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Effects of different live feed on ballan wrasse (*Labrus bergylta*) larval hepatocyte and enterocyte development

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Marine Coastal Development
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Abstract

Salmon lice infestations are one of the biggest challenges for Norwegian salmon farming today. Delousing agents have been the solution to these infestations in the past, but overuse and wrong dosages have caused the lice to become resistant to the chemicals and thus rendering them ineffective. As a result cleaner fish have been proposed as an all-natural delousing agent. The ballan wrasse has been proposed as the ideal cleaner fish for Norwegian waters and is being caught in the wild and put in the fish pens with the salmon. To be able to use ballan wrasse on a larger scale and in a sustainable way, it has been proposed to begin farming of the ballan wrasse. This will secure a stable supply of this fish to the salmon farms. One of the keys to successfully rearing ballan wrasse is to figure out the ideal feeding regime in the larval stage of development when the larvae are dependent on live feed. The traditional live feed of rotifers and artemia do not meet the nutritional requirements of marine fish larvae and has to be enriched to meet these requirements. Copepods has been suggested as a more suitable live feed as it is the natural prey of most wild marine fish and meets their nutritional requirements thus not needing to be enriched.

In this study the traditional live feed of rotifers and artemia was compared with using intensively reared copepods (*Acartia tonsa*) exclusively through the whole live feed period of ballan wrasse larvae. The Rotifer-group was fed enriched rotifers from 4 dph until 30 dph, and was co-fed enriched rotifers and enriched artemia between 30 dph and 40 dph. From 40 dph until the end of the experiment (45 dph) the Rotifer-group was fed enriched artemia exclusively. The Copepod-group was fed intensively reared copepods exclusively throughout the experiment (4-45 dph). The ballan wrasse larvae were fed *Acartia tonsa* nauplii until 15 dph, then as the larvae grew they were fed copepodits from 15 dph until 30 dph. From 30 dph to 45 dph the larvae were fed a combination of copepodits and adult *Acartia tonsa* in a 2:1 ratio. The two groups were compared to see if the nutritional differences of the live prey had an effect on different growth parameters and how it affected the gut and liver development in the ballan wrasse larvae.

The results from this study indicates that intensively reared *Acartia tonsa* was a better live feed for ballan wrasse larvae compared to enriched rotifers and enriched artemia. The larvae that were fed copepods showed better growth and had better liver and gut development than the larvae fed enriched rotifers and artemia.

Contents

1. Introduction

1.1 Background

With the rising number of people on the planet, food production has to be expanded to meet the future requirements and aquaculture is said to play an important role is this development. Aquaculture, especially of salmon, has become an important industry in Norway. With a production of 1.16 million tons of salmon in 2013 (Norwegian Directorate of Fisheries, 2014), it has become an important industry in Norway. The export of salmon from Norway was valued at 40 billion NOK (Statistics Norway, 2014) making it an important export. The salmon industry has developed a lot since the beginning in the 1970`s. The technological progress has made large scale fish farms a possibility and today one fish farm can contain 200 000 fish (Taranger et al., 2014). Although these intensive fish farms are highly successful there are several challenges with high density fish rearing. The spreading of diseases, the escape of farmed salmon, which interfere with the wild salmon population, and increased numbers of salmon lice in the Norwegian fjords, are some of the challenges the industry faces today (Taranger et al., 2014).

1.2 Salmon lice and cleaner fish

The salmon louse is an ectoparasite (Kabata 1979) that feeds on the skin of its host (Pike & Wadsworth, 2000). This damages the skin of the salmon and cause lacerations that can become fatal, especially when a salmon is infected with a large number of lice (Grimnes & Jakobsen, 1996). In addition they can infect the wild salmon and cause problems for the wild populations (Bjørn et al., 2001; Butler, 2002). The industry has tried to combat this problem for a long time and in the past the main solution has been to use delousing agents to remove the lice from the salmon. Overuse and wrong dosages have caused the lice to become resistant to several kinds of delousing remedies (Jones et al., 1992; Sevatdal & Horsberg, 2003; Lees et al., 2008) and it is now hard to effectively get rid of the lice using just chemicals.

A solution has been to use wrasse and other species of cleaner fish for delousing the farmed salmon. They can be put into the pens with the salmon and will eat the lice off the fish. Wrasse has been used as a cleaner fish since the late 1980`s (Espeland et al., 2010), but has not been widely used until later years. In Norwegian waters the species that is considered to be the most suited for this purpose is the ballan wrasse. It has a big appetite, it will effectively clean the lice off the salmon (Ottesen et al., 2008), and it is active at lower temperatures than

other wrasse species (Kvenseth & Øien, 2009). As the chemical delousing agents gets less and less effective the more one has to rely on the natural delousing effects of the wrasse. Most of the wrasse used in Norwegian fish farms today, are caught in the wild (Kvenseth et al., 2003), but in a longer perspective one has to think of more sustainable ways of supplying the wrasse to the farms. Farming of ballan wrasse would help relieve the pressure on the wild populations and help ensure stable supplies of wrasse to the salmon farms.

1.3 Ballan wrasse and live feed.

When starting to farm a new species there is several challenges and needs for knowledge. How do we get the fish to breed in captivity, what is the best hatching conditions, how big are the larvae when they hatch etc. One of the biggest challenges with marine fish larvae is their general small size at hatching. The salmon is so developed at hatching that it can be fed with formulated feed right away, but the smaller ballan wrasse is not as developed at hatching and therefore has to be fed live feed (Conceicao et al., 2010). The traditional live feed regime used on several marine species, consists of first a period of feeding with rotifers, and as the larvae grows bigger, they are weaned on to the larger artemia, before they are big enough to be weaned on to formulated feed (Southgate, 2003; Conceiao et al., 2010). One of the challenges with these species as food for the fish larvae is that they do not naturally meet the larvae's requirements for essential fatty acids (EFAs). Therefore they have to be enriched before they are fed to the larvae (Evjemo & Olsen, 1997; Conceicao et al., 2010). EFAs like eicosapentaenoic acid (20:5n-3; EPA), docosahexaenoic acid (22:6n-3; DHA) and arachidonic acid (20:4n-6; ARA) is said to be particularly important for the growth and development of marine fish larvae (Sargent et al., 1999; Bell et al., 2003). Not only is the amount of these fatty acids important, but the ratio between them are also important, the optimal ratio will vary from species to species (Sargent et al., 1999). EFAs stored in the polar lipid fraction is said to be more easily available for the fish than fatty acids stored in the neutral lipids (Izquierdo et al., 2000; Gisbert et al., 2005; Wold et al., 2009). When the rotifers and artemia are enriched, the essential fatty acids are stored in the neutral lipid fraction (Rainuzzo et al., 1994a; Rainuzzo et al., 1994b; Nerhus, 2007) and are therefore less available for the developing larvae (Izquierdo et al., 2000; Kjørsvik et al., 2009; Wold et al., 2009). As an alternative to the more traditional live feed regime it has been proposed to feed the larvae with copepods. Both naturally harvested and intensively reared copepods have been proven to be a good live prey option for marine fish larvae. When compared to feeding with rotifers and artemia, copepods as live feed has resulted in better growth, survival and pigmentation

(Evjemo & Olsen , 1997; Luizi et al., 1999; Shields et al., 1999; Imsland et al., 2006; Eidsvik, 2010) . Copepods are the natural pray of many wild marine fish larvae (Hunter, 1981) and are more suited to meet the nutritional requirements of the fish larvae. They do not need to be enriched as the amount and ratio of essential fatty acids meets the requirements and is more available to the larvae as it is stored in the polar lipid fraction (Evjemo & Olsen, 1997; Evjemo et al., 2003; van der Meeren et al., 2008; Overrein et al., 2010)

1.4 Gut and liver development

As mentioned marine fish larvae like the ballan wrasse are quite small and undeveloped at hatching (Osse et al., 1997; Yufera & Darias, 2007). The mouth and anus opens in time for exogenous feeding and the gut itself is an undifferentiated tube with no functional stomach (Kjørsvik et al. 1991). As the larvae grow the gut will differentiate and will get more and more developed over time (Yufera & Darias, 2007, Govani et al,. 1986; Waagbø et al., 2001). The ballan wrasse will never develop a functional stomach (Hamre & Sæle, 2011) and therefor the functional development of the gut is important for the larvae to be able to digest and absorb the required nutrients for growth and survival.

The liver is not developed at hatching and appears at the start of exogenous feeding. It will grow over time as the fish larvae grows and become more and more developed (Hoehne-Reitan & Kjørsvik, 2004; Kjørsvik et al., 2004; Young et al., 2006). The liver catabolizes fat, carbohydrates and protein and stores energy like glycogen and lipids (Lie et al., 1986; Young et al., 2006). The liver is easily affected by malnutrition or starvation and is a good indicator of the nutritional quality of a live feed (Hoehne-Reitan & Kjørsvik, 2004).

1.5 Aim of the study:

The aim of this study is to compare two different live feed programs to see if using intensively reared copepods as live feed for ballan wrasse larvae will give better growth and development than the more traditional live feed of enriched rotifers and artemia. We will have two groups of three replicas each were one group is fed enriched rotifers and then artemia, while the other group is fed intensively reared copepods of increasing size exclusively throughout the experiment. To measure any difference between the two groups we are looking at different growth parameters as standard length, myotome height, dry weight and daily weight increase.

In terms of larval development we are going to look at the liver and gut development of the two groups to see if the difference in nutritional quality of the live feed will give different development in the alimentary system. In the liver and gut we will look at area size of nuclei and mitochondria, the area fraction of nuclei, mitochondria and glycogen, the general appearance of the liver and gut tissue and the appearance of mitochondrial membranes and cristae. We will also look at the microvilli density and height in the gut.

2. Materials and methods

2.1 First feeding experiment.

The first feeding experiment was performed on ballan wrasse larvae (*Labrus bergylta*) and was conducted in the CodTech lab at NTNU Sealab in Trondheim, Norway. The experiment consisted of two different feeding regimes with three replicates of each treatment.

The first group (the Copepod-group) was fed *Acartia tonsa* exclusively throughout the whole experiment. Starting with nauplii until 15 days post hatch (dph) then as the larvae grew they were fed copepodits from 15 until 30 dph. From 30 to 45 dph the larvae were fed a combination of copepodits and adult *Acartia Tonsa* in a 2:1 ratio.

The other group (the Rotifer-group) were fed rotifers (Brachionus Sp.) enriched on Multigain (BioMar, Norway) until 40 dph. From 30 to 40 dph the Rot group was co-fed enriched rotifers and enriched Brine Shrimp (Artemia franciscana) before the larvae were fed Brine shrimp exclusively until the end of the experiment.

Ballan wrasse larvae (Labrus bergylta) from Nofima (Sunndalsøra, Norway) was placed in a holding tank with gentle aeration for acclimatization (12 °C, 250 L) when they were 2 dph. The following day (3 dph) the larval density was measured to get an estimated count of number of larvae. The larvae were transferred to six 100 L conical bottomed, cylindrical tanks with an estimated number of 8200 larvae per tank. From 2 dph to 4 dph the larvae were kept in darkness and from 4 dph and throughout the experiment the larvae were kept in constant illumination (MASTER TL-D 90 Graphica, 18W/965, Philips, Netherlands). Temperature and oxygen levels were measured every day (ProODO Optical Dissolved Oxygen Meter, YSI Inc, OH, USA), being kept at 12-16 °C (table 2.1) and 80 % saturation respectively. The seawater that was pumped into the tanks, was filtered through a sandfilter and through a 1µm mesh to get rid of particles (Skjermo et al. 1997), and was microbially matured to stabilize the bacterial content. The water exchange was gradually increased from 2 to 8 times a day through the experiment. Ceramic clay (Vingerling K148, Sibelco, Germany) was added to the water to increase the visual contrast and reduce the bacterial load. With increasing water exchange the amount of clay added to the water was increased from 1.6 g per feeding at the beginning of the experiment to 3 g per feeding at the end of the experiment (table 2.1).

Table 2.1: Experimental setup and parameters for the start feeding experiment of ballan wrasse larvae.

Days post hatch	3	4	5	6	7	8	9	10	11	12	13	14	15	16-21	22	23	24	25	26	27	28	29	30	31	32-35	36	37	38	39	40	41	42	43	44	45
Temperature (°C)	12	13	13	13	14	14	14	15	15	15	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16	16
Day degrees	36	49	62	75	89	103	117	132	147	162	178	194	210	226	242	258	274	290	306	##	338	354	370	386	388-530	466	482	498	514	530	546	562	578	594	610
Clay (g/ tank /day)	-	3,2									4,8				7,5			8			8,5		9												
Water exchange rate	2	2	2	2	2	2	2	2	2	2	2	4	4	4	4	4	4	4	4	4	6	6	6	6	6	6	6	8	8	8	8	8	8	8	8
Feeding frequency	2 times a day										3 times a day																								

Rotifer-group: 3 tanks		Enriched rotifers
Individuals per feeding	-	3500 / 5000 / 12000
		Enriched artemia
Individuals per feeding		3000

Copepod-group 3 tanks		Copepods																																	
Individuals per feeding	-	5000																																10000	
Stage		n												c			n	c	n	c														67 % n + 33 % a	

N:nauplii, c:copepodits, a: adult

2.2 Live feed production

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

Rhodomonas baltica (Clone NIVA 5/9 Cryptophycea: Pyrenomondales) were grown in 160 and 200 litre plexiglas cylinders. In periods of increased production 300 litre plastic bags were also used. The pH in the cylinders was kept between 7.5 and 8.5 (pH/mV-meter, WTW pH 315i, Germany) and the culture was continuously aerated with 1-2% CO_2. When overgrowing of the cylinder walls was observed, the tanks were drained of algae and cleaned with hot water and chlorine, and then rinsed. To replenish the cleaned cylinder 50 % of the algae content of a neighboring tank was transferred to the clean tank, the cylinder filled to the top with seawater and Conway medium (1 ml per liter seawater) was added. The water used in the algae cultivation was filtered through a sand filter, heated to 20°C , and filtered through a 1 µm mesh, before being chlorinated (10-15% NaOCl, 0.25 ml per litre, no aeration, >5 hours) and dechlorinated ($Na_2S_2O_3$, 3 g per 100 litre, aeration, >5 hours).

50 % of the algae in a cylinder were harvested daily. The remaining culture was diluted with seawater and 1 ml Conway medium per liter seawater was added.

2.2.2 Cultivation of copepods (*Acartia tonsa*)

2.2.2.1 Egg production

Acartia tonsa (clone DFH.AT1) was cultivated under constant illumination in three 1700 liter tanks, with flow through of seawater (sand filtered, heated to 20°C and filtered through a 1µm mesh) at a water exchange rate of once per day. The sieve covering the water outlet was cleaned every morning to keep the sieve from becoming clogged. Temperature, O_2 levels, pH and salinity was measured every morning (ProODO Optical Dissolved Oxygen Meter, YSI Inc., OH, USA; LH-T28, Lohand, China) and kept at 19-22°C, over 4 mg L-1 dissolved O_2, 7.6-8 and 34-35 ppt respectively. Harvested algae (*Rhodomoas baltica*) were transferred to a 1000 L tank and were fed continuously (Nesse, 2010) to the copepod tanks by an electromagnetic dosing pump (AXS602, Seko, Italy).

The copepod eggs were collected daily by siphoning along the bottom of the tanks. The water collected was filtered through two sieves with 120 µm and 100 µm mesh to rinse out any waste, before the water was filtered through a 64 µm sieve to concentrate the eggs. The eggs were rinsed with seawater before being stored in seawater in NUNC EasyFlasks™ (Nunc A/S, Denmark.) at 2°C.

2.2.2.2 Production of copepod nauplii for feed

The amount of eggs needed was emptied from a NUNC flask into 100 L white plastic tanks and was kept under moderate aeration and at 20-22°C. After 24 h when the eggs hatched the nauplii were fed Rhodomonas 3 times a day until harvesting.

When collecting the nauplii needed for feeding, the density was estimated by taking out a water sample from the tank, fixating the sample with Lugol`s solution and taking out 7 drops of 50 µl. The individuals in each drop were counted, the highest and the lowest count was cancelled and the density was estimated by calculating the mean of the 5 remaining drops. The desired amount was harvested and concentrated by a 64 µm sieve and stored in a 100 L plastic tank under moderate aeration and 8°C until they were fed to the larvae(>12 hours). Before feeding the nauplii density in the holding tank was estimated, the desired amount for one feeding was taken out and concentrated before being transferred to the larval tanks.

2.2.3 Cultivation, enrichment and harvesting of rotifers (Brachionus sp.)

Brachionus sp. was cultured in three 250 L tanks with conical bottoms in seawater at 34 ppt and a temperature of 19-23°C. The rotifers were fed Baker`s yeast (2.6 g per million rotifers day^{-1}) dissolved in seawater that was continuously pumped into the tanks. For long term enrichment Multigain (0.14 g per million rotifers) blended with seawater was added to the rotifer tanks twice a day. Debris was removed from the tank daily by flushing from an outlet at the bottom of the tank for 5 seconds.

Due to a high amount of particles in the water (yeast, Multigain, wastes) the rotifer tanks had to be cleaned once a week. The rotifers were transferred into a holding tank while the rotifer tanks were cleaned out. The tanks were filled with clean water and the rotifers were put back in.

The density and egg ratio of each culture was estimated every morning. From water samples taken from each tank 12 drops of 25 µl was taken out and the number of individuals and number of eggs were counted in each drop. The highest and lowest count was cancelled out and the amount in the remaining 10 drops was added together to get the egg ratio and further multiplied to get the number of individuals per liter.

The desired amount of rotifers were then taken out from a tank and concentrated and washed in a 64 µm sieve. The rotifers were then put in a 100 L tank with seawater (24°C and moderate aeration) and were short term enriched with Multigain (0.14 g per million

individuals) for 2 hours. Before feeding them to the larvae the density was double checked before the desired amount for one feeding was taken out, washed and concentrated. The remaining rotifers were stored cold (8°C and under moderate aeration) to keep the nutritional value stable until the following feedings.

2.2.4 Cultivation of Artemia franciscana

Dry Artemia cysts were hydrated in fresh water (4.9 liter of water for 450-500 g cysts, 15-25°C) under heavy aeration for one hour before being decapsulated. The decapsulation process was done according to Sorgeloss et al. (1977). 59.4 g NaOH dissolved in 150 ml of water and 1.44 L of NaOCl was added to the hydrated cysts. The process was stopped when the cysts changed color from brown to orange. The decapsulated cysts were filtered through a 125 µm mesh net and rinsed under running water for about 5 minutes. The cysts were then concentrated and stored at 5°C and used within 6 days.

