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Effects of different live feed on larval growth and development in ballan wrasse (*Labrus bergylta* Ascanius, 1767)

- A metabolomics study

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Abstract

The use of ballan wrasse (*Labrus bergylta*) for the treatment of salmon lice (*Lepeophtheirus salmonis*) in salmon farming is rapidly growing due to resistance to chemical treatments. The demand for high quality and large amounts of ballan wrasse has thus led to the development of ballan wrasse farming. Due to the small size of the newly hatched larvae, the first-feeding of the larvae has become a bottleneck area. The size and nutritional properties of the live feed chosen can greatly affect both growth and development. Using copepods as live feed in the first part of the feeding regime should give a positive effect on the larval growth and development as they are the natural prey of marine fish larvae and are nutritional superior to rotifers.

The aim of this study was to use 1H-NMR to examine the differences in larval metabolic composition as a function of the different types of live feed, as well as to find the most suited method for preparation of ballan wrasse samples for metabolic analysis. To examine this, ballan wrasse larvae was fed using four different feeding regimes. The live feed used was different between the four groups for the first 30 days post hatch. After this all groups were fed the same diet. Larvae in the Copepod group were fed only the copepod *Acartia tonsa*. The Cop7 was fed copepods the first 7 days, then enriched rotifers. The RotMG group was fed enriched rotifers, and the RotChl group was fed unenriched rotifers. After 30 days post hatch, all treatments was fed enriched *Artemia* before weaning to formulated feed.

Results from the present study indicate that the use of copepods in first-feeding of ballan wrasse resulted in an increase in growth compared to larvae fed rotifer diets. Metabolic analysis of the larvae show that changes in the levels of certain metabolites can be used as biomarkers for growth and development. Differences seem to be correlated to feed composition, with TMAO and taurine standing out as reliable biomarkers for growth differences

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1. Introduction

Since the early 1970s farming of Atlantic salmon (*Salmo salar*) has been practiced in Norway, and since 1977 salmon has been the main species in Norwegian aquaculture (Statistikk, 1987). The salmon lice (*Lepeophtheirus salmonis*) (Krøyer 1937) has become an increasing problem for both wild and farmed salmon (Heuch & Mo, 2001). Salmon lice are parasites living on the salmon, attached to the skin and feeding on skin and flesh. The adult parasite is mobile and can move around on the salmon. Feeding on the mucus, skin and blood of the fish, the lice will cause open wounds. Mortality from salmon lice is mainly caused by the wounds this feeding inflicts (Pike, 1989).

Different treatments are used to control the infestation of lice, both chemical and biological. The delousing chemicals are added either by bathing the fish in the chemical treatment (Brandal & Egidius, 1979) or administering the treatment through the feed (Roy et al., 2000). The chemicals being used include hydrogen peroxide (Treasurer & Grant, 1997), Neguvon® (Brandal & Egidius, 1979), azamethiphos (Roth et al., 1996) and emamectin benzoate (Roy et al., 2000). Although chemical treatments have an effect in the control of the lice, there are also problems such as high cost, problematic dispersal and death caused by the toxicity of the chemicals (Roy et al., 2000). With resistance against chemical feed of bath distributed treatments developing in the lice (Jimenez et al., 2012), alternative lice treatments are used.

Biological treatment of salmon lice is carried out through the use of cleaner fish, mostly from the wrasse family. In Norway we commonly find six different species of wrasse capable of cleaner activity. These species are goldsinny wrasse (*Ctenolabrus rupestris*), ballan wrasse (*Labrus bergylta*), corkwing wrasse (*Symphodus melops*), rock cook *(Centrolabrus exoletus*), cuckoo wrasse (*Labrus bimaculatus*) and scale-rayed wrasse (*Acantholabrus palloni*). Of these species the ballan wrasse is the most commonly used (Espeland et al., 2010).

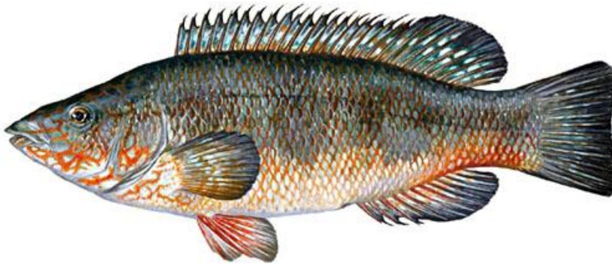

Figure 1: Adult ballan wrasse

Ballan wrasse (*Labrus bergylta*, with Norwegian common name berggylt, Figure 1) is the biggest of the wrasse species found in Norwegian waters. The fish may attain a length of 60 cm, but normally they are found to be between 30-50 cm. With a lifespan of up to 25 years they are also the wrasse species that has the longest lifespan (Sayer & Treasurer, 1996). They are found from Morocco and up to the middle of Norway (Trondheimsfjorden) (Sayer & Treasurer, 1996 ; Espeland et al., 2010).

1.1 Ballan wrasse as cleaner fish

The first time wrasse were used in Norwegian aquaculture was in the middle of 1970s in Batalden in Sogn (Kvenseth, 2011). Through continuous grazing the cleaner fish will provide a continuous control of the lice level. The wrasse are most effective on big lice, and adult female lice are clearly preferred, keeping a control on the salmon lice egg production (Kvenseth et al., 2003a). The interaction between the wrasse, the amount of lice, the net mesh size, the dead fish haul mechanism and the availability of alternative feed are factors interacting to determine the effectiveness of the wrasse's cleaning ability in the farming cage (Kvenseth et al., 2003b). It is recommended to use wrasse hideouts in the salmon pens, providing a less stressful environment for the wrasse. As the wrasse is active in the daytime, they also use the hideouts for rest during the night time (Kvenseth et al., 2003b).

The first catch of goldsinny wrasse in Norway started in 1988, with approximately 1000 fish caught. In 1997 the number of wrasse caught had risen to 3.5 million. Because of the many chemical delousing treatments that became available, the number of caught wrasse dramatically decreased to 1 million fish from 2005 to 2006. After discovering that resistance to the chemical treatments developed (Jimenez et al., 2012), wrasse fishing again became more popular, and in 2009 4.4 million wrasse was caught in Norway. Assuming that the average weight of a wrasse is approximately 50 grams, this would be a catch of 220 tons of wrasse. In 2010 , 440 tons of wrasse were caught (18-20 million wrasse), which is a doubling from 2009 and ten times as much as in 2008. If this trend of wrasse fishing continues in the

same manner as it has in the last years, the wild population of wrasse will probably be heavily influenced as these local populations seem to be small and prone to overfishing (Espeland et al., 2010). To prevent high fishing strain on the wrasse populations and ensure a steady supply of fish, the most logical solution is to farm wrasse for use in salmon farming. Not only will this help loosen the strain on the wild population, it will also give a more stable delivery of fish, with regards to amount, size, fish health and seasonality.

1.2 Farming ballan wrasse

When farming ballan wrasse, the brood stock is being kept in big tanks with spawning mats in the bottom. It is not possible to strip the ballan wrasse in the same way as with cod or halibut, so the spawning occur naturally (Skiftesvik & Bjellland, 2003). The natural spawning season for the ballan wrasse is between May and June, but with manipulation of the light conditions it is possible to make the fish spawn also at other times (Skiftesvik et al., 2011). The tanks have to be deep for the ballan wrasse to be able to perform their spawning behavior. Sufficient hideouts are necessary for the brood stock fish to thrive, and plastic bags that have been cut and tied to lines to resemble kelp have been used with good results for many years (Skiftesvik et al., 2011 ; Skiftesvik, 2009). When the ballan wrasse spawn, the eggs sink to the bottom of the tank where they are collected on spawning mats. These mats are then moved to incubation tanks where the eggs will hatch (Skiftesvik et al., 2011).

The newly hatched larvae are moved to another tank to avoid pollution from the hatching debris. The larvae are reared between 12-16°C (Skiftesvik et al., 2011). Newly hatched ballan wrasse larvae are almost transparent and have an average size of 3.6 mm (Skiftesvik & Bjellland, 2003). After approximately 4-5 days post hatch (dph) the yolk sack is absorbed and the larvae need to start feeding. The larvae are fed live feed organisms such as rotifers, copepods and *Artemia* (Skiftesvik et al., 2011). To trigger the larval start feeding, algae or clay is being added to the tank. Adding clay or algae has also been found to have a positive microbial effect on the water quality in the tank. (Skiftesvik et al., 2011 ; Skiftesvik, 2009 ; Attramadal et al., 2012). 40 dph all organs and fins are developed and approximately 60 dph the larvae can start the weaning period from live feed to formulated feed (Skiftesvik & Bjellland, 2003 ; Skiftesvik, 2009).

1.3 Live feed and first-feeding

The ballan wrasse larva is an altricial larva, meaning that when the yolk sac is absorbed when the larva remain relatively immature (Govoni et al., 1986). The transition from endogenous to exogenous feeding is found to be a bottleneck area in the first feeding of marine fish larvae such as Atlantic cod (*Gadus morhua*) (Baskerville-Bridges & Kling, 2000), Atlantic halibut (*Hippoglossus hippoglossus*) (Olsen et al., 2000) and green wrasse (*Labrus viridis*) (Kožul et al., 2011). Using the live feed with the right size and the right nutritional composition, depending on the larval size and developmental stage, is crucial for survival, optimal growth and development of the larvae (Bengtson, 2003). Marine fish larvae show that natural copepods are a better larval feed than rotifers and *Artemia* sp. (Imsland et al., 2006 ; Overrein, 2010 ; Rajkumar & Kumaraguru vasagam, 2006). Feeding zooplankton to larval Atlantic cod gives higher growth rate and better development than rotifers (Imsland et al., 2006). The biochemical composition, nutritional value and size decides how well suited the different species of live feed is for the rearing of marine fish larvae (Evjemo & Olsen, 1997 ; Helland et al., 2003 ; van der Meeren et al., 2008)

The content of lipids in marine pelagic copepods may vary greatly with latitude, season and food availability, ranging from 2 to 61% in medium and low latitude species, and 8 to 73% in the high latitude species. The lipid content of rotifer are 34-43% phospholipids and 20-55% triacylglycerols, and small amounts of sterols, sterolesters, mono- and di- acylglycerols and free fatty acids (Nagata & Whyte, 1992 ; Rainuzzo et al., 1997). Phospholipids (PL) are polar lipids, and serves as a important source of energy in the fish (Sargent et al., 1993 ; Sargent et al., 1997). The rate of larval biosynthesis of PL does not meet the larvas requirements for PL to support optimal growth. Because of this, an optimal amount and composition of the phospholipids in the live feed is important to optimize growth (Geurden et al., 1995). The fatty acid composition of the phospholipids is important as some are essential to ensure optimal growth and development of the larvae (Evjemo et al., 2003 ; Watanabe & Kiron, 1994). Without the ability to synthesize these essential fatty acids, the larvae have to get it through their diet (Bell et al., 2003).

Eicosapentanoic (EPA)(20:5n-3) and docosahexaenoic (DHA)(22:6n-3) are essential fatty acids for development and survival of marine fish larvae (Watanabe et al., 1983). Marine fish have EPA and DHA in the phospholipids of the cellular membrane, but cannot synthesize

these from linolenic acid (18:3n-3), this makes them essential dietary constituents. Marine copepods have a relatively low lipid level, but the phospholipid fraction is found to be high in EPA and DHA (45-60 % of the total fatty acids)(Olsen, 2004). Reared rotifers lack the adequate amounts of EPA, DHA and ARA (arachidonic acid) that the marine fish larvae nutritionally require. Enrichment with these fatty acids is required using algae or lipid emulsion (Sargent et al., 1997). *Artemia* nauplii contain between 0.4 and 33.6% linolenic acid. The EPA levels of the *Artemia* sp. seem to be inversely related to linolenic acid levels (0.6 to 14.7% EPA) (Dhont & Van Stappen, 2003). The amount and quality of the lipid content of the rotifer is regulated using long or short enrichment periods and with a variety of lipid emulsions in the diets (Rainuzzo et al., 1997). The protein content of a rotifer ranges from 28 to 63% and the lipid content ranges from 9-28% of the dry weight (Nagata & Whyte, 1992 ; Rainuzzo et al., 1997 ; Øie & Olsen, 1997). The protein content of the copepods ranges from 24 to 82% of dry weight, with the medium latitude species having the highest values (Båmstedt, 1986). *Artemia* sp. nauplii have a protein content ranging from 41 to 62% of the dry weight. Lipid content is between 12 and 28%, and the carbohydrate content between 4 and 11% of DW (Dhont & Van Stappen, 2003). The high content of DHA and EPA found in the PL of copepods (Evjemo & Olsen, 1997 ; Evjemo et al., 2003 ; Overrein, 2010 ; van der Meeren et al., 2008) make then ideal feed organisms for rearing of marine fish larvae.

The differences in nutritional properties, movement and size between the different types of live prey, could result in differences in growth and development between larvae fed different combinations of these preys. To study this, an analysis of selected bio markers should give an overview of the metabolic status of the larva at the sampling point. Comparing metabolic status from larva fed different live prey and at different sampling points could then be used to compare the nutritional effect of different live prey at different developmental stages. To analyze metabolic bio markers, metabolomics is the most relevant analysis method.

1.4 Metabolomics

The most commonly used definition for metabolomics is “the scientific study of chemical processes involving metabolites. Specifically, metabolomics is the “systematic study of the unique chemical fingerprints that specific cellular processes leave behind”, the study of their

small-molecule metabolite profiles" (Daviss, 2005). The metabolome represents the collection of all metabolites in a biological cell, tissue, organ or organism, which are the end product of cellular processes (Jordan et al., 2009). There are different platforms used for the detection of metabolites. These methods involve gas chromatography (GC) or liquid chromatography (LC) coupled with subsequent mass spectrometry analyses (MS) of the separated molecules. MS is also possible to use directly without chromatographic separation(Sparkman et al., 2011). Another analysis principle is Nuclear magnetic resonance spectroscopy (NMR). NMR is a non-destructive, non-targeted fingerprinting technique which can be used to detect a wide range of different classes of low molecular weight metabolites with differing charges, stability or volatility. One of the most attractive traits of NMR is the ability to measure non-destructively, i.e. vivo intact tissue samples. NMR provides high resolution structural information about metabolites for unambiguous identification (Hemminga, 1992). The main advantage of NMR is high analytical reproducibility and simplicity of sample preparation, it is however not as sensitive as the MS based techniques (Roessner & Beckles, 2009). Metabolomics data are often raw data fingerprints or lists of metabolite levels with corresponding metabolomics approaches being metabolite fingerprinting and metabolite profiling, respectively (Goodacre et al., 2004). Different software is available for analysis of the results both from NMR and MS. Following the chosen analytical method, chemometrics is a predominant part of metabolomics, with methods such as principal component analysis (PCA) or partial least squares regression (PLS) (Trygg et al., 2007). Principal component analysis is a mathematical method used to organize information from data sets acquired from samples. PCA reduces larger numbers of variables into relatively few new variables, called principal components (PC's), these PC's account for the majority of the variability found in the data. Using such analysis method enables the information to be described with fewer variables than originally obtained. In a data set containing many thousand variables, approximately 20 PC's can contain almost all the metabolic information of the sample. The first PC is the direction through the data that explains most of the variability in the data. The second PC must be at right angle to the previous PC and describes the maximum amount of the remaining variability. PC's are described by scores and loadings. Scores are the new variables and loadings describe how the variables differ in correspondence with differences in the scores. Each sample will have a

score for each PC, in the same way as the value for each of the wavelength variables in the original data set (Davies & Fearn, 2005).

The method chosen for sample preparation is important. It is critical to terminate enzyme activity to get an accurate measure of the metabolic profile. By flash freezing the sample it is possible to get the exact metabolic data from the accurate sample point. If the sample is left unfrozen the metabolic activity will continue for some time and the results will be inaccurate. When preparing the samples it is also important to avoid thawing of the sample. The loss of metabolites through thawing and drip loss can affect the metabolic profile (Martinez et al., 2005). Lin et al. (2007) examined the preparation methods of fish tissue with focus on different types of homogenization methods and different types of extraction solvents. They examined the results from the different methods in relation to metabolic yield, reproducibility, ease and speed. They found that when comparing grinding and mechanical homogenizing there were few differences. Regarding speed and ease of preparation, mechanical homogenization would be preferred, and using methanol/chloroform/water was found to give the best solvent extraction. The method also has the benefit of extracting the hydrophilic and hydrophobic metabolites into different fractions (Lin et al., 2007). Wu et al. 2008 analyzed three different styles to do this preparation. Stepwise preparation (adding solvents and mixing in many steps), Two steps (adding solvents and mixing in two steps) and All-in-one (adding all the solvents and mixing the sample in one step). They also used a new method for homogenizing the samples using plastic tubes and ceramic beads in a Precellys homogenizer with room for 24 samples at the same time, drastically cutting the time spent homogenizing and consequently reducing the chance of thawing during manual homogenizing. The results showed that the all-in-one method gave metabolic profiles with peaks from lipids, indicating a high content of lipids in the sample. The main difference between the all-in-one method and the other extraction methods was due to enhanced extraction of lipids into the polar phase of the all-in-one method. As a result this lipid peaks in the spectra might hide smaller peaks from metabolites of low concentration. There was little difference between the stepwise and two-step extraction methods. But with the focus also on rapid sample preparation they conclude that the two- step extraction method would be the best suited method for high-throughput tissue extraction (Wu et al., 2008).

1.5 Metabolomics in aquaculture

The use of metabolomic analyses on aquatic organisms has been dominated by environmental studies. Environmental metabolomics is defined as "the application of metabolomics to characterize the interactions of living organisms with their environment". Pioneering work in environmental metabolomics has included characterizing of responses in organisms to toxic stress, and thus discovering novel biomarkers for use in environmental diagnostics (Bundy et al., 2009). Some species used for aquaculture farming have been studied, like the Atlantic salmon (*Salmo salar*)(Gribbestad et al., 2005), Atlantic cod (Martinez et al., 2005) and the Atlantic halibut (Sitter et al., 1999), examining metabolic composition and properties.

