NTNU – Trondheim
Norwegian University of
Science and Technology

The effect of different live prey types on growth and muscle development, in ballan wrasse (*Labrus bergylta*, Ascanius 1767) larvae

Martin Berg

Marine Coastal Development
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Supervisor: Elin Kjørsvik, IBI
Co-supervisor: Gunvor Øie, SINTEF
Trina Galloway, SINTEF

Norwegian University of Science and Technology
Department of Biology

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Martin Vinje Berg

Abstract

Sea lice have been causing problems in Norwegian salmon farming industry for decades; increasing mortality and reducing the welfare of farmed and wild fish. Various chemical agents have been used to combat sea lice, but increased tolerance and resistance have shifted the focus more onto biological combatants in the form of cleaner wrasse. The ballan wrasse (*Labrus bergylta*) is the most promising species for aquaculture, but problems with poor larval growth and survival suggests that the feeding practices in use today remain suboptimal.

At present, the feeding regimes consist of enriched rotifers, followed by *Artemia sp.* and weaning onto formulated feed. However, the nutritional profile of copepods is known to be highly beneficial for marine fish larvae, and increased larval growth, survival and development have been reported when copepods are administered as live feed. In the present study the fish larvae were fed intensively cultivated copepod nauplii (*Acartia tonsa*) in order to evaluate the effects on somatic growth, survival and muscle development, compared to when being fed rotifers (*Brachionus ibericus*). Four different feeding regimes were used; the Copepod treatment received copepod nauplii 4-30 dph, while larvae from the Cop7 treatment received copepod nauplii 4-10 dph, followed by short-term enriched rotifers (11-30 dph). Larvae from the treatments RotMG and RotChl were given short-term enriched and cultivated rotifers respectively, 4-30 dph. Larvae from all treatments went through a transition phase onto *Artemia sp.* 24-30 dph, and were weaned onto formulated feed 40-50 dph. The larval somatic growth and survival were studied from start to end (2-61 dph), while muscle development was studied until 21 dph, in order to address the direct effects of copepod nauplii vs. rotifers.

The results in the present study revealed that larvae fed copepod nauplii achieved higher growth rates initially, and greater dry weight already at 8 dph (also at the end of the experiment) compared to larvae fed rotifers. From 4-21 dph the increase in muscle mass was significantly larger in larvae from the Copepod treatment; accounted for by higher hyperplastic and hypertropic growth. The number of red and white fibres, size of deep white fibres, and total muscle area was found to correlate strongly with larval standard length.

Contents

1. Introduction

1.1 Background

Norwegian salmon farmers became the first to experience disease outbreaks caused by sea lice, among their farmed fish (Pike, 1989). These ectoparasitic organisms represent a serious threat both to animal welfare and economy, in North Atlantic salmon farming industry. Wounds inflicted by sea lice often cause osmoregulatory stress upon the host, and in severe cases - death (Muncaster et al., 2010). Due to vast expansion of Norwegian aquaculture over the past decades, sea lice have kept its position as a pest-organism for the salmon farmers (Heumann et al., 2012). Heavy sea lice infections affect wild populations as well as farmed animals, because infected individuals inside sea cages can function as contamination pathways to the outside (Bjørn & Finstad, 2002 ; Frazer, 2009).

Despite being a continuous threat, the infection levels of sea lice have greatly been reduced due to treatment and management strategies in place today (Pike & Wadsworth, 2000). While effective vaccination procedures are yet unsuccessful, the common way of controlling sea lice have been chemical treatment. However, the discharge of chemicals into the environment have raised speculations, regarding consequences for non-target species and the environment itself (Dempster et al., 2011). This issue has inspired Norwegian salmon farmers to search for alternative solutions in the war against sea lice, and one promising approach includes biological control.

Biological control involves using other living organisms to control pests and weeds, a practice that has been used in terrestrial agriculture since the 1930s (Pimentel, 1991 ; Treasurer, 2002). However, the first reports of biological control in aquaculture came in 1973, and involved cleaning activity by certain species of wrasse (Potts, 1973). Later, cleaning of Atlantic salmon (*Salmo salar*) by wrasse were observed in aquaria in 1987, and this lead to successful experimental trials in sea cages the following years (Bjordal, 1990 ; Treasurer, 2002). To date, the use of cleaner fish remains the only successful technique for controlling sea lice biologically (Treasurer, 2002). Among farmers, the interest and usage of cleaner fish have increased the last couple of years, after being down to a minimum between 2004 and 2007. In this period, a feed based delousing agent known as “Slice® vet” (emamectin benzoate) was the preferred choice against sea

lice, and dominated the Norwegian aquaculture industry (Skiftesvik, 2009). However, increased tolerance to emamectin benzoate was reported in several areas, and thus reduced the effectiveness of Slice® vet (Intervet, 2010). In Norway, the use of cleaner fish is therefore considered as a counteractive measure to combat chemically resistant sea lice (Skiftesvik, 2009). At present, Norwegian salmon farmers are focusing resources at several species of cleaner fish, but particularly the ballan wrasse (*Labrus berggylta*).

Ballan wrasse is a common inhabitant along the east Atlantic coast, from Morocco in south to Trondheim in north. It is the largest of the European wrasses (up to 60 cm), and has proven to be an effective cleaner of sea lice from farmed Atlantic salmon (Muncaster et al., 2010). Ballan wrasse is the preferred species for several reasons, especially fast growth and higher activity at low temperatures compared to other wrasses. It is also more tolerant to handling stress, and is generally the species that has seen the most progress regarding cultivation (Kvenseth & Øien, 2009). The natural diet of ballan wrasse (adults) consists of a wide variety of invertebrate organisms, including gastropods, bivalves, molluscs, crustaceans, hydroids, bryozoans, zooplankton, ectoparasites and algae (Treasurer, 2002 ; Westneat & Alfaro, 2005). The ballan wrasse is also a sequential hermaphrodite, meaning that it changes sex during its life cycle. It starts life as female, and changes into male after several years, when it has reached a size around 30 centimetres (EFSA, 2010).

Today the majority of ballan wrasse for louse control is fished from the wild, but the stocks are limited and their numbers are uncertain (Nodland, 2009). Due to this, the wild stock of ballan wrasse is vulnerable to overfishing. In addition, fishing ballan wrasse from the wild is unpredictable, because it offers no guarantee to obtain rightly sized individuals at the right time of the year. At present there is a growing interest in commercializing ballan wrasse farming in Norway, to provide a sustainable supply of cleaner fish to the salmon farmers (Muncaster et al., 2010). As the first in the field, Marine Harvest Labrus (Bergen) started their ballan wrasse farming in 2009, and have made good progression since then. However the challenges are many, and one of the main bottlenecks in production seems to be high mortalities during first-feeding and weaning onto formulated feed (Nodland, 2009). As of yet, little is known about the nutritional requirements of ballan wrasse larvae. The traditional live feed used for intensive cultivation of marine fish larvae have proved to be suboptimal for ballan

wrasse (Skiftesvik, 2009), and it is therefore important to explore alternatives when it comes to feeding regimes and live feed organisms. An optimal diet is important to ensure normal development, and to ensure that individuals are physically fit to function as lice removers inside salmon cages.

1.2 Live feed organisms

The most common live feed used in rearing of marine fish larvae are rotifers (*Brachionus spp.*) and the brine shrimp (*Artemia sp.*) (Evjemo & Olsen, 1997). These organisms are easily mass cultivated, which makes them the most convenient of all live preys currently available (Lie et al., 1997 ; Conceição et al., 2010). However, rotifers and *Artemia* are known to be poor in several nutrients required for normal growth and development, compared to marine zooplankton (Sargent et al., 1999b ; Bell et al., 2003).

Zooplankton constitutes a major part of the natural diet for many fish species (Evjemo et al., 2003). Marine fish larvae reared on zooplankton (mainly harvested copepods) have been characterized by high growth and survival, as well as normal pigmentation patterns and little occurrence of skeletal deformities (Berg, 1997 ; Evjemo & Olsen, 1997 ; van der Meeren et al., 2008). However, the copepods used in today's fish hatcheries are usually harvested from the sea, or grown in outdoor ponds under semi-intensive conditions (Hamre et al., 2002 ; Evjemo et al., 2003). Outdoor systems are highly influenced by season, so there is no year-round production (Støttrup, 2000 ; Evjemo et al., 2003). In addition the production volumes are unpredictable, and there is little to no control of parameters such as copepod size, nutritional value and species composition, which may affect the growth and survival of reared fish larvae (Berg, 1997). Outdoor systems are also at risk of parasitic contamination, and some copepod species are known to be hosts of several teleost parasites (Støttrup, 2000).

Methods for intensive cultivation of the copepod *A. tonsa* have been developed, which involves harvesting and storing eggs that can be hatched whenever necessary (Støttrup et al., 1986). Intensive production can secure a stable supply of copepods regardless of season, and offers a controlled environment compared to outdoor, semi-intensive systems. To date, intensive production of copepods remain small-scale (Støttrup et al., 1986), but due to improvements seen in fish larvae fed on copepods, there is an

increasing interest to make it viable in commercial scale (Overrein, 2010). In fact, SINTEF Fisheries and Aquaculture are doing pioneer work when it comes to up-scaling and further developing intensive copepod production; thereby making first feeding experiments with cultivated copepods possible (Gunvor Øie, SINTEF, pers. Comm.).

Copepods are believed to cover the nutritional requirements of most marine fish larvae, and may therefore function as a nutritional reference when producing rotifers and *Artemia* (Evjemo & Olsen, 1997). Enrichment techniques are being used to increase the nutritional value of rotifers and *Artemia*, but still they remain inferior to copepods (van der Meeren et al., 2008 ; Conceição et al., 2010). The main constituents of fish eggs are proteins and lipids (Watanabe, 1982), and these macronutrients are paramount for normal growth and development in fish larvae (Sargent et al., 1999a ; Hilton et al., 2008). Protein and lipid composition is also the main bottleneck regarding quality of rotifers and *Artemia*.

1.3 The importance of macronutrients

1.3.1 Lipids

Lipids have a wide range of functions, and are known to have great importance to fish. Certain lipid classes are neutral (triacylglycerols and wax esters), and function as energy storage, while others are polar (sterols and phospholipids), and play a central role in cell membrane formation and membrane fluidity control (Sargent et al., 1999a ; Hilton et al., 2008). Lipids also supply essential, highly unsaturated fatty acids (HUFAs) that fish larvae require for normal development and growth. These fatty acids are mainly eicosapentaenoic acid [20:5n-3; EPA], docosahexaenoic acid [22:6n-3; DHA] and arachidonic acid [22:4n-6; ARA] (Watanabe, 1982 ; Ajiboye et al., 2011), and can be part of both phospholipids and neutral lipids. Evidence indicates that developing marine fish larvae cannot synthesize sufficient amounts of phospholipids *de novo*, and therefore have an absolute requirement for dietary phospholipids (Bell et al., 2003).

When cultivated copepods (*A. tonsa*) are fed an adequate diet of microalgae (*Rhodomonas baltica*), they have proved to be excellent sources of HUFAs, compared to rotifers and *Artemia* (Støttrup & Jensen, 1990 ; Overrein, 2010 ; Ajiboye et al., 2011). The total lipid content of copepods is relatively low, but their fraction of phospholipids

is high compared to enriched rotifers and *Artemia* (McEvoy et al., 1998 ; van der Meeren et al., 2008). Also, the HUFAs in copepods are mainly located in the phospholipid fraction, while enriched rotifers and *Artemia* mainly incorporate the HUFAs in neutral storage lipids (Coutteau & Mourente, 1997). It has been shown that HUFAs in phospholipids are more available to fish larvae, and easier digested, compared to HUFAs supplied through neutral lipids (Coutteau & Mourente, 1997 ; Izquierdo et al., 2000 ; Kjørsvik et al., 2009). This is proposed as one of the main reasons why copepods remain nutritionally superior, compared with rotifers and *Artemia* (Bell et al., 2003).

1.3.2 Proteins

Proteins and free amino acids (FAA) are important for growth in all fish (Hilton et al., 2008), because growth is mainly a result of protein deposition within muscle tissue (Carter & Houlihan, 2001). Marine pelagic fish eggs contain a large pool of FAA at fertilization, which function as an important energy source for fish larvae during the yolk-sac stage (Rønnestad et al., 1999). It seems that FAA may be an absolute necessity to support further growth and survival at first feeding, because the ability to break down protein is low in early fish larvae. This is largely due to the fact that marine pelagic fish larvae have immature digestive systems (Govoni et al., 1986 ; Helland et al., 2003). In addition to FAA, live feed organisms usually contain a large fraction of soluble, intact proteins. It has been suggested that soluble proteins are more available for larval digestion and absorption than insoluble proteins (Srivastava et al., 2006). Fish larvae in general have a great potential for growth (Osse et al., 1997), as demonstrated by newly hatched larvae of turbot, mackerel, anchovy, herring and cod, increasing their dry body mass 100-fold in three weeks (Nellen, 1986). The protein requirements of fish larvae are usually higher than that of adults, due to rapid growth (Hamre et al., 2003).

Copepods are known to contain higher amounts of protein and FAA than rotifers and *Artemia* (Helland et al., 2003 ; Conceição et al., 2010). Rotifers contain relatively high amounts of protein, but approximately 50 % exists in insoluble form. Therefore, the availability of rotifer protein to marine fish larvae is reduced (Srivastava et al., 2006). *Artemia* are usually enriched with lipids in an attempt to cover their deficiency of HUFAs, and this results in high amounts of neutral lipids, at the expense of protein (Conceição et al., 2010).

Since a large part of growth in fish is due to protein deposition in muscle tissue, it seems likely that a high protein diet (copepods) should have a marked effect on larval growth and muscle development, compared to rotifers and *Artemia*. The majority of studies on growth and muscle development in fish larvae have focused on abiotic factors (such as the effect of temperature), while fewer studies have focused on nutritional effects (Galloway et al., 1999 ; Kjørsvik et al., 2011).

1.4 Growth and development of muscle tissue

Studies have shown that muscle growth in early life may affect the rate of growth in later life stages, as well as the ultimate size (Weatherley, 1990). The larvae of teleost fish spend most of their resources into developing organs associated with food intake and swimming (Osse et al., 1997). Fast acquisition of a greater size is very important to fish larvae, as it allows faster swimming, increased foraging capacity and better avoidance to predators, which all have a great influence on survival (Galloway et al., 1998). Skeletal swimming musculature is the predominant tissue component in fish, and represents approximately 20% and 60 % of total body mass, in newly hatched larvae and adult fishes, respectively (common carp) (Alami-Durante, 1990 ; Osse & van den Boogaart, 1995).

1.4.1 Fibre types and organization

The skeletal swimming musculature is organized into segments called myotomes, and comprises the main edible tissue in fish (Bone, 1978 ; Weatherley et al., 1988). The building blocks within myotomes are different types of muscle fibres, and the main fibre types in teleost skeletal musculature are red, white and intermediate (pink) fibres. Red fibres are also called slow fibres, because they power slow-paced activity such as migration and foraging. Red fibres are highly vascular and utilize aerob metabolism (Johnston, 1999). White fibres account for more than 90 % of the muscle mass, and are classified as fast fibres. They utilize anaerobic metabolism to generate power for high speed swimming, a necessity during escape and predation behavior (Bone, 1978 ; Altringham & Johnston, 1990). Pink muscle fibres have characteristics of both red and white fibres, and operate at intermediate swimming speeds. They also contain a distinct type of myosin (Scapolo & Rowlerson, 1987).

When comparing the organization of skeletal muscle fibres in fish and terrestrial vertebrates, the different fibre types show greater extent of segregation in fish (Bone, 1978). There is also a difference between larval stage and adult form when it comes to skeletal muscle fibre organization in fish (Galloway, 1999). At hatching most fish larvae have a superficial monolayer of fibres, surrounding an inner muscle mass. This inner mass develops into white fibres, while the superficial layer will become red fibres (El-fiky et al., 1987). The extent of fibre differentiation at hatching varies between species, and is influenced by factors such as body length, larval maturity and locomotion capacity (Galloway, 1999). In adult fish the red fibres tend to cluster laterally near the horizontal septum, where they are separated from the inner bulk of white fibres by a pink or intermediate zone. Red fibres rarely exceed 10 % of the total cross-sectional area (Johnston, 1999).

The maximum diameter of mature red and white muscle fibres is estimated to be 50 and 200 μm respectively, so growth to a large body size can only be attained by recruitment of new fibres (Weatherley et al., 1988). As an example, in Atlantic salmon (*Salmo salar*) the number of white fibres per myotome is around 5000 at hatching, 180 000 at smoltification and over 1 000 000 in an adult weighing 4 kg (Johnston, 1999). The number of muscle fibres recruited to reach a given girth varies between species and different strains of the same species (Weatherley et al., 1979), and can be influenced by environmental factors such as temperature and diet (Kiessling et al., 1991).

