1
RotChl	1	2	1.41	0	1	0
RotChl	1	3	1.63	0	0	1
RotChl	1	4	1.33	0	1	0
RotChl	1	5	1.43	0	0	0
RotChl	1	6	1.91	0	0	0
RotChl	1	7	1.92	0	0	1
RotChl	1	8	2.00	0	3	0
RotChl	1	9	0.34	0	0	0
RotChl	1	10	2.00	0	3	0
RotChl	2	1	1.41	0	0	0
RotChl	2	2	0.12	0	0	0
RotChl	2	3	1.35	0	1	0
RotChl	2	4	1.50	1	12	3
RotChl	2	5	1.22	1	7	0
RotChl	2	6	1.39	1	1	0
RotChl	2	7	2.00	0	0	0
RotChl	2	8	1.97	0	0	0
RotChl	2	9	1.64	0	6	0
RotChl	2	10	0.13	0	0	1
RotChl	3	1	1.09	0	7	1
RotChl	3	2	1.31	0	0	0
RotChl	3	3	1.92	0	0	0
RotChl	3	4	0.28	0	0	0
RotChl	3	5	1.46	0	1	0
RotChl	3	6	0.36	0	7	1
RotChl	3	7	0.32	0	0	1
RotChl	3	8	1.24	0	1	0
RotChl	3	9	0.02	0	0	0
RotChl	3	10	0.38	2	4	0
Average			1.18	0.17	1.87	0.33