The desired amount of decapsulated cysts was put in a tank with 60 L of seawater (25-28°C) with heavy aeration for 24 hours for hatching. The hatched Artemia was washed in an Artemia-washer for 10 minutes. The washed nauplii was then concentrated and transferred to a new tank. The Artemia was enriched twice with Multigain (10 g/60L; Biomar) during the next 24 hours. The Artemia was then harvested, rinsed and concentrated before being fed to the fish larvae.

2.3 Larval sampling and fixation

Fish larvae were randomly sampled from the tanks using a ladle. The ladle was rinsed with hot fresh water between each sampling to avoid transferring bacteria. The first sampling done on 3 dph was done before the larvae were transferred to the different treatment tanks. After this point an equal number of individuals were sampled from each tank on sample days. The collected larvae were anesthetized using tricaine methanesulfonate (MS-222, Finquel, Agent Chemical Laboratories Inc., USA) and then rinsed in distilled water. The fish sampled for histological analysis were fixated in 4 % paraformaldehyde (PFA) in 0.11M hepes buffer and stored cold (4°C) in glass vials.

2.3.1 Standard length and myotome height

Measurement on standard length (SL) and myotome height (MH) were done on larvae collected on 6 different days: 3, 8, 15, 28, 36 and 45 dph. On 3 dph 20 larvae were sampled from the egg incubator before the remaining larvae were distributed to the different treatment tanks. On the other days 10-15 larvae were sampled from each tank.

The larvae were anaesthetized and SL and MH were measured from pictures by using a Nikon SMZ1000 stereomicroscope and Infinity 1-3C camera. SL was measured from the tip of the mouth to the end of the notochord on small larvae and to the start of the tail fin on larger larvae. MH was measured perpendicular to the axial skeleton, right behind the anus. SL and MH measurements were performed by Stine Wiborg Dahle (SINTEF). After image analysis the same larvae were rinsed in distilled water and sampled for CN-analysis.

2.3.2 Dry weight and daily weight increase

The same larvae used for SL and MH measurements were used for CN-analysis. The larvae were analyzed for carbon content by using an Elemental Combustion Analyzer (Costech Analytical Technologies Inc., USA) with acetanilide as a standard (analysis done by Marthe Schei, SINTEF). Larval dry weight was calculated by using the equation:

$DW = (\mu g\ carbon\ larvae^{-1}) * 2.34$ (Reitan et al., 1993)

Specific growth rate (SGR) and daily weight increase (%DWI) was further calculated using these equations according to Ricker (1958):

$$SGR = \frac{ln\, W_2 - lnW_1}{t_{2-t_1}}$$

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

where W_1 and W_2 are the dry weight at time t_1 and t_2.

2.3.3 Survival

From 15 dph dead fish larvae were siphoned off the bottom of the tanks and counted every second/third day. At the end of the experiment (45 dph) the remaining fish in the tanks were counted. The data was corrected for sampled larvae and the larvae that were accidentally removed during cleaning of the tanks. The mortality up to 15 dph was considered to show a negative linear regression with time.

2.4 Histological analysis

5 fixated larvae from 3 dph and 5 fixated larvae from each treatment from 6, 8 and 15 dph were embedded in EPON (Appendix 1).

Sections were made through the liver and the midgut (Figure 2.1) for further analysis in a light microscope (1 µm thick sections) and in a transmission electron microscope (70 nm thick sections) with a Leica Richert Ultracut microtome (Leica Microsystems, Germany). The 70 nm thick sections for electron microscopy were performed by Tora Bardal, NTNU.

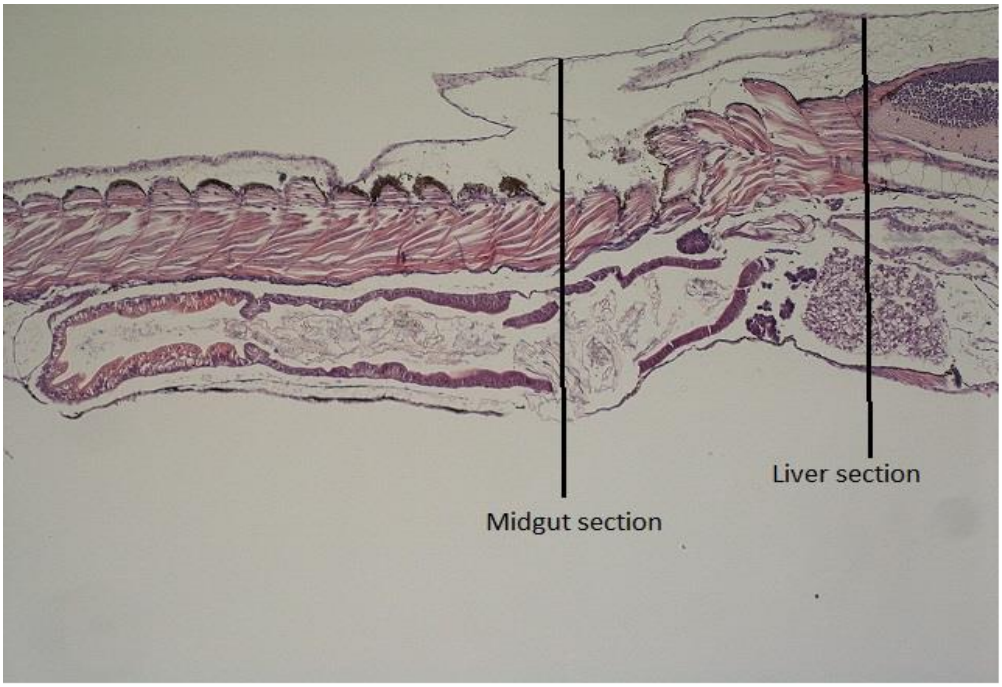

Figure 2.1: Ballan wrasse larvae 8 dph showing where the liver and gut sectioning were performed. Photo: Maren Ranheim Gagnat

2.4.1 Light microscope

The sections were stained with Toluidine Blue and Borate buffer and studied under a Zeiss Axioscope2 plus microscope (Zeiss Inc., Germany) equipped with a JVC TK-C1381 color video camera (JVC, Japan). The sections were analyzed with an image processing program called Image-J.

On both the sections from the liver and the gut the nucleus area size was measured using Image J.

2.4.2 Transmission electron microscope

The sections were contrasted with lead citrate and observed in a Jeol JEM-1011 transmission electron microscope (Jeol LTD, Japan) at an accelerating voltage at 80 kV by Tora Bardal (NTNU). The photos taken from the transmission electron microscope was further analyzed with the image processing program Image J. In Image J a point grid was located on randomly selected photos (x10 000 magnification) taken of the liver and gut sections. Each point covered a given area. On the liver sections the total area covered by mitochondria, lipid, glycogen, endoplasmic reticulum (ER), nucleus and general cytosol was measured. On the photos of the gut sections the area covered by mitochondria, ER, nucleus, general cytosol and vacuole 1 and 2 was measured. This gave a two-dimensional quantification and the area fraction of the different organelles was calculated. On the photos of the liver and gut sections mitochondria size was measured and a qualitative study on mitochondrial structure and appearance was done. On the sections of the gut microvilli height and density was also measured.

2.5 Statistical analysis

Data that consisted of percentage values were transformed using an arc-sine transformation before being statistically tested. Difference of means was tested using a one-way ANOVA ($P<0.05$). An F-test were performed to check for homogeneity of variance ($p=0.05$). A T-test for equal variance was performed if there was homogeneity of variance and a T-test for unequal variance if there was not. The statistical testing was done in Microsoft Office Excel and graphs were made in SigmaPlot 12.5 (Systad Software Inc., USA 2013).

3 Results

3.1 Growth and survival

3.1.1 Dry weight and daily weight increase

Mean dry weight (DW) of the ballan wrasse larvae at the beginning of the experiment was 50.54 ± 2.07 µg. At 8 dph there was already a difference in DW between the larval groups, and at 15 dph the Copepod-group had a significantly higher DW of 191 ± 11 µg larva^{-1} versus 82 ± 0.69 µg larva^{-1} for the Rotifer-group (Figure 3.1). The difference between the Rotifer and the Copepod-group continued past 28 dph (figure 3.2), but after 30 dph, when the Rotifer-group were weaned on to Artemia sp., the difference decreased and there was no significant difference between the groups from 36 dph until the end of the experiment. Both groups had a slowdown in the increase of dry weight between 28 and 36 dph, with it increasing again between 36 and 45 dph. For the Rotifer-group this was the beginning of the weaning period on to artemia which could mean that they had a hard time accepting the new feed at first. The slowdown in dry weight increase in the Copepod-group could be due to sub-optimal numbers and size of the copepods they were fed. When the feeding was changed from copepodits to copepodits and 1/3 adult copepods at 36 dph the mean dry weight increased more rapidly again.

The daily weight increase (DWI) of both larval groups was negative in the first period of the experiment prior to exogenous feeding (Figure 3.3). In the period of 8-15 dph both groups had a positive DWI with a significantly higher DWI in the Copepod-group with 18.8 ± 2.5 % versus 8.7 ± 2 % in the Rotifer-group. Over the next two time intervals, 15-28 and 28-36 dph there was a decrease in DWI for the Copepod-group before it increased again at 36-45 dph. This can be explained by the issue of the sub-optimal feeding regime discussed earlier. When the adult copepods were introduced in the diet at 36 dph, the DWI increased again. The decrease in DWI in the Rotifer-group at 28-36 dph can be explained by the weaning onto Artemia in this time period. After the larvae were fully weaned onto artemia the DWI increased again at 36-45 dph, and was at the same level as the Copepod-group at around 15 %. Looking at the mean DWI for the whole start feeding period (3-45 dph) there was no significant difference between the groups and it was at 10.5 % for the Copeopod-group and 9.25 % for the Rotifer-group.

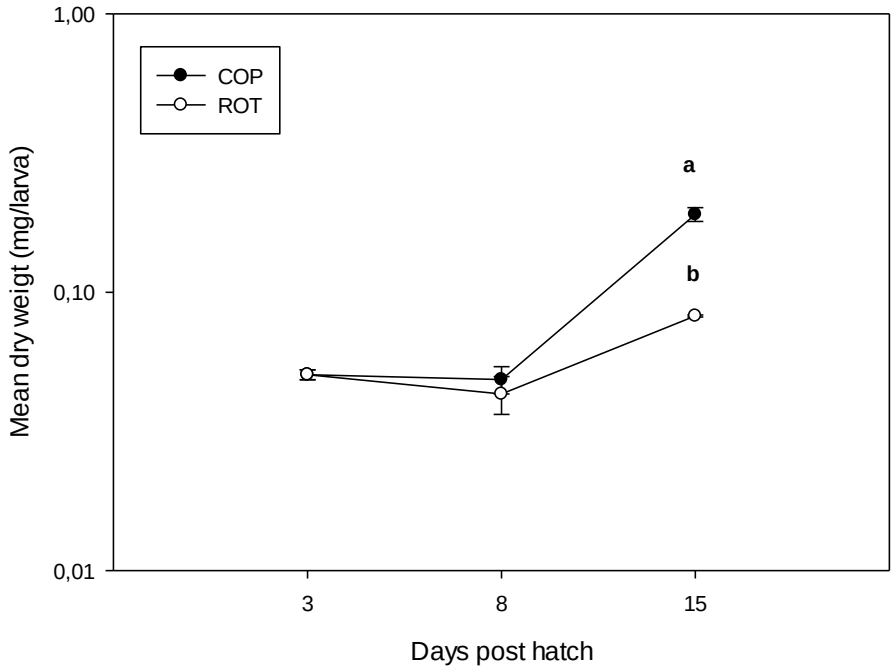

Figure 3.1: Mean dry weight (mg larva^{1}) of ballan wrasse larva from 3 to 15 dph were Rotifer group were fed rotifers and Copepod-group fed copepod nauplii (y-axis presented in logarithmic scale). Data are mean ±SE (n=20-35). Letters indicates significant difference.

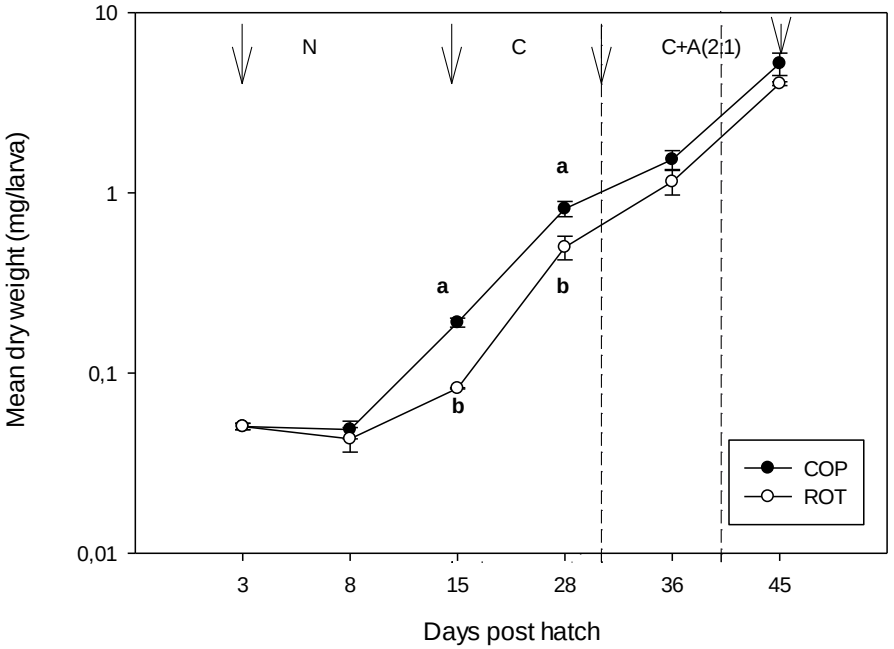

Figure 3. 1: Mean dry weight (mg larva^{-1}) for the whole start feeding experiment of 45 days (y-axis presented in logarithmic scale). Letters indicates significant difference between the groups. Error bars indicates ± SE. The area between the dashed lines marks the time of co-feeding with rotifers and artemia (30-41 dph). The arrow heads marks the time of feeding with copepod nauplii (N) 3-15 dph, copepodits (C) 15-30 dph and copepodits and adult copepods (A) 30-45 dph.

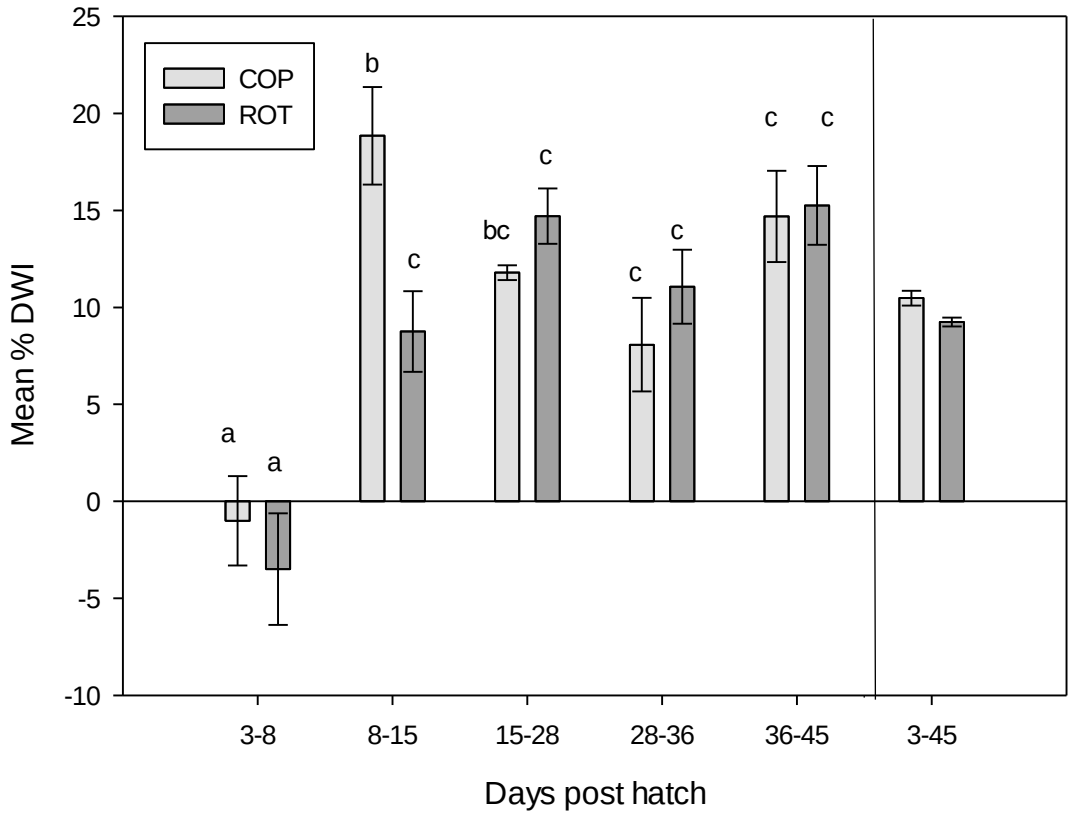

Figure 3.2: Daily weight increase (DWI, %) for the ballan wrasse larvae during the whole start-feeding experiment consisting of 45 days split into five intervals and one for the whole experiment. Letters indicates significant difference between treatments and within treatments. Error bars indicate ± SE.

3.1.2 Standard length (SL) and myotome height (MH)

In the beginning of the experiment from 3 to 8 dph there was a negligible increase in SL in both larval groups, and no significant difference between the groups, 3.93±0.07 for the Copepod-group and 3.87±0.02 for the Rotifer-group (Figure 3.4). From 15 dph and throughout the experiment the Copepod-group had a significantly higher SL than the Rotifer-group.

The myotome height of the Copepod larvae was significantly higher than that of the larvae from the Rotifer-group at 8 dph and throughout the whole experiment (Figure 3.5). The Rotifer-group didn't have a significant increase in myotome height before 28 dph.