1.4.2 Growth mechanisms

Muscle growth in fish involves two principal mechanisms. Hypertrophy is the term for an increase in fibre size, while hyperplasia is the term for fibre recruitment (increase in number) (Rowlerson & Veggetti, 2001a). Muscle development in fish is different from mammals, in that fish muscle continues to grow throughout the animal's life, both by hypertrophy and hyperplasia. In mammals, muscle growth occur mainly by hypertrophy of the fibres formed prior to birth (Rowe & Goldspink, 1969).

The development of muscle tissue is called myogenesis, and involves some fundamental events common to all vertebrates. These events include specification of stem cells to a myogenic fate (myoblasts), proliferation, cell cycle exit, differentiation, migration and fusion (Rescan, 2001 ; Rescan et al., 2001). Studies have shown that red and white

muscle fibres originate from different populations of myoblasts (Devoto et al., 1996 ; Blagden et al., 1997). The muscle fibres differentiate during or soon after formation, and begins producing contractile proteins (myofibrils), which eventually constitute the majority of the fibre volume (Koumans & Akster, 1995). When muscle fibres expand (hypertrophy) they absorb nuclei from myoblasts, in order to maintain a near constant nuclear to cytoplasmic ratio (Koumans et al., 1994). New fibres (hyperplasia) form on the surface of existing fibres by the fusion of myoblasts, forming a multinucleated myotube which matures into a new muscle fibre (figure 1.1).

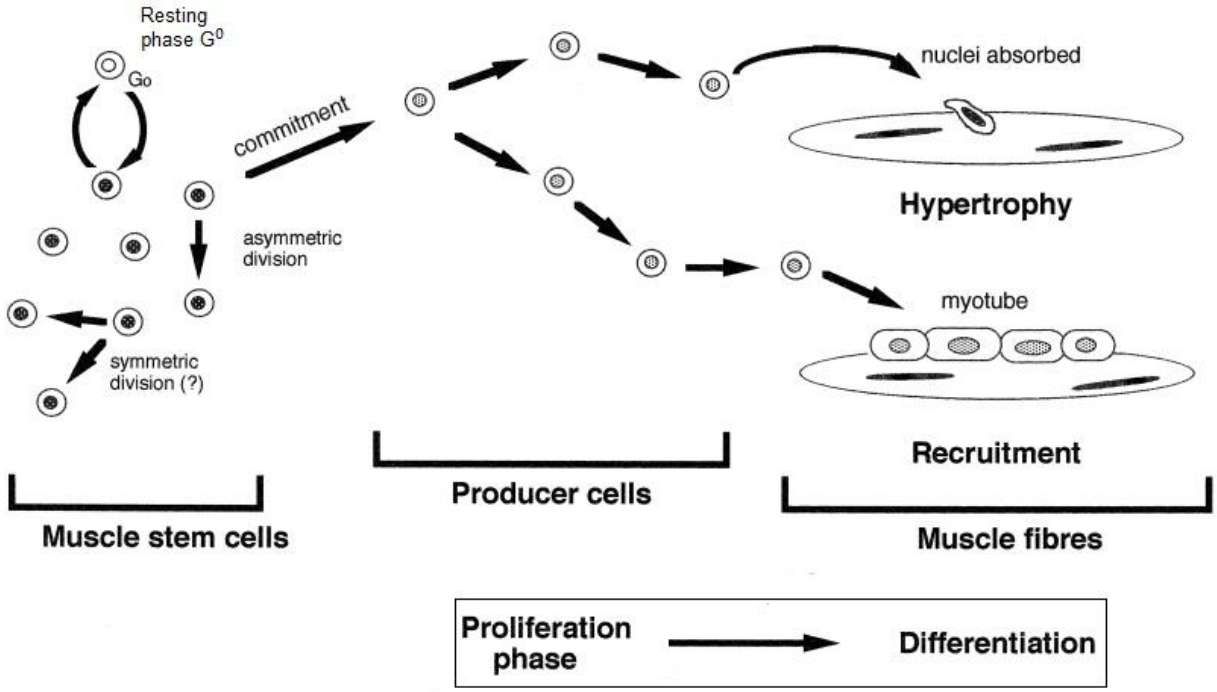

Figure 1.1: A model for the cellular basis of muscle growth in fish. Muscle stem cells exist either in a resting cycle (G^0), or become activated to undergo asymmetric division, regenerating the original stem cell and a "producer cell". Producer cells are myoblasts committed to terminal differentiation, and their fate is controlled by a balance of proliferation- and differentiation signals. The myoblasts are either absorbed within existing muscle fibres, or they fuse together on the surface of existing fibres forming a myotube, which matures into a new muscle fibre (Johnston, 1999).

In teleosts there are three main phases of myogenesis. The first wave of myogenesis takes place during the embryonic stage, when stem cells become committed to a myogenic fate and forms the initial muscle fibres (Johnston, 2006). A second phase of myogenesis begins late in the embryonic stage, and continues during (at least parts of) the larval stage (Rowlerson & Veggetti, 2001a). This phase completes the formation of the main muscle layers which were initiated in the previous phase, while also generating new white fibres along distinct germinal zones at the dorsal, ventral and lateral borders of the myotome (Matsuoka & Iwai, 1984 ; Akster et al., 1995). The generation of new fibres along a distinct layer has given rise to the name "stratified" hyperplasia, and is the main mechanism for increased fibre number after hatching (Johnston, 2006). A third phase of myogenesis takes place later, in fish that grow to a large final size (Rowlerson & Veggetti, 2001a). Myoblasts located between the larger white fibres become activated, giving rise to a mosaic pattern of large and small fibre diameters (known as mosaic hyperplasia) (Johnston, 2006). Mosaic hyperplasia is the main mechanism for increasing white fibre number in the juvenile and adult stages of most species, continuing until around 40 % of maximum fish length (Weatherley et al., 1988). Mosaic hyperplasia is absent in species which remain small (Rowlerson & Veggetti, 2001a).

As previously mentioned, the nutritional effects on muscle development in fish larvae remains poorly documented, except for a few species including Atlantic cod (*Gadus morhua*, African catfish (*Clarias gariepinus*), common carp (*Cyprinus carpio*), pike perch (*Sander lucioperca*) and Pacu (*Piaracrus mesopotamicus*) (Akster et al., 1995 ; Alami-Durante et al., 1997 ; Galloway et al., 1999 ; Ostaszewska et al., 2008 ; Leitao et al., 2011). Common to all these studies is that different diets were administered to fish larvae, in order to evaluate the effects on somatic- and muscle growth. Further, the diets which gave the highest somatic growth rates were associated with the greatest extent of white fibre hyperplasia. With the goal of commercializing ballan wrasse as a sea lice combatant, the dietary effects on its growth and muscle development requires further investigation; particularly because of the indications that early muscle development affects growth and performance in later life stages.

1.5 Aim

The aim of this study was to investigate the effects of different live prey types, in terms of somatic growth and muscle development, in ballan wrasse larvae. The different feeding regimes were composed according to what is commercially used (short-term enriched rotifers), and according to what is thought as optimal (copepod nauplii) and suboptimal (cultivated rotifers). The hypothesis was that larvae fed copepod nauplii (*A. tonsa*) would achieve better survival and growth, and increased muscle development compared to larvae fed rotifers (*B. ibericus*). Muscle development was evaluated in larvae at 4, 8 and 21 dph (in order to study the direct effects of copepod nauplii vs. rotifers) based on the following histological analyzes:

- Muscle mass of red and white fibres, measured as total cross-sectional area covered by red and white fibres in one bilateral half of the myotome
- Hyperplasia; measured as increase in number of red and white fibres in one bilateral half of the myotome
- Hypertrophy; measured as increase in cross-sectional area of individual white fibres, both deep white fibres and recruitment fibres in one bilateral half of the myotome

If the proposed hypothesis is backed up by the results, copepods are more optimal than rotifers as live feed for ballan wrasse larvae. Such information will be valuable for the farmers of both ballan wrasse and salmon, because optimal feeding of ballan wrasse would produce healthy, physically fit individuals, which would have the best terms to function as effective lice removers within salmon cages. The number of skeletal deformities would be reduced; further improving the health and functionality of the fish. Optimal feeding would also increase the effectiveness and predictability of production, and thus shorten the time needed for the fish to reach commercially desired size. Consequences may further manifest in economic profit, as well as increased animal welfare within salmon cages (due to earlier onset of ballan wrasse cleaning activity).

2. Materials and methods

The first-feeding experiment and analyses took place at Sealab (Trondheim), in the laboratories of SINTEF Fisheries and Aquaculture and NTNU. The experiment was part of the project "Produksjon av berggylte", a co-operation between SINTEF, NTNU, Nofima, NIFES and the Institute of Marine Research. Three other master students were involved in the experiment, namely Maren Ranheim Gagnat (organ development), Maria Oknes Sørøy (functional development) and Martin Almli (metabolomics). The experiment lasted from late June to August 2011.

2.1 Larval rearing and experimental setup

The ballan wrasse larvae used in the experiment were supplied by Marine Harvest Labrus (Bergen). Upon arrival, the fish larvae (2 days post hatch) were gathered in a reservoir tank (250 L). Larval density was calculated by counting subsamples (50 mL), before larvae were distributed to their respective treatment tanks (100 ind. L^{-1}). The 12 treatment tanks were circular in shape and contained 100 L of sea water, subject to gentle aeration. Before reaching the tanks, the sea water was sand filtered, microbially matured, heated and filtered through a 1 µm mesh (Skjermo et al., 1997)(modified). In addition, the tanks were equipped with sieves and surface skimmers. The oxygen level was always kept above 80 %, by adjusting the rate of water exchange. Water exchange were 2 times day^{-1} up to 14 dph, 4 times day^{-1} up to 25 dph, 6 times day^{-1} up to 32 dph and 8 times day^{-1} the last three weeks. Water temperature in the beginning were 12 °C, and was raised to 13 °C, 14 °C, 15 °C and 16 °C at 6, 12, 15 and 23 dph respectively. The lights were kept off the initial two days, before being switched on for the rest of the experiment (two 18 W fluorescent tubes per tank).

Four different feeding regimes were used in the first feeding experiment, and each treatment was replicated 3 times (Table 2.1). Larvae in the **Copepod** treatment were fed copepod nauplii from 4-30 dph. **Cop7** received copepod nauplii the first week (4-10 dph), followed by short-term enriched rotifers from 11-30 dph. The treatments **RotMG** and **RotChl** were fed short-term enriched and cultivated rotifers respectively, from 4-30 dph. All treatments were given enriched *Artemia sp.* nauplii from 24-50 dph., and all treatments were weaned onto formulated feed (Nofima)(Appendix 1), starting at 40 dph and lasting until the termination of the experiment (60 dph).

Table 2.1: Experimental setup of the first-feeding experiment, including feeding regimes, schedule for temperature, water exchange and mesh size (central sieve in the fish tanks) and sampling days.

Dph (days post hatch)	**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**	**35**	**36**	**37**	**38**	**39**	**40**	**41**	**42**	**43**	**44**	**45**	**46**	**47**	**48**	**49**	**50**	**51-60**
Day degrees	49	62	75	89	103	117	132	147	162	178	194	210	226	242	258	274	290	306	322	338	354	370	386	402	418	434	450	466	482	498	514	530	546	562	578	594	610	626	642	658	674	690	706	722	738	754	770	786-930
Water exchange	2 times day^{-1}											4 times day^{-1}											6 times day-1							8 times day-1																				
Clay (g tank^{-1} day^{-1})	5				10							13.3				16.7							20				25																					0
Temperature (°C)	12			13					14			15											16																										
Sieve size (µm)	200																			500																													750
Copepod	Copepod nauplii																																																
																					Artemia (Multigain)																												
																																				Formulated feed (Nofima)												
Cop7	Copepod nauplii											Rotifers (short-term enriched)																																				
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RotMG	Rotifers (short-term enriched)																																															
																					Artemia (Multigain)																												
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RotChl	Rotifers (cultivated)																																															
																					Artemia (Multigain)																												
																																				Formulated feed (Nofima)												
Sampling																																																
CN/DW	x				x				x			x						x							x				x							x									x		
Survival										x			x			x			x			x		x			x			x			x			x			x			x			x	x
SL/MH	x				x													x												x						x									x		x
Muscle analysis	x				x				x			x						x						x						x						x									x		x

Table 2.2: Overview of the co-feeding period for larvae in the Cop7 treatment; includes days and received fractions (%) of copepod nauplii and rotifers.

Cop7 treatment						
Dph	4-7	8	9	10	11	12
Copepod nauplii fraction (%)	100	67	67	100	33	0
Rotifer fraction (%)	0	33	33	0	67	100

Copepod nauplii and rotifers were manually distributed to the fish tanks, at a density of 12 000 ind. L^{-1}. From 4-19 dph the larvae were fed three times a day, and thereafter four times daily. Due to a lack of copepod nauplii, larvae in the Cop7 treatment were co-fed with short-term enriched rotifers from 8-11 dph (table 2.2). The manual feeding lasted until 30 dph, which was the last day the fish larvae received copepod nauplii and rotifers as feed. From then on, a feeding robot (Storvik Aqua AS, Norway) was used for automatic feeding of *Artemia sp.* nauplii (3000 ind. L^{-1}), and for continuous distribution of formulated feed (10 g $tank^{-1}$ day^{-1}). The formulated feed came in two different particle sizes (300-600 and 600-800 µm). The smallest particles were distributed through the robot, and the bigger particles were manually distributed to the fish tanks as a supplement, at dph ≥ 48. The robot was also used to add clay to the fish tanks, as a means of improving contrast, as well as removal of organic matter and bacteria (Attramadal et al., 2012). 5 g clay $tank^{-1}$ day^{-1} was added at 4 dph, and the amount was adjusted upwards with increased water flow. From 30-50 dph the amount of clay used was 25 g $tank^{-1}$ day^{-1}. Following 50 dph, no more clay was added to the tanks. From 13-49 dph the bottom of the fish tanks were cleaned (by siphon) every second day, and 13 dph was also the first day with counting of dead fish. Following 49 dph, the tanks were cleaned on a daily basis.

2.2 Live feed production

2.2.1 Microalgae

The red algae *R. baltica* was cultivated in plastic cylinders of two different sizes, as well as in larger plastic bags. The volumes of the sylinders were 160 and 200 L, and 300 L for the bags. The cultivation was semi-continuous, and each day 40-50 % of the algae were harvested for feeding. Algal density at harvest was approximately 1 million cells ml^{-1}. Seawater (34‰ salinity) was added to replace the harvested fraction, and the cultures were also given Conwy medium based on the amount of added seawater (1 ml Conwy L^{-1} seawater). The seawater used for refilling were kept in large reservoirs (1000 L and 700 L), where it was disinfected before use.

The disinfection is a two-step process, and consists of chlorination and dechlorination. First chlorine was added (100 ml chlorine L^{-1} seawater), and after a minimum of 5 hours

sodium thiosulphate was added as a dechlorination agent (3 g thiosulphate L^{-1} seawater). The dechlorination also proceeded for 5 hours minimum, under heavy aeration, before the water was ready for use. pH in the cultures was kept between 7,5 and 8,3, and anomalies were countered by regulating the amount of CO_2 entering the cultures. Each cylinder and bag had its own light sources covering the sides.

New cultures were started every 10-14 days. Between each restart, all cylinders were flushed out and washed thoroughly, and chlorinated/dechlorinated. Old bags were taken down and new ones put up, depending on the need for algae. New cultures were set up in the smaller cylinders, by adding 10 L of algae from a stock solution to 150 L of seawater, with the addition of Conwy medium.

2.2.2 Copepods

Egg production: Adult copepods (*A. tonsa*) were cultivated in two circular tanks, with volume capacities of 1000 and 1600 L respectively. The physical environment consisted of moderately aerated seawater (34‰ salinity), which had been sand filtered and filtered through a 1 µm mesh. The water temperature was kept at 20°C. The copepods received microalgae (*R. baltica*) continuously through an automatic feeding pump, and algal density was kept between 10-30 000 cells ml^{-1} (Skogstad, 2010). The algal density was checked by sampling from the run-out water, and running the sample through a cell counter (Multisizer ™ 3 Coulter counter, Beckman Coulter Inc., USA). Each day eggs were gathered by a rotating arm in the bottom of the tanks, and harvested using a siphon. The eggs were washed through two sieves (120 µm and 60 µm respectively), before transfer to NUNC EasyFlasks™ (NUNC A/S, Denmark) (75 cm^3) with seawater. The flasks were put on cold storage (2°C), and the water portions were exchanged every 3-4 weeks. Hatching success was tested for every batch of eggs, in order to estimate the required amount of eggs to set for hatching.