The use of metabolomic analysis in the aquaculture could help develop methods for both better farming of the species and also better processing techniques with quality of the product in mind. The quality of the aquaculture product depends on a range of factors like the developmental phase, feeding regimes, environmental temperature and the composition of the feed. The changes of these factors can be traceable through metabolic profiles, and this would give the possibility to analyze the differences in metabolic concentrations and profiles given by the different conditions the species are living under. A metabolic study of different dietary regimes of the finfish cobia (*Rachycentron canadum*) showed that glucose, tyrosine, betaine and lactate in particular, indicated differences in growth and development caused by the feed composition (Schock et al., 2012). A metabolic analysis of gilthead sea bream *(Sparus aurata*) found a range of metabolites such as inosine, inosine 5'-monophosphate, glycine, alanine, histidine and glycogen proved to be reliable biomarkers that could be used for distinguishing amongst the farming systems and also for providing information about the fish storage time (Savorani et al., 2010). Karakach et al. used both 1H-NMR and mass spectrometry to find metabolic responses of Atlantic salmon juveniles when exposed to long-term handling stress. Perhaps the most interesting and relevant example showed that the different types of stress handling could be found as differences in metabolite concentrations (Karakach et al., 2009).

2. Aims of the study

- **Establish an extraction method suitable for metabolic analysis of ballan wrasse (*Labrus bergylta*).**

  Testing different sample preparation methods to determine the most reproducible and effective way of handling the samples. The metabolic yield, preparation time and spectrum base line interference should be considered.

- **Compare the effects of copepods and rotifers as part of a feeding regime in start feeding of ballan wrasse (*Labrus bergylta*) by studying growth and metabolic status using metabolomics.**

  Analyze NMR spectra from ballan wrasse larvae reared under controlled conditions, and fed different feeding regimes. A comparison of the metabolic components should indicate metabolic composition and variability of different metabolites during the larval stage as a function of growth, development and the dietary composition of different feeding regimes.

3. Material and methods

3.1 The first-feeding experiment

The ballan wrasse larvae arrived 2 dph from the hatchery Marin Harvest Labrus (Bergen, Norway). All larvae were kept in one holding tank (250 liter) before being transferred to the rearing tanks. The larval density in the holding tank was calculated and the larvae were distributed to 12 tanks (84 larvae L^{-1}). The initial seawater temperature was 12°C and was gradually increased to 16°C (Table 1). The salinity of the seawater was 34 ppm. The seawater used for the fish tanks was sand filtered and then filtrated through a 1 µm mesh filter. Microbial maturation was performed according to Skjermo et al., using a continuous degasser and then filtrating with a 1 µm filter before being added to the tank (Skjermo et al., 1997). All tanks were moderately aerated and a surface skimmer was used to clean the water surface. The water exchange rate started at 2 exchanges per day in the beginning and ended with 8 exchanges per day. The lights were kept off for the 2 first days after arrival (4 dph), after which constant light (2x18W/965 fluorescent light per tank) was used for the remainder of the experiment. The experiment was terminated at 61 dph.

3.2 Larval feeding regime

The fish larvae were divided in 4 different feeding regimes with triplicate tanks (Table 1). The four feeding regimes were Copepod, Cop7, RotMG and RotChl:

Copepod: The larvae were fed newly hatched nauplii of the copepod *Acartia tonsa (clone DFH.AT1)* from 3 dph to 23 dph. Between 23 dph and 30 dph there was a co-feeding period with both copepods and *Artemia* sp. nauplii.

Cop7: The larvae were fed copepod nauplii (*Acartia tonsa)* from 3 dph to 10 dph. After 10 dph the diet was switched to rotifers (*Brachionus ibericus,* Cayman), enriched with Multigain (Biomar) (Table 2). At 23 dph a co-feeding period with enriched rotifers and *Artemia* sp. nauplii started, lasting until 30 dph.

RotMG: This group was fed the rotifer (*Brachionus ibericus,* Cayman) cultivated on *Chlorella* (Pacific Trading) and enriched with Multigain, from 3 dph until 23 dph. From 23 dph until 30 dph they were co-fed with a mix of rotifers and *Artemia* sp. nauplii enriched with Multigain.

RotChl: The RotChl group were fed rotifers *(Brachionus ibericus,* Cayman*)* cultivated on *Chlorella*, without any enrichment, from 3 dph until 23 dph. From 23 dph until 30 dph they were co-fed unenriched rotifers and Multigain enriched *Artemia* sp. nauplii.

After 30 dph all groups were fed in the same way. From 30 dph to 40 dph the larvae were fed exclusively on *Artemia* sp. nauplii enriched with Multigain (Biomar). From 40 dph to 50 dph there were a co-feeding period with *Artemia* sp. nauplii and formulated diet and from 50 dph to 61 dph the larvae were fed only formulated feed (Nofima) (Appendix 1).

The copepods and the rotifers were distributed manually in the start feeding tanks, with a density of approximately 12000 ind/L^{-1}. The larvae were fed 3 times daily from 4 dph until 19 dph. From 20 dph the larvae were fed 4 times per day. Copepod nauplii used were grown to have approximately the same biomass as rotifers when fed to the larvae. *Artemia* sp. nauplii were distributed to the tanks using a feeding robot at night (Storvik Aqua AS, Norway), and manual distribution in the daytime. After the *Artemia* stage of the regime was completed the feeding robot was used for the weaning and dry feed period (10 g $tank^{-1}$ day^{-1}).

From 4 dph until 50 dph, clay (Vingerling K148, Sibelco, Germany) was added to the tanks. The amount of clay used was adjusted to match the increasing water exchange rate through the experiment, starting with 5g tank $^{-1}$ day $^{-1}$ on 4 dph, ending up with 25g $tank^{-1}$ day $^{-1}$. This was done to create an environment that might make it easier for the larvae to see the live feed during the initial first feeding (Attramadal et al., 2012).

Table 1: Feeding regime layout including sampling overview.

DPH	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34-39	40	41	42	43	44	45	46	47	48	49	50	51-60	61
Day degrees	12	24	36	48	60	73	86	99	112	125	138	152	166	180	195	210	225	240	255	270	285	300	316	332	348	364	380	396	412	428	444	460	476	492-572	588	604	620	636	652	668	684	700	716	732	748	764-908	924
Water exchange rate	2 times day $^{-1}$														4 times day $^{-1}$											6 times day $^{-1}$							8 times day $^{-1}$														
Temperature (°C)	12					13						14			15								16																								
Clay (g tank $^{-1}$ day $^{-1}$)	0			5			10								13.33				16.7							20				25																0	
Sieve	200 µm												500 µm																																	750 µm	
Copepod 3 tanks				Copepods																															Formulated feed (Nofima)												
																								Artemia (enriched with Multigain)																							
Cop7 3 tanks				Copepods							Rotifers (enriched with Multigain)																								Formulated feed (Nofima)												
																								Artemia (enriched with Multigain)																							
RotMG 3 tanks				Rotifers (enriched with Multigain)																															Formulated feed (Nofima)												
																								Artemia (enriched with Multigain)																							
RotChl 3 tanks				Rotifers (not enriched)																															Formulated feed (Nofima)												
																								Artemia (enriched with Multigain)																							
Sampling																																															
CN/ Dry Weight		X		X				X				X			X						X							X					X		X							X					X
Mortality														X		X		X		X		X		X		X		X		X		X		X		X		X		X		X		X	X	X	X
SL/MH				X				X													X							X						X												X	
NMR								X				X			X						X						X						X		X							X					X

Table 2: Distribution between feed, nauplii age and nauplii stage in the Cop 7 group.

Cop7 treatment

DPH	4-7	8	9	10	11	12-23
Copepod nauplii (%)	100	67	67	100	33	0
Rotifers (%)	0	33	33	0	67	100
Age of nauplii (days)	3	3	3	3	4	-
Stage of nauplii	III-IV					-

3.3 Cultivating algae

The micro algae *Rhodomonas baltica* was cultivated in 160L and 200L transparent plastic tubes. Additional algae were produced in 300L transparent plastic bags mounted in metal frames. The plastic tubes were illuminated by fluorescent lighting from three sides (3x2x58 watt). The temperature was approximately 20°C and the algae were aerated with air mixed with 1-2% CO_2. Every day 40-50% of the culture was harvested and replaced with disinfected seawater (34 ppt) and Conwy medium (1ml Conwy L^{-1} added seawater)(Walne, 1974)(Appendix 2). The algae cultures were kept at a density of approximately 1 million cells L^{-1}. The seawater used for the algal culture was treated with sodium hypochlorite (100ml 10-15% NaOCl L^{-1}) for approximately 6 hours before being dechlorated using thiosulphate (3g thiosulphate 100 ml^{-1} chlorine) under heavy aeration.

3.4 Copepod egg production

The copepod *Acartia tonsa* was cultivated in 700L and 1000L tanks. The tanks have a flat bottom with an automatic cleaning arm attached in the bottom center. The salinity of the seawater was 34 ppt and the temperature was approx. 20°C. The water exchange rate was 100% exchange in 24 hours, and the lights were always on. Aeration was moderate to low using 4-5 small aeration stones giving little turbulence in the water column. The copepods were fed the algae *Rhodomonas baltica*. The amount of feed used was 2x60L for the 700L tank, fed out over the duration of 24 hours using a peristaltic pump. The 1000L tank was fed approximately 3x60L from a feed reservoir using a peristaltic pump over the duration of 24 hours. The density of the algae fed was between 800 000 – 1.2 million cells ml^{-1} .The eggs produced by the copepods were collected once a day using a glass rod to siphon the eggs up from the bottom of the tank into a bucket. Using a 200µm filter first, and then a 64 µm filter, the eggs were filtered out from the waste products and washed in seawater. The eggs were stored in Nunc- bottles with seawater in refrigerated temperature of 2 °C

3.4.1 Hatching copepod eggs

The copepod eggs were hatched in 100L plastic tanks (4-6 tanks). Seawater (20-22°C, 34 ppt) was used to fill the tanks, and stored eggs were mixed together and added to the tanks. After 24 hours the water in the tanks was gently mixed. Approximately 1 liter of algae were added every day to trigger more hatching. After 48 hours the water was mixed once more

and more algae added. 72 hours after incubation, egg shells and waste was siphoned out by using a filter (64µm), and some of the water was removed to concentrate the copepods. The hatched nauplii were then moved to a reservoir (250L) where they were held until fed to the larvae.

3.5 Production and enrichment of rotifers

The rotifer *Brachionus ibericus,* Cayman was cultivated in 260L conical glass fiber tanks with a water volume of approximately 250L. The system was flow through (constant water exchange) with a valve in the bottom allowing flushing of the waste. Temperature was between 19°C and 25°C, with a water salinity of 34ppt. The tank was aerated moderately to keep the rotifers evenly mixed. The internal filter was removed and rinsed daily, and the bottom waste was removed through the valve.

Samples from the tank was counted (12 drops à 50µl) to calculate density and egg ratio and the culture was fed with algae (*Chlorella*, Pacific trading). The algae were mixed in a bucket and fed out with a peristaltic pump over the duration of 24 hours. When reaching a density of 750 ind. ml^{-1} the culture was diluted. Once a week the tank was cleaned. The volume of the tank was transferred to a filter net (64µm) and the rotifers were concentrated and washed using seawater before being moved to a clean tank.

The rotifers were transferred to a net and washed before transfer to a tank. In the enrichment tank the water was heated and the enrichment (Multigain) was added (0,15g Multigain million $rotifers^{-1}$). The rotifers were enriched for 2 hours under heavy aeration. After enrichment the rotifers were washed and then added to a known volume of water allowing calculation of the needed amount of rotifers for larval feeding. The enriched rotifers not being used immediately was kept refrigerated at 8°C (maximum 18 hours) for later use.

3.6 Production and enrichment of *Artemia*

Decapsulation of *Artemia franciscana* were performed according to Sorgeloos et al., (1977). The cysts were kept refrigerated for maximum one week. Cysts were transferred to 60L conical hatching tanks for 24 hours. After hatching the *Artemia* sp. were washed to remove hatching debris. The hatched nauplii were moved back to the tanks for enrichment. Enrichment was done using Multigain (10g $60L^{-1}$) (Biomar, Norway) twice within 24 hours at a temperature between 25-28°C, 34 ppt seawater and with heavy aeration. After 24 hours of enrichment the *Artemia* sp. were washed again before being fed to the fish larvae.

3.7 Live feed analysis

Samples of *Acartia tonsa,* enriched rotifers (Multigain), unenriched rotifers *(Chlorella*) and enriched *Artemia* sp. was analyzed for nutritional values (Table 3). Excess water was removed before the samples were put in individual sampling tubes. The samples were stored at -80°C and freeze dried before being sent to National Institute of Nutrition and Seafood Research (NIFES) for analysis. Analysis was performed according to accredited methods.

Table 3: Nutritional values of different live feed types used in the first-feeding of ballan wrasse larvae.

	Protein g/100g DW	Lipids g/100g DW	DHA %	EPA %	ARA %	DHA:EPA ratio
Acartia tonsa	67.8	8.8	22.4	4.2	0.8	5.3
Enriched rotifer (Multigain)	42.3	11.6	19.8	6.5	0.9	3.0
Unenriched rotifer (*Chlorella*)	44.4	11.9	14.4	8.5	0.9	1.7
Artemia sp. enriched (Multigain)	34.9	27.6	21.5	5.3	2.5	4.0

3.8 Sampling

3.8.1 Dry weight

Dry weight larval samples were taken at 2, 4, 8, 12, 15, 21, 27, 33, 40, 47 and 61 dph. At 2, 4, 8, 12 and 15 dph, 12 larvae were sampled from each tank. 20 larvae were sampled on 21 dph, and 30 larvae were sampled on 27, 33, 40 and 47 dph. The last sampling day, 61 dph, 50 larvae were sampled from each tank. The larvae were rinsed with freshwater and then put in pre-weighed tin capsules, one larva in each capsule. The samples were kept at 60°C for 48 hours before analysis. Larvae from 2, 4, 8, 12, 21 and 27 dph were analyzed for carbon and nitrogen content (CN) using an Elemental combustion analyzer (Costech Analytical Technologies Inc.). The carbon content of the samples was multiplied using a factor of 2.34 (average carbon content of fish larvae) to calculate the dry weight of the larvae (Reitan et al., 1993). Samples were weighed on Mettler Toledo XA 204 Delta Range or Mettler Toledo UMX 2 (Not CN samples).

The daily weight increase (%DWI) was calculated using equation according to (Ricker, 1958):

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

Growth coefficient *g* is:

$g = (lnW_2-lnW_1)/(t_2-t_1)$

W_1 and W_2 are dry weight at day t_1 and t_2

3.8.2 Larval growth

Standard Length (SL) was measured between the tip of the snout to the end of the notochord. Myotome Height (MH) was measured immediately posterior to anus (Figure 2)

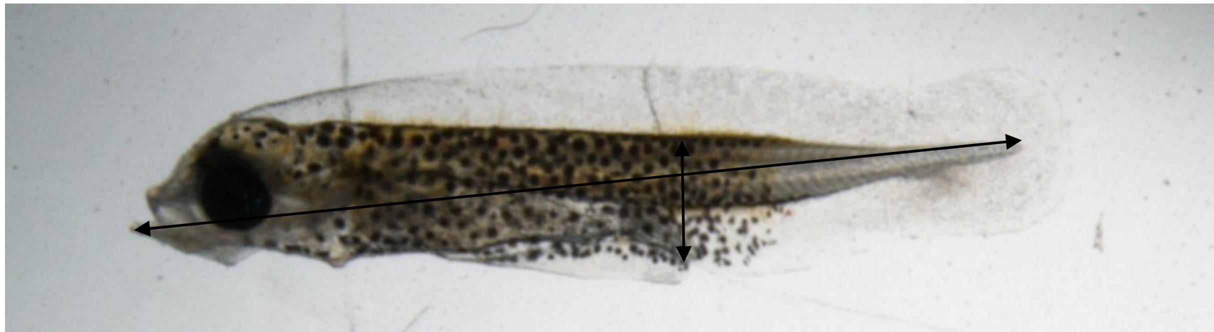

Figure 2: Ballan Wrasse larvae with arrows indicating measuring points for standard length (horizontal line) and myotome height (vertical line).

3.8.3 Survival

The bottom of the tanks was rinsed by siphoning into 10L buckets. The rinsing was performed daily from 13 dph. Dead larvae were counted and registered. Live larvae accidentally caught in the siphoning process were also counted as dead larvae and killed. 13 dph was the first day of dead larvae counting. From 13 dph the tanks were cleaned and dead larvae counted.

3.8.4 NMR Sampling

NMR larvae samples were taken at 8, 12, 15, 21, 27 and 33 dph (n=3), 40, 47 and 61 dph (n=30). Larvae used for NMR analysis were randomly sampled from the tank and then sedated using MS-222 (Finquel®, Agent Chemical Laboratories Inc). The larvae were rinsed in freshwater and then dried in a sieve on paper to remove excess of water. From the sieve the fish larvae were transferred to Precellys tubes and flash frozen using liquid nitrogen (N_2) and stored at -80 °C until preparation.

3.8.5 Preparation of NMR samples

Methanol (MeOH, 67%) was added to the tube and the sample was homogenized using a Precellys homogenizer (program 5)(Precellys 24 lysis & homogenization, Bertin Technologies). After homogenization the sample was centrifuged for 15 min (Centrifuge 5415R, Eppendorf) at 4°C with max rotation (16,1x1000 RCF). Supernatant (800 µl) was transferred to a GC/HPLC glass using an automated pipette. Chloroform ($CHCl_3$) (533 µl) and distilled water (H_2O) (213 µl) was added to the GC/HPLC glass and then mixed with the supernatant using a vortex mixer for 2x30 seconds. The sample was then kept on ice for 15 minutes, before being centrifuged for 5 minutes (Universal 16A, Hettich zentrifugen). The polar phase (700 µl, on the top) was transferred to an Eppendorf tube before it was vacuum centrifuged at 30 °C for 30 minutes (Concentrator plus, Eppendorf). The sample was then frozen again at -80 °C before it was freeze-dried over night. Before freeze-drying, the top of the Eppendorf tube was removed and a parafilm pierced with small holes was added to prevent pressure buildup in the tubes. After freeze-drying, the samples were frozen at -80 °C again.