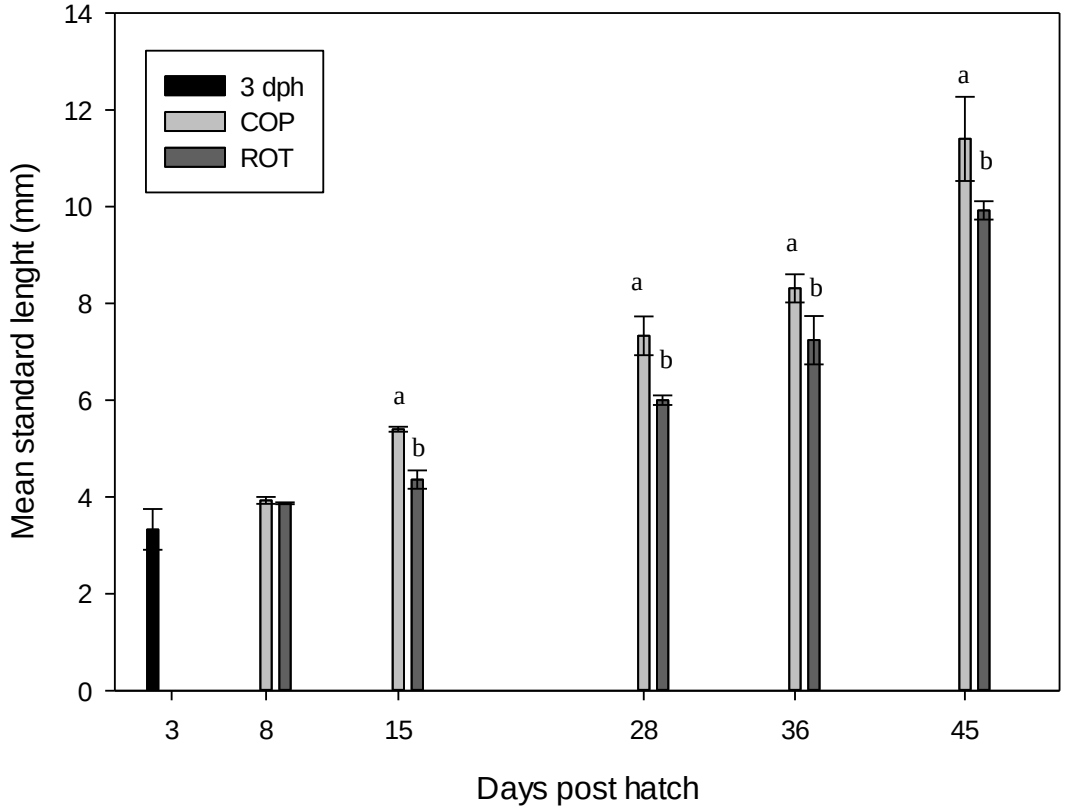

Figure 3.3: Measured standard lenght (SL) at 3 dph, 8 dph, 15 dph, 28 dph, 36 dph and 45 dph (n=12 for 3 and 8 dph, n=20 for 15, 28, 36 and 45 dph). Letters indicates significant difference between treatments. Error bars indicate ± SE.

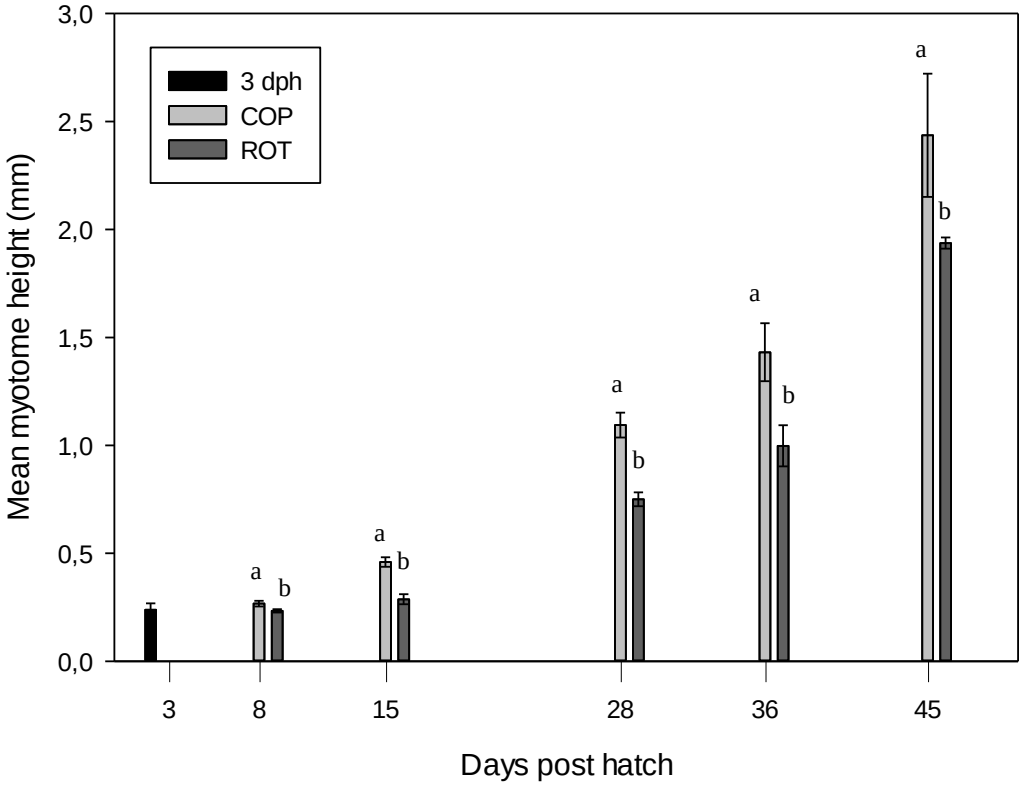

Figure 3.4: Measured myotome height (MH) at 3 dph, 8 dph, 15 dph, 28 dph, 36 dph and 45 dph (n=12 for 3 and 8 dph, n=20 for 15, 28, 36 and 45 dph). Letters indicate significant differences between treatments. Error bars indicates ± SE.

3.1.3 Survival

The highest mortality was at the beginning of the first feeding experiment. From 15 dph and throughout the experiment there was no significant difference in % survival between the two groups. At 45 dph when the experiment was terminated, the Copepod-group had a slightly higher survival than the Rotifer-group, but the difference was not significant.

Table 3.1: Mean survival (%) ± SE of each treatment at 15, 22, 29, 37 and 45 dph (n=3). No significant difference was found between the two treatments

Mean survival (%) ± SE		
DPH	**ROT**	**COP**
15	15 ±1	15 ±1
22	13 ±0	13 ±2
29	11 ±1	12 ±2
37	9 ±1	12 ±2
45	9 ±1	11 ±2

3.2 Liver morphology

3.2.1 Light microscopy

The liver tissue in the larvae at 3 dph seemed quite undeveloped with irregularly shaped nuclei without clearly defined nucleoli. In all of the larvae tested there was residue of yolk and syncytium around the liver tissue, and in two of the larvae tested liver tissue was not found at all. At 8 dph the liver tissue was more developed and organized as lobules with visible blood vessels and blood cells (Figure 3.6 B-C). The nuclei had a rounder shape with clearly defined nucleoli. The liver in the Rotifer-group at 8 dph seemed less developed with less liver tissue compared to the Copepod-group. At 15 dph the liver tissue in both groups was more developed compared to the earlier sample dates. The liver was larger in volume with visible blood vessels and glycogen deposits throughout the tissue (Figure 3.6 D-E).

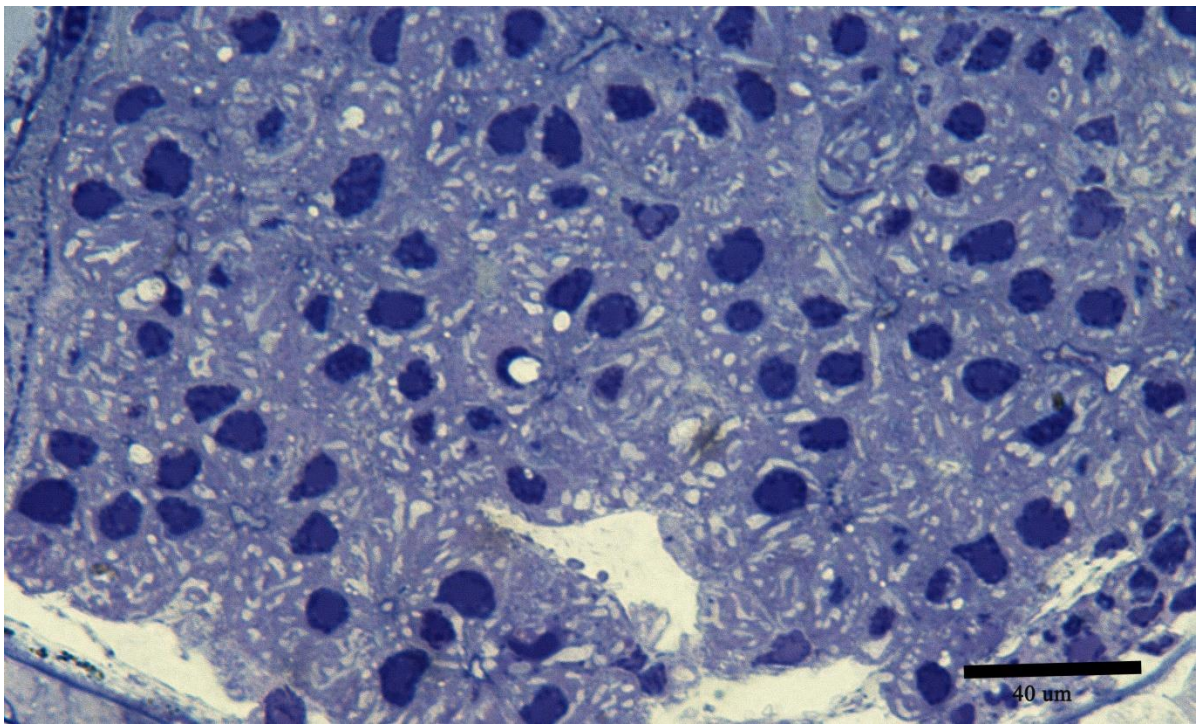

Figure 3.6 A: Photo micrograph of the liver at 3 dph. The tissue is undeveloped with no visible blood vessels and irregularly shaped nuclei without defined nucleoli.

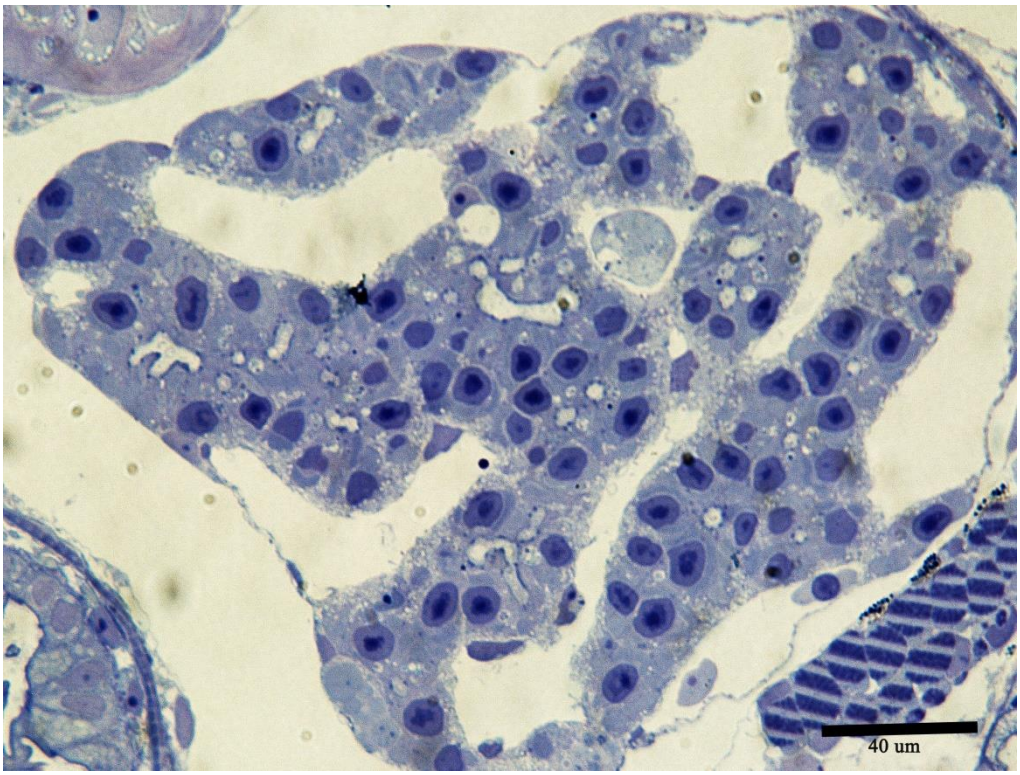

Figure 3.6 B: Photo micrograph of the liver of larvae at 8 dph from the Rotifer-group. The liver is organized into lobules but the tissue seems more immature than the liver tissue in the Copepod-group. The nuclei are rounder in shape and have defined nucleoli.

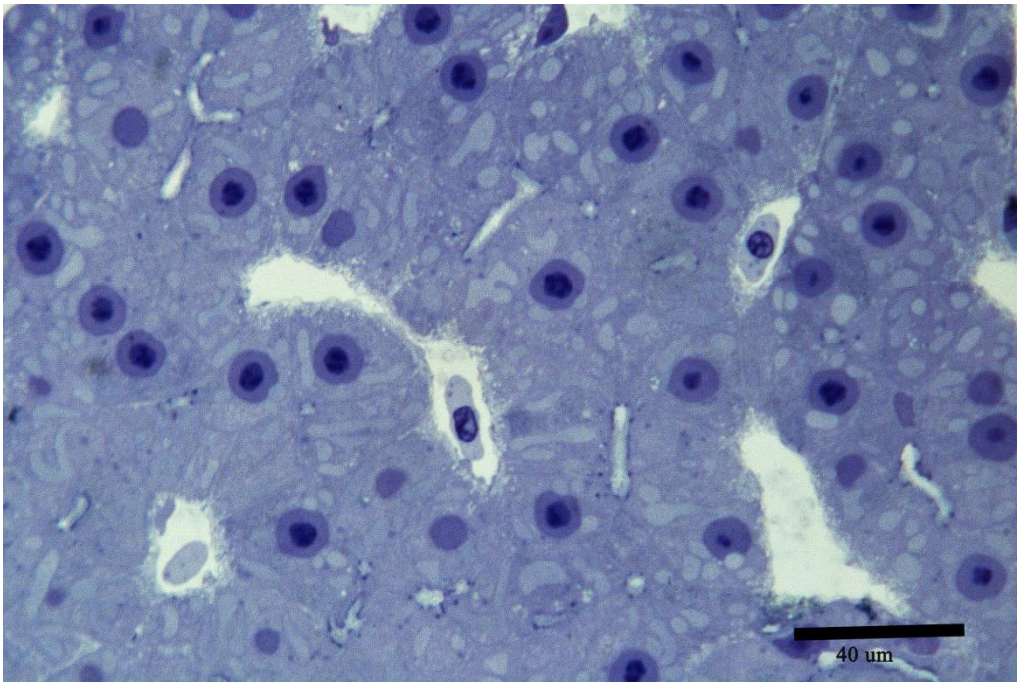

Figure 3.6 C: Photo micrograph of the liver of larvae at 8 dph from the Copepod-group. The tissue seems more developed and organized than at 3 dph and compared to the liver tissue from the Rotifer-group. There are visible blood vessels and blood cells. The nuclei are rounder in shape with defined nucleoli.

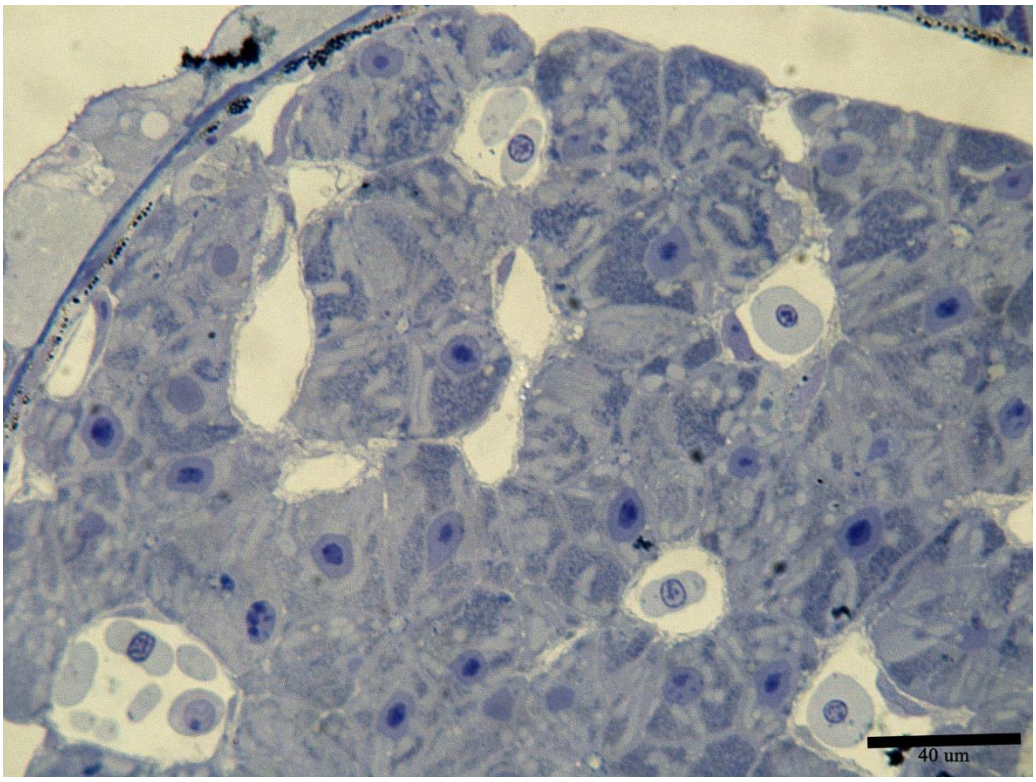

Figure 3.6 D: Photo micrograph of the liver of larvae at 15 dph from the Rotifer-group. The tissue is more developed than at the earlier sample dates. There are visible blood vessels and blood cells, and visible glycogen deposits.