Nauplii production: The harvested copepod eggs were used to produce copepod nauplii, which served as live feed for the fish larvae. Hatching of eggs took place in circular plastic tanks (100 L). The physical conditions in the hatching tanks were the same as in the egg production tanks, except more gentle aeration. The hatched nauplii received feed (*R. baltica*) from 2-4 dph, in order to reach a size similar to the rotifers (180 µm). Density of nauplii was estimated by extracting a subsample (3 mL) and

counting manually in a microscope. Upon feeding, the required amount of nauplii were harvested from the tanks and concentrated, using a siphon and a sieve (60 μm). Finally the nauplii were distributed to the fish tanks, according to the desired density.

2.2.3 Rotifers

Rotifers of the type *B. ibericus (Cayman)* were cultivated in a flow through system, using 300 L tanks with conical bottoms. The cultures were subject to temperatures between 23-25 ˚C, seawater (34 ‰ salinity) and moderate aeration. The rotifers were fed DHA *Chlorella* (Chlorella, Pacific Trading) continuously based on density, using a feeding factor of 2,4 (2,4 mL feed million^{-1} rotifers). Rotifer density in the cultures varied from 200-1000 ind mL^{-1}, and cultures exceeding 750 ind mL^{-1} were diluted 25-40 %. Prior to feeding, rotifers were harvested using a siphon and concentrated in a sieve (40 μm). Once a week the rotifer cultures and respective tanks were washed.

As for short-term enrichment, the rotifers received Multigain (Biomar) (0,2 g million^{-1} rotifers). The enrichment took place in 100 L tanks with heavy aeration, for a period of 2 hours. The temperature was 22-24 °C, and maximum rotifer density during enrichment was 400 ind mL^{-1}. After enrichment the rotifers were washed and distributed to the fish tanks, at the desired density.

2.2.4 Artemia

Artemia sp. nauplii were cultivated in 100 L tanks with conical bottoms, and subject to heavy aeration. The water temperature was kept between 25 and 28 ˚C, and this was achieved by warming the seawater (34‰ salinity) in advance, using electrified heaters. Decapsulation of *Artemia* cysts (EG® INVE Aquaculture, Belgium) was done by following standard SINTEF protocol (Sorgeloos et al., 1977). The decapsulated cysts were washed thoroughly to remove capsules and remaining chlorine. Cysts were then stored in a fridge for a maximum of one week, and portions of this stock were taken out for daily hatching. After hatching (24 hours) the *Artemia* were washed in a concentrator to remove eggshells, before transferring them to a new tank for short-term enrichment. Multigain (Biomar) was used as enrichment medium, and added twice in a 24-hour period (20 g Multigain 60 L^{-1} seawater day^{-1}). After enrichment the *Artemia* were washed once again before feeding them out to the wrasse larvae, at the desired density.

2.3 Live feed analysis

The nutritional contents of the live feed were analyzed by NIFES (Nasjonalt Institutt for Ernærings- og Sjømatforskning). Total lipid content was determined by acid hydrolysis, and protein content was determined by Leco - nitrogenanalyzer - Dumans/Liebig method – thermal conductivity detection. In addition, the method ICP-MS (inductively-coupled plasma mass spectrometry) (Wolf, 2005) was used to find the contents of various elements, including calcium (Ca), potassium (K), magnesium (Mg) and phosphorus (P).

The nutritional analysis of the live feed organisms revealed that the copepod nauplii contained the highest content of protein (67.8 %), but lowest content of lipid (8.8 %) (table 2.3). The HUFAs DHA and EPA constituted 26.6 % of total fatty acids in the copepod nauplii, which is similar to the values observed in the short-term enriched rotifers and *Artemia*. The copepod nauplii also had the highest observed ratio between DHA and EPA (5.3). The short-term enriched and cultivated rotifers contained equal amounts of protein and lipid, but the percentage of DHA+EPA were higher in the short-term enriched rotifers (26.3 vs 22.8 %). The *Artemia* nauplii contained the lowest content of protein (34.9 %), but highest content of lipid (27.6 %).

Table 2.3: Contents of protein, lipids, essential fatty acids (DHA and EPA), calcium, potassium, magnesium and phosphorus in the different live feed organisms.

	Organism			
Contents	Copepod nauplii	Rotifers (short-term enriched)	Rotifers (cultivated)	Artemia nauplii
Protein (g/100g dw)	67.8	42.3	44.4	34.9
Lipid content (g/100g dw)	8.8	11.6	11.9	27.6
DHA (% of total FA)	22.4	19.8	14.4	21.5
EPA (% of total FA)	4.2	6.5	8.5	5.3
DHA:EPA ratio	5.3	3	1.7	4.1
Ca (mg/kg dw)	1390	1300	1030	1760
K (mg/kg dw)	13800	11100	12500	13900
Mg (mg/kg dw)	2120	3000	2930	2020
P (mg/kg dw)	13200	11400	12400	11400

2.4 Larval sampling and analysis

Sampling was done from all fish tanks by random selection, using a scoop or landing net as equipment to catch individuals. Larvae from each treatment tank were transferred to plastic cups, and killed by an overdose of metacaine (MS-222, Finquel®, Argent Chemical Laboratories Inc., Redmond, USA) before fixation.

2.4.1 Dry weight

Larvae were sampled for dry weight (DW) analysis at 4, 8, 12, 15, 21, 27, 33, 40, 47 and 61 dph. In the beginning (dph ≤ 21) 12 larvae from each tank were sampled each sampling day. At 27 dph, 24 larvae from each tank were taken out, and from 33 to 47 dph the number of sampled larvae increased to 30 per tank. The last sampling day was at 61 dph, and 50 larvae were sampled from each tank. One of the tanks (RotChl-3) suffered from elevated mortality, and after the sampling at 40 dph there were no larvae left in the tank.

The sampled larvae were rinsed in distilled water and transferred individually to pre-weighed tin capsules, before a drying period at 60°C for 48 hours. Samples up to 27 dph were analyzed for carbon and nitrogen (CN) content in an Elemental combustion analyzer (Costech Analytical Technologies Inc., USA), using acetanilide as standard (CN analysis was conducted by Marte Schei, SINTEF). The carbon content (µg C $larva^{-1}$) was multiplied with a factor of 2,34 (based on the average carbon content of fish larvae), to obtain the larval dry weight (Reitan et al., 1993). Samples from 33 to 61 dph were weighed with a micro balance (UM3 and UMX2, Mettler-Toledo Inc., USA).

Daily weight increase (% DWI) was calculated according to (Ricker, 1958):

$$\% \text{ DWI} = 100(e^{SGR} - 1) \qquad \text{with} \qquad SGR = (\ln DW_2 - \ln DW_1) / (t_2 - t_1)$$

where SGR is the specific growth rate and DW_1 and DW_2 are the mean larval dry weights at times t_1 and t_2 respectively (Galloway, 1999).

2.4.2 Standard length and myotome height

Standard length (SL) and myotome height (MH) was measured on fixated larvae from 4, 8, 21, 28, 39 and 58 dph (n = 12, 12, 12, 30, 30, 30). The SL was measured from the tip of the snout to the base of the caudal fin, and to the end of the notochord in smaller larvae where the caudal fin basis weren't visible (figure 2.1). MH was measured perpendicular to the skeleton, in an area directly posterior to the anus.

Figure 2.1: Photograph of a ballan wrasse larva (15 dph). The inserted horizontal and vertical lines indicate SL and MH respectively. The vertical line also mark the area from where transverse sections were analyzed Photo: Maren Gagnat.

2.4.3 Muscle histology

Analysis of muscle development and growth were performed on larvae from 4, 8 and 21 dph (n = 6 larvae treatment^{-1} day^{-1}), which had been fixed in a 4 % paraformaldehyde solution w/phosphate buffer (Apotekproduksjon AS, article nr: 32 98 47), for approximately two months. Initially the larvae were removed from the fixative and rinsed in phosphate buffered saline (PBS). The larvae were then individually measured for SL and MH, using pictures and the software CAST 2 (Olympus Inc., Denmark). Following measurement, the larvae were oriented in blocks of agarose gel (3 per block) together with a piece of sewing thread, as a means of keeping track of the individuals (figure 2.2). The blocks of agarose were placed in a Tissue Processor (Leica TP 1020) for dehydration.

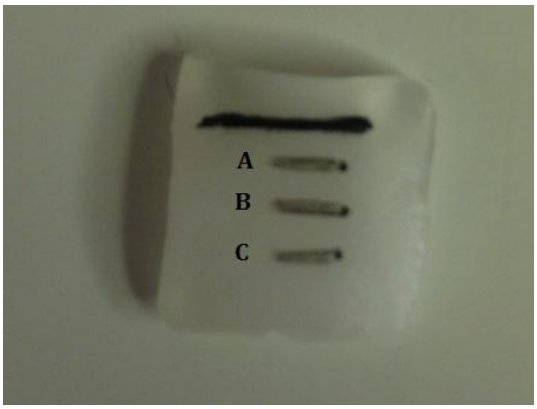


Figure 2.2: Three ballan wrasse larvae oriented in a block of agarose, together with a piece of thread.

After dehydration, the blocks of agarose were oriented for transverse sectioning, and embedded into larger blocks of paraffin. The sectioning was accomplished using a Leica 2055 Autocut microtome (Leica Microsystems, Germany). The embedded larvae were sectioned from caudal fin towards head, until reaching the area directly posterior to the anus. This was accomplished by sectioning past the actual area, and then using previous sections to pinpoint the right location. Visibility of the intestine was used as an indicator to know whether or not the sectioning had progressed too far. Sections were cut with 2 µm thickness, and stained with 0.1 % Toluidine Blue for 30 seconds, and mounted with a water based medium (polyvinyl alcohol). A different staining method was tested (hematoxylin and eosin), but this resulted in pictures of poorer quality which could not be used for the following analysis. The main reason was that the pictures didn't reveal the borders between muscle fibres sufficiently (an absolute requirement for the counting and measuring work).

The sections were photographed using a Zeiss Axioskop 2 plus microscope (Zeiss INC., Germany), equipped with a Nikon Digital Sight DS-5M color video camera (Nikon corp., Japan). Photographs of the sections were uploaded and processed in the software ImageJ. A LCD Tablet drawing board, DTK-2400 (Wacom, China), was used to count all red and white muscle fibres in one bilateral half of the myotome, and to measure the total cross-sectional area of red and white muscle in this area. In addition, the cross-sectional area of all the white fibres was measured individually. In areas with small, tightly packed fibres of similar size, the mean fibre size was calculated by measuring the whole area, and dividing by the number of fibres within the area. The mean cross-

sectional area of individual red fibres was calculated by dividing total cross-sectional area by fibre number.

Growth by hyperplasia was measured as an increase in red and white fibre number, while hypertrophic growth was measured as an increase in fibre size within the deep white fibre zone. Within this zone, the mean size of the fifty largest fibres was calculated; a number which was based on the amount of white fibres present in the larvae shortly after hatching (4 dph). In addition, the dynamics of hypertrophy were studied by assigning all the white fibres into size intervals; quantifying the number of fibres for each interval, and comparing between treatments.

2.4.4 Statistics

All statistical analyses were done using the software IBM SPSS Statistics 19 for Windows (IBM®, USA). Normality in the data was tested using a Shapiro-Wilk's test ($n<100$), or a Kolmogorov-Smirnov test ($n>100$). For normally distributed data, the means were compared using one-way ANOVA. Post hoc testing was done using a Student-Newman Keuls test for homogenous data, and a Dunnet T3 test for non-homogenous data. Data which deviated from a normal distribution were tested using a non-parametric Kruskal-Wallis test, with subsequent post hoc testing using a Mann-Whitney test. Data presented in percent were arcsin transformed before statistical analyses were carried out. $P<0.05$ was used as the critical value for significance. Correlation between variables was tested using a Pearson correlation test.

3. Results

General observations

Larvae from one tank in the Copepod treatment had very poor survival and growth throughout the experiment, compared with the other tanks from the same treatment. At the end of the experiment, larvae from this one tank had the lowest observed growth within any of the treatments, and it was decided that the data from this tank were to be omitted from the mean values regarding survival and growth.

3.1 Survival and growth

3.1.1 Survival

Most of the fish mortality happened during the first week of feeding, before registering of dead larvae had started (13 dph). From 23 dph and onwards, larvae from the Cop7 treatment had significantly higher survival than larvae from the RotChl treatment (table 3.1). When the experiment was terminated (61 dph) the survival was significantly lower in the RotChl treatment (5 %), compared to all other treatments (10-12 %).

Table 3.1: Mean survival (% ± SE) for all treatments at selected days. n=3 tanks treatment^{-1} for all except Copepod, where n=2. Different letters mark significant differences between the treatments.

	Treatment			
Dph	Copepod	Cop7	RotMG	RotChl
13	16 ± 2.6	24 ± 5.3	17 ± 2.7	12 ± 1
23	14 ± 1.5 ^{ab}	20 ± 4.1 ^{a}	13 ± 1.6 ^{ab}	7 ± 1 ^{b}
33	13 ± 1.5 ^{ab}	17 ± 3.5 ^{a}	11 ± 1.5 ^{ab}	6 ± 0.6 ^{b}
43	13 ± 1.4 ^{a}	16 ± 3.1 ^{a}	10 ± 1.5 ^{ab}	5 ± 0.6 ^{b}
53	12 ± 1.1 ^{a}	14 ± 2.3 ^{a}	10 ± 1.5 ^{a}	5 ± 0.6 ^{b}
61	11 ± 0.5 ^{a}	12 ± 1.4 ^{a}	10 ± 1.3 ^{a}	5 ± 0.6 ^{b}

3.1.2 Dry weight and daily weight increase

Larvae from the Copepod and Cop7 treatment had a significantly higher DW than larvae from both rotifer treatments, at 8 and 12 dph (figure 3.1). Larvae from the Copepod treatment also had a significantly higher DW on 15-33 dph, compared to larvae from all other treatments. After the transition from copepod nauplii to rotifers, larvae from Cop7

experienced a decrease in growth rate (12-21 dph), compared to larvae from the other treatments. At 21 dph the Cop7-larvae were no longer significantly different to larvae from RotMG, in terms of DW. Larvae from both the RotMG and RotChl treatment had very similar growth patterns during the whole experiment. During the weaning period onto formulated feed (40-50 dph), no significant differences in DW were found. However, at the end (61 dph) larvae from the copepod treatments again had significantly higher DW than larvae from both rotifer treatments. When the experiment was terminated, the mean dry weight (mg/larva) of the larvae was 19.6 ± 0.0, 17.1 ± 2.7, 13.8 ± 1.4 and 13.0 ± 1.8 for the treatments Copepod, Cop7, RotMG and RotChl respectively. In comparison to the initial larval DW, this constitutes a 490 to 325-fold increase for the respective treatments.

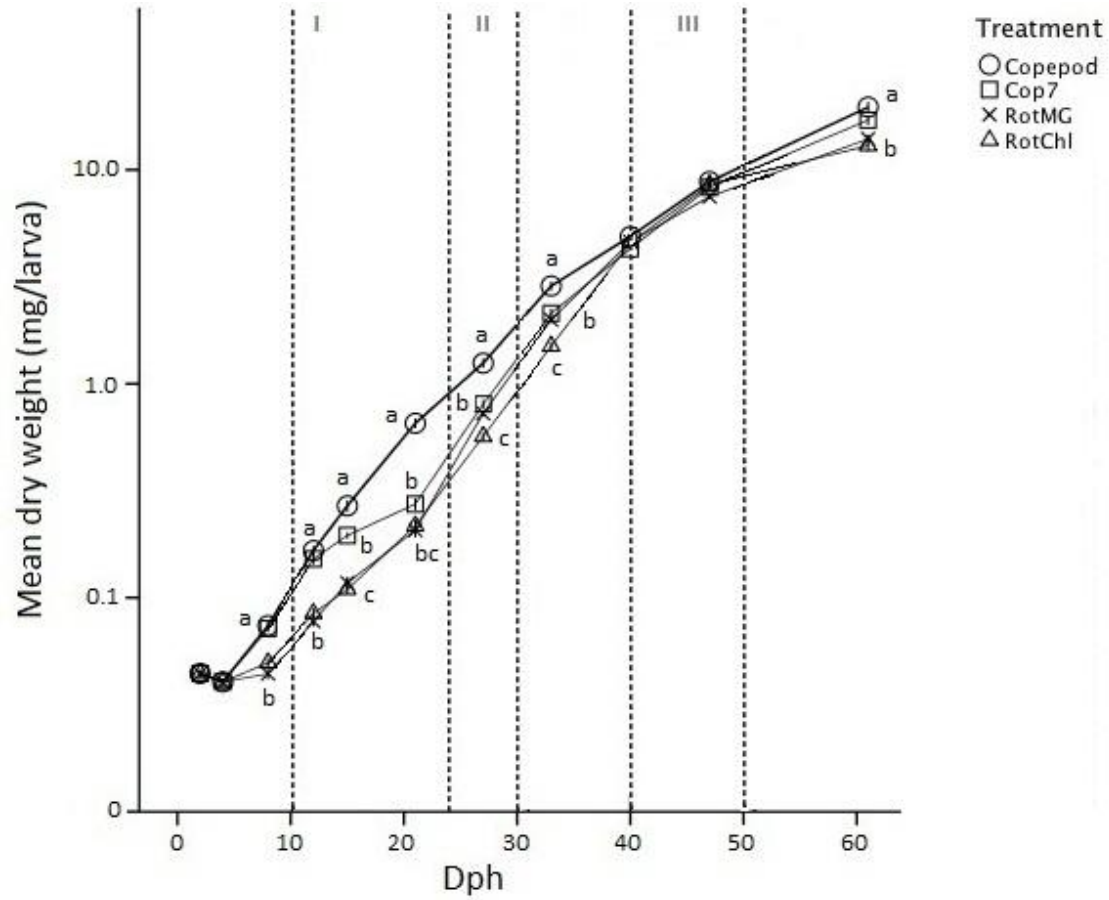


Figure 3.1: Mean dry weight of the ballan wrasse larvae from 2-61 dph, measured as mg/larva. N=12 (dph < 21), n=24 (dph=27), n=30 (dph=33-47) and n=50 (dph=61). Logarithmic Y-axis. Inserted vertical lines indicate changes in feeding regime, and are marked by roman numerals. I: switch from copepod nauplii to short-term enriched rotifers in the Cop7 treatment. II: co-feeding period with *Artemia,* III: weaning period onto formulated feed. Different letters mark significant differences between the treatments, and error bars indicate ± 1 standard error.