24 hours before the NMR analysis the frozen samples were dissolved in 200 µl solution (D2O, PBS pH 7.4, 1mM TSP), and transferred from the Eppendorf tube to a NMR sample tube. The samples were randomized to prevent trends caused by equipment error or failure.

During NMR sample preparation almost all samples from 61 dph were lost due to an error in the labeling of the samples.

3.8.6 NMR spectroscopy

NMR was performed on a Bruker DRU 600 spectrometer (Bruker BioSpin GmbH, Rheinstetten) resonating at 600.23 MHz for ^{1}H. Typically 48 free induction decays (FIDs) were averaged for each samples into 64 K data points using the Bruker library pulse program noesygppr1d.

3.8.7 Multivariate analysis of NMR data

For principal component analysis (PCA) multivariate analysis, the NMR data were imported into MATLAB using ProMetab software from Mark Viant (Viant, 2007). The analyses were performed in PLS-toolbox (Eigenvector research ltd.).

3.9 Statistics

All data were normality tested ($P \geq 0.05$). Standard error (SE) was calculated for all data. Extreme outliers were removed from dataset if appropriate. For all normally distributed data, results were compared using One-Way- ANOVA test. Test of homogeneity of variance were performed (homogeny > 0.05 < no homogeny). Using a Student-Newman Keuls test significant variance was determined. For non-homogeny data a Dunette T3 test were applied to determine significant variance.

All statistical tests were performed in IBM© SPSS© Statistics Version 19 (SPSS Inc. USA)

4. Results

General Observations

During the experiment one of the Copepod group tanks, Copepod Tank 1, was performing poorly compared to all other tanks in the experiment. It was obvious that something was wrong with this tank, but no specific source of the bad development was found. The day degree for all Copepod tanks was calculated (Tank1: 924 day degrees. Tank2: 929 day degrees. Tank3: 939 day degrees), showing only minor differences in this parameter, indicating that differences in the temperature in the tanks are not the reason for this development. Because of this very obvious source of error, Copepod Tank 1 was removed from growth and survival results.

One tank from the RotChl treatment was terminated at 40 dph because all larvae in the tank were dead.

Metabolic samples were not marked with tank numbers, only treatment and dph. Because it was impossible to determine what larvae were from Copepod tank 1, larvae from this tank were included in the metabolomic results.

The first batch of larvae that arrived from Marin Harvest Labrus all died within 24 hours after arrival. The second batch of larvae did much better and survived the handling.

4.1 Larval growth and survival

4.1.1 Dry Weight

From 2 dph, the larvae increased its dry weight from 0.0442±0.00 mg larva^{-1} to a mean value of 19.63±1.11 mg larva^{-1} for the Copepod group, 17.08± 0.86 mg larva^{-1} for the Cop7 group, 13.95±0.7 mg larva^{-1} for the RotMG group and 12.97±0.74 mg larva^{-1} for the RotChl group at 61 dph (Appendix 3).

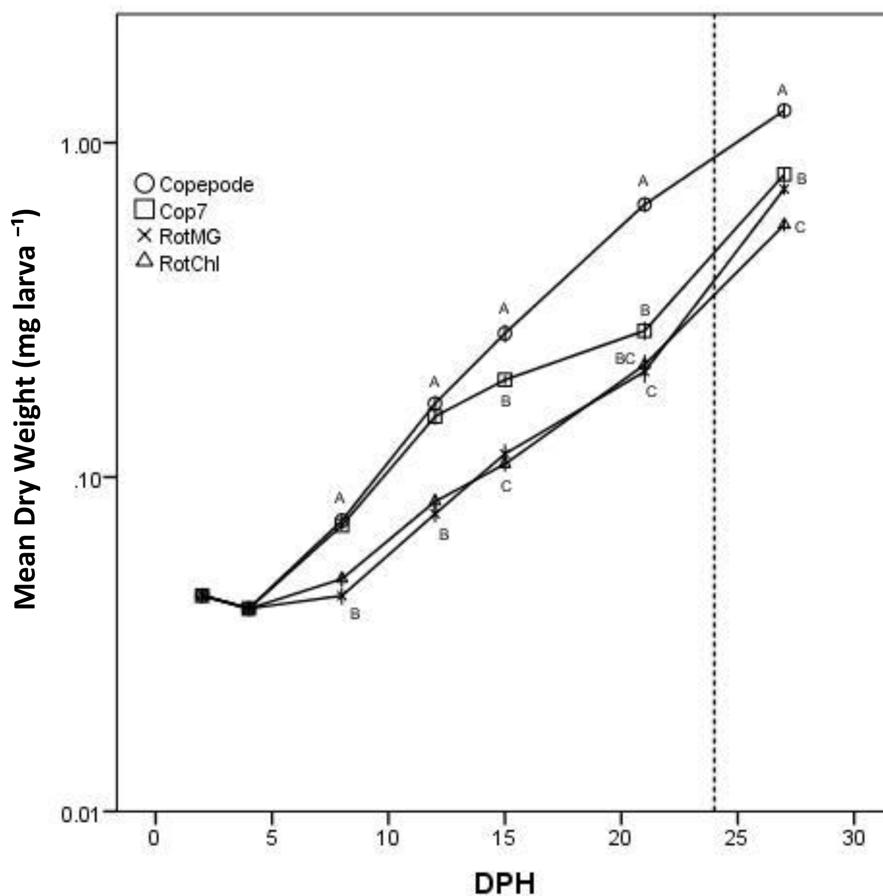

Figure 3: Mean dry weight (mg larva^{-1}) for 2 to 27 dph. The Y axis is represented in logarithmic scale (n=12-150). Significant differences found between results are marked by letters. Error bars = ± SE. Dotted line indicates change in feeding regime.

At 8 dph the copepod-fed groups had significantly higher mean dry weight than the groups fed rotifers (Figure 3). At 15 dph significant differences were found between the copepod feeding groups, with the copepod group having significantly higher mean dry weight than the Cop7 group. At 21 dph, with the Cop7 group switching diet from copepods to enriched rotifers, the mean dry weight of the Cop7 group had stagnated compared to the Copepod group larvae. The weight of these larvae was found to be much closer to the rotifer-fed groups. After 23 dph all groups started co-feeding with *Artemia* sp. nauplii. At this point dry weights started to even out between the groups. The weaning period with formulated feed started at 40 dph, and at 40 and 47 dph there were no significant differences found in the dry weights between groups. At the last sampling day, 61 dph, significant difference was found between the copepod-fed and rotifer-fed groups with copepod-fed larvae having a significant higher dry weight than the rotifer-fed groups (Figure 4).

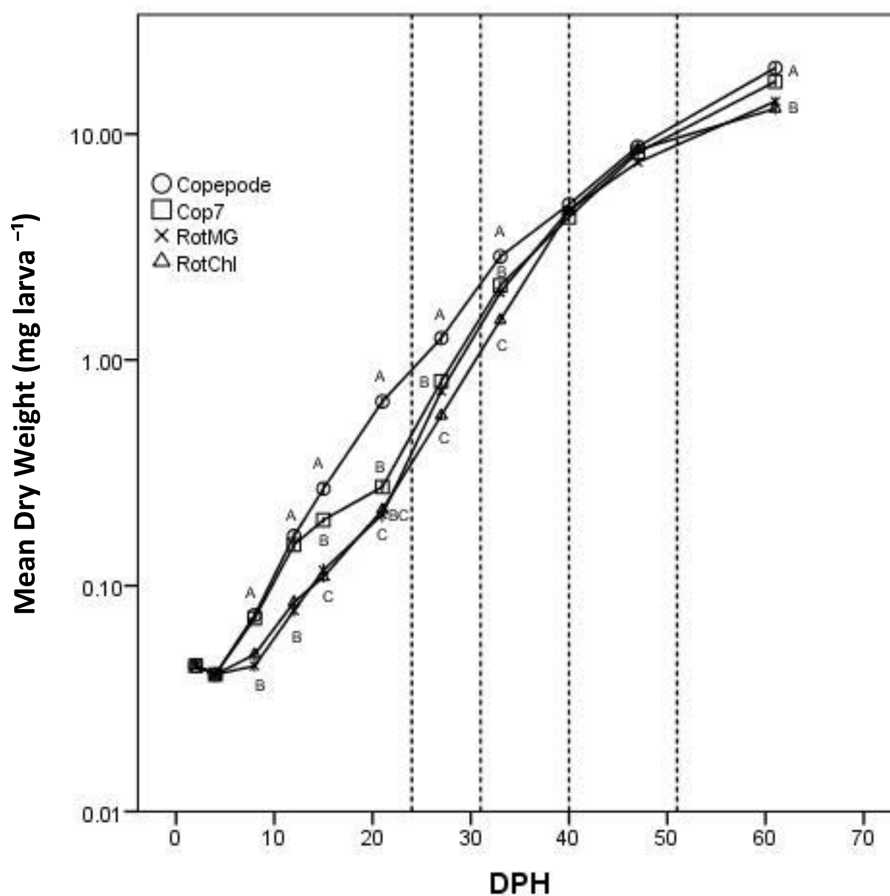

Figure 4: Mean dry weight (mg larva^{-1}) for the start feeding period (2 dph-61 dph). The Y axis is represented in logarithmic scale (n=12-150). Significant differences found between results are marked by letters. Error bars = ± SE. Dotted lines indicate change in feeding regime.

4.1.2 Dry weight increase

Using the sampling points used for dry weight analysis, these intervals were chosen to try to observe changes in dry weight increase as the feed types in the feeding regimes changed. During the 4-12 dph interval all groups were fed different diets. The copepod groups, only being fed copepods during this period, had the highest % dry weight increase (Figure 5). The rotifer-fed groups had significantly lower % DWI compared to the Copepod group in this interval. In the next interval all groups started a co-feeding period with feed regime determined live feed types and enriched *Artemia* sp. nauplii. During this interval the rotifer-fed groups had the highest % dry weight increase. The Cop7 group, changing diet from copepods to rotifers during this interval, had the lowest % dry weight increase. With all larval groups being fed enriched *Artemia* sp., the rotifer-fed groups have the highest % dry weight increase in the 27-40 dph interval. With the weaning period to formulated feed starting in the 40-47 dph interval, most of the differences in the % dry weight increase

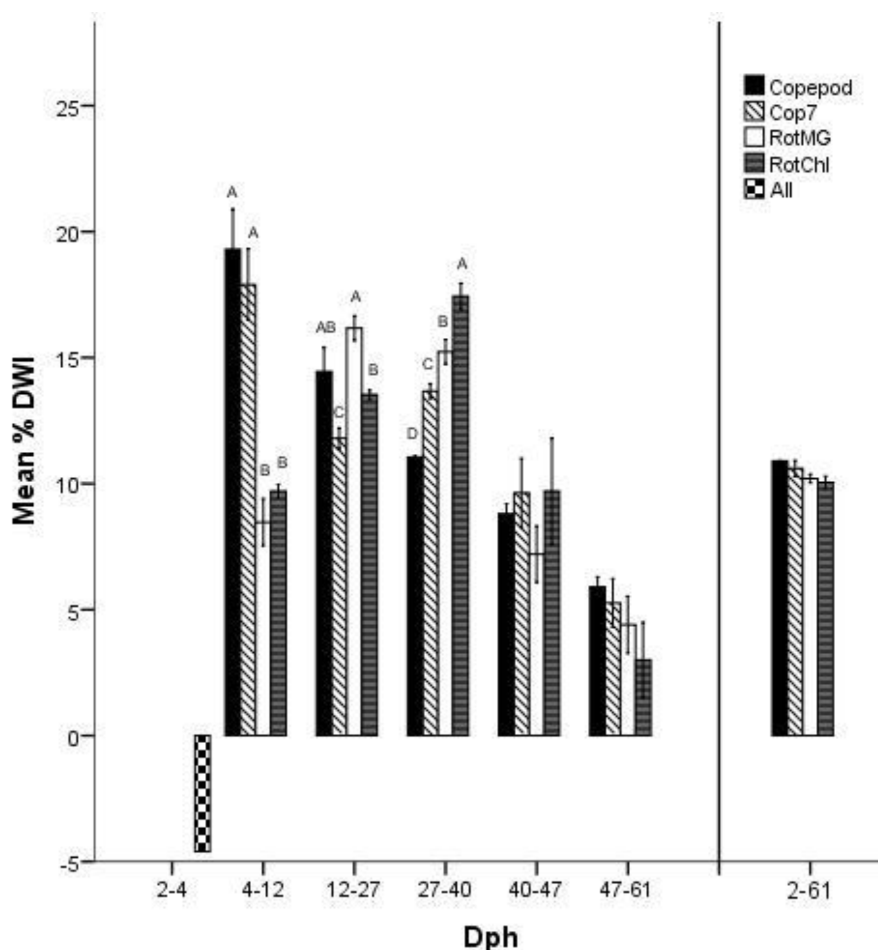

Figure 5: Mean % dry weight increase (DWI) for all measured groups (n= 1-3). Significant differences found between results are marked by letters. Error bars = ± SE.

between the groups disappeared. For interval 40-47 and 47-61 dph, no significant differences were found between the groups. Also for the overall interval between 2-61 dph there were no differences.

4.1.3 Standard length and myotome height

Measurements of the standard length and myotome height were performed at six different sampling days. From the standard length measurements small differences at 8 dph were found (Figure 6). The groups fed copepods were longer than the rotifer-fed groups. With the Cop7 group changing from copepods to rotifers 10 dph, it was found significant differences between the Copepod group and the three groups fed rotifers at 21 dph. At sampling point 28 dph the groups had started co-feeding with *Artemia* sp.. During this point and the 39 dph sampling point, which is in the end of the *Artemia* sp. part of the feed regime, the Copepod

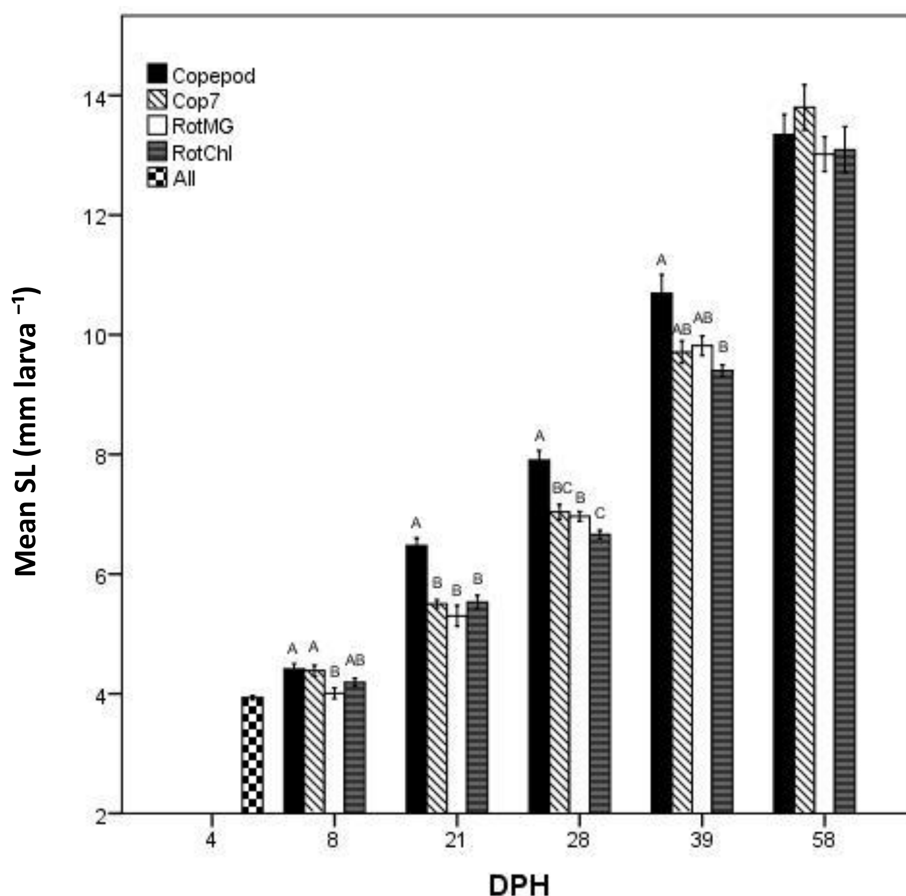

Figure 6: Mean standard length (mm larva^{-1}) for all days post hatch (dph) (n= 12-30). Significant differences found between groups are marked by letters. Error bars = ± SE.

group was significantly longer than the other groups. Differences between the groups were practically the same for these sampling days. The last sampling day, 58 dph, no significant differences were found between any of the groups. At this point all the groups were fed formulated feed, and earlier differences had disappeared.