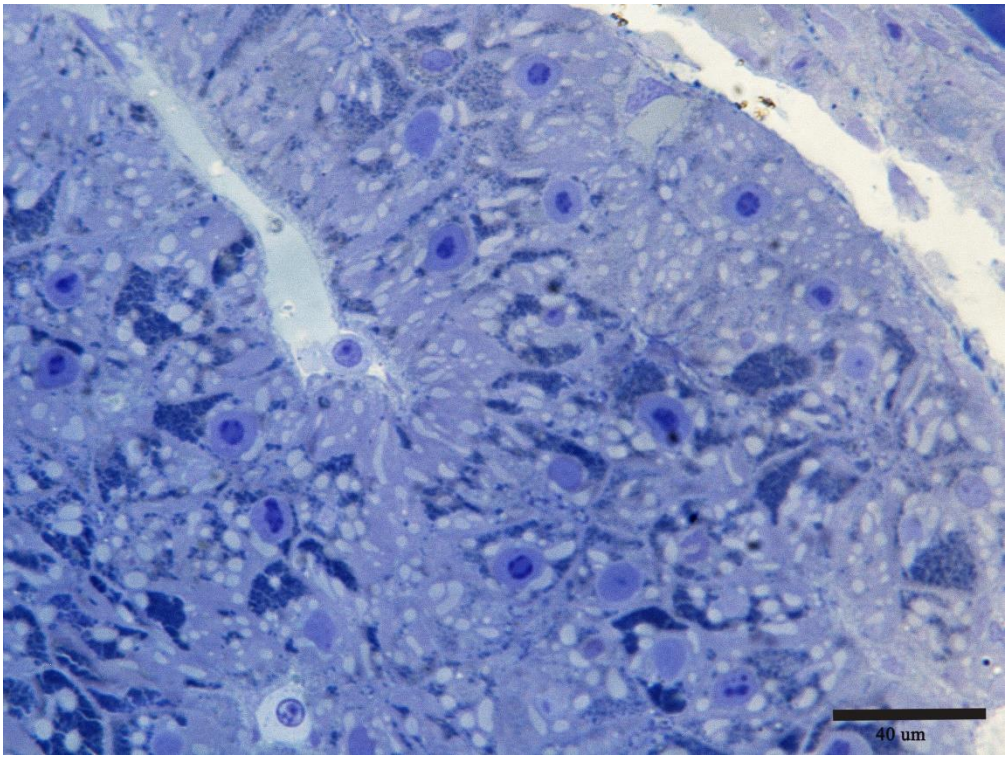

Figure 3.6 E: Photo micrograph of the liver of larvae at 15 dph from the Copepod-group. The tissue is more developed with visible blood vessels. Glycogen deposits are also visible throughout the tissue.

3.2.2 Transmission electron microscopy

The hepatocytes were seen as polygonal cells with large, spherical nuclei (Figure 3.7). Visible organelles consisted of nuclei, many mitochondria and rough endoplasmic reticulum (ER) which were more defined at 15 dph than at 3 dph. Glycogen was observed in the liver already at 3 dph, although at low amounts. The amount of glycogen in the liver gradually increased over time and was most abundant in the Rotifer-group at 15 dph (Figure 3.7 D). In the larvae from the Rotifer-group at 15 dph the glycogen was scattered throughout the cells while in the larvae from the Copepod-group the glycogen was more clustered together. At 3 and 8 dph the mitochondrial membranes was generally more rugged than at 15 dph with not much difference between the groups at 8 dph. At 15 dph the Copepod-group had a larger proportion of smooth outer mitochondrial membranes than the Rotifer-group. At 3 dph there were a much larger proportion of mitochondria with less dense cristae than at 8 and 15 dph (Figure 3.7 A). At 15 dph the ER was much more abundant and organized than on 3 and 8 dph and is seen enveloping the mitochondria (Figure 3.7 D-E).

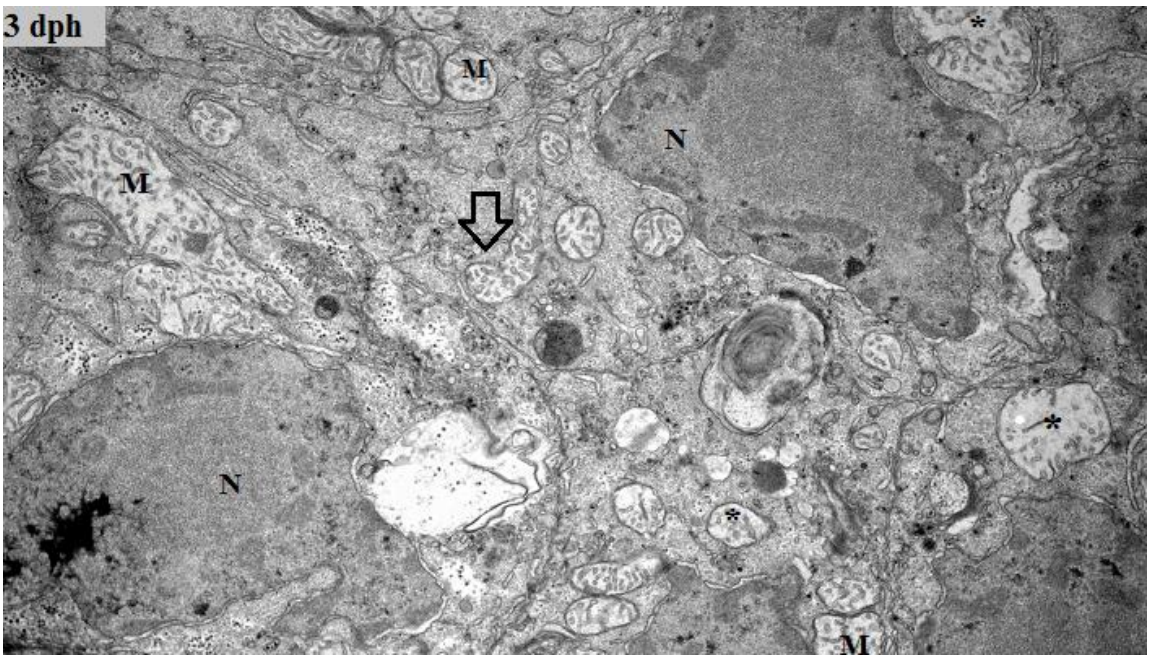

Figure 3.7 A: Micrograph that shows the general morphology of liver tissue from larvae at 3 dph. Nuclei (N) and mitochondria (M) are shown on the photos. The photos show a difference in mitochondrial appearance, abundance and organization of ER and occurrence of glycogen over time. Mitochondria membranes are more rugged (arrow) and cristae less dense (star) at 3 dph than at the later sample dates. Endoplasmic reticulum is more abundant and organized at 15 dph and glycogen was seen in both groups from 8 dph.

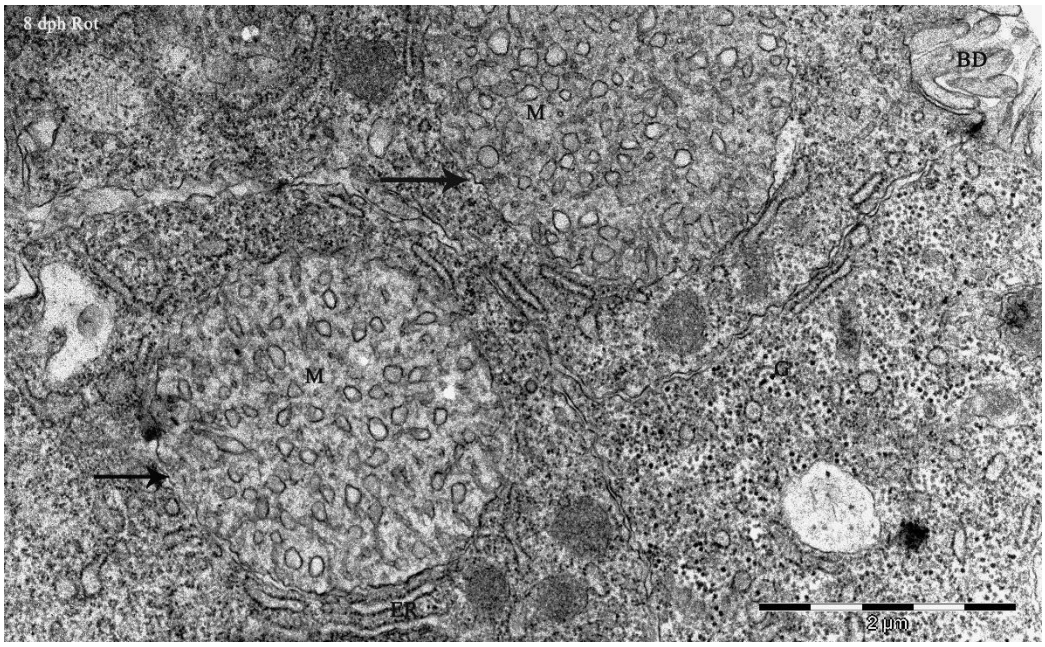

Figure 3.7 B: Micrograph that shows the general morphology of liver tissue from larvae from the Rotifer treatment at 8 dph. Mitochondria (M) and bile ducts (BD) are shown on the photos. Rugged membranes marked with arrows.

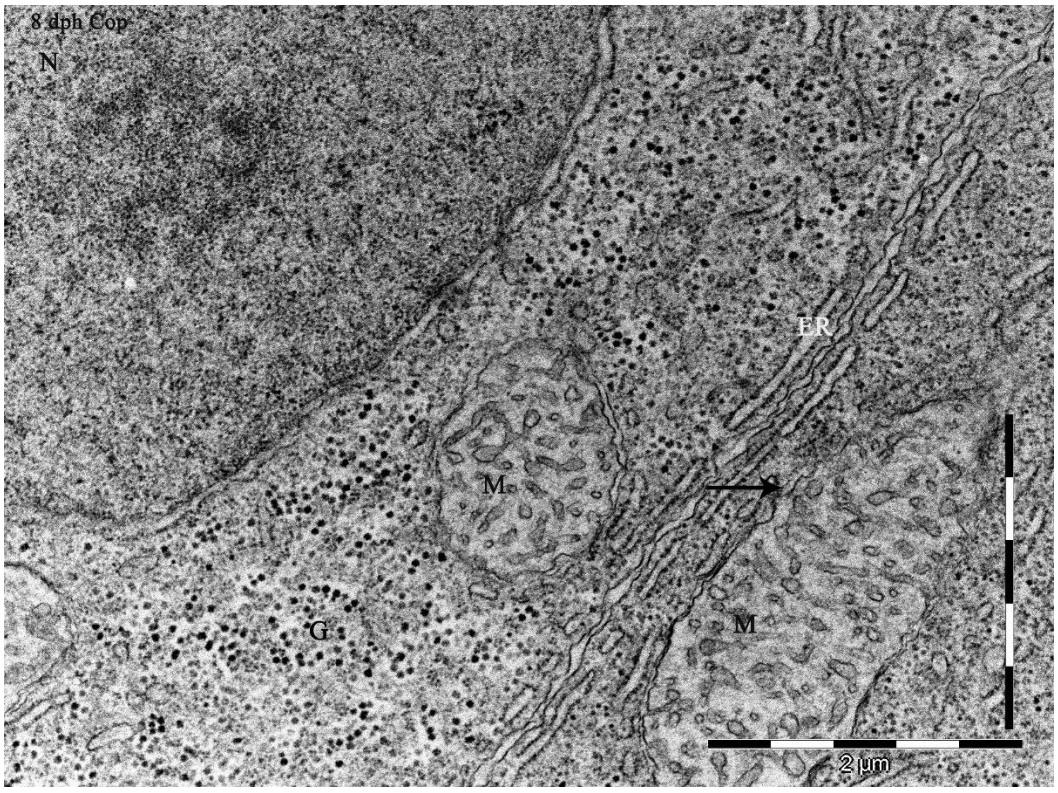

Figure 3.7 C: Micrograph that shows the general morphology of liver tissue from larvae from the Copepod treatment at 8 dph. Nuclei (N), mitochondria (M) and glycogen (G) and endoplasmic reticulum (ER) are shown on the photos. Rugged membrane marked with arrow.

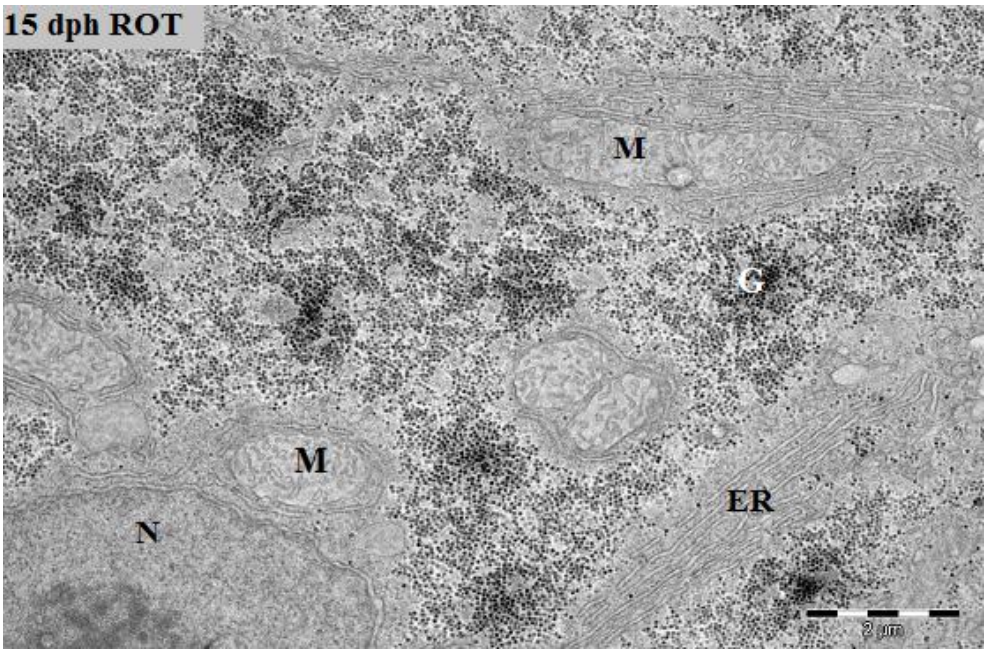

Figure 3.7 D: Micrograph that shows the general morphology of liver tissue from larvae from the Rotifer treatment at 15 dph. Nuclei (N), mitochondria (M), glycogen (G) and endoplasmic reticulum (ER) are shown on the photos.

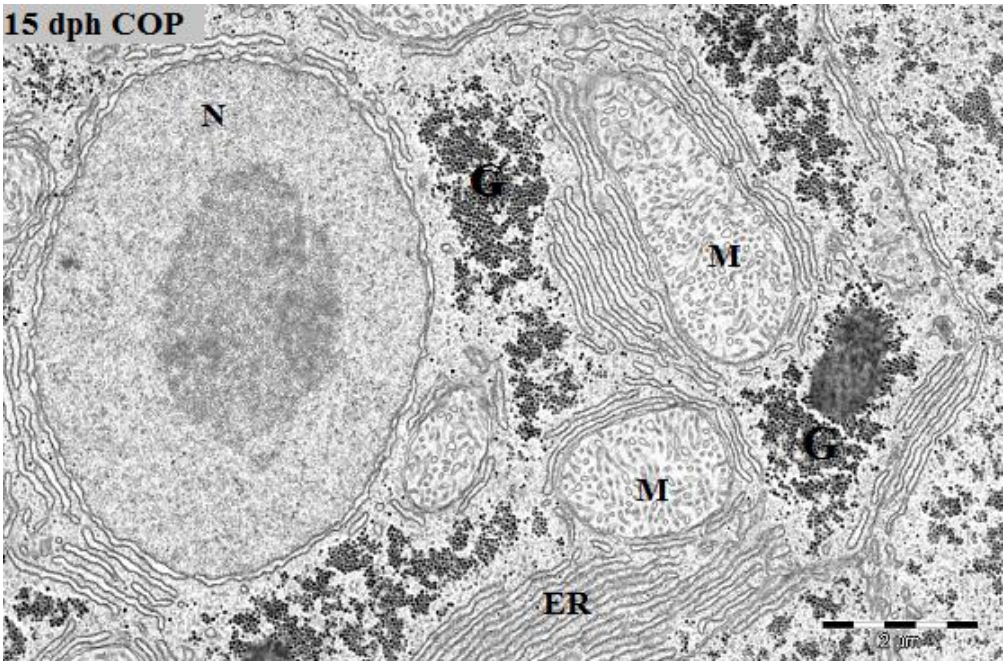

Figure 3.7 E: Micrograph that shows the general morphology of liver tissue from larvae from the Copepod treatment at 15 dph. Nuclei (N), mitochondria (M), glycogen (G) and endoplasmic reticulum (ER) are shown on the photos.

3.2.3 Surface area fraction of hepatocyte components

Glycogen was found in the liver already at 3 dph, although in low levels, 1.2 ± 0.4 %. The surface area fraction of glycogen increased over time in both groups and the highest fraction was found in the Rotifer-group at 15 dph with 38.3 ± 3 % (Figure 3.7).

There was no significant difference in the surface area fraction of mitochondria between or within the two treatments over time. The surface area fraction of the nuclei decreased over time for both treatments and the nucleus area fraction on 3 dph was significantly higher than the nucleus area fraction at 15 dph.

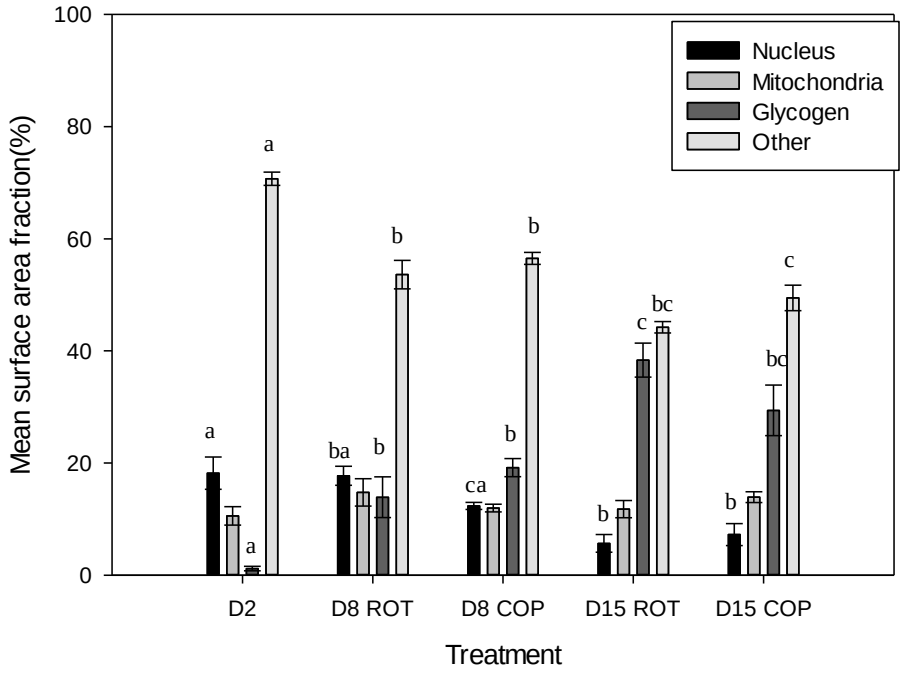

Figure 3.8: Mean surface area fraction (%) of hepatocyte cell components (nuclei, mitochondria, and glycogen) on 3 dph, 8 dph and 15 dph for both treatments (n=5). Letters indicates significant difference between and within treatments. Error bars indicates ± SE.