Prior to feeding (2-4 dph) the daily weight increase (% DWI) was negative, due to depletion of the larvae's yolk reserves (fig. 3.2). Larvae fed copepod nauplii had a significantly higher % DWI than larvae fed rotifers, on 4-12 dph. During the following period (12-27 dph), copepod nauplii were fed only to larvae from the Copepod treatment. At this point, larvae from Cop7 had significantly lower % DWI than larvae from all other treatments. During the *Artemia* period (27-40 dph), the % DWI differed significantly between all treatments. Larvae from the RotChl treatment now had the highest % DWI, followed by larvae from RotMG, Cop7 and Copepod respectively. After weaning onto formulated feed and for the rest of the experiment (40 =< dph <= 61), no more significant differences were found between treatments. For the experiment as a whole (2-61 dph), % DWI was almost identical for all treatments.

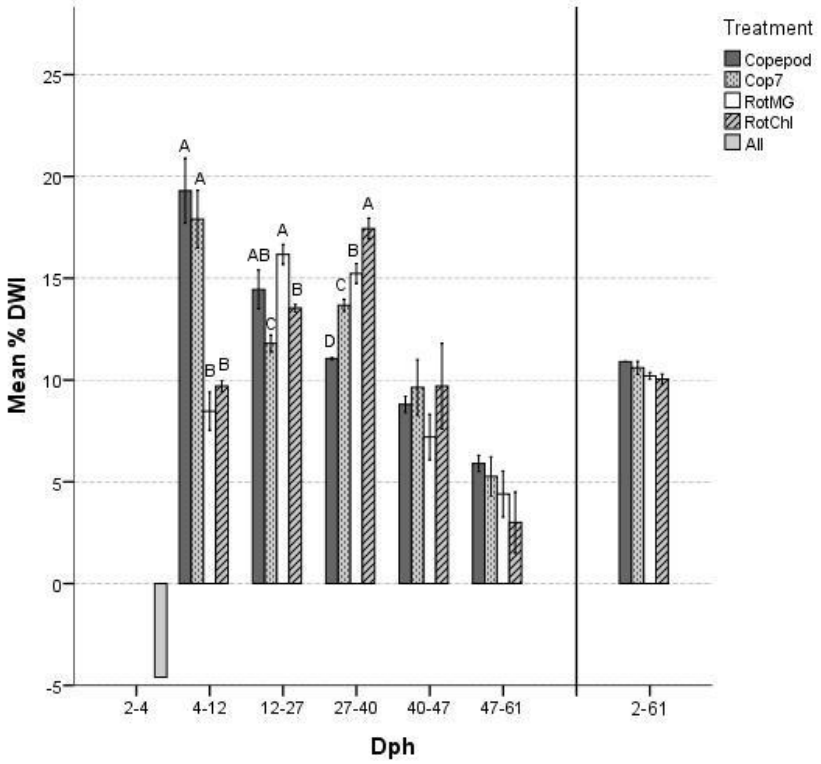
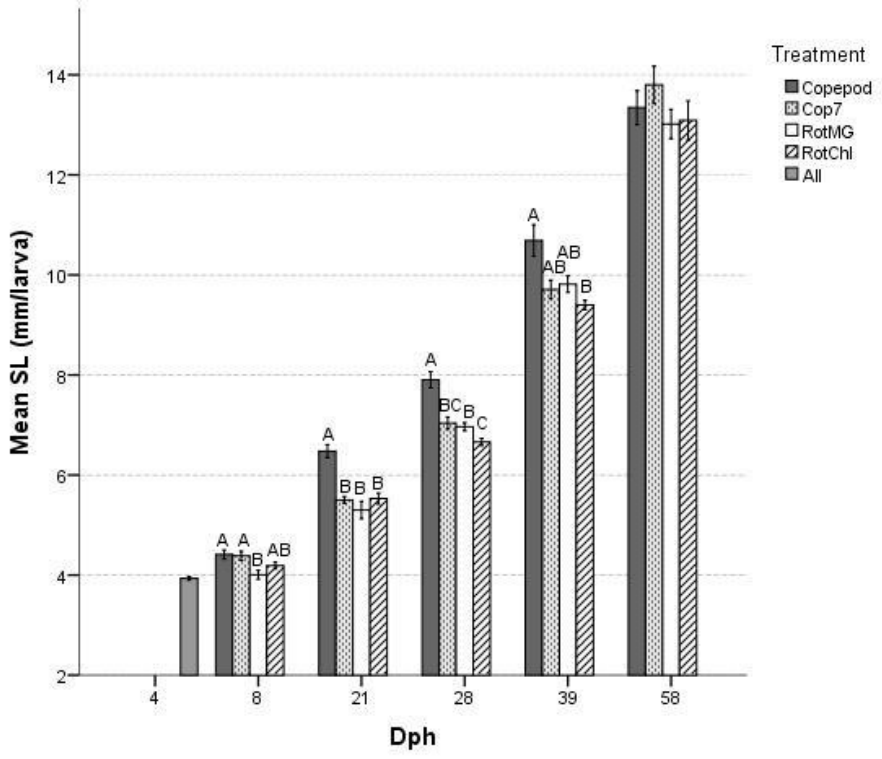


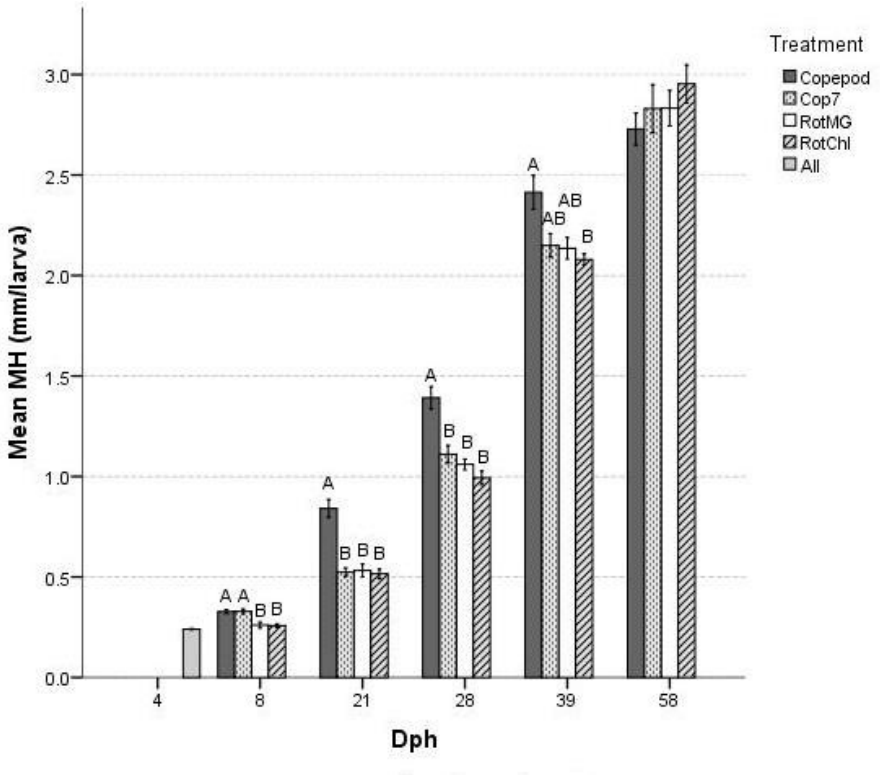
Figure 3.2: Daily weight increase (% DWI) for the ballan wrasse larvae, from 2-61 dph. The intervals are based on changes in the feeding regime: 2-4 dph is prior to feeding, and all larvae were sampled from one tank. 4-12 dph is the period when both copepod treatments received copepod nauplii, while the rotifer treatments received short-term enriched and cultivated rotifers, respectively. From 12-27 dph, all but the Copepod treatment received rotifers. 27-40 dph is the period of co-feeding with copepod nauplii/rotifers and *Artemia* nauplii, as well as exclusive feeding of *Artemia* nauplii. From 40-47 dph the larvae were weaned onto formulated feed, and 47-61 dph is the period when larvae received formulated feed only. Different letters mark significant differences between the treatments, and error bars indicate ± 1 standard error.

3.1.3 Standard length and myotome height

Larvae from both copepod treatments had significantly higher SL and MH than larvae from the RotMG treatment, at 8 dph (fig. 3.3 + 3.4). Prior to, and shortly after co-feeding with *Artemia* (21 and 28 dph), larvae from the Copepod treatment had significantly higher SL and MH than larvae from all other treatments. At this point, larvae from Cop7 were no longer significantly different from larvae in the rotifer treatments (in terms of SL and MH), a trend which continued for the rest of the experiment. Before weaning onto formulated feed, and until the last sampling day (39 and 58 dph), the only significant difference were between the Copepod and RotChl treatments (39 dph), where SL and MH were significantly higher in the first-mentioned. At 58 dph there were no significant differences in larval SL between the treatments, with a mean value of 13.3 ± 1.8 mm. This constitutes more than a 4-fold increase, compared to the initial mean larval SL. Also there were no significant differences in larval MH at this point, with a mean value of 2.8 ± 0.5 mm; which constitute more than a 11-fold increase compared to the initial mean larval MH.



Figur 3.3: Standard length measured on larvae at 4, 8, 21, 28, 39 and 58 dph. Different letters mark significant differences between the treatments, and error bars indicate ± 1 standard error.

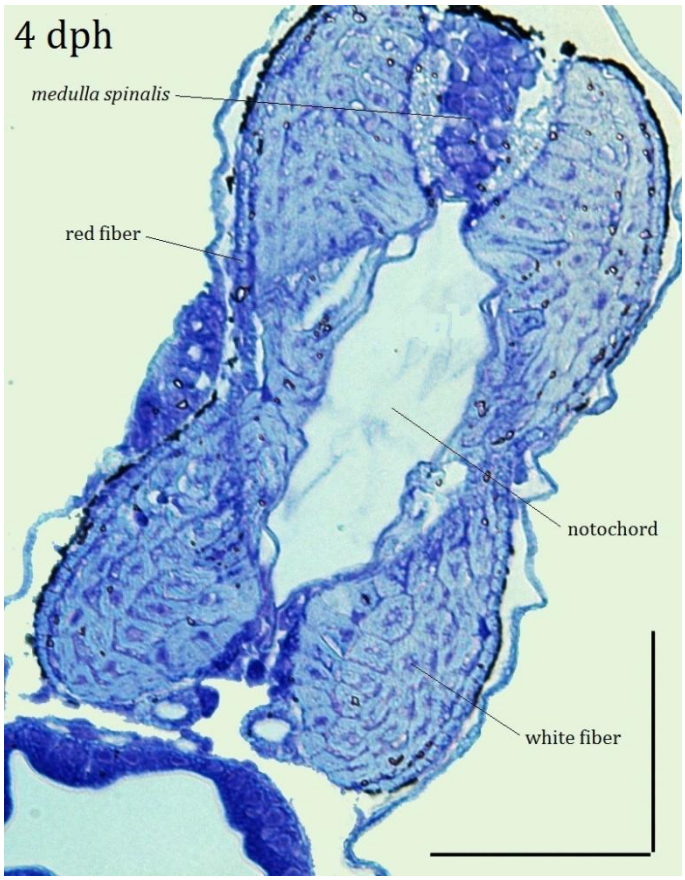


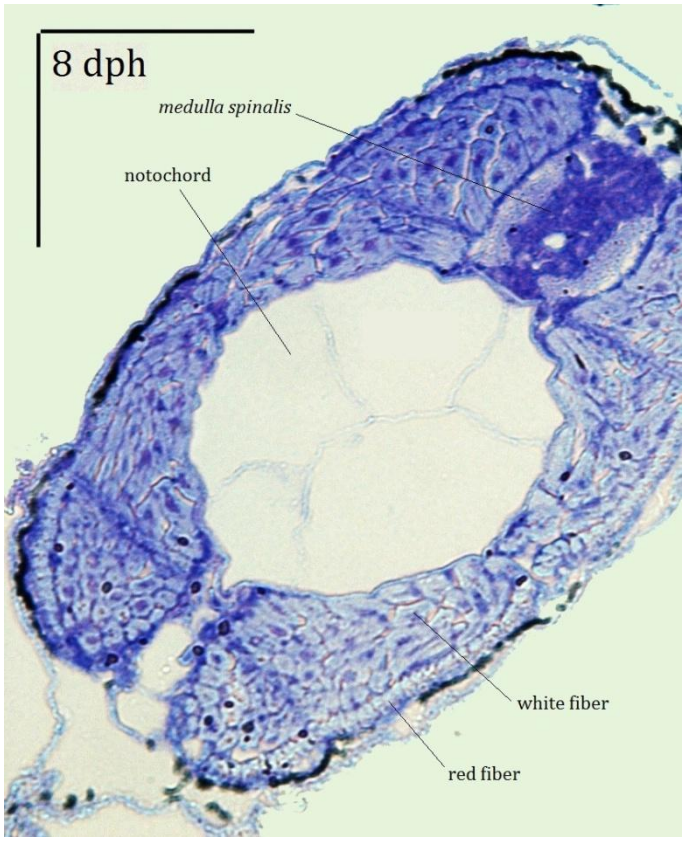
Figur 3.4: Myotome height measured on larvae at 4, 8, 21, 28, 39 and 58 dph. Different letters mark significant differences between the treatments, and error bars indicate ± 1 standard error.

3.2 Muscle analysis

3.2.1 Morphology

At 4 and 8 dph the larvae had a single layer of red fibres covering an inner mass of white fibres, and the latter seemed to be quite uniform in size (figure 3.5). The red fibres also formed a single layer at 21 dph, but the white fibres now displayed a clear size gradient. The largest white fibres (deep white) made up the inner core of the myotome, while fibre size became progressively smaller towards the dorsal, ventral and lateral regions. Zones of fibre recruitment could be recognized directly beneath the sheet of red fibres, especially at the apical regions, and also around the horizontal septum. Mosaic hyperplasia was not observed in any larvae at any of the days.