For the myotome height measurements the same trends were observed as in the standard length measurements. As long as the groups were fed different types of live feed, the copepod-fed groups had the highest myotome height (Figure 7). But when all groups were fed the same type of feed (*Artemia* sp.), the differences between the groups stayed the same. At the last sampling point, when all groups were fed formulated feed, all significant differences between the groups had disappeared

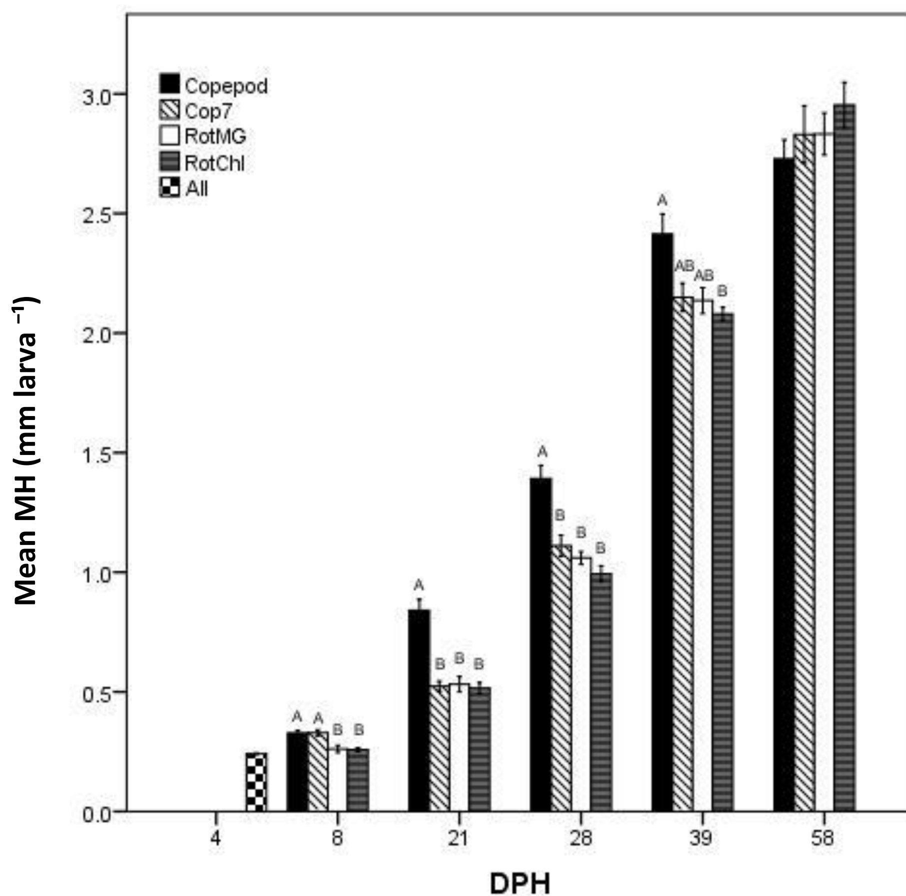

Figure 7: Mean myotome height (mg larva^{-1}) for all days post hatch (dph) (n= 12-30). Significant differences found between groups are marked by letters. Error bars = ± SE.

4.1.5 Survival

First day of registering dead larvae was 13 dph. Because of the low number of samples (n = 2 in Copepod group) the significant difference was not calculated for survival. At the end of the experiment (61 dph) the Cop7 group had the highest mean survival % of 12±1, while the RotChl group had a mean survival % of 5±1, the lowest survival rate of all groups (Table 4).

Table 4: Mean Survival % and standard error at feed regime changes (Table 1) for all groups and at end of experiment.

Days post hatch	Group	Mean Survival (%) ± SE
13	Copepod	16±3
	Cop7	24±5
	RotMG	17±3
	RotChl	12±1
24	Copepod	14±2
	Cop7	20±4
	RotMG	13±2
	RotChl	7±1
31	Copepod	13±2
	Cop7	18±4
	RotMG	12±1
	RotChl	6±1
40	Copepod	13±1
	Cop7	16±3
	RotMG	10±2
	RotChl	5±1
51	Copepod	13±1
	Cop7	15±3
	RotMG	10±2
	RotChl	5±1
61	Copepod	11±1
	Cop7	12±1
	RotMG	10±1
	RotChl	5±1

4.2 Metabolomics

4.2.1 Metabolomics extraction method test

To determine which metabolite extraction method to use, two different types were tested. Extracting with the methanol/water method gave high lipid peaks in the spectrum, some of them high enough to hide other metabolic peaks in the ppm scale (Figure 8). By using the two phase extraction method with methanol/chloroform/water the lipid peaks became a lot smaller and noticeable lipid peaks at 0.5, 1.3 and 5.3 ppm disappeared, giving a much cleaner baseline for the specter. For all extractions the methanol/chloroform/water method were used.

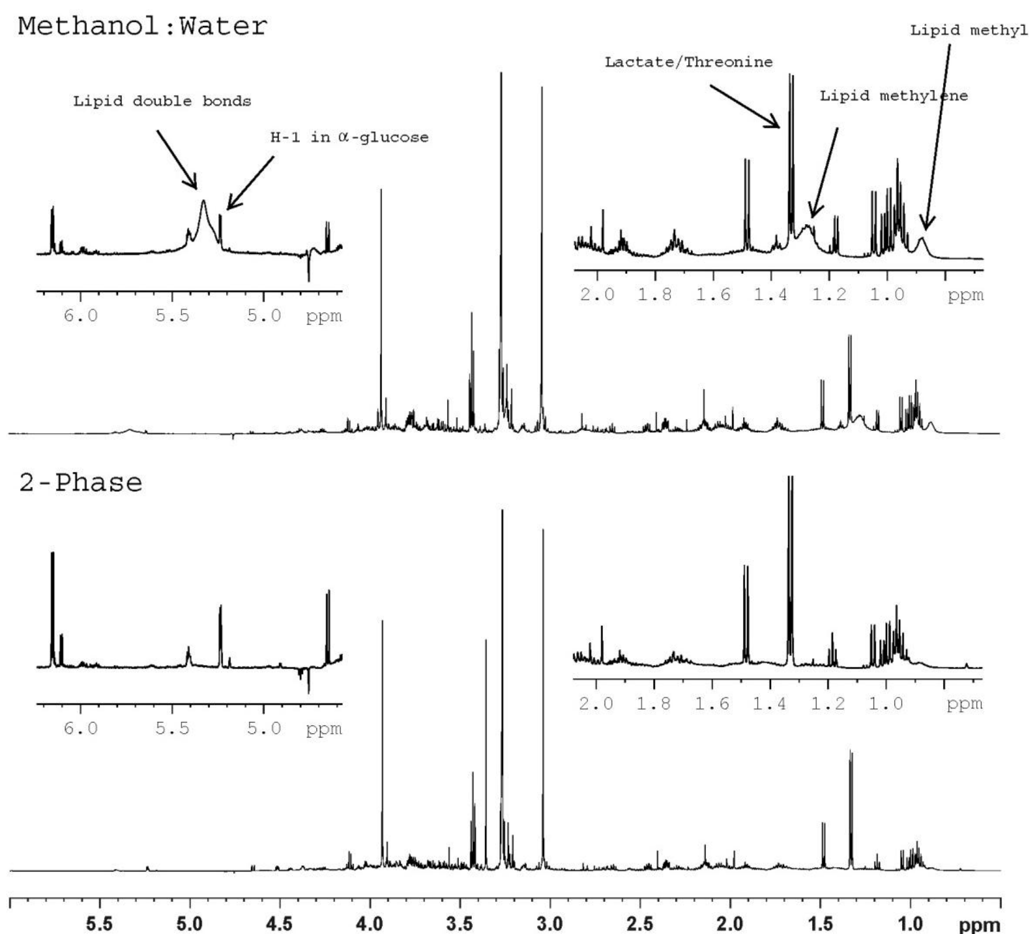

Figure 8: NMR-spectrum of ballan wrasse treated with two different extraction methods. Upper: methanol/water extraction method creating lipid peaks. Lower: 2 phase (methanol/chloroform/water) extraction method giving spectra without lipid peaks.

4.2.2 Identifying metabolites

Within the spectrum of the ballan wrasse, 23 different metabolites were identified. These were the metabolites that could be identified with certainty from the obtained spectrum. Different metabolites were identified by the ppm range of the specific peak and by the peak shape (Figure 9). It was possible to confirm the metabolite by examining the other peaks associated with this metabolite (Figure 10) and by using 2D NMR, NMR software for identification and quantification of metabolites in NMR data (Chenomx Inc.). The ppm range and shape of the peaks were found in metabolic databases containing almost all metabolic compounds.

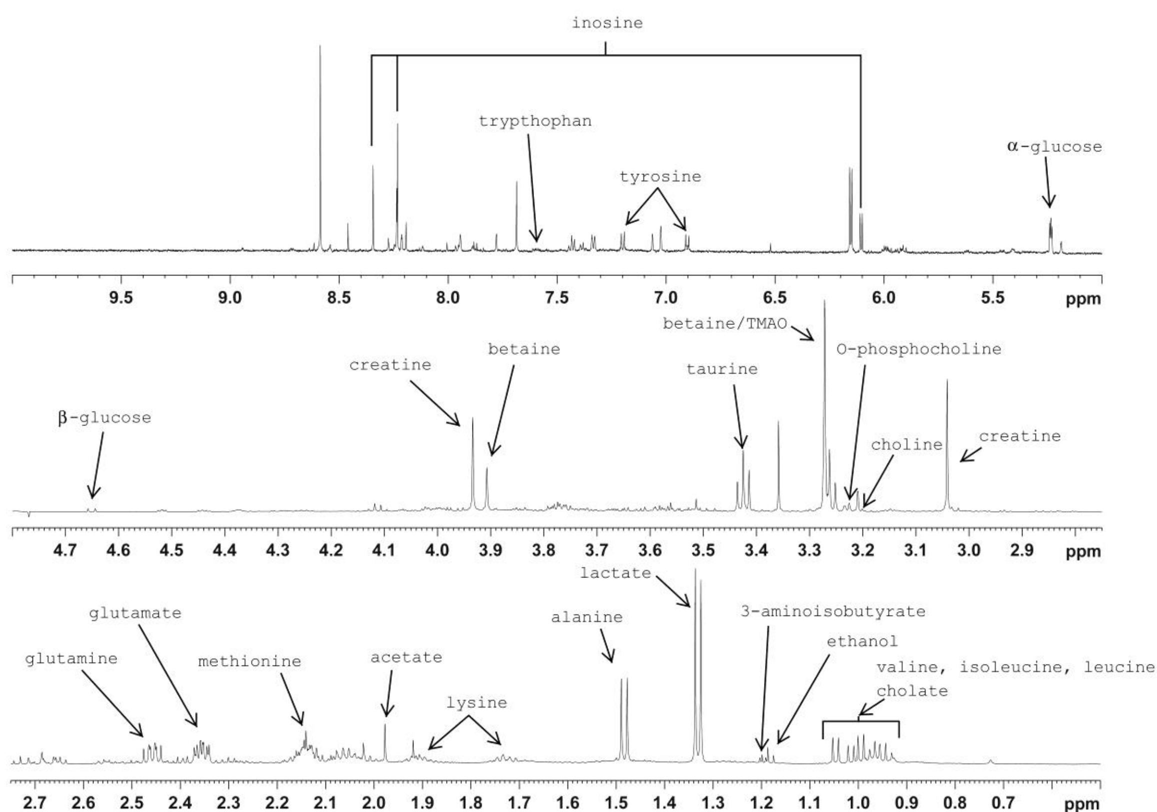

Figure 9: NMR spectrum of Ballan wrasse with twenty three identified metabolites.

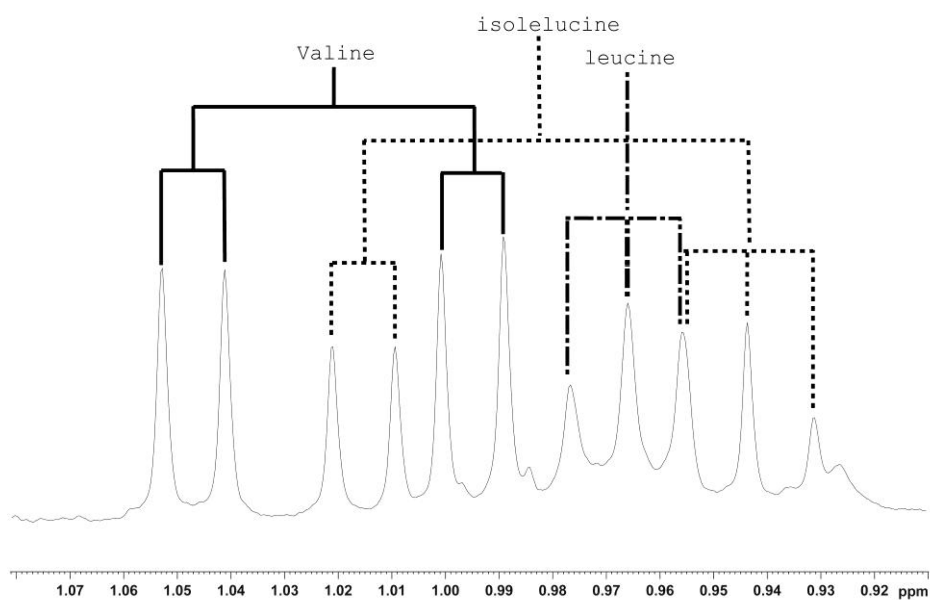

Figure 10: Showing the complexity of the metabolite signal. One metabolite may have more than one peak, and these peaks may be singlets, doublets, triplets and other peak shapes.

4.2.3 Principal Component Analysis (PCA)

The principal component analysis (PCA) (Figure 11) separated groups along both PC1 and PC2. Three clusters were evident in the score plot. The Copepod and Cop7 groups had a higher PC1 score than the RotMG and RotChl groups. In the PC2 direction the RotMG and RotChl groups had a higher value than the Copepod and Cop7 groups, fed copepods. A clustering of samples was found at values approaching 0 in the PC1 and PC2 axis.

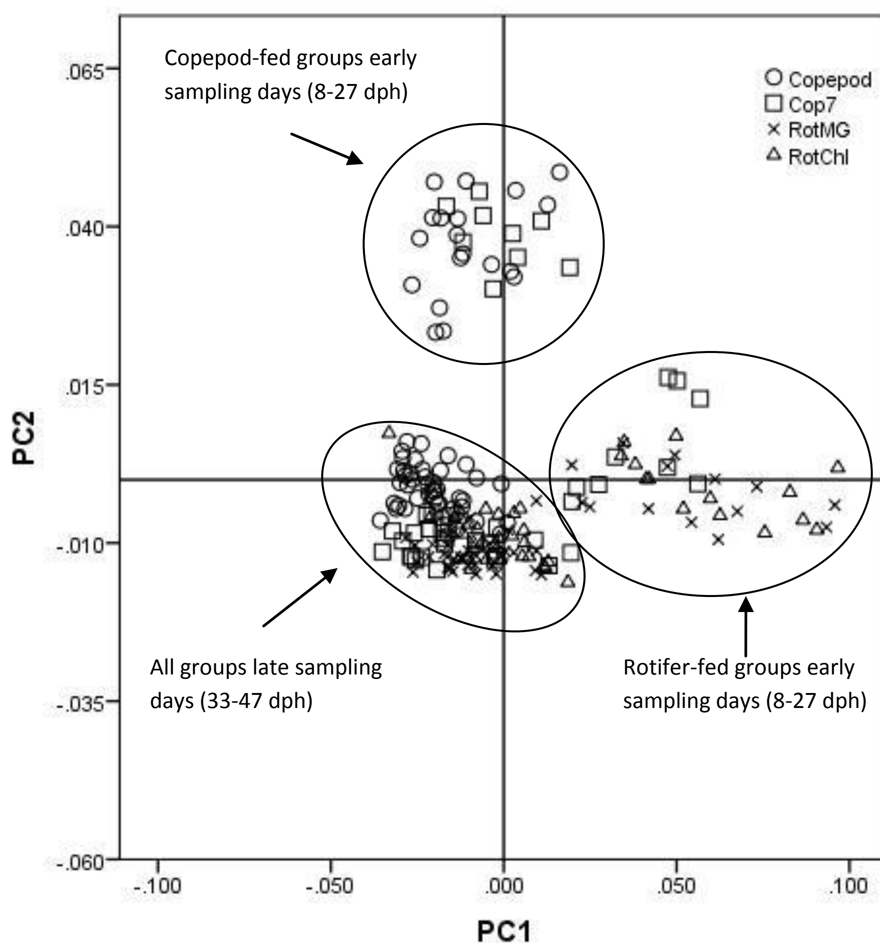

Figure 11: Principal Component Analysis (PCA) score plot of ballan wrasse for all days and all groups (n=203). Circles/ellipses are drawn manually to identify the different groupings of samples. These are not confidence ellipses

The PC1 loading from the PCA analysis showed that the samples with a positive value in the PC1 direction had a higher content of betaine and trimethylamine-N-oxide (TMAO) than the average (Figure 12). One of the peaks indicating the presence of betaine and the single peak for TMAO were close to each other. The partial overlap of betaine and TMAO peaks at approximately the same place, made distinguishing between

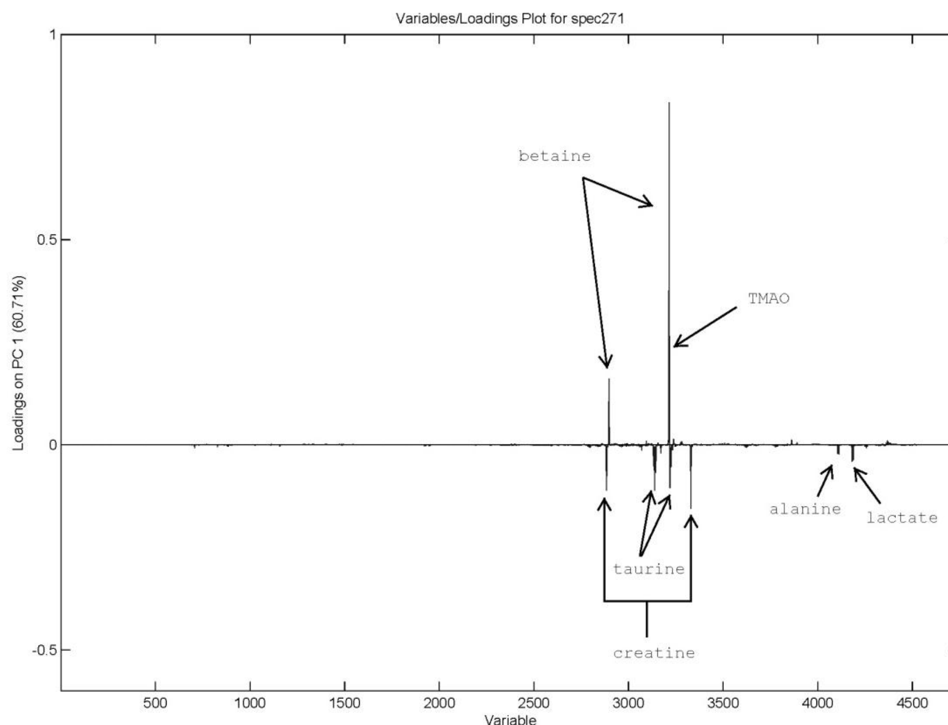

Figure 12: PC1 loading from PCA for ballan wrasse. Positive or negative sample values from the PCA analysis was used to identify the metabolites in the PC1 direction of the PCA. Sample values were compared to an average of all samples in the PCA analysis.

them challenging. However, betaine does have a second peak, which makes identification possible. Samples with positive PC1 values had higher content of betaine than of TMAO relative to each other. Samples on the negative side of the scale in the PC1 loading were samples containing higher values than average of creatine, taurine, lactate and alanine. Taurine and creatine were both identified by two peaks, with higher amounts of creatine present compared to the amount of taurine in the loading. Lactate and alanine was present

in small amounts, but the values and peaks were high enough to make them relevant for the loading.