3.2.4 Area size of nucleus and mitochondria

The nucleus area size was the smallest at 3 dph (91.2 ± 2.2 µm^2), but it is not significantly smaller than the nucleus size in either group at 8 dph (Figure 3.9 A). There was a slight increase in nucleus area size over time but only significantly for the Copepod-group at 15 dph (118.6 ± 2.6 µm^2).

The mitochondria were the smallest at 3 dph (0.74 ± 0.07 µm^2), and there was a significant increase in mitochondria size from 3 dph to 8 dph for both groups (Figure 3.9 B). The mitochondria size for the Rotifer-group was significantly higher than the Copepod-group on 8 dph and both groups on 15 dph (2.85 ± 0.9 µm^2), but the high standard error indicates great variation in the sample. At 15 dph there was no difference in mitochondria size between the groups, with a size of around 2 ± 0.3 µm^2 for both groups.

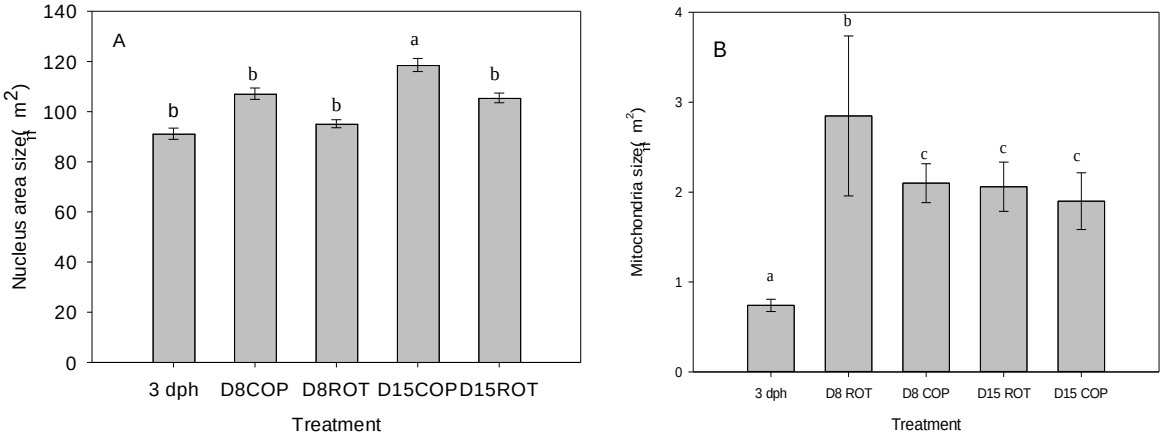

Figure 3.9: Nucleus area size (A) and mitochondria size (B) in the hepatocytes of larvae from 3 dph and from both treatments at 8 and 15 dph. Letters indicates significant difference between and within the groups. Error bars indicates ± SE.

3.2.5 Mitochondrial characteristics

Over time there is an overall decrease in the occurrence of rugged outer mitochondrial membranes. At 3 dph most of the mitochondria had a rugged outer membrane (96.6 ± 3 %). At 8 dph the proportion of smooth outer membrane had increased but still the majority had a rugged appearance. At 15 dph there was a significant difference between the two groups, where the Copepod-group had a bigger proportion of smooth outer membranes than the Rotifer-group.

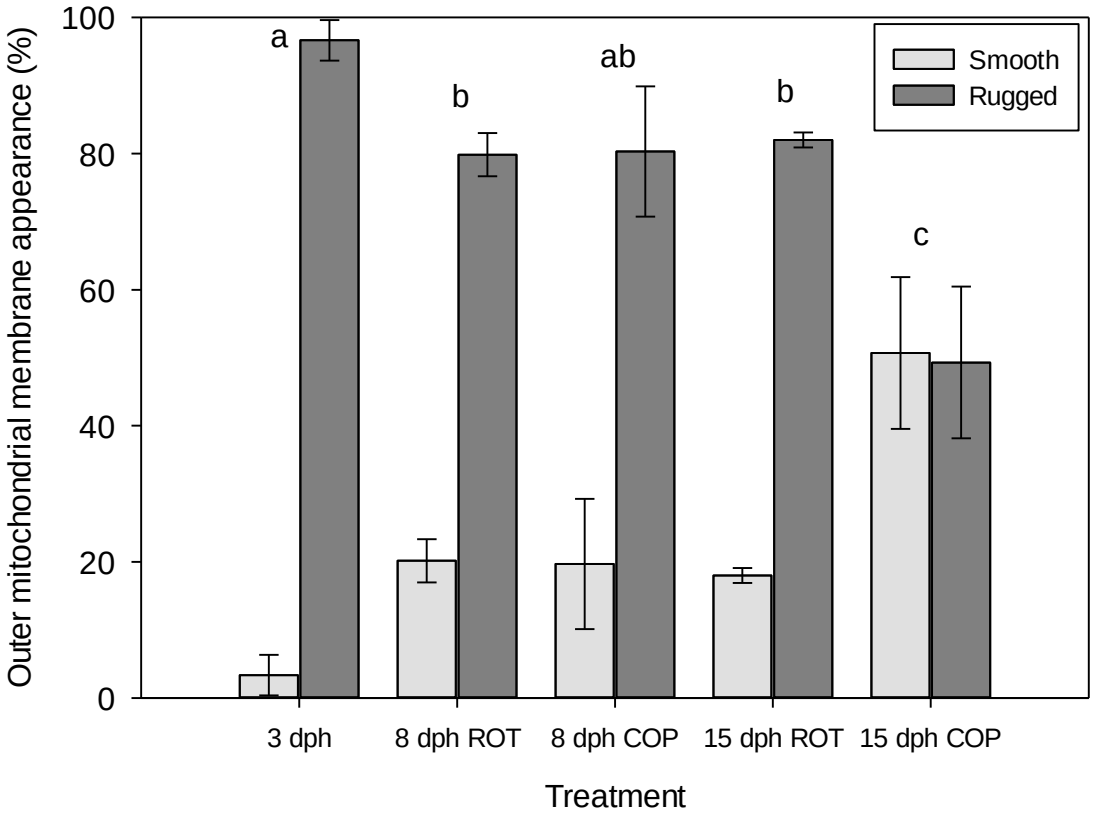

Figure 3.10: Mean outer mitochondrial membrane appearance (%) in the liver of larvae at 3 dph and from both treatments at 8 and 15 dph (n=5). The outer mitochondrial membrane is classified as either smooth or rugged. There was a significantly higher occurrence of rugged membrane structure at 3 dph than at the later sample days and the highest occurrence of smooth membrane structure was found on 15 dph in the Copepod-group. Letters indicates significant difference between and within treatments. Error bars indicates ± ER.

Over time there was significant decrease in the occurrence of less dense cristae and a significant increase in the occurrence of denser cristae. At 8 dph both groups had a significant increase in the occurrence of denser cristae when compared to 3 dph, where the Rotifer-group a significantly higher proportion of denser cristae than the Copepod-group. At 15 dph both groups had a high proportion of dense cristae where the Copepod-group had a significantly higher proportion than the Rotifer-group.

Interdigitated cristae were found in individuals from the Rotifer-group both at 8 and 15 dph with the highest occurrence at 15 dph (14 ± 4 %).

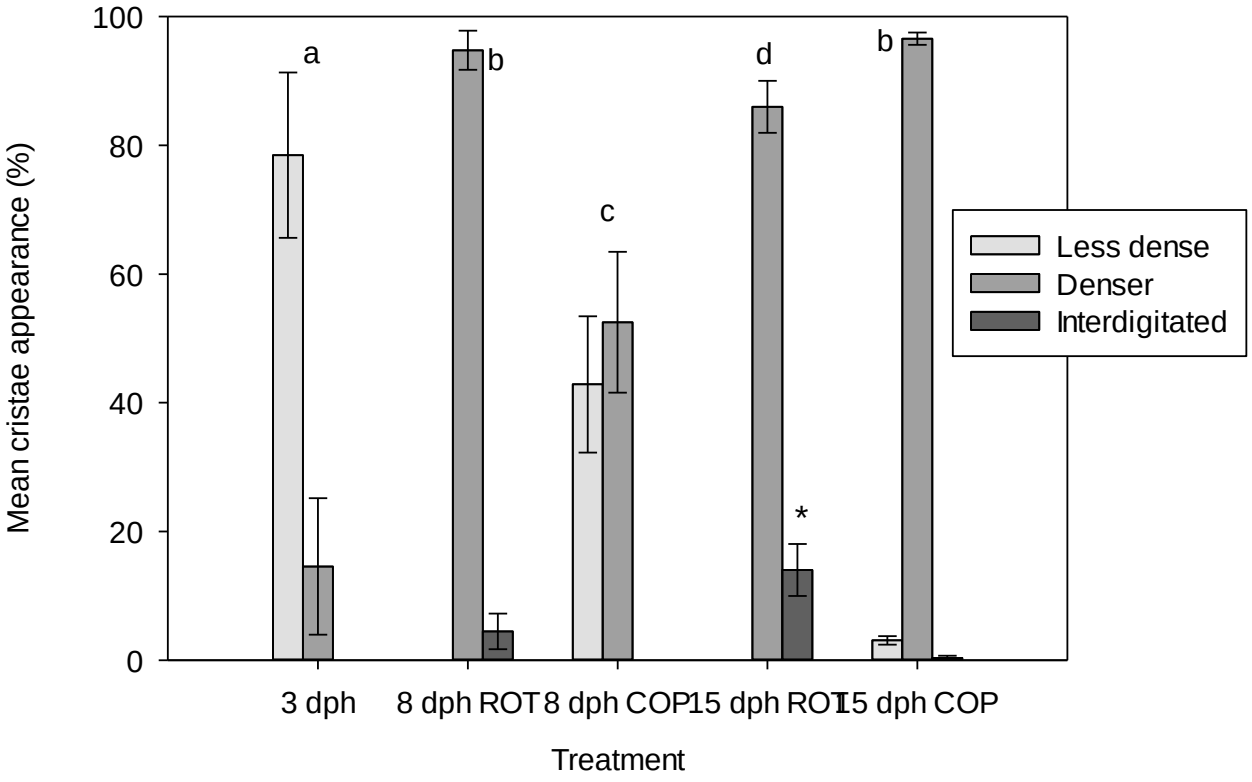

Figure 3.11: Mean cristae appearance (%) in hepatocytes of larvae from 3 dph and from both treatments at 8 and 15 dph (n=5). There was significant difference between the treatments at both 8 and 15 dph and significantly less dense cristae at 3 dph. Interdigitated cristae was mostly found in the Rotifer-treatment with the highest occurrence at 15 dph (*). Letters indicates significant difference between and within treatments. Error bars indicates ± SE.

3.3 Gut morphology

3.3.1 Light microscopy

At 3 dph the digestive tract was short and narrow with thick intestinal epithelium. The enterocytes were long and narrow and the nuclei were oblong in shape with no distinguishable nucleoli (Figure 3.12 A). At 8 dph the digestive tract was wider than at 3 dph and the enterocytes were more distinguishable as columnar cells with round nuclei (Figure 3.12 B-C). The intestinal epithelium in the Copepod-group was starting to fold more than the epithelium in the Rotifer-group. Two of the larvae from the Rotifer-group at 8 dph had a thinner intestinal epithelium with less dense (Figure 3.19) and shorter microvilli (Figure 3.18), exhibiting signs of starvation. At 15 dph the epithelium was thicker and had more folds than at 8 dph for both groups. The microvilli border on the luminal surface was longer in the Copepod-group than the Rotifer-group at 15 dph and both groups at 8 dph (Figure 3 18). At the base of the enterocytes in the Rotifer larvae from 15 dph (Figure 3.12 D) numerous lipid droplets were visible. Lipid droplets were also seen in the enterocytes of the Copepod-group but they were more dispersed throughout the cells and not as numerous.

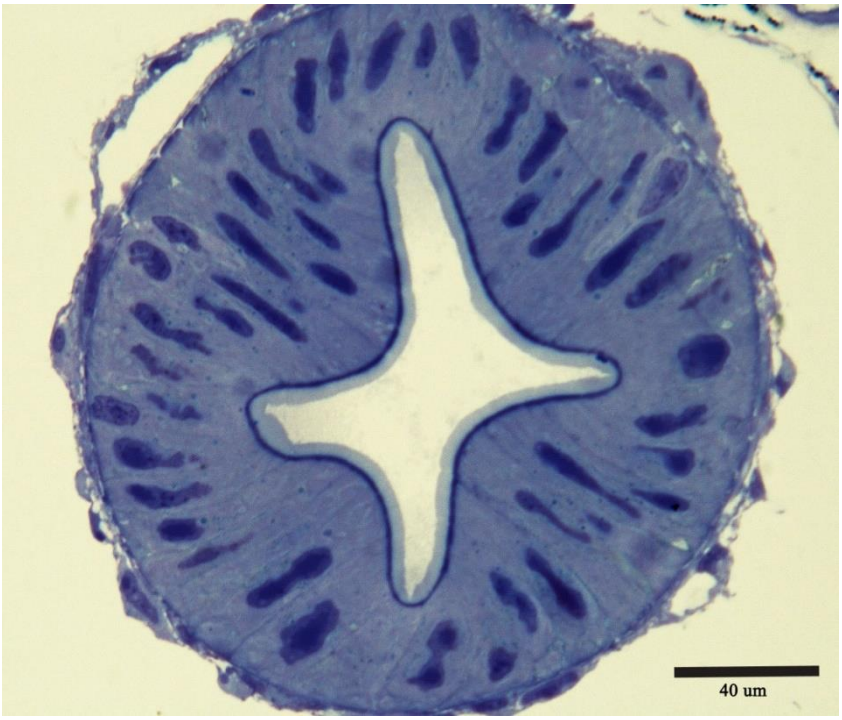

Figure 3.12 A: Photo micrograph of the midgut of a larva at 3 dph. The digestive tract is narrow with thick intestinal epithelium. Visible microvilli border towards the digestive tract. The enterocytes and nuclei are long and narrow.

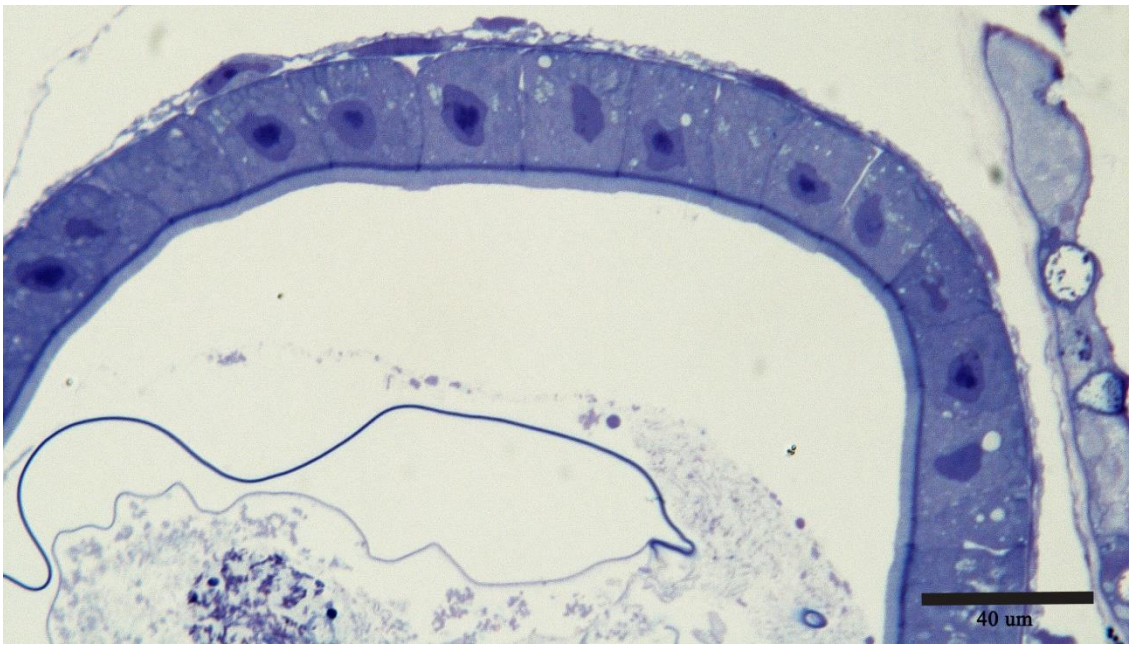

Figure 3.12 B: Photo micrograph of the midgut of a larva at 8 dph from the Rotifer-group. The digestive tract is wider and the intestinal epithelium is narrower than at 3 dph. The cells are more quadratic in shape and the nuclei have become more round. Visible microvilli border towards the digestive tract.