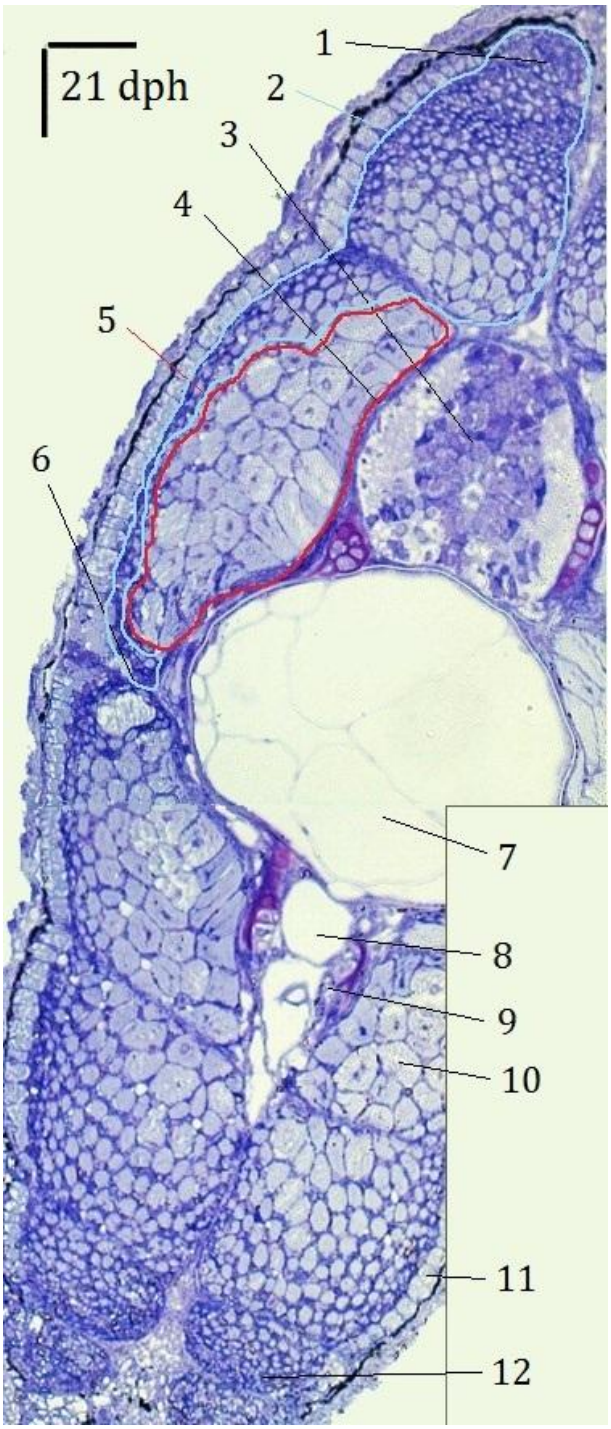


Figure 3.5: Light micrographs showing stained sections of skeletal muscle in ballan wrasse larvae at 4, 8 and 21 dph, directly posterior to the anus. Red fibres can be seen as a single layer covering the outer edge of the muscle area, while several layers of white fibres make up the inner mass. Features and number explanations regarding 21 dph: 1. dorsal apex, 2. recruitment fibres, 3. *medulla spinalis*, 4. neural arch, 5. deep white fibres, 6. horizontal septum, 7. notochord, 8. haemal canal, 9. haemal arch, 10. white fibre, 11. red fibre, 12. ventral apex. The largest white fibres (deep white) constitute the innermost part of the fibre area, and fibre size decreases gradually toward the outer edges. Magnifications are 64x, 64x and 40x for 4, 8 and 21 dph respectively. Scale bars equal 50 µm.

3.2.2 Muscle cross-sectional area and fibre number/size

Larvae from the Copepod treatment had significantly larger total muscle cross-sectional area at 8 dph, compared to larvae from the RotMG treatment (figure 3.6). At 21 dph, larvae from the Copepod treatment had over twice as large a muscle area as larvae from the second largest group (Cop7), and differed significantly from all other treatments. The mean total cross-sectional area (μm^2) of one bilateral half of the myotome increased from 5 563 ± 369 at 4 dph, to 102 073 ± 12 115, 43 942 ± 7 745, 33 342 ± 4 661 and 21 592 ± 2 235 at 21 dph, for the treatments Copepod, Cop7, RotMG and RotChl respectively. These values constitute more than an 18-fold increase in total muscle cross-sectional area for the Copepod treatment, and a 4-8-fold increase for the other treatments. Regardless of treatment, total muscle cross-sectional area correlated well with larval SL (figure 3.7).

The mean fraction of white fibres was 85 % of the total cross-sectional area at 4 dph, 78-83 % at 8 dph and 79-89 % at 21 dph (table 3.2). Larvae from the Copepod treatment had the highest percentage of white fibres at 8 and 21 dph, and differed significantly from larvae from the RotChl treatment at 8 dph, and all other treatments at 21 dph. At 21 dph there was also a much better correlation between white fibre fraction and larval SL, than at 8 dph. Larvae from the Cop7 treatment had significantly more white fibres than larvae from the RotMG treatment, at 8 dph (figure 3.8). At 21 dph, larvae from the Copepod treatment had significantly more white fibres than all other treatments, and over twice the number as larvae from the RotMG treatment. The mean number of white fibres in one bilateral half of the myotome increased from 84 ± 5 at 4 dph, to 783 ± 94, 550 ± 64, 384 ± 45 and 256 ± 38 at 21 dph, for the treatments Copepod, Cop7, RotMG and RotChl respectively. From lowest to highest, this constitutes a 3-9-fold increase for the different treatments. Regardless of treatment, the number of white fibres correlated well with larval SL (figure 3.9).

The white fibres were categorized according to size (μm^2), and the intervals were < 50, 50-100, 100-200 and > 200. Initially (4 dph), nearly all the white fibres had cross-sectional areas below 100 μm^2 (figure 3.10). At 8 dph, larvae from the Copepod treatment had fewer fibres in the smallest category, and more fibres in the "50-100" and "100-200" category than larvae from all other treatments. Several fibres also belonged to the largest category, and fibres of such size were only observed for larvae in the

Copepod treatment at this stage. At 21 dph, larvae from the Copepod treatment had more fibres in the smallest and largest categories, and fewer fibres in the "50-100" category than larvae from the other treatments. The deep white fibres (fifty largest) were significantly larger in larvae from the Copepod treatment, with a mean size (μm^2) of 341 ± 6, compared to 224 ± 4, 178 ± 4 and 138 ± 4 for the treatment Cop7, RotMG and RotChl respectively. Thus, hypertrophic growth in the deep white fibre zone was 56, 91 and 147 % higher in larvae from the Copepod treatment, compared to the same respective treatments. Regardless of treatment, the size of the deep white fibres correlated well with larval SL (figure 3.12).

Larvae from the RotMG treatment had significantly less red fibres than larvae from all other treatments, at 8 dph (figure 3.13). At 21 dph, larvae from both copepod treatments had significantly more red fibres than larvae from the rotifer treatments. The mean number of red fibres increased from 54 ± 2 at 4 dph, to 91 ± 3, 94 ± 2, 80 ± 4 and 68 ± 3 at 21 dph for the treatments Copepod, Cop7, RotMG and RotChl respectively. The number of red fibres correlated well with larval SL (figure 3.14). Larvae from the Copepod treatment had significantly larger (55-78 %) red fibres at 21 dph, compared to larvae from all other treatments (figure 3.15). The mean size (μm^2) of individual red fibres increased from 16 ± 1 at 4 dph, to 116 ± 8, 75 ± 5, 69 ± 5 and 65 ± 6 at 21 dph, for the treatments Copepod, Cop7, RotMG and RotChl respectively. Regardless of treatment, the mean size of red fibres correlated well with larval SL (figure 3.16).

Table 3.2: Mean fractions of white fibres (% ± SE) in proportion to the total cross-sectional area of muscle, for larvae in the different treatments at 4, 8 and 21 dph. The correlation with larval SL is also shown. Different letters mark significant differences between the treatments.

Dph	**Treatment**				**Correlation with larval SL (R^2)**
	Copepod	Cop7	RotMG	RotChl	
4	85 ± 1				
8	83 ± 1 ^{a}	81 ± 1 ^{ab}	81.5 ± 1 ^{ab}	78 ± 1 ^{b}	0.183
21	89 ± 1 ^{a}	84 ± 1 ^{b}	83 ± 1 ^{b}	79 ± 1 ^{c}	0.741

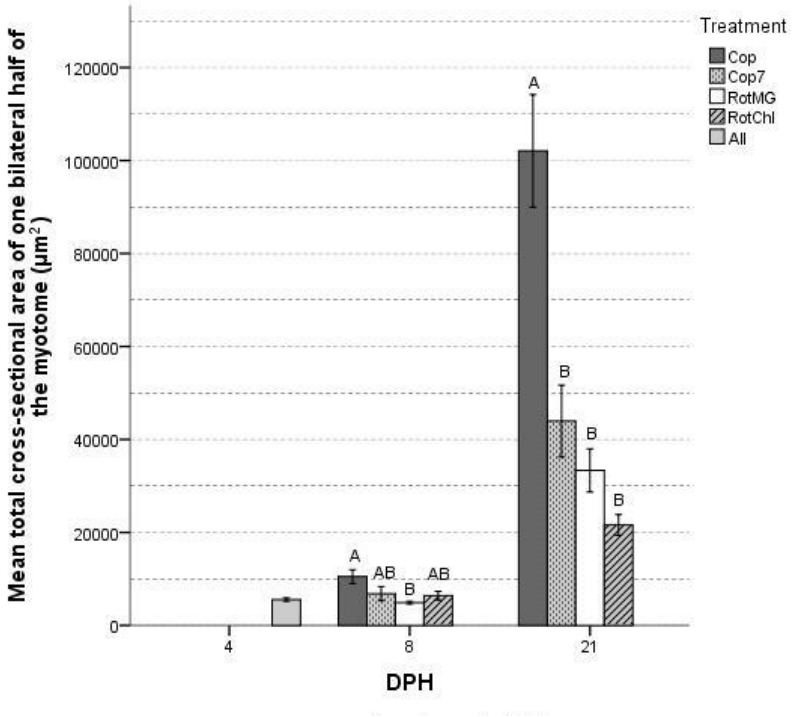


Figure 3.6: Mean total cross-sectional area of one bilateral half of the myotome, for larvae in the different treatments at 4, 8 and 21 dph (n = 6 larvae treatment^{-1} at 4 dph, n = 6, 5, 6, 6 at 8 dph and n = 6, 6, 6, 6 at 21 dph)**.** Different letters mark significant differences between the treatments, and error bars indicate ± 1 standard error.

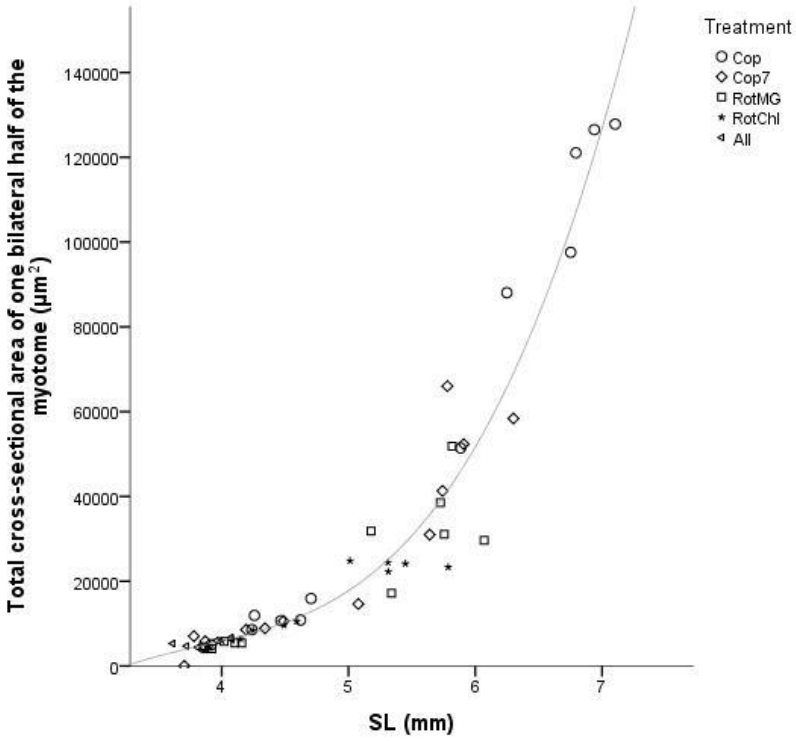


Figure 3.7: Total cross-sectional area of one bilateral half of the myotome, for larvae in the different treatments at 4, 8 and 21 dph (n = 6 larvae treatment^{-1} at 4 dph, n = 6, 5, 6, 6 at 8 dph and n = 6, 6, 6, 6 at 21 dph)**.** Each point in the graph represents one larva. Cubic fit line added at total, R^2 = 0,943.

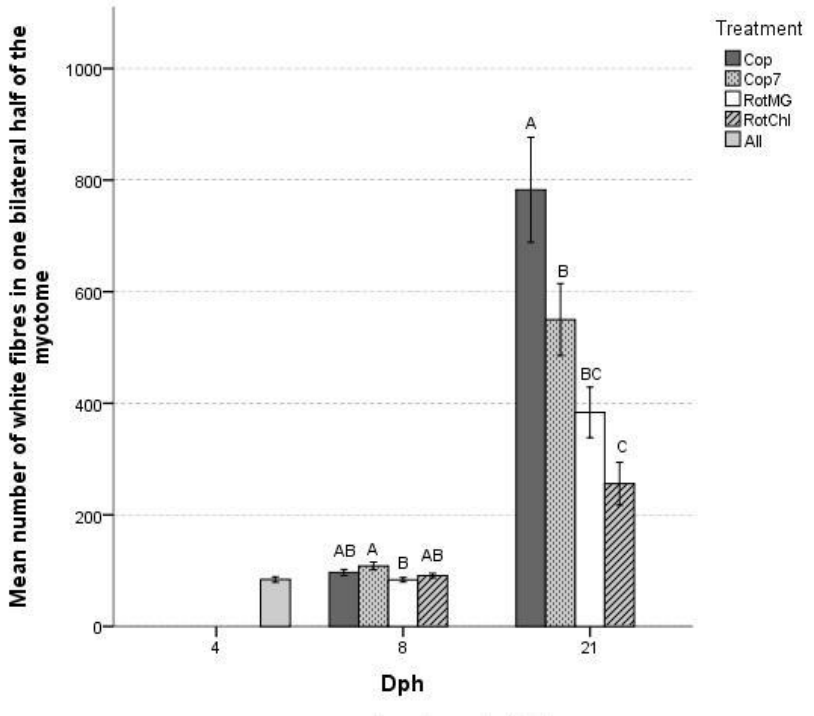


Figure 3.8: Mean number of white fibres present in one bilateral half of the myotome, for larvae in the different treatments at 4, 8 and 21 dph (n = 6 larvae treatment^{-1} at 4 dph, n = 6, 5, 5, 5 at 8 dph and n = 5, 5, 5, 5 at 21 dph)**.** Different letters mark significant differences between the treatments, and error bars indicate ± 1 standard error.

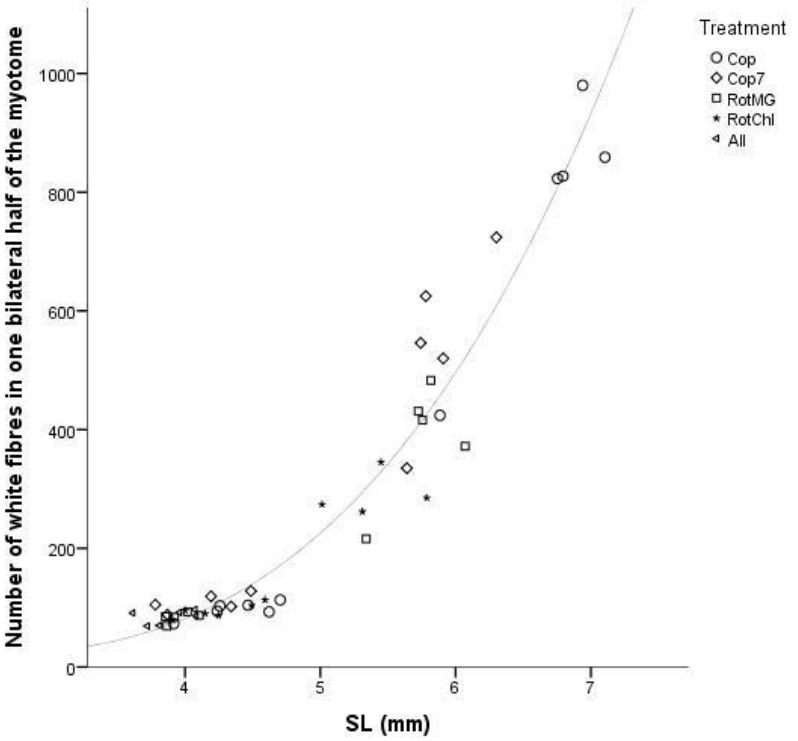


Figure 3.9: Number of white fibres in one bilateral half of the myotome, for larvae in the different treatments at 4, 8 and 21 dph (n = 6 larvae treatment^{-1} at 4 dph, n = 6, 5, 5, 5 at 8 dph and n = 5, 5, 5, 5 at 21 dph)**.** Each point in the graph represents one larva. Cubic fit line added at total, R^2 = 0,941.