Figure 13 shows the PC1 values for each group at the different sampling points. Values above 0 indicated that the sample had a content of betaine higher than average. 8 dph the rotifer-fed groups were found to have higher PC1 values than the two copepod-fed groups. The RotChl group was the group with the highest PC1 value the first sampling day. 12 dph all groups had declining PC1 values. As the Cop7 group stopped copepod feeding and started feeding enriched rotifers, a leap up in the PC1 value was observed. The other groups fed rotifers made a leap up at this point. 21 dph all groups increased in PC1 value, before declining again at the next sampling point at 27 dph. When all groups started the weaning period with *Artemia* sp., a rapid decline in the PC1 values was observed in all rotifer-fed groups. The last sampling day, 47 dph, all groups had started the dry feed weaning period and PC1 values was approximately the same for all groups.

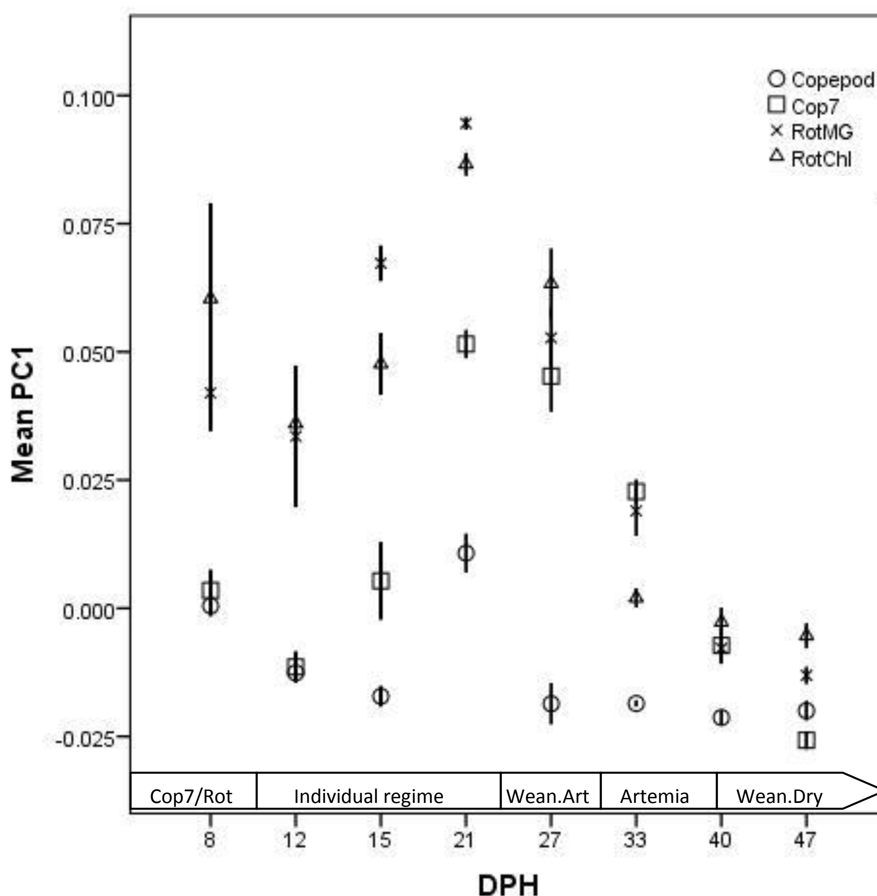

Figure 13: Mean PC1 value from ballan wrasse, plotted for all sampling days post hatch (DPH)(n=203). Error bars = ± SE. Changes in feeding regimes indicated in bottom of figure.

The PC2 loading showed that samples with positive values had higher than average amounts of TMAO mainly (Figure 14). Negative samples in the PC2 loading were samples with higher amount of creatine, taurine, lactate and alanine than the average samples. For negative samples the creatine levels were found to be slightly higher than the taurine levels. Small amounts of lactate and alanine were also observed in the negative samples.

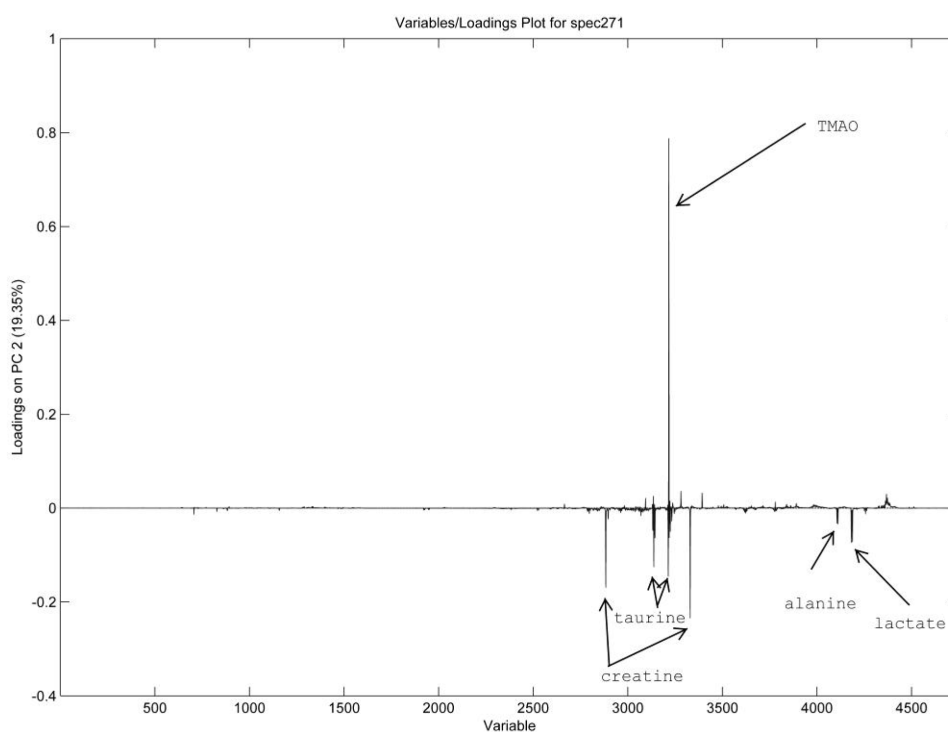

Figure 14: PC2 loading from PCA for ballan wrasse. Positive or negative sample values from the PCA analysis were used to identify the metabolites in the PC2 direction of the PCA. Sample values were compared to an average of all samples in the PCA analysis.

Positive PC2 loading values indicated a higher than average content of TMAO (Figure 15). Negative samples were found to have above average amounts of taurine, creatine, alanine and lactate. At the first sampling day 8 dph, the copepod-fed groups (Copepod and Cop7) had a positive PC2 value, while the rotifer-fed groups (RotMG and RotChl) had negative values. At the end of the individual live feed regimes (21 dph), when the Cop7 group had switched from copepods to enriched rotifers, a decline in the PC2 value was observed for this group. After the feeding period with *Artemia sp.* started, all PC2 values for the copepod-fed groups declined. The last two sampling days Cop7, RotMG and RotChl showed very similar values, with the Copepod group having a slightly higher PC2 value at these days.

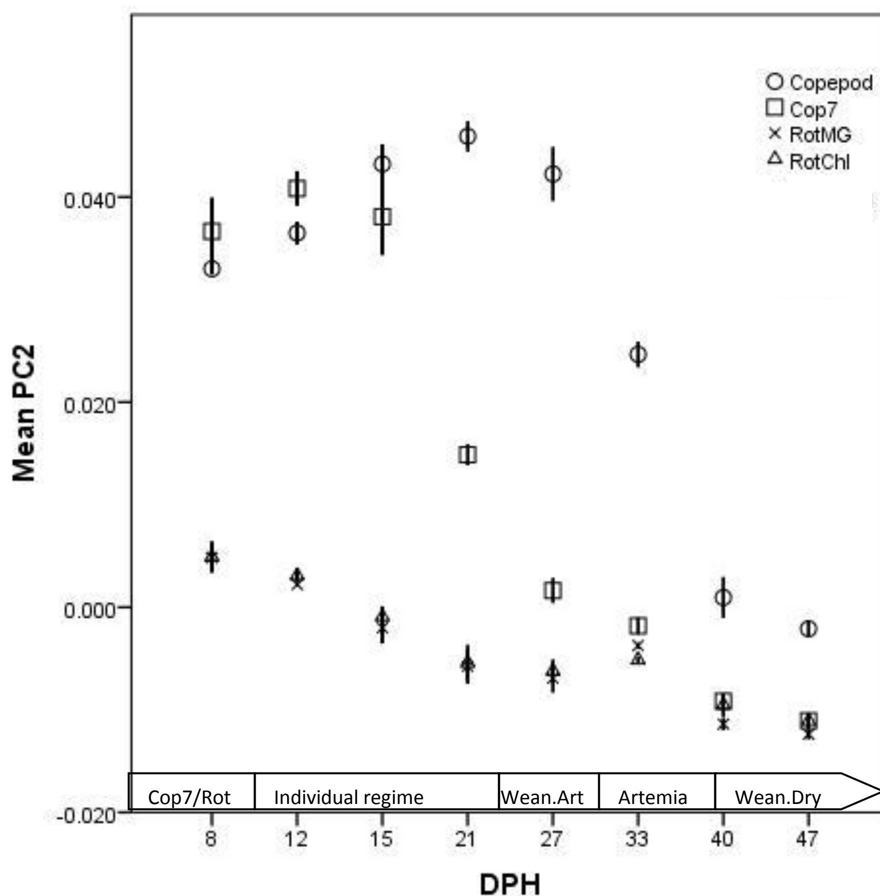

Figure 15: Mean PC2 value from ballan wrasse, plotted for all sampling days post hatch (DPH) (n=203). Error bars = ± SE. Changes in feeding regimes indicated in bottom of figure.

4.2.4 Principal Component Analysis (PCA) for individual sampling days

PC1 loadings from the PCA analysis made for all sampling days showed that positive valued samples contained higher amounts of betaine than average for all samples (Figure 16). Negative samples had higher than average amounts of TMAO, taurine, creatine, lactate and alanine. The first three sampling days (8, 12 and 15 dph), the RotMG and RotChl groups had a positive PC1 loading value indicating a higher value of betaine than average (Figure 17a, 17b, 17c). For the Copepod and Cop7 groups a negative PC1 loading value indicated a higher value of TMAO than average. At 12 dph the Copepod and Cop7 groups showed a higher value of taurine (Figure 17b) and at 15 dph a creatine peak started to emerge in these groups (Figure 17c). On 21 dph the Cop7 groups showed values close to 0, indicating a average score for all metabolites (Figure 17d), while the Copepod group showed high

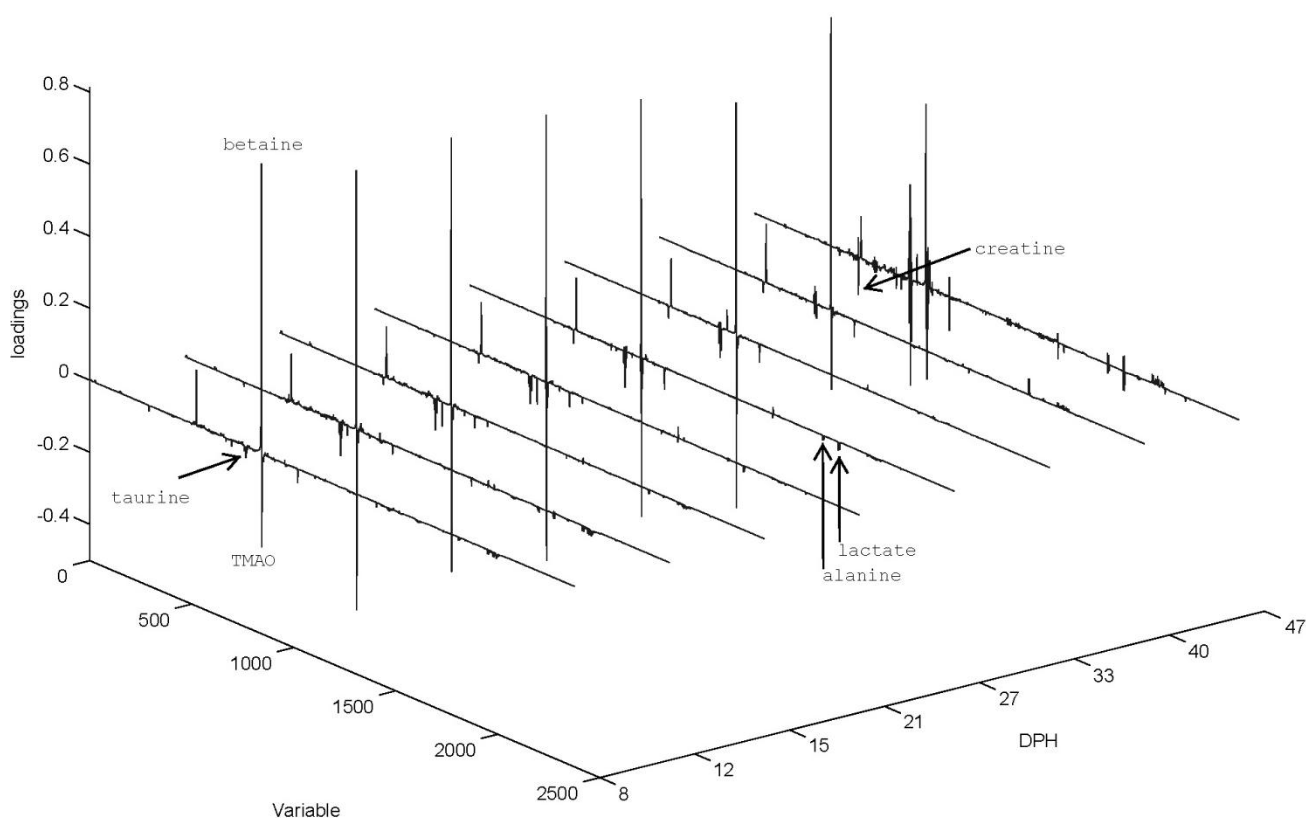

Figure 16: PC1 loadings for all individual PCA score plots (8, 12, 15, 21, 27, 33, 40 and 47 days post hatch). Metabolites were identified and named.

negative values indicating TMAO and taurine. Creatine and small amounts of alanine and lactate were found in negative valued groups at this stage. The rotifer-fed groups still showed high positive loading values associated with betaine. The Cop7 group moved to the positive side of the score plot at 27 dph, indicating a rise in the betaine values (Figure 18a). 33 dph the RotMG, RotChl and Cop7 groups showed average values indicating average concentration of metabolites (Figure 18b). The Copepod group still showed a higher negative value indicating TMAO, taurine and betaine. The alanine and lactate peaks were almost gone from the loading at this stage. For the last two sampling days, 40 and 47 dph, no clear separation of groups were found (Figure 18c, 18d).

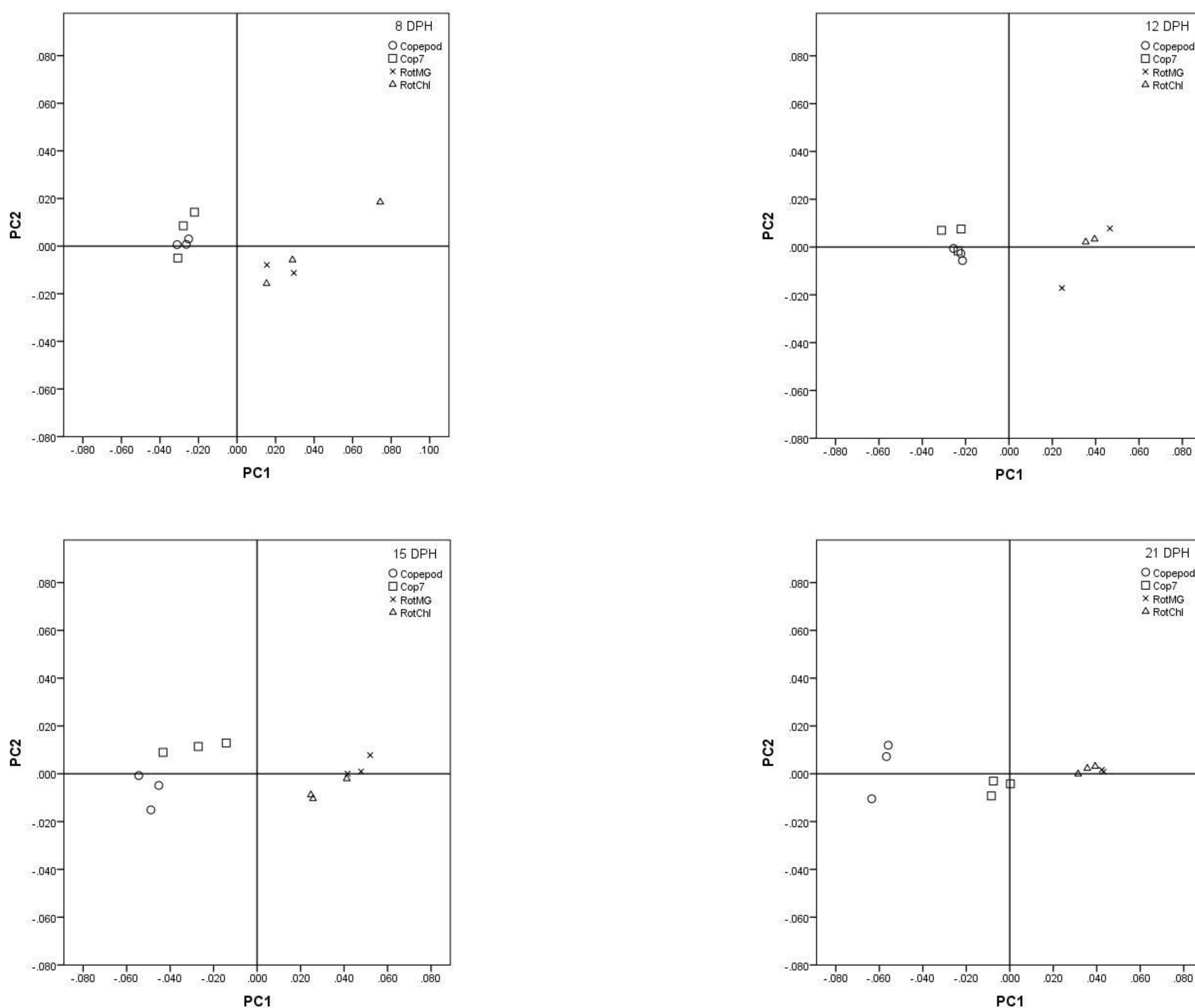

Figure 17: PCA score plot for individual sampling days. PC1 loading 8, 12, 15 and 21 days post hatch for all groups (n=10-12).