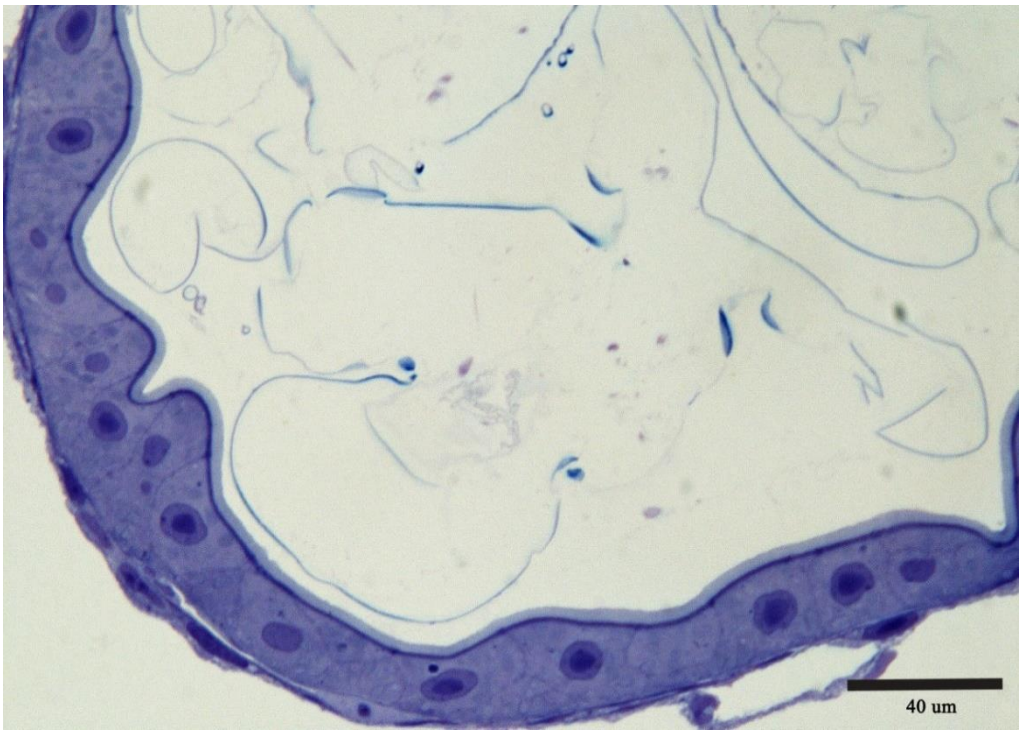

Figure 3.12 C: Photo micrograph of the midgut of a larva at 8 dph from the Copepod-group. The digestive tract is wider than on 3 dph. The epithelium is more folded than in the Rotifer-group. Here the nuclei are also more round than at 3 dph. Visible microvilli order towards the digestive tract.

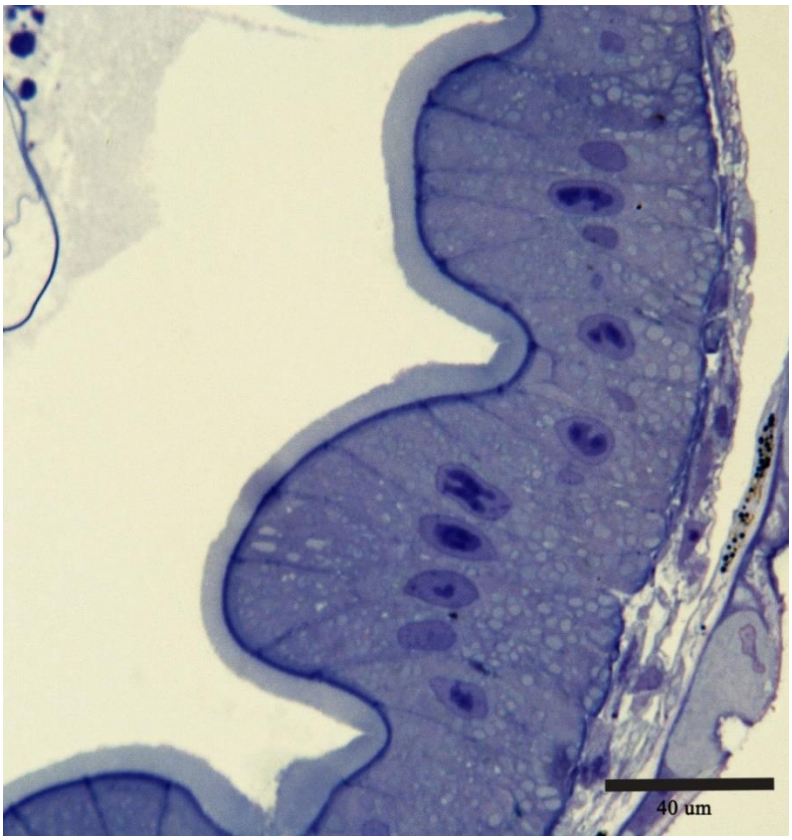

Figure 3.12 D: Photo micrograph of the midgut of a larva at 15 dph from the Rotifer-group. The intestinal epithelium is thicker and more folded than on earlier sample dates. Numerous lipid droplets are visible at the base of the enterocytes. .

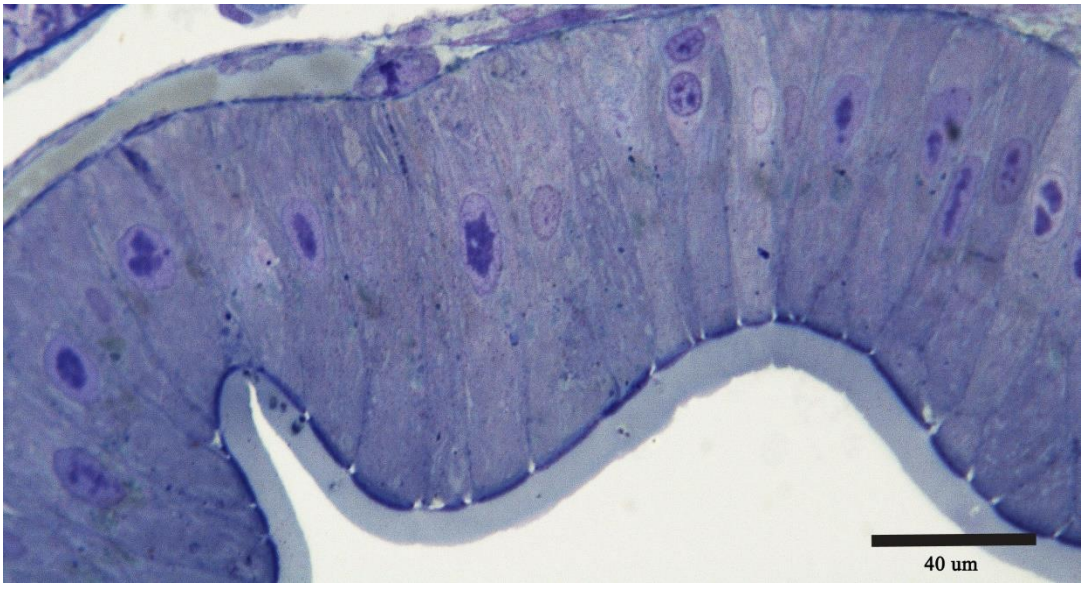

Figure 3.12 E: Photo micrograph of the midgut of a larva at 15 dph from the Copepod-group. The intestinal tissue is thicker and more folded than on earlier sample dates. The microvilli border is thicker and longer than in the Rotifer group. .

3.3.2 Transmission electron microscopy

The intestinal epithelium of the larvae consisted of tall columnar enterocytes with the nuclei at the base of the cells and a border of microvilli at the top. Visible organelles were nuclei, mitochondria and endoplasmic reticulum (ER). The outer mitochondrial membranes were more rugged at 3 dph than at later sample dates (Figure 3.13 A). At 8 dph both groups have a larger proportion of rugged than smooth membranes but less so than at 3 dph. At 15 dph both groups have smoother outer mitochondria membranes with no big difference between the groups. There was no big difference in cristae density over time either within or between groups, though more incidents of less dense cristae at 3 dph than at later sample dates (Figure 3.13 A). At 15 dph the ER was much more abundant and organized in the enterocytes of the Copepod-group than for the Rotifer-group and both of the groups at 8 dph (Figure 3.13 E). Two unidentified vacuoles were observed in the enterocytes of the Rotifer-group at 8 and 15 dph. Vacuole 1 was round with white droplets inside (Figure 3.13 B), while vacuole 2 had grey color with a darker outer ring.

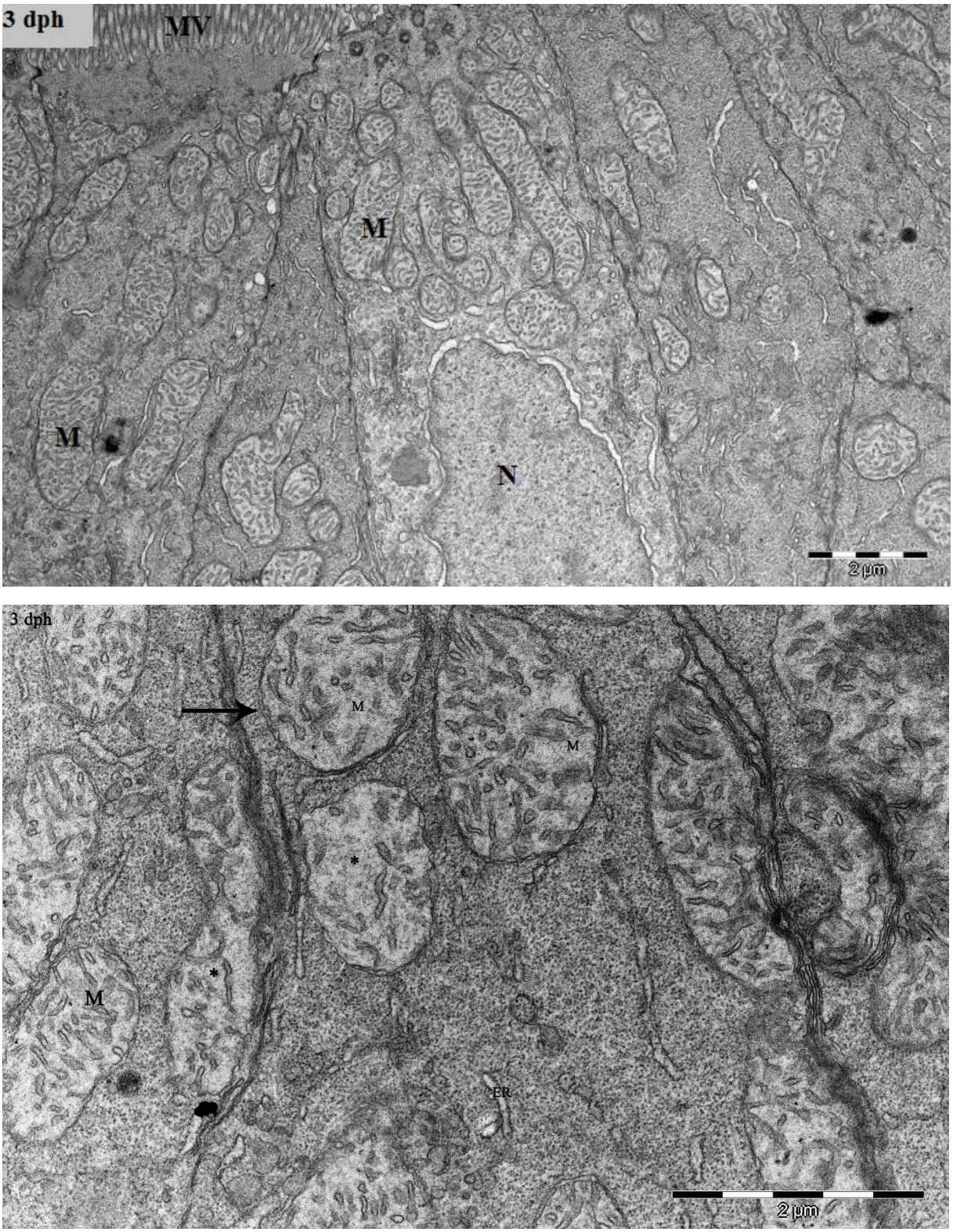

Figure 3.13 A: Micrograph that shows the general morphology of intestinal tissue in the midgut from larvae at 3 dph. Nuclei (N), mitochondria (M) and microvilli (MV) are shown on the photos. The photos show a difference in mitochondrial membrane structure and cristae density over time and endoplasmic reticulum gets more abundant and organized over time. At 3 dph the outer mitochondrial membranes are more rugged (arrow) and the cristae less dense (*) than at 15 dph.

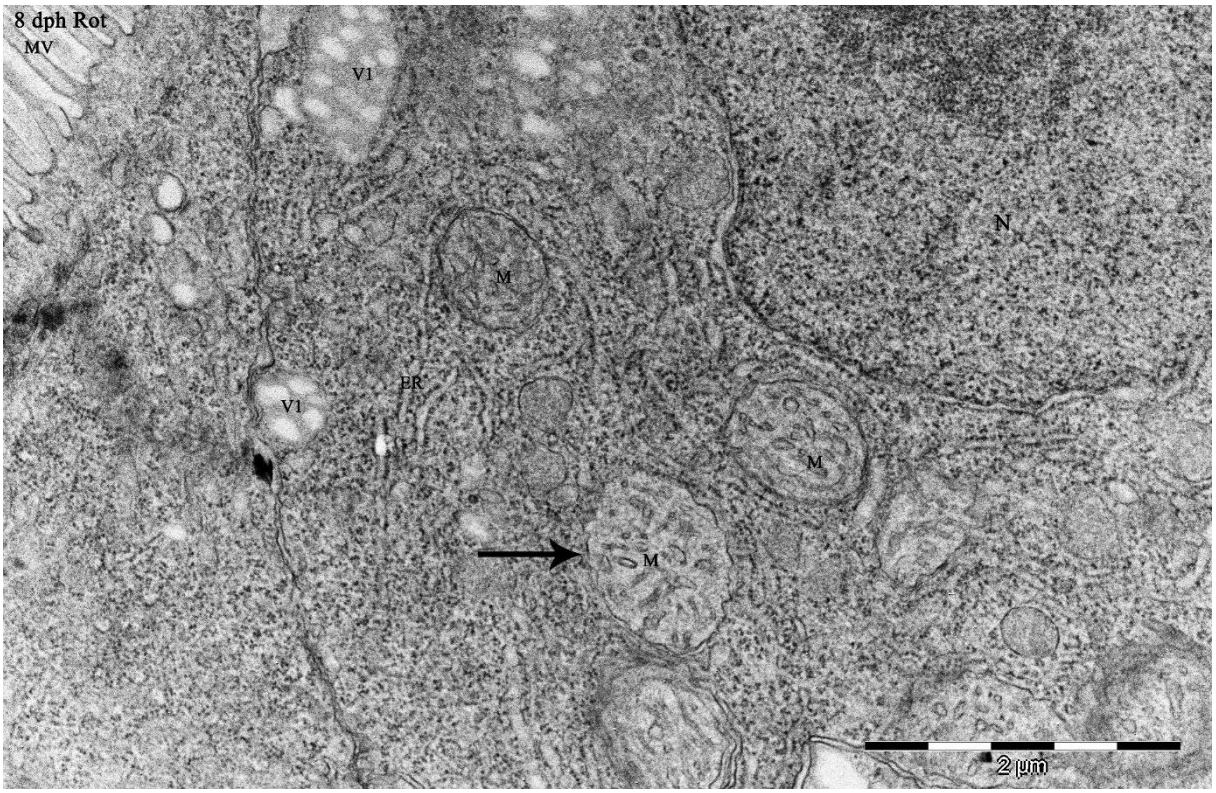

Figure 3.13 B: Micrograph that shows the general morphology of intestinal tissue in the midgut from larvae from the Rotifer-treatment at 8 dph. Nuclei (N), mitochondria (M), vacuole 1 (V1) and microvilli (MV) are shown on the photo. The arrow marks a mitochondria with rugged outer membrane.

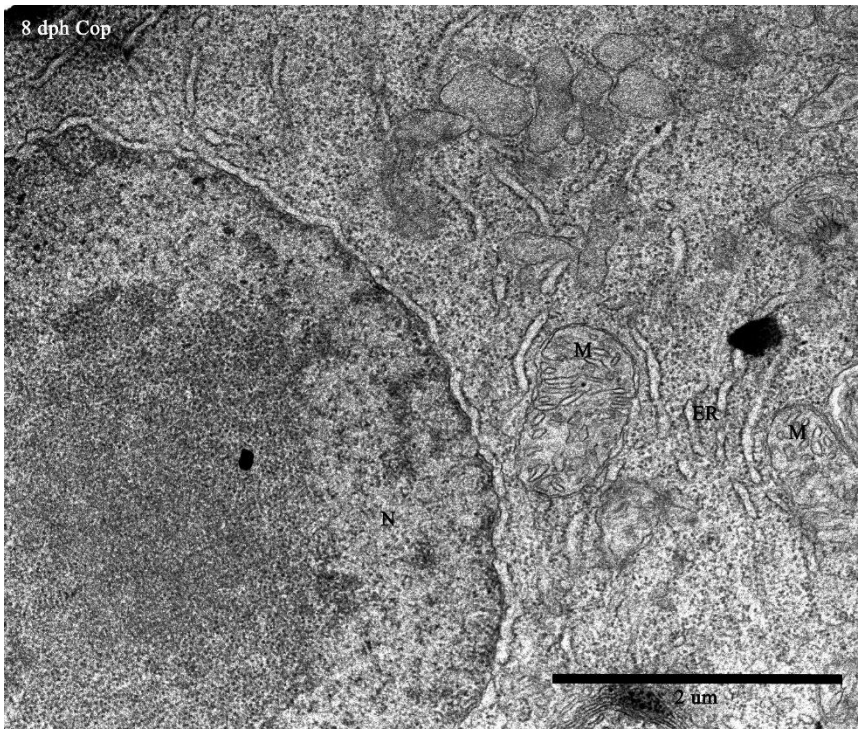

Figure 3.13 C: Micrograph that shows the general morphology of intestinal tissue in the midgut from larvae from the Copepod-treatment at 8 dph. Nuclei (N) and mitochondria (M) are shown on the photo.