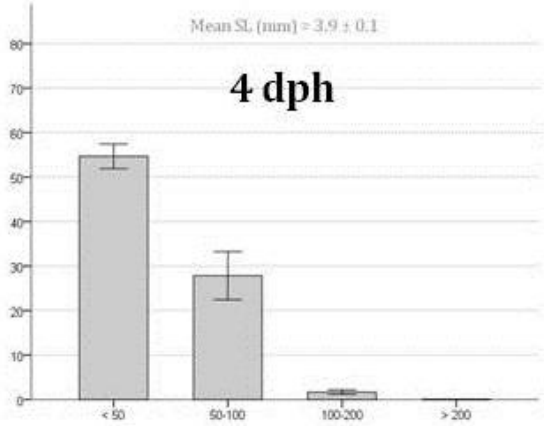
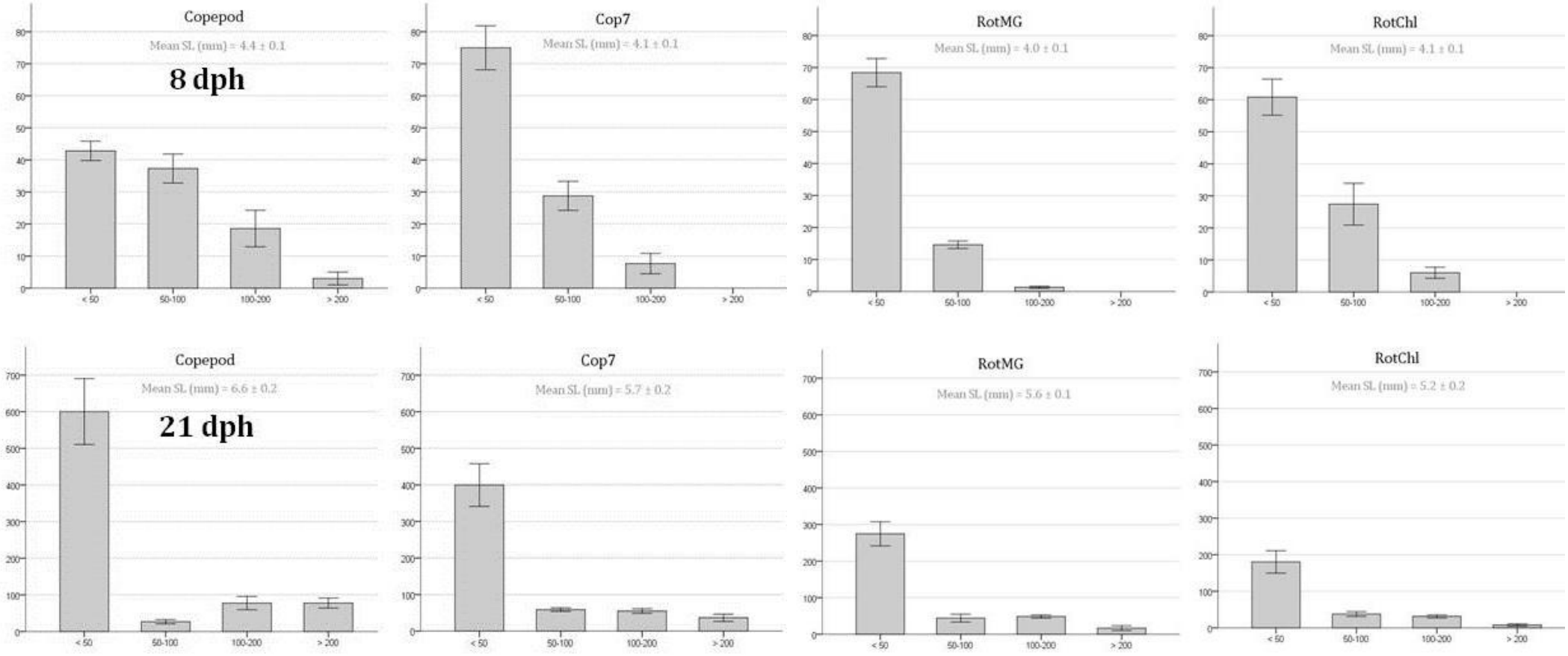


Figure 3.10: Mean number of white muscle fibres within size intervals (< 50, 50-100, 100-200 and > 200 µm²) for larvae in the different treatments at 4, 8 and 21 dph. Top row represents 4 dph, center row represents 8 dph and bottom row represents 21 dph. The mean standard length ± SE of larvae is shown for each treatment., and error bars indicate ± 1 standard error.



Mean absolute number of white fibres

Fibre size (µm²)

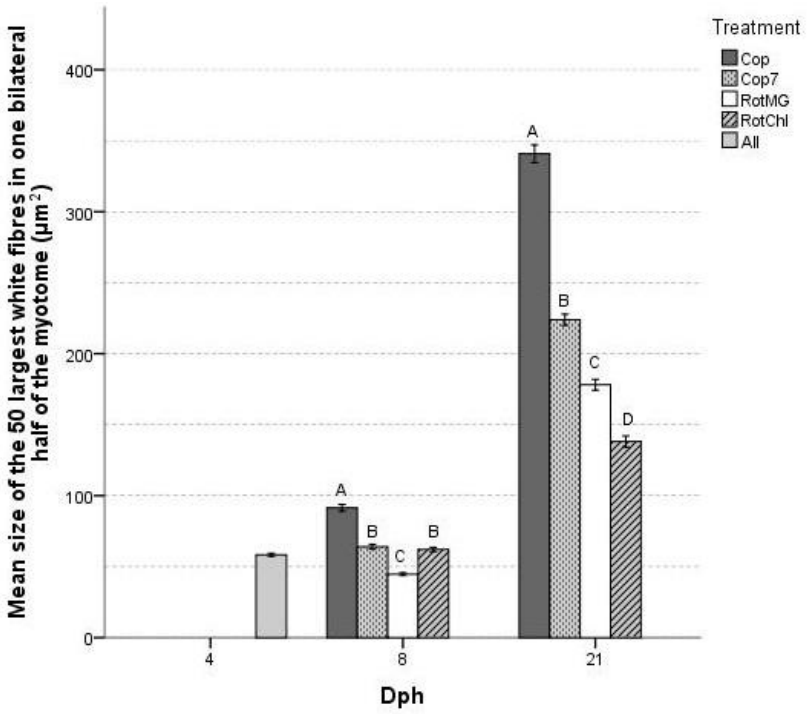


Figure 3.11: Mean size of the 50 largest white fibres present in one bilateral half of the myotome, for larvae in the different treatments at 4, 8 and 21 dph (n = 6 at 4 dph, n = 6, 5, 5, 5 at 8 dph and n = 6, 5, 6, 6 at 21 dph)**.** Different letters mark significant differences between the treatments, and error bars indicate ± 1 standard error.

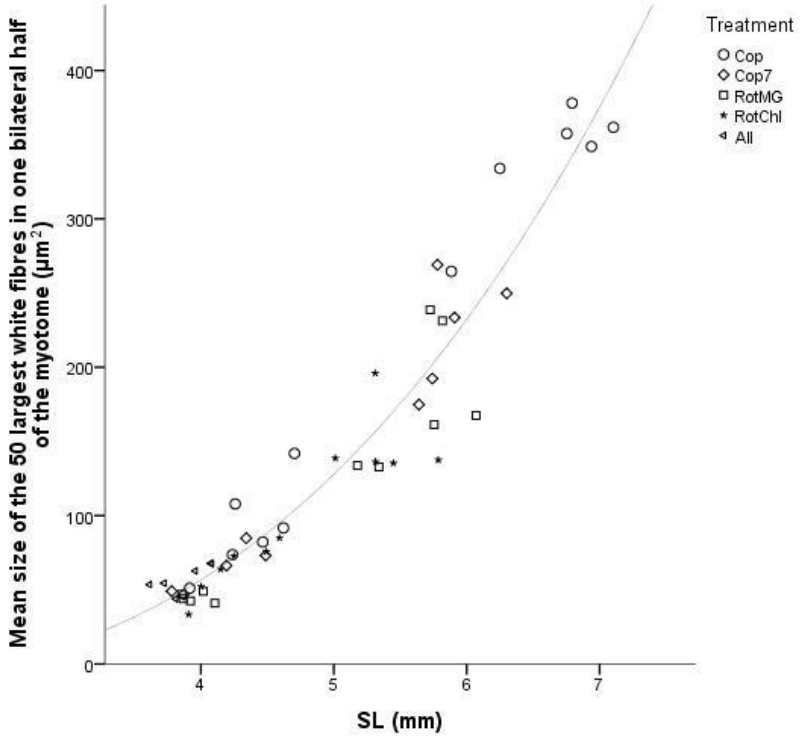


Figure 3.12: Mean size of the 50 largest white fibres present in one bilateral half of the myotome, for larvae in the different treatments at 4, 8 and 21 dph (n = 6 at 4 dph, n = 6, 5, 5, 5 at 8 dph and n = 6, 5, 6, 6 at 21 dph). Each point in the graph represents a larva. Cubic fit line added at total, R^2 = 0,922.

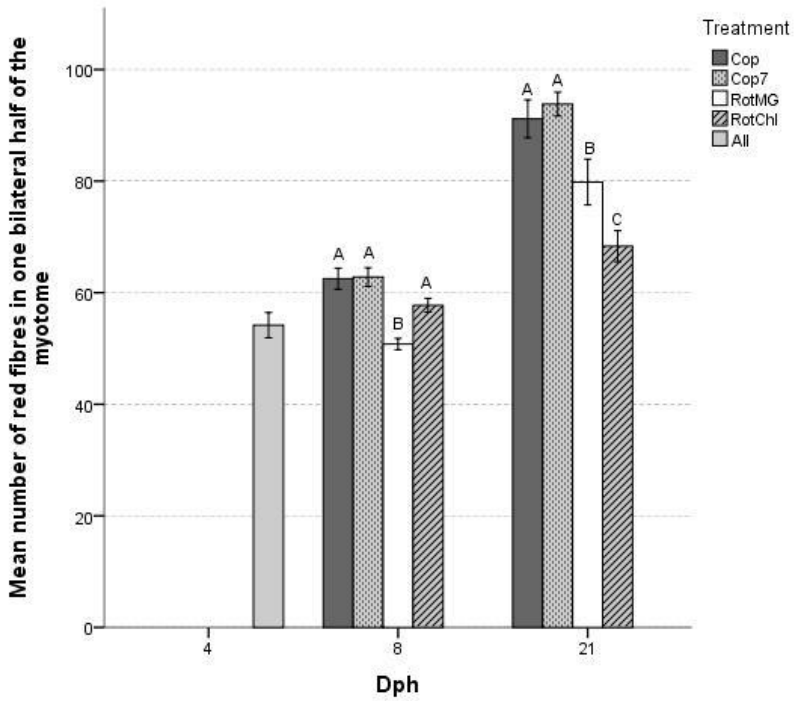


Figure 3.13: Mean number of red fibres in one bilateral half of the myotome, for larvae in the different treatments at 4, 8 and 21 dph (n = 6 larvae treatment^{-1} at 4 dph, n = 6, 5, 5, 4 at 8 dph and n = 6, 5, 6, 6 at 21 dph)**.** Different letters mark significant differences between the treatments, and error bars indicate ± 1 standard error.

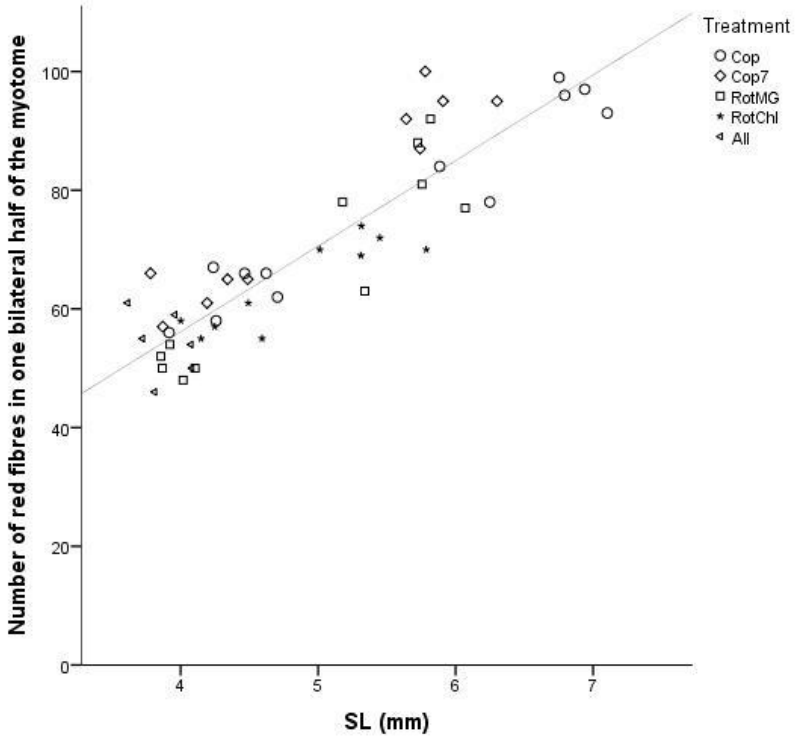
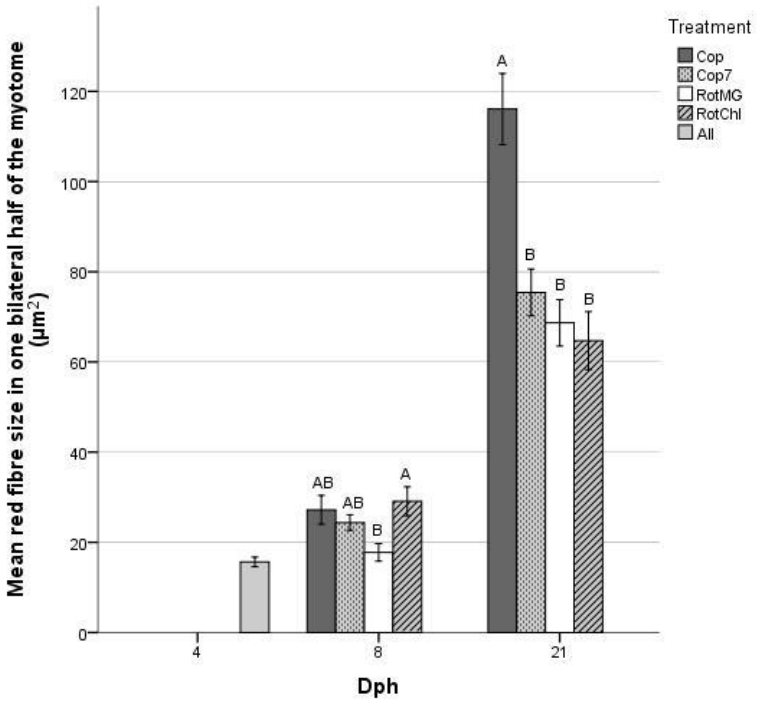
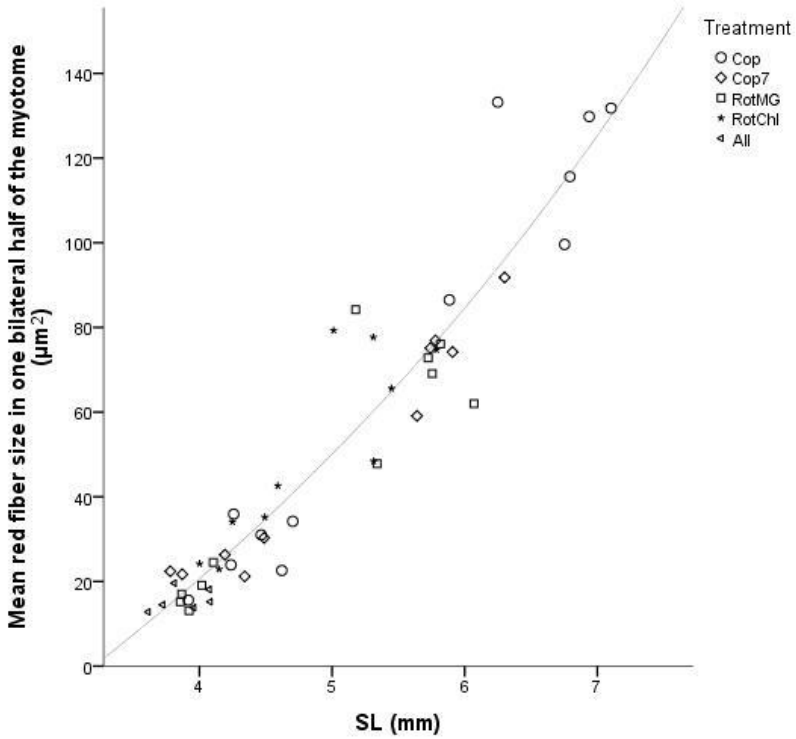


Figure 3.14: Number of red fibres in one bilateral half of the myotome, for larvae in the different treatments at 4, 8 and 21 dph (n = 6 larvae treatment^{-1} at 4 dph, n = 6, 5, 5, 4 at 8 dph and n = 6, 5, 6, 6 at 21 dph)**.** Each point in the graph represents one larva. Linear fit line added at total, R^2 = 0,813.



Figur 3.15: Mean size of the red fibres in one bilateral half of the myotome, for larvae in the different treatments at 4, 8 and 21 dph (n = 6 larvae treatment^{-1} at 4 dph, n = 6, 5, 5, 4 at 8 dph and n = 6, 5, 6, 6 at 21 dph)**.** Different letters mark significant differences between the treatments, and error bars indicate ± 1 standard error.