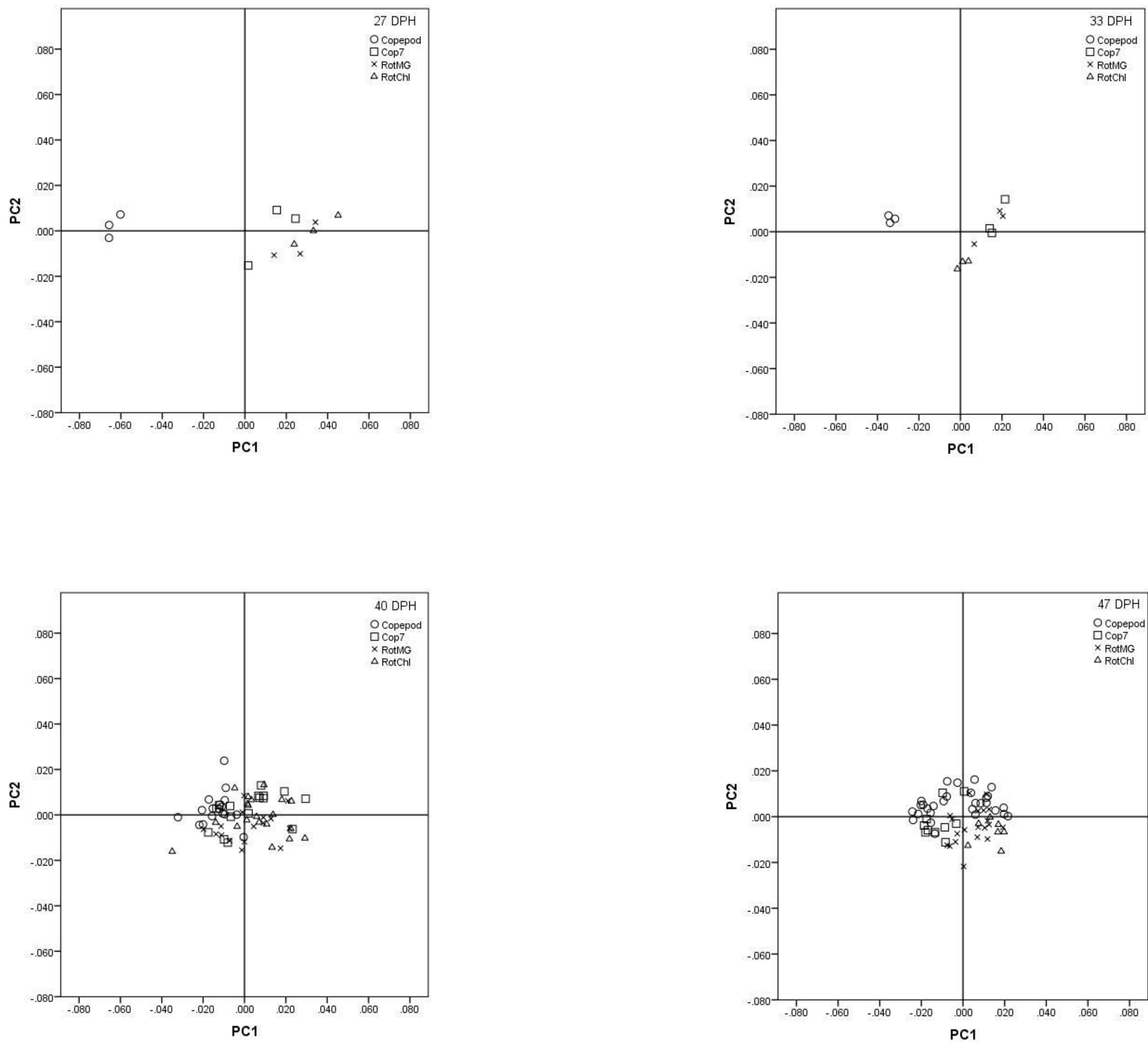

Figure 18: PCA score with PC1 loading 27, 33, 40 and 47 days post hatch for all treatments (n=12-69).

This analysis was performed to see how important TMAO was for the differentiation between the different treatments in the experiment. Leaving TMAO out of the analysis gave a PCA score plot without any pattern between the groups or within a special sampling day (Figure 19). The higher PC1 values found in the RotChl and RotMG groups at the early sampling days, and the high PC2 values found for the Copepod and Cop7 groups at early sampling days (Figure 11), disappeared with the removal of the TMAO peak.

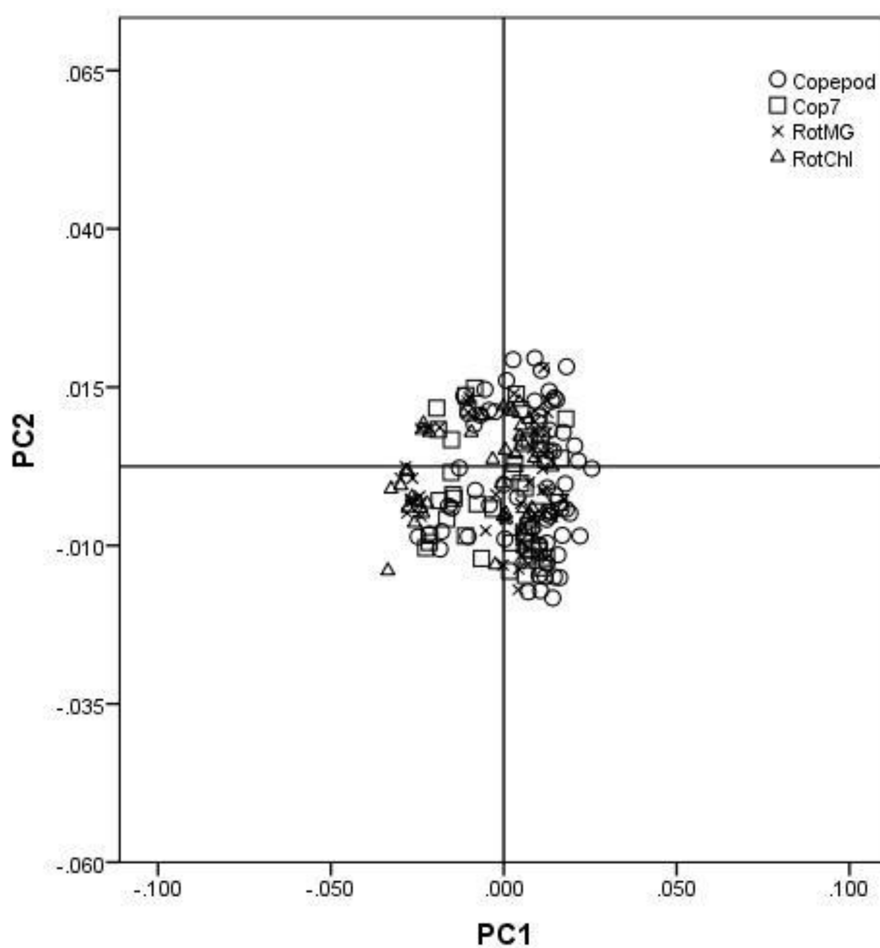

Figure 19: Principal Component Analysis (PCA) score plot from ballan wrasse for all days and all treatments. Leaving TMAO out of the analysis gave a PCA score plot without the influence of TMAO.

4.2.5 Metabolic analysis of live feed

Using NMR spectroscopy the four different types of feed was analyzed. The feed types were analyzed to identify the possible content and concentration of the metabolites taurine, betaine and TMAO (Figure 20). The results showed that betaine was found in all live feed species. The highest betaine concentration was found in the unenriched rotifer, containing 614.10 nmol/mg DW, while the smallest amount was found in *Artemia* sp. which had 49.96 nmol/mg DW betaine (Table 5). TMAO was only identified in the copepods. The concentration in the copepods was found to be 166.50 nmol/mg DW TMAO. In the other live feeds TMAO was not present or the concentration was too small to detect. Taurine was found in both copepods and *Artemia* sp.. 30.31 nmol/mg DW taurine was found in the *Artemia* sp.. In copepods the double amount of taurine was found, with a concentration of 61.63 nmol/mg DW.

Table 5: Concentrations of betaine, taurine and TMAO found in the four different live feed types used in the experiment. Concentration in nmol mg DW^{-1}. ND = Not detected.

Treatment	**Betaine**	**Taurine**	**Trimethylamine N-oxide**
Artemia sp.	49.96876	30.31146	ND
Unenriched Rotifer (*Brachi. Ibericus* Cayman)	614.1039	ND	ND
Enriched Rotifer (*Brachi. Ibericus* Cayman)	489.648	ND	ND
Copepod (*Acartia Tonsa*)	174.1971	61.63429	166.5019

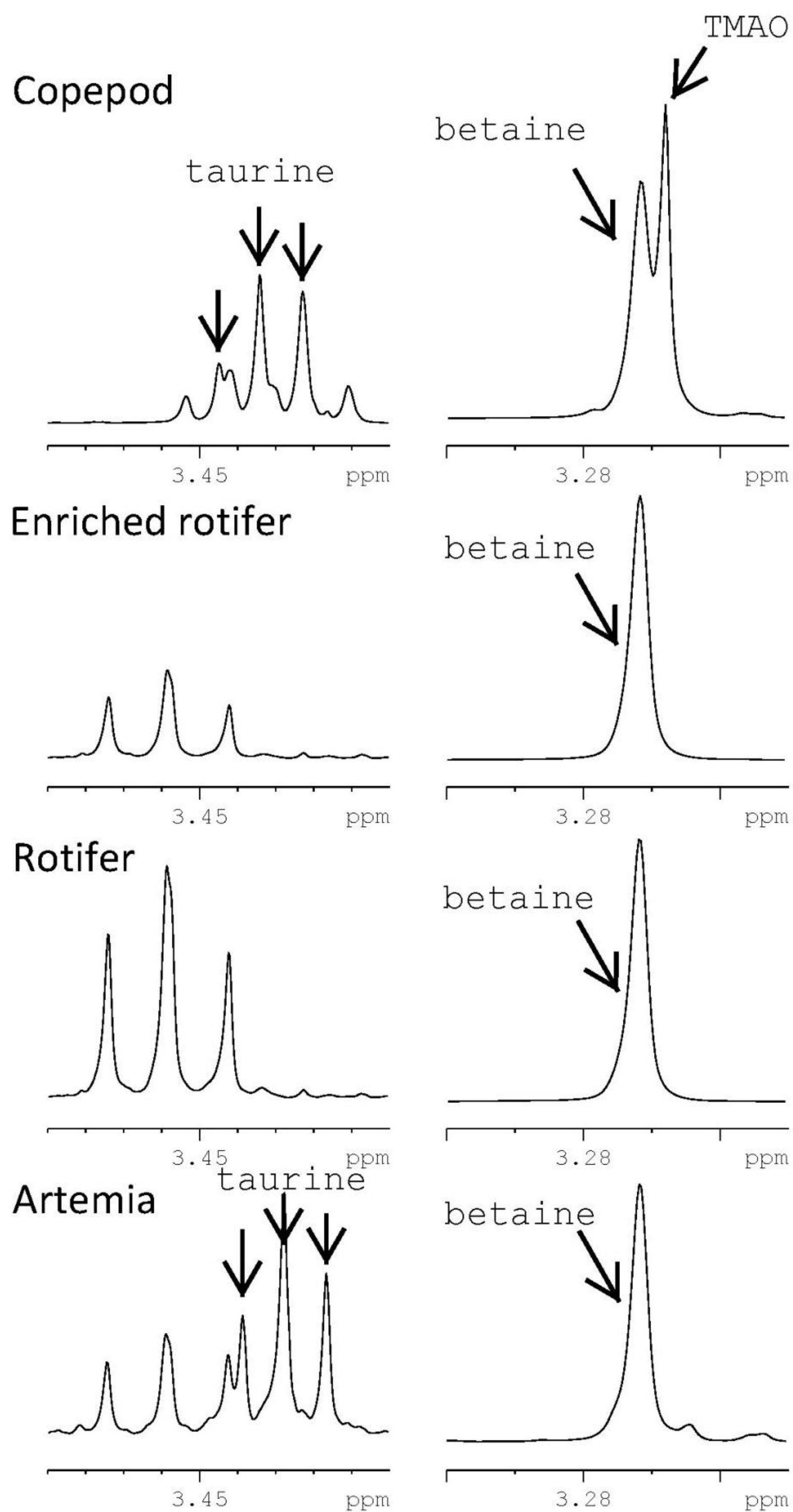

Figure 20: NMR spectra for all feed types. Spectra show ppm ranges associated with taurine (left) and TMAO/betaine (Right).

5. Discussion

5.1 Growth and survival

Growth differences in the early sampling days were closely linked to the type of live prey provided for the larvae. The Cop7 group was fed copepods for only 7 days, ending at 10 dph. Measurements done at 15 dph showed that the Cop7 group was having a lower increase in the dry weight compared to the Copepod group. The reason why the Cop7 group did not continue to grow at the same rate as the Copepod group was probably because at 10 dph the Cop7 group changed feed from copepods to rotifers. The lower nutritional values of the rotifer and its lipid composition could heavily impact the change in somatic growth (van der Meeren et al., 2008). The same trend could be seen in the mean % DWI figure (Figure 5), where the Cop7 group scored high in the first interval (4-12 dph). At the next interval (12-27 dph) the Cop7 group scored the lowest, indicating that the high increase in somatic growth halted after the copepod feeding stopped. Examining the dry weight for this interval, both of the rotifer groups and the Cop7 group had similar weight development, with lower growth in the RotChl group than in the others. By 40 dph all groups had approximately the same dry weight, with no significant differences found between the groups. This change in the growth pattern coincides with the change in the feeding regime from rotifer or copepod diet to *Artemia* diet. Analysis of the live feed used, performed by NIFES, showed that the lipid values of *Artemia* was higher than the values of rotifers (van der Meeren et al., 2008). This would probably result in a higher growth rate for the fish larvae changing from rotifer to *Artemia* diet. Copepods had a higher nutritional value than both rotifers and *Artemia* (van der Meeren et al., 2008). They had twice the amount of lipids compared to *Artemia*, which is probably the reason why the Copepod group had a decrease in somatic growth compared to the other groups when changing to a nutritionally inferior feed. Similar results have been found in cod, where larvae fed copepods as first-feeding diet showed higher growth rate compared to rotifer-fed larvae (Koedijk et al., 2010). The same study also found that if the larvae were switched to a sub-optimal diet (e.g. copepod to rotifer) this nutritional deficiency could result in disruption of growth and development (Koedijk et al., 2010). These findings suggest that the Cop7 group loses some of its growth momentum as a result of the diet change from copepods to rotifers.

For the Copepod group a little decline in the growth curve was observed around the onset of the copepod to *Artemia* co-feeding period. The same decline was found for this group after the weaning period between *Artemia* and formulated feed had started. This may be a consequence of the larvae not preferring their new type of food. This might make them selective to the old type of feed at the start of the co-feeding period even though there is less of this feed type available than earlier. This type of selective behavior has also been found in turbot larvae (*Scophthalmus maximus* L.) given a co-feeding diet of copepods and rotifers, with the preference clearly towards the copepods even though the amount of copepods was small (Kuhlmann et al., 1981). In the weaning period from *Artemia* to formulated feed the decline in growth could also be partly explained by the hardness or the taste of the formulated feed. When the larvae were changing from a thin exoskeleton with high water content to the much harder formulated feed, the formulated feed might be less palatable to the larvae once it has been taken into the mouth. A study of gilthead seabream preference in food type and hardness has indicated the same trend, with the formulated feed not being preferred at the start of the co-feeding period (Fernandezdiaz et al., 1994 ; Conceição et al., 2010). Measurements of standard length and myotome height showed the same trend for both measurements, with significant differences between the copepod-fed and the rotifer-fed groups in the first sampling day. 21 dph the Cop7 group had the same values as the rotifer groups, probably as a result of the diet change from copepods to rotifers. Also for the next two sampling days (28 and 39 dph) the same trend is found with the Copepod group being larger than the rotifer-fed larvae. On the last sampling day all significant differences has disappeared, leaving all groups with larvae of approximately the same standard length and myotome height. Similar results have been found studying Atlantic cod, where at the end of the experiment the larvae showed significant differences in somatic growth, but no significant differences were found in the measurements of standard length and myotome height (Galloway et al., 1999). It is suggested that the cod larvae would prioritize rapid growth to reach a certain length rather than gaining muscle mass if a choice is necessary(Galloway et al., 1999). This may explain why the ballan wrasse larvae from the present experiment all ended up at approximately the same length and height, but significant differences were found between the groups regarding somatic growth.

Within 24 hours after the first batch of newly hatched larvae arrived, all were found dead. The cause for this mass death could either be related to stress or bad environmental conditions. Too high surface aeration or residual tank cleaning chemicals are the two most likely reasons. After the arrival of the second batch of larvae the surface aeration was lowered, resulting in many larvae surviving the first 24 hours. The water in the tanks was also exchanged, which might have removed some chemical residues still remaining in the tanks.