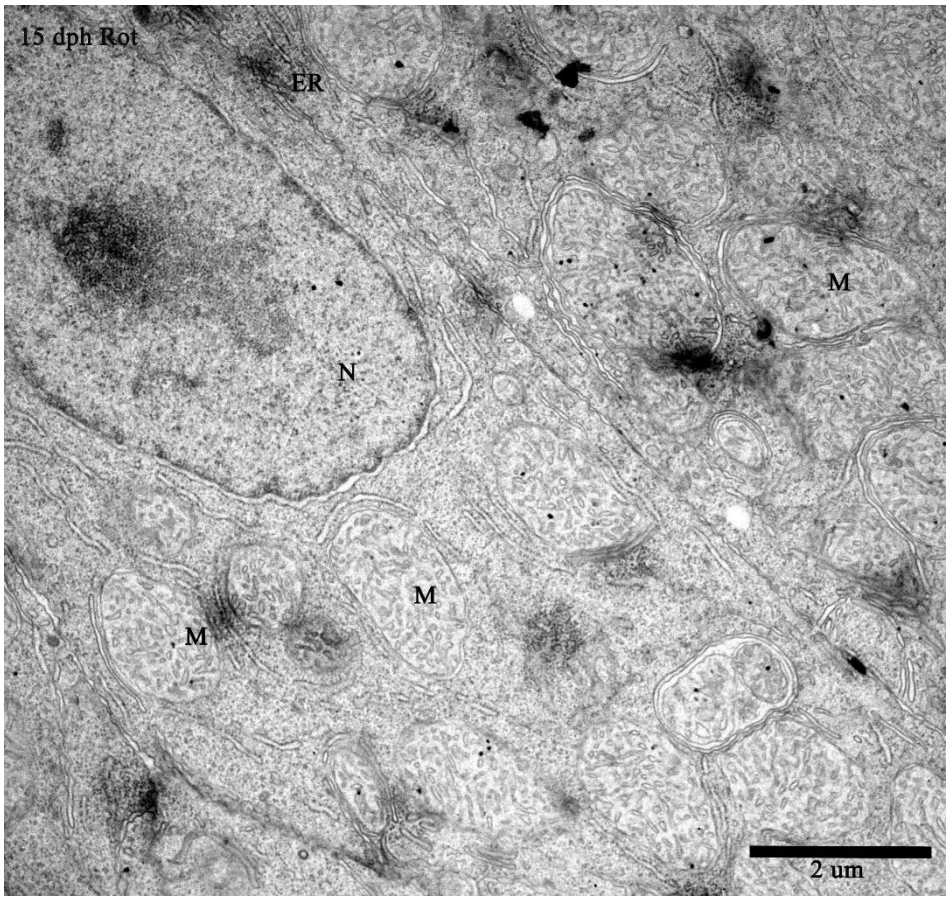

Figure 3.13 D: Micrograph that shows the general morphology of intestinal tissue in the midgut from larvae from the Rotifer-treatment at 15 dph. Nuclei (N), mitochondria (M) are shown on the photo.

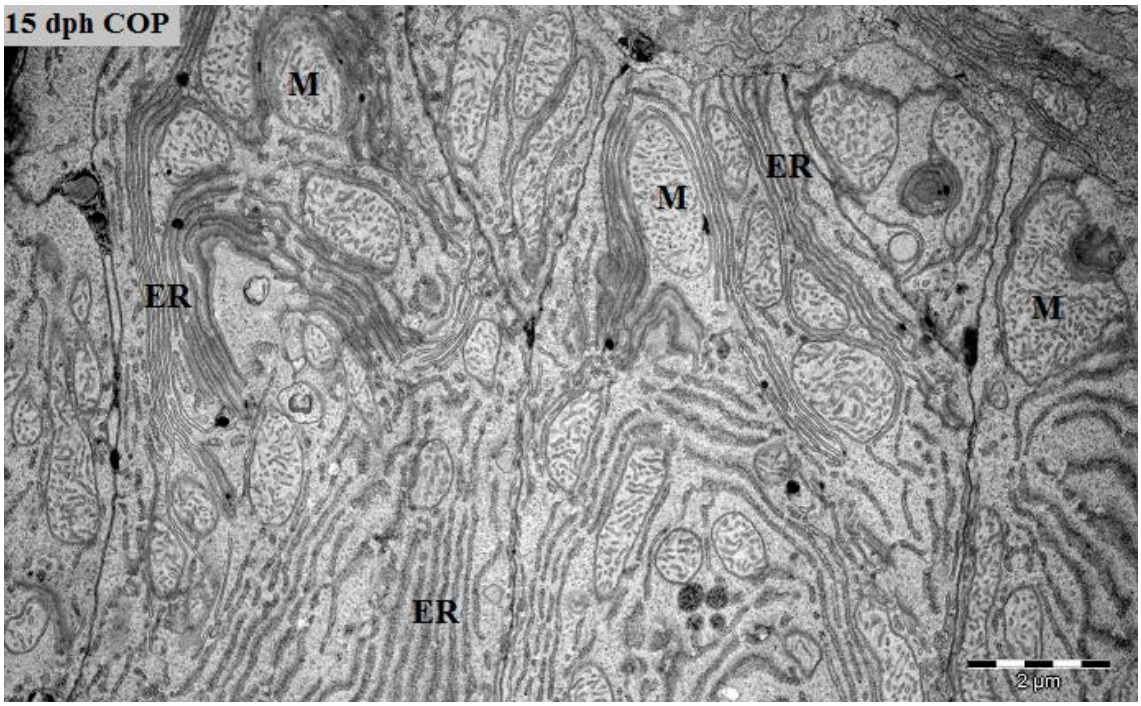

Figure 3.13 E: Micrograph that shows the general morphology of intestinal tissue in the midgut from larvae from the Copepod-treatment at 15 dph. Mitochondria (M) and endoplasmic reticulum (ER) are shown on the photo.

3.3.3 Surface area fractions of enterocyte components

The surface area fraction of nuclei was highest at 3 dph (20.6 ± 2.3 %) and decreased slightly over time for both groups but there was no significant difference between or within treatments (Figure 3.14).

The surface area fraction of mitochondria decreased slightly over time and there was a significant difference between 3 dph and both treatments on 8 and 15 dph (Figure 3.14).

The surface area fraction of endoplasmic reticulum (ER) in the enterocytes of the Rotifer-group decreases slightly from 3 to 8 dph but then seems to stabilize at around 25 %. In the Copepod-group there also is a decrease in the ER area fraction from 3 to 8 dph, but at 15 dph there is a significant increase in the fraction of ER from 23.5 to 34.2 %.

Vacuole 1 and vacuole 2 was unidentified vacuoles and were only found in individuals from the Rotifer treatment at 8 and 15 dph. The highest value of vacuole area fraction was found on 8 dph for vacuole 1 (8.4 ± 1 %), which was significantly higher than on 15 dph (3.6 ± 1 %). The surface area fraction of vacuole 2 had a slight increase from 8 to 15 dph but the difference was not significant.

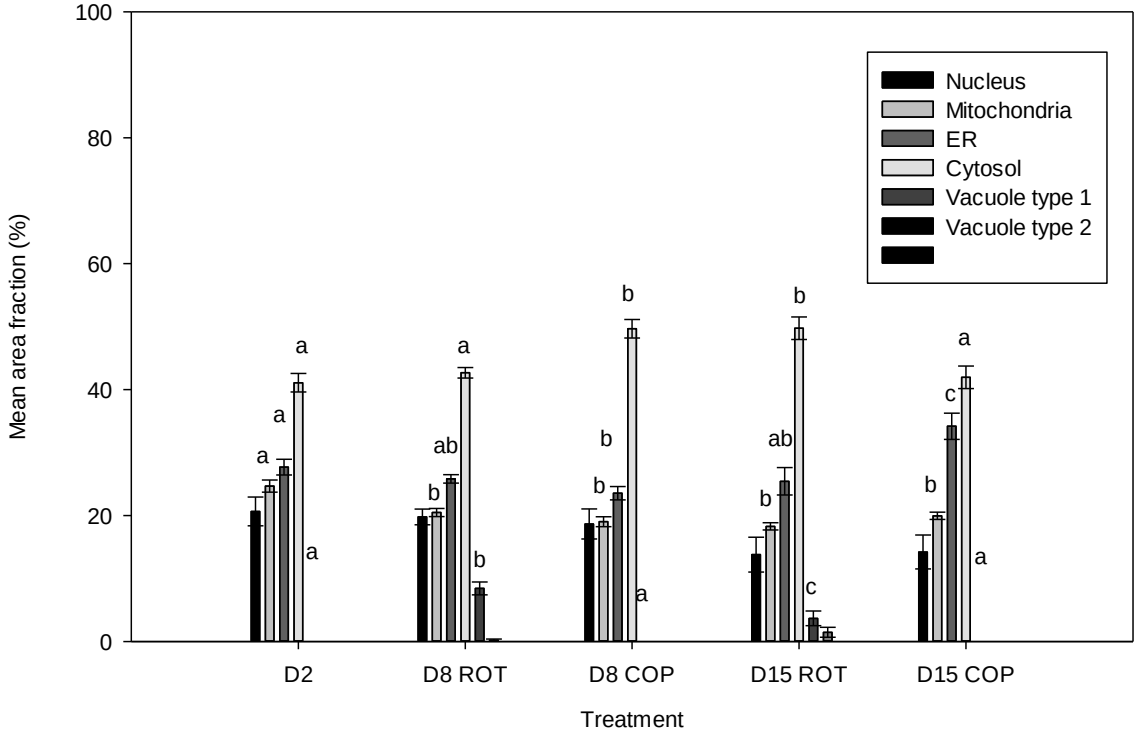

Figure 3.14: Mean surface area fraction (%) of enterocyte cell components (nuclei, mitochondria, endoplasmic reticulum (ER), general cytosol, vacuole 1 and 2 and microvilli) on 3 dph, and for both treatments at 8 dph and 15 dph (n=5). Letters indicates significant difference between and within treatments. Error bars indicates ± SE.

3.3.4 Area size of nucleus and mitochondria

There was an overall increase in nucleus area size over time for both groups. The smallest nucleus area size was found in the Rotifer-group (89.2 ± 2 μm^2) at 8 dph which was actually smaller than at 3 dph (102.6 ± 3.4 μm^2). The largest nucleus area size was found in the Copepod-group at 15 dph (137.8 ± 3.2 μm^2) which was significantly larger than the nuclei at 3 dph and the nucleus area size in the Rotifer group at 8 dph.

The general trend for mitochondrial area size for both groups was a decrease in size over time but there was no significant difference between the groups or between the groups and 3 dph. The largest mitochondrial area size was at 8 dph in the Rotifer-group (1 ± 0.08 μm^2) and the smallest in the Rotifer-group at 15 dph (0.73 ± 0.06 μm^2).

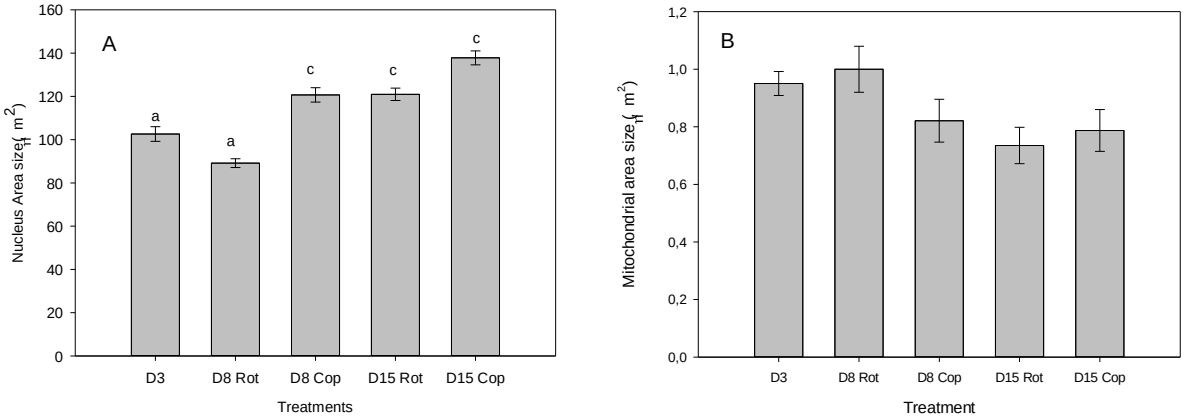

Figure 3.15: Nucleus area size (A) and mitochondria size (B) in the enterocytes of larvae from 3 dph and from both treatments at 8 and 15 dph. Letters indicates significant difference between and within the groups. No significant difference in mitochondria size was found. Error bars indicates ± SE.

3.3.5 Mitochondrial appearance

The highest occurrence of rugged outer mitochondrial membranes was seen at 3 dph and there seemed to be a general decrease in the occurrence of rugged membranes over time for both groups. At 15 dph the larvae from the Copepod-group seemed to have more mitochondria with smooth outer membranes than the larvae from the Rotifer-group, but the difference between the groups were negligible.

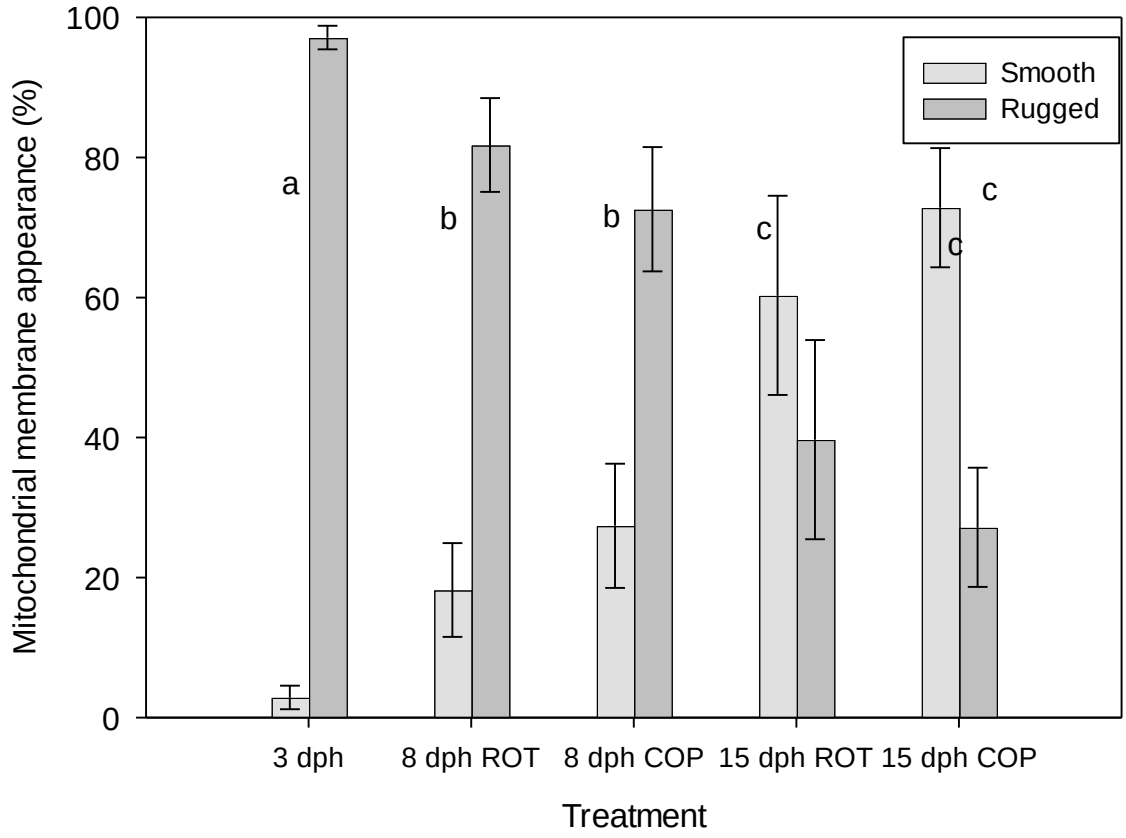

Figure 3.16: Mean outer mitochondrial membrane appearance (%) in the enterocytes of larvae at 3 dph and from both treatments at 8 and 15 dph (n=5). There was a significantly higher occurrence of rugged membrane structure at 3 dph than at the later sample days and the highest occurrence of smooth membrane structure was found on 15 dph in the Copepod-group. Letters indicates significant difference between and within treatments. Error bars indicates ± ER.

The cristae density was generally high for both groups over time, and there is no significant difference between the groups. Interdigitated cristae were observed in the Rotifer-group at 8 dph and in the Copepod-group at 15 dph but only a small number in one individual for both groups.

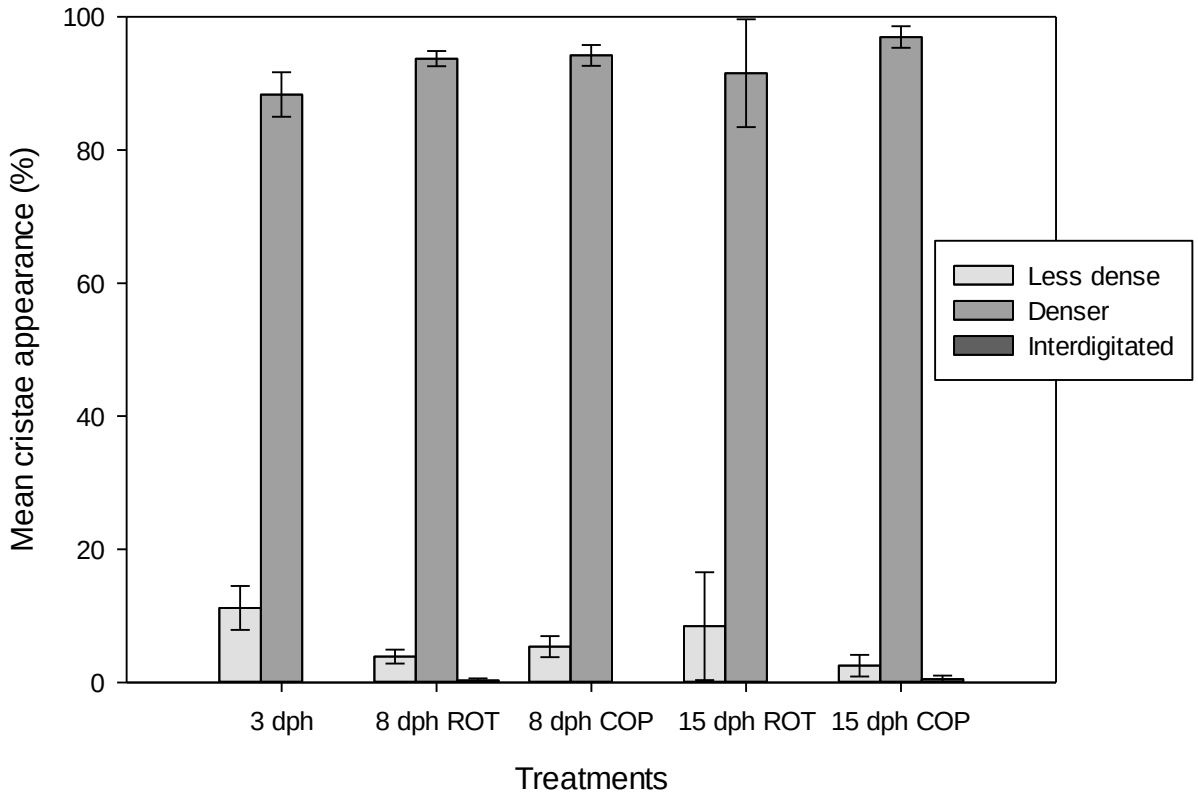

Figure 3.17: Mean cristae appearance (%) in the enterocytes of larvae from 3 dph and from both treatments at 8 and 15 dph (n=5). There was no significant difference in cristae density between or within the two treatments. Error bars indicates ± SE.