Figur 3.16: Mean size of the red fibres in one bilateral half of the myotome, for larvae in the different treatments at 4, 8 and 21 dph (n = 6 larvae treatment^{-1} at 4 dph, n = 6, 5, 5, 4 at 8 dph and n = 6, 5, 6, 6 at 21 dph)**.** Each point in the graph represents one larva. Cubic fit line added at total, R^2 = 0,900.

4. Discussion

It is well known that different diets may result in highly different somatic growth rates, between fish larvae of the same genetic origin (Claramunt & Wahl, 2000). The current hypothesis was that larvae fed copepod nauplii would achieve better survival and growth, and increased muscle development, compared to larvae fed rotifers.

4.1 Effects of prey type/quality on somatic growth and survival

Already at 8 dph, larvae from the copepod treatments were larger than larvae from the RotMG treatment, in terms of DW, SL and MH. Larvae from both copepod treatments kept a solid growth advantage in the period of receiving copepod nauplii (4-10 and 4-30 dph), but experienced a clear reduction in growth rate when their diet changed to short-term enriched rotifers and *Artemia*. The subsequent growth rate of larvae from Cop7 was actually lower than that of larvae from both rotifer treatments, indicating that the suppressed growth was not attributed only to nutritional quality. Other experiments have shown that when ballan wrasse larvae are fed copepod nauplii initially, they don't seem to appreciate a swap in live prey type to rotifers and *Artemia*; seen as reduced feeding activity (Elin Kjørsvik, pers. comm.). This may indicate that copepod nauplii are more attractive (physically and/or chemically) to the fish larvae than rotifers and *Artemia*, and that the increased larval growth rate may be a result of greater consumption of prey organisms, and not only due to differences in nutritional quality.

A reduction in growth rate associated with dietary change has also been observed in cod larvae. A short period (14 days) of dietary change from enriched rotifers to natural zooplankton had a positive effect on growth; and a negative effect when the change was reversed (Koedijk et al., 2010). When the feeding regime switched from copepod nauplii/rotifers to *Artemia* (present study), larvae from the different treatments became progressively more alike, and by the onset of weaning (40 dph) the larvae were practically identical regarding DW, SL and MH. At the end of the experiment (60 dph), larvae from the copepod treatments had gained significantly higher DW than larvae from the rotifer treatments, due to faster growth late in the formulated feed period. Early diet may have a long-term effect on growth (Imsland et al., 2006), so the increased growth (towards the end of the experiment) of larvae from both copepod treatments,

may be a result of being fed copepod nauplii initially. However, a two-month experiment is not adequate for studying long-term effects, so analysis of older larvae is required to further evaluate such effects. It should be mentioned that the size of copepod nauplii were not adjusted as the fish larvae grew larger, so the nauplii size may eventually have become too small to be energetically optimal; possibly preventing the larvae to grow at their full potential.

The positive effects of using zooplankton as live feed for fish larvae have been documented in many studies. When fed copepods (adult or nauplii), larvae of cod and sea bass achieved faster growth rates, compared to when being fed rotifers and *Artemia* (Rajkumar & Vasagam, 2006 ; Koedijk et al., 2010 ; Overrein, 2010 ; Hansen, 2011). Copepod nauplii have also shown to be the best live feed for flatfish, in terms of survival and normal development (Bell et al., 2003). The improvements seen in growth, survival and development are mainly linked to nutritional quality of the feed, and copepods are known to contain high amounts of protein and HUFAs, compared to rotifers and *Artemia* (Støttrup & Jensen, 1990 ; Helland et al., 2003 ; Overrein, 2010). In the present study, the nutritional analysis of the live feed revealed that the copepod nauplii contained approximately 50 and 90 % more protein than the rotifers and *Artemia*, respectively. The copepod nauplii also had the lowest lipid content, but the HUFAs DHA and EPA constituted 26.6 % of total fatty acids; a value similar to the short-term enriched rotifers and *Artemia*. Furthermore, the HUFAs in copepods are known to be easier available to fish larvae, due to mainly being located in phospholipids (PL), and less in neutral storage lipids (NL) (Izquierdo et al., 2000 ; Gisbert et al., 2005). Experiments on larvae of cod and sea bass (*Dicentrarchus labrax*) showed that the essential HUFAs were more beneficial for growth and development, when they were incorporated in dietary PL rather than NL (Gisbert et al., 2005 ; Kjørsvik et al., 2009).

The larger fraction of phospholipids in copepods (Fraser et al., 1989 ; van der Meeren et al., 2008), combined with easier availability of HUFAs, should imply that fish larvae fed copepod nauplii would get developmental advantages compared to larvae fed rotifers and *Artemia*. Other results from our experiment showed that larvae from the Copepod treatment (28 dph) caught prey more efficiently, were more tolerant to stress and had less skeletal deformities (twisted neural arches excluded), than larvae from both rotifer treatments (Sørøy, 2012). The improved ability to catch prey may be a result of

increased swimming capacity due to greater muscle mass, but it may also indicate a more highly developed sensory apparatus in the larvae fed copepod nauplii. Studies have shown that DHA is essential for neural development and function, including that of the retina (Bell & Tocher, 1989 ; Brown, 1994). It is therefore likely that DHA has a key role regarding visual perception in fish larvae, which undoubtedly would affect their ability to spot and catch prey. In a stress test conducted at 29 dph, it was found that larvae from the Copepod treatment had significantly lower mortality (after 24 hours) than larvae from the RotChl treatment. There were large differences in mortality between larvae from both rotifer treatments, and whether an individual survived or not was found to correlate positively with larval SL. All tested larvae from the Copepod treatment survived after being stressed, and these larvae were also largest in terms of SL (Sørøy, 2012). This clearly demonstrates the importance of minimizing the stress inflicted upon fish larvae in a rearing facility, particularly in their earliest stages of life.

Based on the live feed analysis, as well as the higher growth rate observed in larvae fed copepod nauplii, it is clear that optimal conditions were not met when feeding larvae with short-term enriched rotifers. During the first week (4-12 dph), larvae fed rotifers achieved only half the growth rate compared to larvae fed copepod nauplii. Gagnat (2012) calculated the daily growth rate of various organs in the ballan wrasse larvae, and found that the total organ growth rate (4-8 dph) was 2-4 times higher in larvae fed copepod nauplii, compared to larvae fed rotifers. The differences in growth rate were particularly high for organs associated with locomotion and digestion, such as skeletal muscle, liver and intestine. In the period 4-21 dph, the present study showed highly increased muscle growth in larvae from the Copepod treatment, which had (on average) 3 times larger muscle area than larvae from the RotMG treatment (21 dph). A substantial part of fish larval growth is increased muscle mass by synthesis and deposition of protein, so the fast muscle growth in larvae fed copepod nauplii was probably triggered by the higher levels of dietary protein and (possibly) free amino acids (FAA) (Rønnestad et al., 1999). The content of FAA in the live feed was not analyzed, but other studies have shown that copepods and copepod nauplii (harvested) have more FAA compared to enriched rotifers and *Artemia* (Moren et al., 2006 ; Hamre et al., 2008 ; van der Meeren et al., 2008 ; Conceição et al., 2010).

In the present study, the protein and total lipid content of rotifers with and without short-term enrichment were almost identical. However, the enrichment boosted the level of DHA in the short-term enriched rotifers, which then achieved a higher DHA:EPA ratio compared to the cultivated rotifers. Diets which contain a high DHA:EPA ratio (> 1.5-2.0) generally seem to result in better survival and growth among fish larvae, compared to diets with lower ratios (Moksness et al., 2004 ; Wold et al., 2009). The copepod nauplii, *Artemia* nauplii and short-term enriched rotifers had DHA:EPA ratios well above this threshold (5.3, 4.1 and 3.0 respectively), while the cultivated rotifers had a ratio of 1.7. At the end of the experiment, the RotChl treatment had significantly lower survival (5 %) than the other treatments (10-12 %), implying that a higher DHA:EPA ratio is beneficial for survival. The difference in ratio between short-term enriched/cultivated rotifers did not seem to affect growth, because larvae from both rotifer treatments had very similar growth patterns throughout the experiment.

Initially there were massive mortalities in all treatments, and by 13 dph more than 75 % of all the larvae had died. Since larvae fed copepod nauplii survived no better than larvae fed rotifers, it seems fair to assume that some other factor(s) than the feed were causing the vast mortality at this point. Considering the fragile state of newly hatched marine pelagic fish larvae, it is possible that the majority of larvae could not cope with the physical stress associated with transportation, and distribution to the tanks. The aeration in the tanks was considered to be gentle, but still it may have been an additional stressor for the fish larvae. In intensive production of marine fish larvae, it is not unusual that mass mortality is caused by opportunistic pathogenic bacteria in the sea water (Skjermo & Vadstein, 1999). The immature immune systems of fish larvae make them extra vulnerable to microbial attack (Hansen & Olafsen, 1999), so harmful microbes may also be a possible explanation for the high initial larval deaths. The overall survival (5-12 %) was lower than what was achieved in a similar experiment on cod (11-20 %) (Halseth, 2011).

4.2 Dietary effects on muscle development and growth

Generally there is a positive correlation between body length and developmental stage within fish larvae (Laurence, 1979). It is known that the swimming performance of fish larvae is related to body length (Johnston & Hall, 2004), and therefore it seems likely that also growth of swimming musculature would be related to body length. The present study did reveal a strong correlation between SL and muscle growth, including total muscle cross-sectional area, hyperplastic- and hypertrophic growth.

Studies on other marine species have shown that increased somatic growth rates are related to increased contributions of white fibre hyperplasia, to larval muscle growth (Akster et al., 1995 ; Alami-Durante et al., 1997 ; Galloway et al., 1999 ; Ostaszewska et al., 2008 ; Leitao et al., 2011). In the present study, the larval muscle growth clearly reflected the differences in somatic growth rate. By 21 dph, larvae from the Copepod treatment had increased their total cross-sectional area of muscle more than 18-fold, while larvae from the rotifer treatments only achieved a 4-6-fold increase. This was supported by a parallel organ study on the ballan wrasse larvae, which found that larvae from the Copepod treatment (on average) increased their total volume of muscle tissue 27 times from 4-21 dph, compared to 8-11 times in larvae from the other treatments (Gagnat, 2012). Skeletal musculature is the largest and most rapidly growing tissue in larval, juvenile and adult fish, and this was also found in our experiment (Alami-Durante, 1990 ; Osse & van den Boogaart, 1995 ; Gagnat, 2012). Muscle growth by hyperplasia was measured as an increase in white fibre number, while hypertrophic growth was measured as an increase in fibre size within the deep white fibre zone. Regardless of treatment, the hyperplastic and hypertrophic growth correlated strongly with larval SL. At 21 dph, larvae from the Copepod treatment were overall largest, in addition to having the greatest number of white fibres and the largest deep white fibres.

Differences in hypertrophy and hyperplasia (between treatments) are best studied in figure 3.10, and differences can be seen already at 8 dph. At this point, larvae from the Copepod treatment had an increased hypertrophic growth compared to larvae from the other treatments, represented by a greater number of large fibres (100-200 and > 200 μm^2) and less small fibres (< 50 μm^2). At 21 dph it is clear that the number of large fibres (100-200 and > 200 μm^2) and small recruitment fibres (< 50 μm^2) increased with

increasing larval length. However, the largest analyzed larvae were all from the Copepod treatment (21 dph), and therefore it is not certain if the increased muscle growth in these larvae is a result of diet or size alone. With a larger sample size it may have been possible to find and compare similarly sized larvae across treatments, which potentially could give more insight regarding dietary effects on muscle growth.

In the youngest larvae (4 dph) the muscle fibres were organized in two layers that were clearly distinguishable. A single layer of red fibres encircled several layers of inner white fibres; a pattern which seems to be common for newly hatched larvae of most teleost fish (Proctor et al., 1980 ; O'Connell, 1981 ; van Raamsdonk et al., 1982 ; Batty, 1984 ; Matsuoka & Iwai, 1984 ; Galloway et al., 1999 ; Ostaszewska et al., 2008 ; Halseth, 2011). In the period 4-21 dph, white fibres covered 76-91 % of the total muscle area in the fish larvae. There was a much better correlation between the fraction of white fibres and larval SL at 21 dph (R^2 = 0.741), than at 8 dph (R^2 = 0.183). Larvae from the Copepod treatment all had white fibre fractions close to 90 %, and these larvae were overall largest in terms of SL (6.6 mm on average). To compare, larvae from the RotChl treatment had white fibre fractions mostly under or close to 80 %, and were overall smallest (5.2 mm on average). For cod larvae at 30 and 60 dph there have been reported white fibre fractions above 90 % (Galloway et al., 1999 ; Halseth, 2011), which is also normal to find in adult fish (Bone, 1978).

From 4 to 21 dph there was a relatively small increase in red fibre number, compared to white. Still, larvae from both copepod treatments had significantly more red fibres at 21 dph, than larvae from the rotifer treatments. In general, the greater muscle mass and number of red and white muscle fibres indicate that larvae from the Copepod treatment would have increased swimming capacity (both when it comes to aerobic endurance and explosivity), and thus be better suited for foraging and catching prey than larvae from the other treatments. However, in addition to muscular propulsion, the swimming capacity of fish larvae is highly related to their length, as described by the Reynolds number (Re). The ratio between inertial and viscous forces affects the flow pattern around an object in a liquid medium, in relation to the objects length and speed (Osse & van den Boogaart, 2004). Re values below 200 indicate that friction due to viscosity is considerable; a reality that fish larvae have to endure until they reach around 12 mm in

length (Johnston & Hall, 2004 ; Osse & van den Boogaart, 2004). The high energy losses associated with friction makes it crucial for fish larvae to increase their length quickly, to ensure a fast escape from the viscous forces acting upon them (Shine, 1978 ; Osse & van den Boogaart, 2004).

New white muscle fibres were recruited between 4 and 21 dph, from distinct zones at the dorsal and ventral apical regions of the myotome, as well as laterally beneath the sheet of red fibres, and close to the horizontal septum. Similar zones of fibre recruitment have been found in other marine species, including Atlantic cod, sea bass, gilthead sea bream (*Sparus aurata*) and plaice (*Pleuronectens platessa*) (Veggetti et al., 1990 ; Brooks & Johnston, 1993 ; Rowlerson et al., 1995 ; Galloway et al., 1999 ; Halseth, 2011). This type of fibre recruitment represents the second phase of myogenesis (stratified hyperplasia), which is the main mechanism for increasing fibre numbers after hatching (Johnston, 2006). However, the third phase (mosaic hyperplasia) was not observed for any larvae at 21 dph. The onset of mosaic hyperplasia varies between species, but it occurs mainly during the juvenile stage (Rowlerson & Veggetti, 2001b). In herring (*Clupea harengus*), the metamorphosis is complete at fish lengths around 37 mm (total length), and mosaic hyperplasia have been found to start in individuals exceeding 25 mm (Johnston et al., 1998). A recent study on cod larvae revealed traces of mosaic hyperplasia in individuals ranging from 19-28 mm (SL) (Halseth, 2011), and the transition from larvae to juvenile cod happen at fish lengths of 20-30 mm (Falk-Petersen, 2005). Metamorphosis in ballan wrasse is complete at fish lengths of 10-11 mm (SL) (Ottesen et al., 2012), and the largest analyzed larvae in the present study (muscle) were 5-7 mm. Considering the apparent relation between metamorphosis and mosaic hyperplasia, it is more likely that mosaic hyperplasia can be observed in larvae sampled later in the experiment.

4.3 Conclusions and future perspectives

Feeding ballan wrasse larvae with intensively cultivated copepod nauplii (4-10 or 4-30 dph) resulted in higher somatic growth, compared to larvae fed short-term enriched rotifers. The rapid decrease in growth observed after the dietary switches from copepod nauplii to rotifers and *Artemia*, strongly suggests that ballan wrasse larvae have problems accepting a different live feed, after being introduced to copepod nauplii. There is a chance that such an issue may be solved by prolonging the co-feeding periods, gradually increasing the fraction of the new live feed organism. However, considering the nutritional benefits of copepod nauplii/adults, I think that future experiments should test diets solely consisting of copepods; and where the sizes of copepods are adjusted according to larval size. In order to achieve better survival, the main focus should be to further minimize all the factors causing physical stress upon the larvae, especially in the first period after hatching.

The larval muscle grew by both hyperplasia and hypertrophy, and clearly reflected the differences in SL. Considering the strong correlation between muscle growth and SL, the increased muscle growth in larvae from the Copepod treatment is likely due to size rather than diet. However, with the lack of equally large larvae across the treatments, dietary effects cannot be ruled out. For similar experiments in the future it is advisable to increase the sample size of larvae, in an attempt to find equally large individuals across treatments, so that possible dietary effects on muscle growth can be separated from size-related effects. It is also important to design experiments in such a way that long-term effects can be evaluated further, because muscle growth in early life stages of ballan wrasse may affect the growth rate later in life, as well as the ultimate size.