The rate of larval death for the first days post hatch was very high. The first day of registering survival was 13 dph. During the first 13 days of the experiment, at least 80% of the larvae were dead. The high mortality at the start of the experiment could be caused by sub-optimal environmental conditions in the tank. With all the larvae from the first batch probably dying as a result of bad environmental conditions, it is possible that this might also have affected these larvae in the start of the experiment. The highest rate of survival was found in the Cop7 group 13 dph, with a mean survival of 24±5%. Lowest survival was found in the RotChl group with a mean survival of 12±1%. At the last sampling day the Cop7 group still had the highest survival of 12±1% and RotChl had the lowest survival with 5±1%. A similar study of live feed effects on Atlantic cod found a survival of 10.8% for cod larvae fed rotifers, and a survival of 8.9% for larvae fed copepods (Koedijk et al., 2010). The survival data showed that all groups had approximately the same decline in survival, indicating that the type of live feed used did not have a direct connection to the ballan wrasse larvae survival during the live feed period. The same trend has been found in Atlantic halibut, where survival is unaffected by the type of live prey used (Hamre et al., 2002).

5.2 Metabolomics

Performing the metabolic profiling and PCA analysis of the larvae treated with different feeding regimes allowed us to analyze the changes in the metabolic status of the larvae as it changed during the experiment. From the PC loadings a few metabolites was identified as the ones having the highest variation of the specific metabolites. Of the 23 different metabolites identified in the ballan wrasse spectrum, the six metabolites with the highest variation in the spectra were selected and investigated. These metabolites were TMAO, betaine, taurine, lactate, creatine and alanine.

The PCA analysis for all groups and all sampling days show that TMAO is the most abundant metabolite separating between the groups being fed the different types of live feed. The Copepod group is found to have high values of TMAO and the Cop7 group also has high values for the first days, while in the other groups betaine is the most abundant metabolite. As the Cop7 group value becomes closer to the rotifer treatments in the dry weight figure (Figure 4), it scores lower in the PC2 loading, which indicates a lower variation of TMAO.

TMAO is an osmolyte found in muscle tissue of marine organisms that is often credited with counteracting protein-destabilizing forces (Seibel & Walsh, 2002). TMAO has also been reported to have a function in cell volume regulation in elasmobranches, offsetting denaturation of functional proteins induced by urea in sharks (Somero, 1986), lowering the freezing point of blood in antarctic teleosts (Raymond & DeVries, 1998), and it has antioxidant properties (Ishikawa et al., 1978). In all marine fish TMAO is found to accumulate in the tissues, especially in muscle tissue. The amount of TMAO may differ depending on such factors as species, age, season and location (Agústsson & Strøm, 1981). It seems that trimetylamine (TMA) is microbiologically formed from dietary choline in the intestinal tract, where TMA is absorbed and converted into TMAO (Niizeki et al., 2003). A study of cod showed that they synthesize TMAO by oxidation of TMA, and that the monooxygenase activity is of the same level as other TMAO producing fish (Agústsson & Strøm, 1981).TMAO is also found in the natural food of marine fish, showing that TMAO is produced by the animals in the lower levels of the marine food chain, such as copepods (Agústsson & Strøm, 1981). Adding dietary TMAO to tilapia feed (*Oreochromis niloticus*) showed that the largest amount of TMAO accumulated when the fish got TMAO directly through the feed, and also when the diet was rich in choline, which was synthesized to TMAO (Niizeki et al., 2003). Heightened levels of TMAO was also found in Chinook salmon (*Oncorhynchus tschawytscha*) when fed dietary TMAO (Benoit & Norris, 1945). Analysis of the live feed used in this experiment showed that TMAO was only found in the copepod (Figure 20). Also, the exoskeleton of the copepod is rich in choline. As both these factors contribute to higher levels of TMAO, this is probably the reason for the high levels of TMAO found in the larvae fed the copepod diet. In the unenriched and enriched rotifers and in the *Artemia* sp., betaine was found to be the dominating osmolyte. Studying the effect of TMAO and betaine on growth in swine (*Sus scrofa domesticus*) (Overland et al., 1999) found that TMAO have a

positive effect on the growth, while betaine seem to have no effect on the growth. With TMAO being the metabolite that separates the groups from each other, it is likely that TMAO functions as a better osmolyte in the ballan wrasse larvae than betaine. The differences in growth can be explained by the synthesizing of TMAO. Larvae fed copepod diets could get the TMAO directly from the feed and from the synthesis of choline, while the larvae fed the diets rich in betaine must use this metabolite as an osmolyte. The larvae could obtain TMAO by synthesizing betaine to TMA and then oxidizing it to TMAO (Ballantyne, 1997). By not having to synthesize TMAO the copepod-fed larvae use less energy to obtain the osmolyte, which they in turn can use for growth instead. Later in the present experiment, when all groups were fed the same diets containing no dietary TMAO, the differences between the groups have faded.

Examining the PC loadings from the PCA analysis, taurine was found in groups with negative values. This indicates that the groups with negative value in the PC loadings are the groups with the highest amounts of taurine. In the PC1 loadings the Copepod group is found to have a negative value, meaning that a heightened taurine value is found in larvae from this group. It is also found that the other groups move to a negative PC2 score in the later sampling days (from 27 dph). Natural live prey for fish larvae, such as copepods, contains high levels of taurine (Conceição et al., 1997 ; van der Meeren et al., 2008). This might suggest that the physiological requirements for taurine in fish is high in the larval/juvenile stage, when the organ systems differentiate and develop (Pinto et al., 2010). In a study of the copepods nutritional biochemical composition it was found that copepods reared in pond systems had from 84 to 102 nmol/mg DW taurine, while intensive reared rotifers contained 3 nmol/mg DW taurine (van der Meeren et al., 2008). Higher levels of taurine in the feed are suggested to result in higher growth rate in turbot larvae (Conceição et al., 1997). Also for the flatfish Senegalese sole (Solea senegalensis) a supplement of taurine resulted in faster growth and better development of the larvae(Pinto et al., 2010). Analysis of the feed used in this experiment showed that the copepod, *Acartia tonsa*, had a taurine level of 61.6 nmol/mg DW (Table 5). The enriched rotifer and the unenriched rotifer used, showed values of taurine that was not detectable. Examining the differences in dry weight between larvae fed the copepod feed and larvae fed the rotifer feed, the first sampling days show significant differences between the groups. In a nutritional study of the *Artemia* it was found taurine

values between 57 and 65 nmol/mg DW (van der Meeren et al., 2008). The taurine value of the *Artemia* used in the present experiment was found to be 30.3 nmol/mg DW. This is much higher than the rotifer values, and might be one of the reasons why the growth differences found between the groups in the early sampling days disappears when larvae from all the groups are fed the same diet of *Artemia*. These findings suggest that taurine is an important supplement to the diet of marine fish larvae with regards to both development and growth.

The appearance of lactate was first found at 27 dph, where it was found in the groups scoring negative in the PC1 loading. At this point only the Copepod treatment has values indicating lactate. In fish, anaerobic processes are mainly characterized by the lactate mechanism (Livingstone, 1983 ; Van Waarde, 1983). Therefore measuring the lactate should indicate the possible contribution of anaerobic processes. In Atlantic halibut an increase in the lactate level was found after 26 dph, corresponding to increased activity of the larvae which suggests anaerobic respiration (Finn et al., 1995). At hatching it is found that most fish larvae have an inner muscle mass surrounded by one layer of superficial fibers. The inner fibers are responsible for bursts of rapid swimming and will develop into white muscle fibers. The superficial fibers in turn are thought to function in respiration and in slow, sustained movements and will develop into red muscle fibers. The differentiation between the red and the white muscle fibers might be important for the finding of lactate. Both superficial red and inner white fibers have an aerobic metabolism in the early larval stage of many fish larvae(El-Fiky et al., 1987). During the larval development the distribution of the adult muscle, including specialization of metabolism in the different muscle fibers develop gradually (Veggetti et al., 1993). As the muscle fibers differentiate, lactate should be found as the white muscle fibers specialize in anaerobic respiration. This is supported by the fact that the Copepod group larvae are the biggest in growth compared to the other groups at the stage where lactate is detected. Therefore they might have reached a developmental stage where the differentiation between the two muscle fibers occurs. Another theory for the finding of lactate is through the intestinal content of the larvae. Lactate bacteria have been known to participate in the normal intestinal microbiota of fish. The lactic acid, or lactate, is a metabolic end product of carbohydrate fermentation (Ringø & Gatesoupe, 1998 ; Gatesoupe, 2008). The exoskeleton of the copepod is made of chitin, which is a polysaccharide, a carbohydrate long chained molecule. The availability of carbohydrates in

the Copepod group could be found as lactate in the intestinal tracks of the ballan wrasse larvae

Due to lipid interference when using MeOH:H_2O extraction the slightly more time-consuming 2-fase extraction method was chosen. This affected the amount of time spent preparing the NMR samples, but is important as lipid resonance may overlap small important resonance peaks with the subsequent loss of metabolic information.

Conclusion and future research

The use of the copepod *Acartia tonsa* in start-feeding of ballan wrasse resulted in an increased growth in early larval development when compared to larvae fed both enriched and unenriched rotifers. Larvae fed diets containing copepods had significantly higher dry weight at the end of the experiment compared to rotifer-fed larvae. Nutritional values and composition of copepods make them the most suitable live feed in the early rearing of ballan wrasse larvae compared with rotifers. This is also supported by the feed analysis performed on the different types of live feed. In future studies, feeding regimes with longer periods of copepod feeding should be investigated, examining how far into the larval development copepods would be the superior live feed organism.

NMR was used to find differences in the metabolite composition in ballan wrasse given different types of live feed. Differences seem to be correlated to feed composition, with TMAO and taurine standing out as reliable biomarkers for growth differences. This is supported by literature, and should be subject to further research. Further studies into taurine and TMAO, as well as other compounds could be utilized to observe metabolic variation caused by feed composition. The non selective nature and simplicity of NMR-based metabolomics makes NMR a good starting point for the study of metabolite compounds in ballan wrasse, which this study has been an example of. Other –omic based research methods have higher sensitivity which could be included in future research.

References

Agústsson I, Strøm AR (1981) Biosynthesis and turnover of trimethylamine oxide in the teleost cod, *Gadus morhua*. *Journal of Biological Chemistry,* **256**.

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

Ballantyne JS (1997) Jaws: The inside story. The metabolism of elasmobranch fishes. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,* **118**, 703-742.

Baskerville-Bridges B, Kling LJ (2000) Larval culture of Atlantic cod (*Gadus morhua*) at high stocking densities. *Aquaculture,* **181**, 61-69.

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

Bengtson DA (2003) Status of marine aquaculture in relation to live prey: past, present and future. In: *Live Feed in Marine Aquaculture*. Blackwell Science Ltd., pp. 1-13.

Benoit GJ, Norris ER (1945) Studies on trimethylamine oxide. II. The Origin of trimethylamine oxide in young salmon. *Journal of Biological Chemistry,* **158**, 439-442.

Brandal PO, Egidius E (1979) Treatment of salmon lice (*Lepeophtheirus salmonis* Krøyer, 1838) with Neguvon® — Description of method and equipment. *Aquaculture,* **18**, 183-188.

Bundy JG, Davey MP, Viant MR (2009) Environmental metabolomics: a critical review and future perspectives. *Metabolomics,* **5**, 3-21.

Båmstedt U (1986) Chemical composition and energy content. In: *The Biological Chemistry of Marine Copepods* (ed. by Corner EDS, O'Hara SCM). Clarendon Press, Oxford, pp. 1-58.

Conceição LEC, van der Meeren T, Verreth JAJ, Evjen MS, Houlihan DF, Fyhn HJ (1997) Amino acid metabolism and protein turnover in larval turbot (*Scophthalmus maximus*) fed natural zooplankton or *Artemia*. *Marine Biology,* **129**, 255-265.

Conceição LEC, Yúfera M, Makridis P, Morais S, Dinis MT (2010) Live feeds for early stages of fish rearing. *Aquaculture Research,* **41**, 613-640.

Davies AMC, Fearn T (2005) Back to basics: the principles of principal component analysis. *Spectroscopy Europe*.

Daviss B (2005) Growing pains for metabolomics. *Scientist,* **19**, 25-28.

Dhont J, Van Stappen G (2003) Biology, tank production and nutritional value of *Artemia*. In: *Live Feeds in Marine Aquaculture*, Blackwell Science Ltd., pp. 65-121.

El-Fiky N, Hinterleitner S, Wieser W (1987) Differentiation of swimming muscles and gills, and development of anaerobic power in the larvae of cyprinid fish (*Pisces*, Teleostei). *Zoomorphology,* **107**, 126-132.

Espeland SH, Nedreaas K, Mortensen S, Skiftesvik AB, Agnalt A-L, Durif C, Harkestad LS, Karlsbakk E, Knutsen H, Thangstad T, Jørstad K, Bjordal Å, Gjøsæter J (2010) Kunnskapsstatus i ett økende fiskeri. *Kunnskapsstatus Leppefisk*.

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

Evjemo JO, Reitan KI, Olsen Y (2003) Copepods as live food organisms in the larval rearing of halibut larvae (*Hippoglossus hippoglossus L.*) with special emphasis on the nutritional value. *Aquaculture,* **227**, 191-210.

Fernandezdiaz C, Pascual E, Yufera M (1994) Feeding-behavior and prey size of gilthead seabream, *Sparus aurata*, larvae fed on inert and live food. *Marine Biology,* **118**, 323-328.

Finn RN, Rønnestad I, Fyhn HJ (1995) Respiration, nitrogen and energy metabolism of developing yolk-sac larvae of Atlantic halibut (*Hippoglossus hippoglossus L.*). *Comparative Biochemistry and Physiology Part A: Physiology,* **111**, 647-671.

Galloway TF, Kjørsvik E, Kryvi H (1999) Muscle growth and development in Atlantic cod larvae (*Gadus morhua L.*), related to different somatic growth rates. *Journal of Experimental Biology,* **202**.

Gatesoupe FJ (2008) Updating the importance of lactic acid bacteria in fish farming: Natural occurrence and probiotic treatments. *Journal of molecular microbiology and biotechnology,* **14**, 107.

Geurden I, Radünz-Neto J, Bergot P (1995) Essentiality of dietary phospholipids for carp (*Cyprinus carpio L.*) larvae. *Aquaculture,* **131**, 303-314.

Goodacre R, Vaidyanathan S, Dunn WB, Harrigan GG, Kell DB (2004) Metabolomics by numbers: acquiring and understanding global metabolite data. *Trends in Biotechnology,* **22**, 245-252.

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

Gribbestad IS, Aursand M, Martinez I (2005) High-resolution 1H magnetic resonance spectroscopy of whole fish, fillets and extracts of farmed Atlantic salmon (*Salmo salar*) for quality assessment and compositional analyses. *Aquaculture,* **250**, 445-457.

Hamre K, Opstad I, Espe M, Solbakken J, Hemre GI, Pittman K (2002) Nutrient composition and metamorphosis success of Atlantic halibut (*Hippoglossus hippoglossus, L.*) larvae fed natural zooplankton or *Artemia*. *Aquaculture Nutrition,* **8**, 139-148.

Helland S, Terjesen BF, Berg L (2003) Free amino acid and protein content in the planktonic copepod *Temora longicornis* compared to *Artemia franciscana*. *Aquaculture,* **215**, 213-228.

Hemminga MA (1992) Introduction to NMR. *Trends in Food Science & Technology,* **3**, 179-186.

Heuch PA, Mo TA (2001) A model of salmon louse production in Norway: effects of increasing salmon production and public management measures. *Diseases of Aquatic Organisms,* **45**, 145-152.

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

Ishikawa Y, Yuki E, Kato H, Fujimaki M (1978) The mechanism of synergism between tocopherols and trimethylamine oxide in the inhibition of the autoxidation of methyl linoleate. . *Agricultural and biological chemistry,* **42**.

Jimenez DF, Heuch PA, Revie CW, Gettinby G (2012) Confidence in assessing the effectiveness of bath treatments for the control of sea lice on Norwegian salmon farms. *Aquaculture*.

Jordan KW, Nordenstam J, Lauwers GY, Rothenberger DA, Alavi K, Garwood M, Cheng LL (2009) Metabolomic characterization of human rectal adenocarcinoma with intact tissue magnetic resonance spectroscopy. *Diseases of the Colon & Rectum,* **52**, 520-525.

Karakach TK, Huenupi EC, Soo EC, Walter JA, Afonso LOB (2009) (1)H-NMR and mass spectrometric characterization of the metabolic response of juvenile Atlantic salmon (*Salmo salar*) to long-term handling stress. *Metabolomics,* **5**, 123-137.

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

Kožul V, Glavić N, Tutman P, Bolotin J, Onofri V (2011) The spawning, embryonic and early larval development of the green wrasse *Labrus viridis* (Linnaeus, 1758) (Labridae) in controlled conditions. *Animal Reproduction Science,* **125**, 196-203.

Kuhlmann D, Quantz G, Witt U (1981) Rearing of turbot larvae (*Scophthalmus maximus L.*) on cultured food organisms and postmetamorphosis growth on natural and artificial food. *Aquaculture,* **23**, 183-196.

Kvenseth PG (2011) Leppefiskhistorie fra 1967 til 2010 - og ti år frem i tid! In: *Norsk Fiskeoppdrett,* pp. 12-14.

Kvenseth PG, Andreassen J, Solgaard J (2003a) Small fish - big benefit. *Norsk Fiskeoppdrett,* **28**, 4-6.

Kvenseth PG, Andreassen J, Solgaard J (2003b) Use of wrasse for small salmon. *Norsk Fiskeoppdrett,* **28**, 12-16.

Lin CY, Wu HF, Tjeerdema RS, Viant MR (2007) Evaluation of metabolite extraction strategies from tissue samples using NMR metabolomics. *Metabolomics,* **3**, 55-67.

Livingstone DR (1983) Invertebrate and vertebrate pathways of anaerobic metabolism: evolutionary considerations. *Journal of the Geological Society,* **140**, 27-37.

Martinez I, Bathen T, Standal IB, Halvorsen J, Aursand M, Gribbestad IS, Axelson DE (2005) Bioactive compounds in cod (*Gadus morhua*) products and suitability of H-1 NMR metabolite profiling for classification of the products using multivariate data analyses. *Journal of Agricultural and Food Chemistry,* **53**.

Nagata WD, Whyte JNC (1992) Effects of yeast and algal diets on the growth and biochemical composition of the rotifer *Brachionus plicatilis* (Müller) in culture. *Aquaculture Research,* **23**, 13-21.

Niizeki N, Daikoku T, Hirata T, El-Shourbagy I, Song X, Sakaguchi M (2003) Mechanism of biosynthesis of trimethylamine oxide in tilapia reared under seawater conditions. *Fisheries Science,* **69**, 74-87.

Olsen AI, Attramadal Y, Reitan KI, Olsen Y (2000) Food selection and digestion characteristics of Atlantic halibut (*Hippoglossus hippoglossus)* larvae fed cultivated prey organisms. *Aquaculture,* **181**, 293-310.

Olsen Y (2004) Live food technology of coldwater marine fish larvae In: *Culture of cold-water Marine Fish* (ed. by Moksness E, Kjørsvik E, Olsen Y). Blackwell Scientific Publications Ltd, pp. 85-136.

Overland M, Rørvik KA, Skrede A (1999) Effect of trimethylamine oxide and betaine in swine diets on growth performance, carcass characteristics, nutrient digestibility, and sensory quality of pork. *Journal of Animal Science,* **77**, 2143-2153.

Overrein I (2010) Copepods in aquaculture. Norwegian university of science and technology.

Pike AW (1989) Sea lice — Major pathogens of farmed atlantic salmon. *Parasitology Today,* **5**, 291-297.

Pinto W, Figueira L, Ribeiro L, Yúfera M, Dinis MT, Aragão C (2010) Dietary taurine supplementation enhances metamorphosis and growth potential of *Solea senegalensis* larvae. *Aquaculture,* **309**, 159-164.

Rainuzzo JR, Reitan KI, Olsen Y (1997) The significance of lipids at early stages of marine fish: a review. *Aquaculture,* **155**, 103-115.

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

Raymond JA, DeVries AL (1998) Elevated concentrations and synthetic pathways of trimethylamine oxide and urea in some teleost fishes of McMurdo Sound, Antarctica. *Fish Physiology and Biochemistry,* **18**, 387-398.

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

Ricker ER (1958) *Handbook of computations for biological statistics of fish populations,* Bulletin of the Fisheries Research Board of Canada, Bulletin of the Fisheries Research Board of Canada.

Ringø E, Gatesoupe F-J (1998) Lactic acid bacteria in fish: a review. *Aquaculture,* **160**, 177-203.

Roessner U, Beckles DM (2009) *Metabolite Measurements,* Springer, New York.

Roth M, Richards RH, Dobson DP, Rae GH (1996) Field trials on the efficacy of the organophosphorus compound azamethiphos for the control of sea lice (Copepoda: *Caligidae*) infestations of farmed Atlantic salmon (*Salmo salar*). *Aquaculture,* **140**, 217-239.

Roy WJ, Sutherland IH, Rodger HDM, Varma KJ (2000) Tolerance of Atlantic salmon, *Salmo salar L.*, and rainbow trout, *Oncorhynchus mykiss* (Walbaum), to emamectin benzoate, a new orally administered treatment for sea lice. *Aquaculture,* **184**, 19-29.

Sargent JR, Bell JG, Bell MV, Henderson RJ, Tocher DR (1993) The metabolism of phospholipids and polyunsaturated fatty acids in fish. In: *Aquaculture: Fundamental and Applied Research*. AGU, Washington, DC, pp. 103-124.

Sargent JR, McEvoy LA, Bell JG (1997) Requirements, presentation and sources of polyunsaturated fatty acids in marine fish larval feeds. *Aquaculture,* **155**, 117-127.

Savorani F, Picone G, Badiani A, Fagioli P, Capozzi F, Engelsen SB (2010) Metabolic profiling and aquaculture differentiation of gilthead sea bream by 1H NMR metabonomics. *Food Chemistry,* **120**, 907-914.

Sayer MDJ, Treasurer JW (1996) North European wrasse: identification, distribution and habitat. In: *Wrasse: Biology and use in aquaculture*. Fishing News Books, Blackwell Science Ltd, pp. 3-12.

Schock TB, Newton S, Brenkert K, Leffler J, Bearden DW (2012) An NMR-based metabolomic assessment of cultured cobia health in response to dietary manipulation. *Food Chemistry,* **133**, 90-101.

Seibel BA, Walsh PJ (2002) Trimethylamine oxide accumulation in marine animals: relationship to acylglycerol storage. *Journal of Experimental Biology,* **205**, 297-306.

Sitter B, Krane J, Gribbestad IS, Jorgensen L, Aursand M (1999) Quality evaluation of Atlantic halibut (*Hippoglossus hippoglossus L*) during ice storage using H-1 NMR spectroscopy. In: *Advances in Magnetic Resonance in Food Science* (ed. by Belton PS, Hills BP, Webb GA). Royal Soc Chemistry, Cambridge, pp. 226-237.

Skiftesvik AB (2009) Oppdrett av berggylte. In: *Norsk Fiskeoppdrett,* pp. 36-37.

Skiftesvik AB, Bjellland RM (2003) Farming of ballan wrasse (*Labrus Bergylta)*. In: *Norsk Fiskeoppdrett,* pp. 41-44.

Skiftesvik AB, Opstad I, Kvenseth PG, Bjelland R (2011) Oppdrett av berggylte. In: *Norsk Fiskeoppdrett,* pp. 42-43.

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

Somero G (1986) From dogfish to dogs: Trimethylamines protect proteins from urea. *Physiology,* **1**, 9-12.

Sorgeloos P, Bossuyt E, Lavina E, Baezamesa M, Persoone G (1977) Decapsulation of *Artemia* cysts - simple technique for improvement of use of brine shrimp in aquaculture. *Aquaculture,* **12**, 311-315.

Sparkman OD, Penton ZE, Kitson FG (2011) Chapter 1 - Introduction and History. In: *Gas Chromatography and Mass Spectrometry: A Practical Guide (Second edition)*. Academic Press, Amsterdam, pp. 2-13.

Statistikk NO (1987) Fiske og Oppdrett av Laks m. v. 1986. Statistisk Sentralbyrå.

Treasurer JW, Grant A (1997) The efficacy of hydrogen peroxide for the treatment of farmed Atlantic salmon, *Salmo salar L.* infested with sea lice (Copepoda: *Caligidae*). *Aquaculture,* **148**, 265-275.

Trygg J, Holmes E, Lundstedt T (2007) Chemometrics in metabonomics. *Journal of Proteome Research,* **6**, 469-479.

van der Meeren T, Olsen RE, Hamre K, Fyhn HJ (2008) Biochemical composition of copepods for evaluation of feed quality in production of juvenile marine fish. *Aquaculture,* **274**, 375-397.

Van Waarde A (1983) Aerobic and anaerobic ammonia production by fish. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry,* **74**, 675-684.

Veggetti A, Mascarello F, Scapolo PA, Rowlerson A, Carnevali MDC (1993) Muscle growth and myosin isoform transitions during development of a small teleost fish, *Poecilia reticulata* (Peters) (Atheriniformes, Poeciliidae): a histochemical, immunohistochemical, ultrastructural and morphometric study. *Anatomy and Embryology,* **187**, 353-361.

Viant MR (2007) Revealing the metabolome of animal tissues using 1H nuclear magnetic resonance spectroscopy metabolomics. **358**, 229-246.

Walne PR (1974) *Culture of Bivalve Molluscs. 50 Years' Experience at Conwy,* West Byfleet.

Watanabe T, Kiron V (1994) Prospects in larval fish dietetics. *Aquaculture,* **124**, 223-251.

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

Wu HF, Southam AD, Hines A, Viant MR (2008) High-throughput tissue extraction protocol for NMR- and MS-based metabolomics. *Analytical Biochemistry,* **372**, 204-212.

Øie G, Olsen Y (1997) Protein and lipid content of the rotifer *Brachionus plicatilis* during variable growth and feeding condition. *Hydrobiologia,* **358**, 251-258.

Appendix 1

Formulated feed production and feed recipe

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

Conwy Medium recipe (Walne, 1974)

$NaNO_3$ (Sodium Nitrate)	100.0gr
Na-EDTA (EDTA disodium salt)	45.0gr
H_3BO_3 (Boric Acid)	33.6gr
$NaH_2PO_4 \bullet 2H_2O$ (Sodium Phosphate, monobasic)	20.0gr
$FeCl_3 \bullet 6H_2O$ (Ferric Chloride, 6–hydrate)	1.3gr
$MnCl_2 \bullet 4H_2O$ (Manganous Chloride, 4–hydrate)	0.136gr
Vitamin B_1 (Thiamin HCl)	0.1gr
Vitamin B_{12} (Cyanocobalamin)	0.05gr
Trace Metal Solution *	1ml
Distilled water (to make)	1 000ml.

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

Trace Metal Stock Solution *

$ZnCl_2$ Zinc Chloride	2.1gr
$CoCl_2 \bullet 6H_2O$ (Cobalt Chloride, 6–hydrate)	2.1gr
$(NH_4)_6Mo_7O_{24} \bullet 6H_2O$ (Ammonium Molybdate, 4–hydrate)	2.1gr
$CuSO_4 \bullet 5H_2O$ (Copper Sulphate)	2.0gr

Distilled water100ml

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

Walne P.R. 1974. Culture of Bivalve Molluscs. 50 Years' Experience at Conwy. Fishing News (Books), West Byfleet, 173 pp.

Appendix 3

Table A1: Mean dry weight, Standard Error and Total N for all days post hatch, treatments and tanks.

Mean Dry Weight (mg larva $^{-1}$)					
DPH	**Treatment**	**Tank**	**Mean**	**SE**	**Total N**
2	All		0.0442	0.0016	12
4	All		0.0409	0.0013	12
8	Copepod	1	0.0728	0.0032	12
		2	0.072	0.0042	12
		3	0.0762	0.0055	12
	Cop7	1	0.0797	0.0033	12
		2	0.0638	0.0043	12
		3	0.0715	0.0042	12
	RotMG	1	0.0515	0.0048	12
		2	0.0393	0.0044	12
		4	0.0417	0.004	12
	RotChl	1	0.0435	0.0043	11
		2	0.0531	0.0033	12
		3	0.0517	0.0053	12
12	Copepod	1	0.1395	0.013	11
		2	0.1482	0.0131	12
		3	0.1837	0.0091	12
	Cop7	1	0.1783	0.0086	12
		2	0.1489	0.0185	12
		3	0.1281	0.0138	12
	RotMG	1	0.0674	0.0093	12
		2	0.0821	0.0056	12
		4	0.0834	0.0069	12
	RotChl	1	0.085	0.0047	12
		2	0.0872	0.0092	12
		3	0.0815	0.011	12
15	Copepod	1	0.2179	0.0211	12
		2	0.2554	0.0215	12
		3	0.284	0.0259	11
	Cop7	1	0.2198	0.0056	12
		2	0.1779	0.0108	12
		3	0.1757	0.0167	12
	RotMG	1	0.1326	0.0137	12
		2	0.119	0.0147	11
		4	0.1005	0.0126	12
	RotChl	1	0.1236	0.0091	12
		2	0.112	0.0095	12
		3	0.0924	0.009	12
21	Copepod	1	0.4417	0.0557	12

		2	0.6246	0.0474	12
		3	0.6832	0.0465	12
	Cop7	1	0.3471	0.0247	12
		2	0.2436	0.0323	12
		3	0.2313	0.0215	12
	RotMG	1	0.263	0.0296	12
		2	0.1713	0.0204	12
		4	0.1859	0.0227	12
	RotChl	1	0.2364	0.0279	11
		2	0.2212	0.0228	12
		3	0.1945	0.0283	12
27	Copepod	1	1.0103	0.0942	24
		2	1.2766	0.0789	24
		3	1.2227	0.1071	24
	Cop7	1	1.1754	0.3014	24
		2	0.7766	0.0423	24
		3	0.757	0.0678	24
	RotMG	1	0.7119	0.0561	24
		2	0.7609	0.0568	24
		4	0.7101	0.0312	24
	RotChl	1	0.5563	0.0322	24
		2	0.5754	0.0511	24
		3	0.572	0.0429	24
33	Copepod	1	2.4936	0.1941	30
		2	3.0184	0.2097	30
		3	2.7133	0.1886	30
	Cop7	1	3.0452	0.1456	30
		2	1.5953	0.1372	30
		3	1.7662	0.1466	30
	RotMG	1	1.901	0.1404	30
		2	2.1472	0.1637	30
		4	1.9265	0.1167	30
	RotChl	1	1.4104	0.0796	30
		2	1.5325	0.1141	30
		3	1.5598	0.1571	30
40	Copepod	1	3.8792	0.2471	30
		2	4.9983	0.3742	30
		3	4.7666	0.4201	30
	Cop7	1	4.9061	0.3827	30
		2	3.8585	0.2765	30
		3	4.0768	0.286	30
	RotMG	1	4.1793	0.1997	30
		2	4.6395	0.2901	30
		4	5.0101	0.2452	29
	RotChl	1	4.7615	0.2233	30

		2	4.1584	0.224	30
		3	4.8416	0.2321	22
47	Copepod	1	7.3508	0.655	30
		2	9.2463	0.8182	30
		3	8.3644	0.7993	30
	Cop7	1	10.9654	0.7002	30
		2	7.1279	0.6523	30
		3	6.7709	0.4328	30
	RotMG	1	7.1253	0.3696	30
		2	8.3293	0.4335	30
		4	7.039	0.4243	30
	RotChl	1	7.9587	0.3552	30
		2	9.0823	0.4469	30
61	Copepod	1	7.754	0.8175	50
		2	19.594	1.7326	50
		3	19.678	1.4085	50
	Cop7	1	21.52	1.7276	50
		2	12.064	1.2437	50
		3	17.662	1.1791	50
	RotMG	1	16.838	1.4258	50
		2	11.126	1.1131	50
		4	13.8918	0.881	49
	RotChl	1	14.722	1.1531	50
		2	11.1898	0.8785	49

Appendix 4

Table A2: Mean % Dry weight increase and Standard Error for all intervals and all treatments.

		% 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

Appendix 5

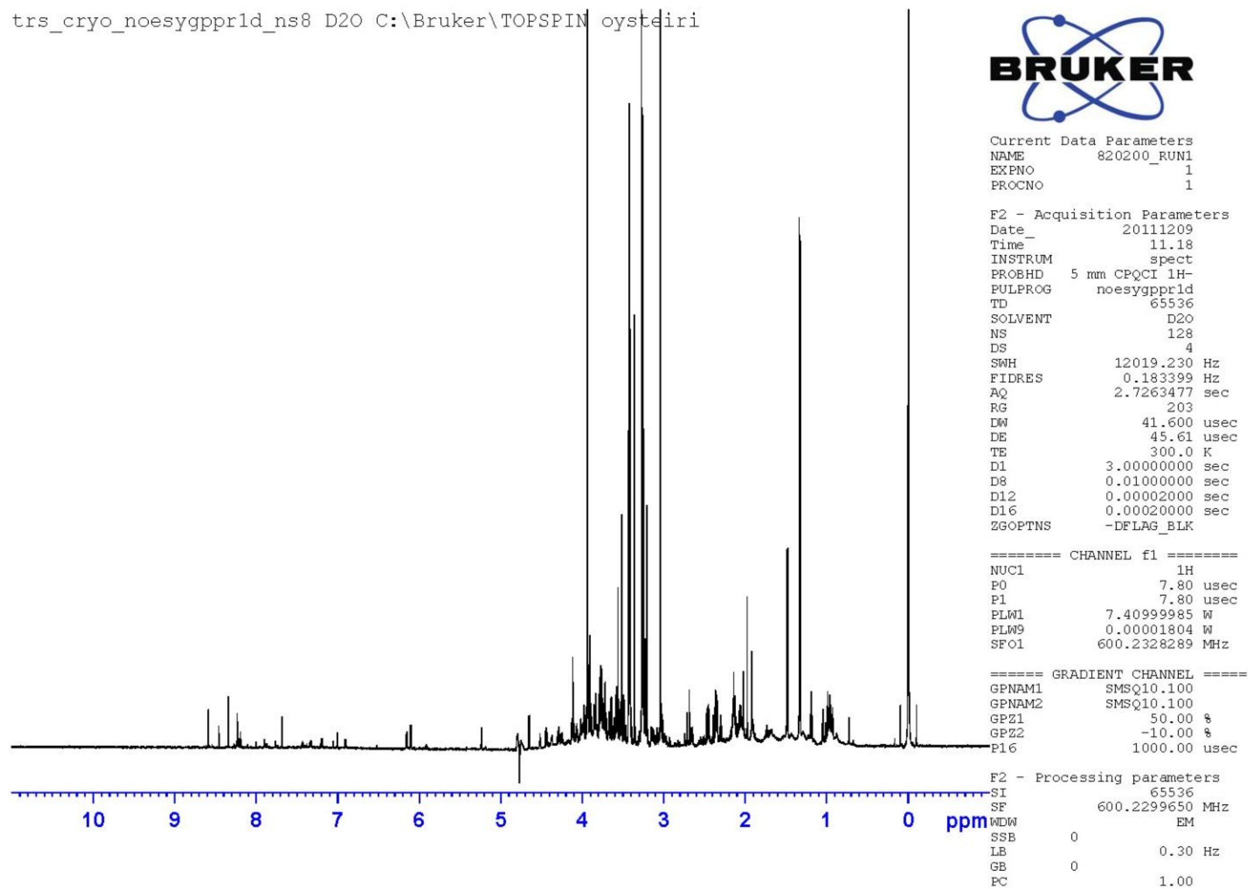

Figure A1: Typical NMR parameters for acquiring spectrum