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APPENDIX 1 – Production and composition of formulated feed

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.

	% (dw)
Protein	60.1
Lipid	13.1
Carbohydrates	14.8
Ash	12.6

Recipe

	% (ww)
Fish meal LT [a]	47,162
Shrimp meal[b]	24
Wheat[c]	17,8
Soy lecithin[d]	3
Cod Powder[e]	5
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]Cod fish powder, product code 0271, Seagarden, Avaldsnes 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 – Muscle analysis

Table A1: Standard length, myotome height, total cross-sectional area of red and white fibres, % red and white fibres of total muscle area, number of red and white fibres and mean size of red and white fibres, of all the analysed larvae at 4, 8 and 21 dph.

							% of total muscle area					
DPH	Treatment	Individual	SL (mm)	MH (mm)	TotA white (μm^2)	TotA red (μm^2)	White	Red	# white	# red	Mean white fibre size (µm2)	Mean red fiber size (µm2)
4	All	A	3.96	0.26	5188.813	816.232	86.4	13.6	92	59	62.7	13.8
4	All	B	4.07	0.24	5747.228	976.11	85.5	14.5	97	54	67.8	18.1
4	All	C	3.72	0.24	3928.732	796.766	83.1	16.9	69	55	54.5	14.5
4	All	D	3.61	0.23	4524.727	781.399	85.3	14.7	91	61	53.6	12.8
4	All	E	4.08	0.25	5460.636	757.258	87.8	12.2	86	50	67.6	15.2
4	All	F	3.81	0.21	3498.778	901.232	79.5	20.5	70	46	43.9	19.6
8	Cop	A	4.24	0.30	6975.423	1604.358	81.3	18.7	94	67	73.7	23.9
8	Cop	B	4.62	0.36	9352.252	1490.07	86.3	13.7	93	66	91.7	22.6
8	Cop	C	3.92	0.24	4139.96	871.696	82.6	17.4	73	56	51.1	15.6
8	Cop	D	4.70	0.37	13790.7	2122	86.7	13.3	113	62	141.9	34.2
8	Cop	E	4.26	0.30	9853.7	2083.9	82.5	17.5	103	58	107.9	35.9
8	Cop	F	4.47	0.35	8644.884	2049.186	80.8	19.2	104	66	82.2	31.1
8	Cop7	A	4.19	0.31	6960	1607.035	81.2	18.8	119	61	66.3	26.3
8	Cop7	B	3.87	0.27	4598.352	1238.472	78.8	21.2	88	57	46.8	21.7
8	Cop7	C	3.78	0.27	5560.664	1480.901	79.0	21.0	105	66	49.1	22.5
8	Cop7	D	4.34	0.30	7495.8	1379	84.5	15.5	102	65	84.8	21.2
8	Cop7	E	4.49	0.34	8641.2	1970	81.4	18.6	128	65	73.1	30.3
8	Cop7	F	3.70	0.19								
8	RotMG	A	3.87	0.21	3297.788	851.677	79.5	20.5	69	50	44	17.0
8	RotMG	B	3.86	0.24	3748.141	788.311	82.6	17.4	85	52	47	15.2
8	RotMG	C	4.02	0.24	4867.228	918.517	84.1	15.9	93	48	49	19.1
8	RotMG	D	3.92	0.23	3365.981	707.369	82.6	17.4	84	54	42.5	13.1
8	RotMG	E	4.11	0.24	4197.476	1223.8	77.4	22.6	87	50	41	24.5
8	RotMG	F	4.16	0.24	4492.905	917.065	83.0	17.0				
8	RotChl	A	3.91	0.19	3380.326	929.715	78.4	21.6	79		33.5	
8	RotChl	B	3.87	0.21	2954.875	766.121	79.4	20.6				
8	RotChl	C	4.00	0.27	4510.304	1404.507	76.3	23.7	95	58	52.3	24.2
8	RotChl	D	4.15	0.24	5155.909	1257.479	80.4	19.6	90	55	64	22.9
8	RotChl	E	4.49	0.32	7541.5	2145.9	77.8	22.2	104	61	75.8	35.2
8	RotChl	F	4.25	0.29	6487.39	1941.526	77.0	23.0	87	57	72.8	34.1

							% of total muscle area				Mean white	Mean red fiber
DPH	**Treatment**	**Individual**	**SL (mm)**	**MH (mm)**	**TotA white (µm^2)**	**TotA red (µm^2)**	**White**	**Red**	**# white**	**# red**	**fibre size (µm2)**	**size (µm2)**
21	Cop	**A**	6.25	0.86	77665.2	10390.3	88.2	11.8		78	334	133.2
21	Cop	**B**	7.10	1.04	115565.9	12258.7	90.4	9.6	859	93	361.7	131.8
21	Cop	**C**	5.88	0.71	44152.2	7268.7	85.9	14.1	424	84	264.6	86.5
21	Cop	**D**	6.79	0.99	109936	11101	90.8	9.2	827	96	378.1	115.6
21	Cop	**E**	6.75	0.93	87712.9	9863.6	89.9	10.1	823	99	357.5	99.6
21	Cop	**F**	6.94	1.05	113934.4	12590.4	90.0	10.0	980	97	348.8	129.8
21	Cop7	**A**	5.64	0.59	25537.2	5439.2	82.4	17.6	335	92	174.8	59.1
21	Cop7	**B**	5.08	0.42	11560	3089.094	78.9	21.1				
21	Cop7	**C**	6.30	0.80	49657.1	8723.2	85.1	14.9	724	95	249.8	91.8
21	Cop7	**D**	5.74	0.66	34768.7	6531.1	84.2	15.8	546	87	192.4	75.1
21	Cop7	**E**	5.78	0.71	58333.5	7687.9	88.4	11.6	625	100	269	76.9
21	Cop7	**F**	5.91	0.78	45277.3	7050.6	86.5	13.5	520	95	233.5	74.2
21	RotMG	**A**	5.18	0.55	25252.3	6565	79.4	20.6		78	133.8	84.2
21	RotMG	**B**	5.73	0.55	32104.5	6410.6	83.4	16.6	431	88	238.7	72.8
21	RotMG	**C**	5.82	0.76	44840.5	7004.8	86.5	13.5	483	92	231.3	76.1
21	RotMG	**D**	6.07	0.61	24869.7	4771.1	83.9	16.1	372	77	167.5	62.0
21	RotMG	**E**	5.76	0.55	25463.6	5598.1	82.0	18.0	416	81	161.3	69.1
21	RotMG	**F**	5.34	0.46	14160.712	3010.352	82.5	17.5	216	63	132.8	47.8
21	RotChl	**A**	5.31	0.49	18676.8	3585.2	83.9	16.1		74	136.4	48.4
21	RotChl	**B**	5.79	0.55	18093.5	5230.8	77.6	22.4	285	70	137.5	74.7
21	RotChl	**C**	5.45	0.55	19442.7	4723.5	80.5	19.5	345	72	135.4	65.6
21	RotChl	**D**	4.59	0.34	8227.8	2345.012	77.8	22.2	113	55	85	42.6
21	RotChl	**E**	5.31	0.45	19052.8	5358.4	78.0	22.0	262	69	196	77.7
21	RotChl	**F**	5.01	0.44	19260.3	5553.2	77.6	22.4	274	70	138.7	79.3

APPENDIX 3 – Copepod hatching test

Prior to testing for hatching success, the eggs were stored in 75 cm^3 NUNC EasyFlasks™. After shaking the flasks, 50 µl was extracted and diluted up to 1 ml with seawater. From the diluted solution, another 50 µl was extracted and transferred to a petri dish. A stereomicroscope (MZ-12.5, Leica Microsystems, Germany) with an equipped camera (DFW-SX900. Sony, Japan) was used to photograph the droplet, and the photograph was printed out in order to count the eggs manually. Then the eggs were set to hatch, by adding 10 ml of seawater and concealing the petri dish with a lid. After hatching (48h) the nauplii were fixated with phytofix and counted in a stereomicrocope, using a peristaltic pump (Watson Marlow Alitea Inc., Sweden).

The flasks with remaining eggs were weighed, and the egg volumes were calculated using equation 1. The number of eggs in each flask was calculated by equation 2, and hatching rate was calculated by equation 3.

1) $V = (F - F_0) \times D_{sw}$
2) $N = X \times V$
3) $HR = (N / E) \times 100$

Symbol explanation:

V = egg volume
F = flask with eggs and water
F_0 = empty flask
D_{sw} = density of seawater: 1.028
X = eggs ml^{-1}
HR = hatching rate
N = number of eggs (2), number of nauplii (3)
E = number of eggs

APPENDIX 4 – Survival

Table A2: Survival (mean % ± SE) for all treatments from 13-61 dph.

Copepod			Cop7			RotMG			RotChl		
Dph	Mean %	Std. Error of Mean	Dph	Mean %	Std. Error of Mean	Dph	Mean %	Std. Error of Mean	Dph	Mean %	Std. Error of Mean
13	14.70	2.26	13	23.71	5.30	13	17.19	2.69	13	12.08	.87
14	14.70	2.26	14	23.71	5.30	14	16.07	1.62	14	11.49	.42
15	14.70	2.26	15	23.71	5.30	15	16.07	1.62	15	10.14	1.30
16	14.27	2.03	16	22.90	5.08	16	15.22	1.59	16	9.78	1.12
17	14.27	2.03	17	22.90	5.08	17	15.22	1.59	17	9.66	1.23
18	14.12	1.99	18	22.23	4.88	18	14.74	1.63	18	9.39	1.15
19	14.12	1.99	19	22.23	4.88	19	14.74	1.63	19	9.29	1.25
20	12.42	1.84	20	20.13	4.18	20	13.45	1.60	20	8.37	.71
21	12.42	1.84	21	20.13	4.18	21	13.45	1.60	21	7.86	1.21
22	12.29	1.80	22	19.69	4.07	22	12.97	1.57	22	7.44	.99
23	12.29	1.80	23	19.69	4.07	23	12.97	1.57	23	7.40	1.03
24	12.29	1.80	24	19.69	4.07	24	12.97	1.57	24	7.40	1.03
25	12.10	1.81	25	19.03	3.85	25	12.50	1.49	25	7.06	.85
26	12.10	1.81	26	19.03	3.85	26	12.50	1.49	26	7.03	.88
27	11.93	1.88	27	18.66	3.80	27	11.89	1.46	27	6.85	.79
28	11.93	1.88	28	18.66	3.80	28	11.89	1.46	28	6.68	.96
29	11.85	1.90	29	18.36	3.83	29	11.54	1.42	29	6.37	.81
30	11.85	1.90	30	18.36	3.83	30	11.54	1.42	30	6.31	.87
31	11.85	1.90	31	18.36	3.83	31	11.54	1.42	31	6.31	.87
32	11.58	1.81	32	17.03	3.46	32	10.73	1.52	32	5.73	.58
33	11.58	1.81	33	17.03	3.46	33	10.73	1.52	33	5.70	.61

34	11.56	1.81	**34**	16.49	3.29	**34**	10.59	1.52	**34**	5.57	.54
35	11.52	1.82	**35**	16.25	3.22	**35**	10.52	1.54	**35**	5.46	.59
36	11.52	1.82	**36**	16.25	3.22	**36**	10.52	1.54	**36**	5.46	.59
37	11.49	1.83	**37**	16.16	3.21	**37**	10.50	1.53	**37**	5.45	.59
38	11.49	1.83	**38**	16.16	3.21	**38**	10.50	1.53	**38**	5.44	.60
39	11.49	1.83	**39**	16.16	3.21	**39**	10.50	1.53	**39**	5.44	.60
40	11.37	1.81	**40**	16.06	3.18	**40**	10.48	1.52	**40**	5.43	.60
41	11.35	1.81	**41**	16.00	3.16	**41**	10.48	1.53	**41**	5.43	.60
42	11.33	1.80	**42**	15.98	3.16	**42**	10.48	1.53	**42**	5.43	.60
43	11.33	1.81	**43**	15.83	3.07	**43**	10.47	1.53	**43**	5.43	.60
44	11.31	1.80	**44**	15.81	3.05	**44**	10.47	1.53	**44**	5.43	.60
45	11.29	1.79	**45**	15.77	3.04	**45**	10.47	1.53	**45**	5.43	.60
46	11.23	1.78	**46**	15.69	3.00	**46**	10.46	1.52	**46**	5.43	.60
47	11.22	1.78	**47**	15.64	2.97	**47**	10.45	1.53	**47**	5.43	.60
48	11.21	1.77	**48**	15.61	2.96	**48**	10.45	1.53	**48**	5.43	.60
49	11.20	1.76	**49**	15.54	2.93	**49**	10.45	1.53	**49**	5.43	.60
50	11.18	1.74	**50**	15.47	2.89	**50**	10.45	1.52	**50**	5.42	.59
51	11.13	1.71	**51**	15.24	2.77	**51**	10.45	1.52	**51**	5.42	.59
52	11.02	1.65	**52**	14.86	2.57	**52**	10.43	1.50	**52**	5.42	.59
53	10.87	1.57	**53**	14.27	2.28	**53**	10.36	1.46	**53**	5.42	.59
54	10.73	1.49	**54**	13.76	2.05	**54**	10.32	1.45	**54**	5.42	.59
55	10.64	1.44	**55**	13.51	1.95	**55**	10.27	1.43	**55**	5.42	.59
56	10.54	1.37	**56**	13.21	1.81	**56**	10.19	1.35	**56**	5.42	.59
57	10.48	1.35	**57**	12.99	1.70	**57**	10.16	1.34	**57**	5.42	.59
58	10.36	1.29	**58**	12.77	1.63	**58**	10.10	1.29	**58**	5.42	.59
59	10.21	1.23	**59**	12.54	1.54	**59**	10.03	1.27	**59**	5.42	.59
60	10.13	1.22	**60**	12.40	1.50	**60**	9.99	1.28	**60**	5.42	.59
61	10.07	1.20	**61**	12.26	1.44	**61**	9.93	1.26	**61**	5.42	.59

APPENDIX 5 – Dry weight

Table A3: Mean dry weight (mg/larva) for each treatment, from 2-61 dph.

		Dry weight (mg)		
DPH	**Treatment**	**Mean**	**SE**	**Total N**
2	All	.0443	.0015	12
4	All	.0403	.0014	12
8	Copepod	.0740	.0022	12
	Cop7	.0716	.0046	12
	RotMG	.0441	.0037	12
	RotChl	.0495	.0031	12
12	Copepod	.1661	.0178	12
	Cop7	.1519	.0146	12
	RotMG	.0776	.0052	12
	RotChl	.0846	.0017	12
15	Copepod	.2696	.0143	12
	Cop7	.1952	.0126	12
	RotMG	.1173	.0092	12
	RotChl	.1094	.0091	12
21	Copepod	.6539	.0293	12
	Cop7	.2740	.0367	12
	RotMG	.2068	.0285	12
	RotChl	.2174	.0122	12
27	Copepod	1.2497	.0270	24
	Cop7	.8054	.0390	24
	RotMG	.7277	.0167	24
	RotChl	.5679	.0059	24
33	Copepod	2.8658	.1526	30
	Cop7	2.1356	.4574	30
	RotMG	1.9916	.0781	30
	RotChl	1.5009	.0459	30
40	Copepod	4.8824	.1158	30
	Cop7	4.2804	.3191	30
	RotMG	4.6096	.2403	30
	RotChl	4.5872	.2156	30
47	Copepod	8.8054	.4410	30
	Cop7	8.2880	1.3426	30
	RotMG	7.4979	.4165	30
	RotChl	8.5205	.5619	30
61	Copepod	19.6360	.0420	50
	Cop7	17.0820	2.7451	50
	RotMG	13.7694	1.3611	50
	RotChl	12.9559	1.7661	50

APPENDIX 6 – Daily weight increase

Table A4: Mean daily weight increase (% DWI) for chosen intervals during the experiment.

		% DWI													
		2-4 dph		**4-12 dph**		**12-27 dph**		**27-40 dph**		**40-47 dph**		**47-61 dph**		**2-61 dph**	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Treatment	**Cop**	-4.6	0	19.3	1.6	14.5	1	11	0.1	8.8	0.4	5.9	0.4	10.9	0
	Cop7	-4.6	0	17.9	1.4	11.8	0.4	13.7	0.3	9.6	1.4	5.3	1	10.6	0.3
	RotMG	-4.6	0	8.5	0.9	16.2	0.5	15.2	0.5	7.2	1.1	4.4	1.1	10.2	0.2
	RotChl	-4.6	0	9.7	0.3	13.5	0.2	17.4	0.5	9.7	2.1	3	1.5	10.1	0.3