3.4 Microvilli density and height

The first 8 days post hatch there seemed to be a decrease in microvilli height in both groups, although only significantly for the Rotifer-group (Figure 3.18). At 15 dph both groups had had an increase in microvilli height and the longest microvilli was found in the Copepod-group, 3.9 ± 0.05 µm vs 2.4 ± 0.02 µm for the Rotifer-group.

Microvilli density decreased significantly for the Rotifer-group when comparing it with the density at 3 dph (8.2 ± 0.14 µm at 3 dph and 6.7 ± 0.09 µm at 8 dph), while remaining stabile for the Copepod-group. At 15 dph there was no difference between the two groups, approximately 7.7 ± 0.2 µm for both.

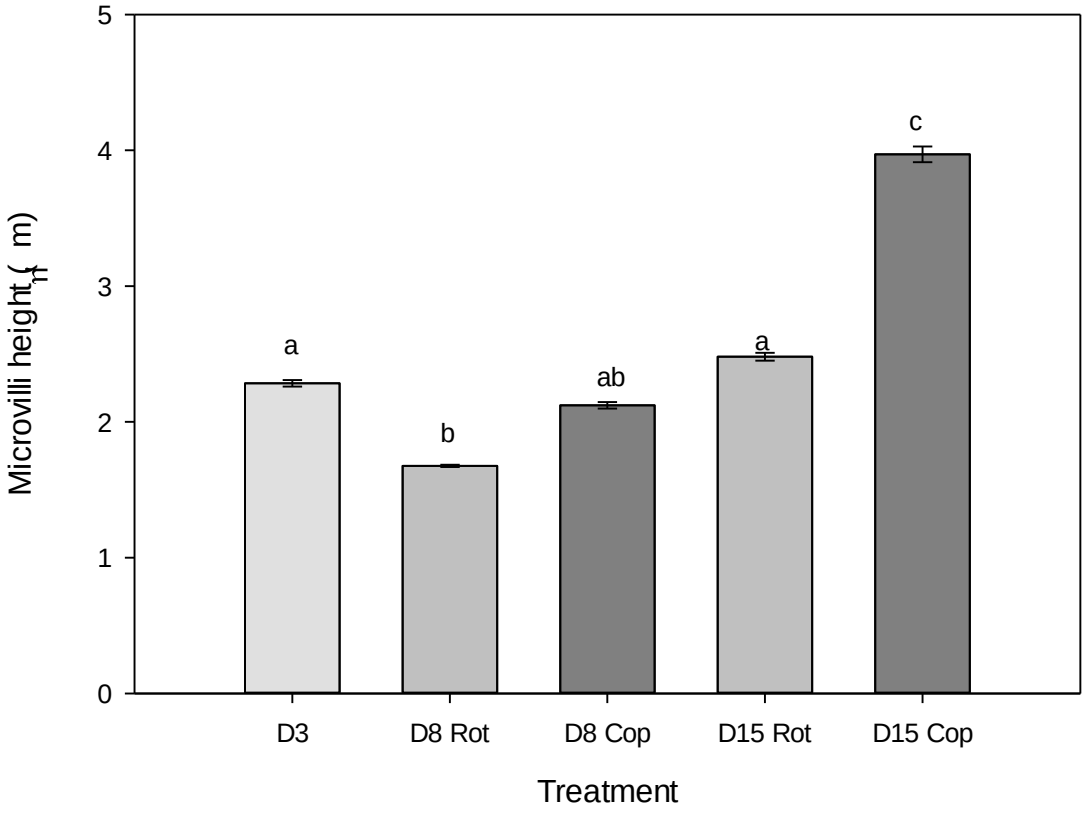

Figure 3.18: Mean microvilli height (µm) in the enterocytes of larvae at 3 dph and from both treatments at 8 and 15 dph. Letters indicates significant difference within and between the groups. Error bars indicates ± SE.

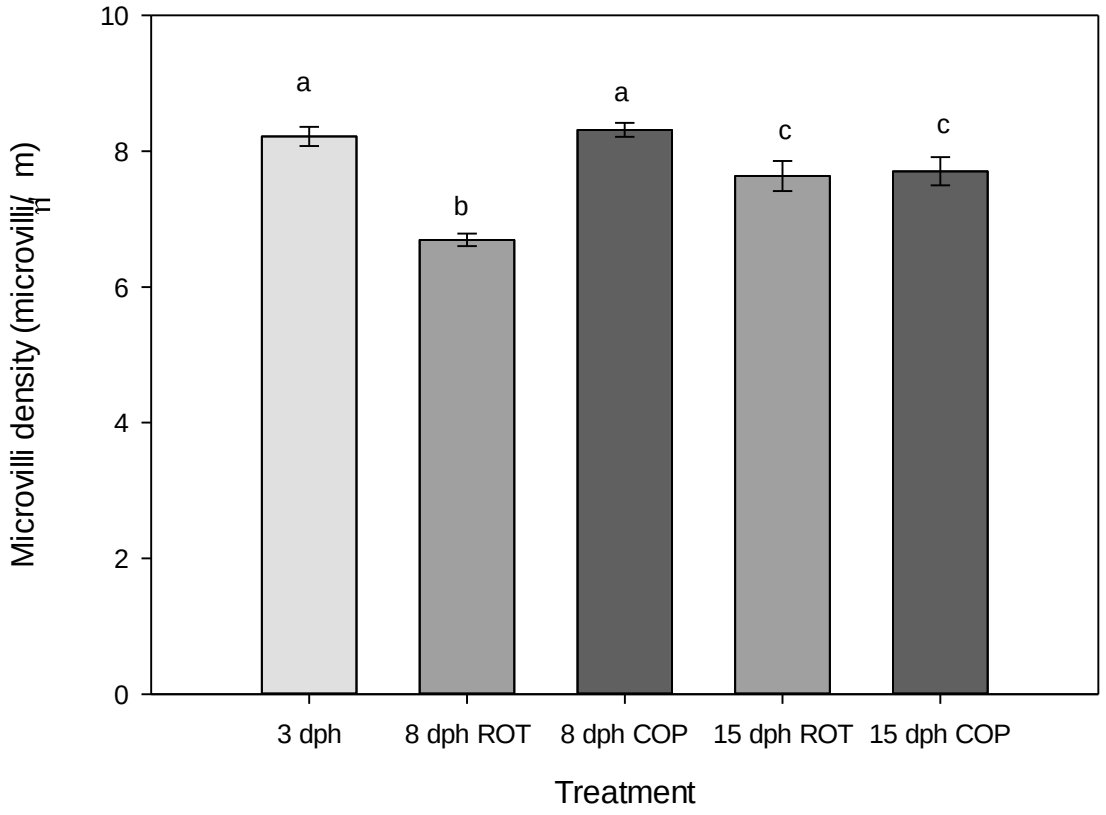

Figure 3.19: Mean microvilli density (microvilli/µm) in the enterocytes of larvae at 3 dph and from both treatments at 8 and 15 dph. Letters indicates significant difference within and between the groups. Error bars indicates ± SE.

4 Discussion

4.1 Larval growth and survival

The present study shows that feeding ballan wrasse larvae intensively reared *Acartia tonsa* exclusively gave better growth than feeding them the traditional live feed diet of enriched rotifers and enriched artemia. This was seen in all three of the growth parameters measured (SL, MH and DW). This implies that intensively reared copepods fit the dietary requirements of the fish larvae better than enriched rotifers and artemia in the first 45 days.

The Copepod-group had a higher dry weight, SL and MH throughout the whole experiment compared to the Rotifer-group. The Copepod-group had significantly higher dry weight at 15 and 28 dph. After the introduction of enriched artemia the difference between the groups decreased but the Copepod-group kept having higher dry weight throughout the experiment, although not significantly. This was also seen in another study done on ballan wrasse (Gagnat, 2012), where the groups weaned from rotifers to artemia showed an increase in growth rate, in turn decreasing the difference between the groups. In that study the group fed copepods was weaned on to artemia at the same time as the other groups which gave a decrease in growth rate. In the present study the Copepod-group was fed copepods exclusively throughout the experiment but we still saw a decrease in growth rate around 30 dph. The slowdown in dry weight increase in the Copepod-group could be due to sub-optimal numbers and size of the copepods they were fed. When the feeding was changed from copepodits to copepodits and 1/3 adult copepods at 36 dph the mean dry weight increased more rapidly again. In future feeding experiments one should probably switch to larger copepodits/adult copepods earlier to satisfy the ballan wrasse larvae's appetite.

In terms of daily weight increase (DWI) both groups showed a negative growth at 3-8 dph. In this early period the larvae are transitioning from endogenous to exogenous feeding so this is probably the cause of the negative weight increase. At 8-15 dph the Copepod group shows a significantly higher DWI than the Rotifer-group. Rapid growth is very important for marine larvae that have a small size at hatching. Larval development is highly connected to size, more so than age. Getting bigger in size means higher development in terms of disease resistance, escaping predators and a more developed digestive system, which in turn gives a better chance of survival.

There was no significant difference in survival between the two groups at the end of the experiment; the same was seen in a similar experiment by Gagnat (2012). 85 % of the larvae

had died before 15 dph (Table 3.1), indicating that the ballan wrasse larvae are quite sensitive in the early days of feeding.

4.2 Effects of different live feed on larval hepatocyte and enterocyte development

The present study showed that there was a maturation of both the hepatocytes and the enterocytes in terms of differences in organelle appearance, abundance and size over time for both groups. The ballan wrasse larvae fed copepods seemed to have better development of the liver and the gut than the larvae fed enriched rotifers.

4.2.1 Nuclei size and appearance

The liver and gut tissue in the ballan wrasse larvae seemed to mature from the start of exogenous feeding and through the first 15 days. At 3 dph the nuclei of the hepatocytes and the enterocytes where irregularly shaped with no clearly defined nucleoli, which can be seen in both the photomicrographs and the micrographs. At the later sample dates one can see a definite change in the appearance of the nuclei with a rounder shape and clearly defined nucleoli. The nucleus area size was also the smallest at 3 dph in both the enterocytes and the hepatocytes. The size of the area of contact between the nucleus and the cytoplasm is decisive for the exchange of macromolecules essential for DNA replication and protein synthesis between the nuclei and the cytoplasm (Ghadially; 1997). Therefore the smaller nuclei at 3 dph can imply a lower metabolic activity and development of the hepatocytes and enterocytes than at the later sample dates when the nuclei were larger. The larvae fed exclusively on copepods had larger nuclei than the larvae fed on enriched rotifers both on 8 and 15 dph. A study done on the Common carp found that larvae fed diets low in phospholipids had smaller liver nuclei than larvae fed diets with high amount of phospholipids (Fontagnè et al. 1998). This fits well with what was found in this study were the larvae fed enriched rotifers, which contain less phospholipids, had the smallest nuclei.

4.2.2 Mitochondrial development

Ghadially (1997) states that there is a positive correlation between the metabolic activity of a tissue and number and size of the mitochondria. As with the nuclei, the mitochondria of the hepatocytes and enterocytes changed in size and appearance over time. The smallest mitochondria were found at 3 dph for both the hepatocytes and the enterocytes, indicating low metabolic activity in these tissues at this stage. From 3 to 8 dph there had been a significant increase in mitochondria size in the hepatocytes, but not such a big difference in the

enterocytes. The size of the mitochondria in the hepatocytes in the Rotifer-group at 8 dph was especially large compared to both the size at 3 dph and the mitochondria of Copepod-group at the same date. Swelling of the mitochondria has been shown to be a compensational reaction to metabolic abnormalities and can lead to reduced function (MacQueen, Leifson et al., 2003; Segner et al., 1984). Photomicrographs of the intestinal epithelium of the same larvae from the Rotifer-group showed that two of the five larvae had very thin intestinal walls. Other studies have seen a reduction in thickness of the intestinal epithelium in starved fish larvae (Kjørsvik et al., 1991; McFadzen et al., 1994). The swelling of mitochondria could therefore be a reaction to starvation and the lack of nutrients necessary to maintain the metabolic activity. Another study from this same first feeding experiment which looked at the expression of genes linked to mitochondrial functions also saw a difference between the two groups at 8 dph. There they found a higher expression of six genes linked to the oxidative phosphorylation process in the mitochondria of the Copepod-group, which implied higher mitochondrial activity, energy generation and release than in the Rotifer-group (Stavrakaki, 2013). The lower activity in the mitochondria paired with the swelling in size seems to indicated that the larvae from the Rotifer-group at 8 dph had reduced function of their mitochondria probably due to starvation. This can also be linked to the difference in DWI between the groups at this date, where the Copepod-group had a significantly higher DWI than the Rotifer-group.

In the hepatocytes there was an initial growth of the mitochondria from 3 to 8 dph, before the mitochondria size seemed to stabilize with no difference between the groups. In the enterocytes there was a general decrease in mitochondria size over time although there was not a big difference either over time or between the groups. This reduction in mitochondria size in the gut could indicate a higher metabolic activity in the tissue at the start of exogenous feeding with a decline in activity as the larvae grew.

Mitochondrial appearance also changed over time. There was a significant difference in cristae density and outer mitochondrial membrane smoothness when comparing the results at 3 dph with the later sample dates. At 3 dph the outer mitochondrial membranes were more rugged and the cristae within less dense making the mitochondria look less mature than at 8 and 15 dph. Similar results were found in a study done on cod larvae were less dense cristae and rugged outer membranes were observed in cod larvae at the start of exogenous feeding (Norheim, 2011). This difference over time signifies a maturation of mitochondria both in the hepatocytes and in the enterocytes.

4.2.3 Endoplasmic reticulum

Another sign of maturation of the liver and intestinal tissue was the change in the abundance of rough endoplasmic reticulum in the cells over time. The endoplasmic reticulum (ER) got more and more abundant over time for both groups but was most abundant in the hepatocytes and enterocytes of larvae fed with copepods exclusively. The ribosome studded rough ER is the site of protein synthesis and packaging, and produces most of the transmembrane proteins for the cells organelles, while the smooth ER is the site for lipid synthesis (Ghadially, 1997). A more abundant ER would signify a larger protein and lipid production, which means a more active and mature cell. The lipids and transmembrane proteins produced in the ER are the components for organelle and cell membranes. When looking at the outer mitochondrial membranes they were much more rugged at 3 dph when the ER was much less abundant in the cells. At the later sample dates, 8 and 15 dph, the ER was getting more and more abundant and the outer membranes are smoother and the mitochondria have a much more rounder shape. Therefore there could be a connection between the maturation of the mitochondria and the abundance of ER in the cells.

4.2.4 Lipid and glycogen storage

Previous studies done on other marine fish larvae have seen a larger frequency of lipid droplets in the liver (Lie et al., 1986; Segner et al., 1994) and the gut (Kjørsvik et al., 1991; Caballero et al., 2003; Wold et al., 2008).

There were few observations of lipid droplets in the hepatocyte of the ballan wrasse larvae from this study. The most lipid droplets were seen in the enterocytes of the larvae from the Rotifer-group at 15 dph where there were numerous droplets at the base of the enterocytes. Lipid droplets were also observed in the enterocytes of the larvae from the Copepod-group but the droplets were more dispersed throughout the cells and not as numerous. The pile up of lipid droplets at the base of the enterocytes could indicate an inability to transport the lipids through the enterocytes and out to the bloodstream fast enough. This could be due to the difference in the origin of the lipids from the feed. Studies have shown that n-3 HUFA from phospholipids can be incorporated more efficiently into larval tissue than fatty acids from neutral lipids (Izquirerdo et al., 2000). Copepods have a high fraction of essential fatty acids in phospholipids, while rotifers have fatty acids in neutral lipids (Olsen et al., 2004). The fact that fatty acids in neutral lipids are harder to incorporate into larval tissue could explain the difficulty of transporting the lipids out of the enterocytes in the larvae fed rotifers.

Studies done on cod larvae showed that there was no stored glycogen in the liver at the start of exogenous feeding (Norheim, 2011), while studies done on turbot and sea bream (Sparus aurata) showed that they had glycogen storage already at hatching (Segner et al., 1994; Guyot et al., 1995). This difference from species to species shows that there is a difference in glycogen storage strategies. This study showed that the ballan wrasse larvae had glycogen stored in the liver at hatching, although at low levels. Both groups had an increase in the amount of glycogen stored over time, and the highest levels of glycogen in the liver were found in the larvae from the Rotifer-group at 15 dph. The difference in the amount of stored glycogen can be linked to the difference in nutrient content of the live prey. Glycogen is a storage form of glucose (Berg et al., 2007) and the amount of glycogen stored will vary with the amount of this carbohydrate in the feed (Waagbø et al, 2001). In rotifers 10.5 to 27 % of the dry weight is carbohydrates (Støltrup, McEvoy, 2003), while the content of carbohydrates in copepods varies from 0.2 to 5.1 % of the dry weight (Corner, O`Hara, 1986). The difference in carbohydrate content of these two live feeds indicates that the larvae fed rotifers would be supplied a larger amount of carbohydrates through the feed making them more able to store glycogen than the larvae fed copepods.

5. Conclusion

This present study showed that using intensively reared *Acartia tonsa* exclusively as live feed for ballan wrasse larvae gave better growth than feeding with enriched rotifers and artemia. The larvae fed copepods were heavier, longer and thicker than the larvae from the Rotifer-group throughout the experiment, indicating that copepods fit the nutritional requirements of the ballan wrasse larvae better than enriched rotifers and artemia. Although the larvae fed copepods showed to have better growth there were no difference in survival between the two groups.

The mitochondria in both the liver and gut seemed to mature over time. The proportion of rugged outer mitochondrial membranes is lower at 15 dph than at 3 dph, and cristae density seems to increase over time. The mitochondria seem to be more mature in the Copepod-group than in the Rotifer-group, indicating that feeding ballan wrasse larvae with copepods is beneficial for mitochondrial development. Copepods have a high content of phospholipids which is important for the development of biomembranes, which can explain the more mature state of the mitochondria in the Copepod-group.

Swelling of the mitochondria at 8 dph in the Rotifer-group, paired with reduced thickness of the gut indicates that these larvae suffered from starvation. It is not clear if this starvation-reaction is caused by unsatisfactory nutritional content of the enriched rotifers or if the larvae are underfed/not able to catch enough prey.

Glycogen was found in the liver of larvae from both groups already at 3 dph, and the surface area fraction of glycogen increased significantly in both groups over time. That the highest levels of glycogen was found in the Rotifer-group can be explained by the difference in carbohydrate content of the live feed. Rotifers contain more carbohydrates than copepods, which mean that the rotifers may provide more stored glycogen in the liver.

The results of the tests done on the liver tissue seemed to show bigger differences between the groups than the tests done in the gut tissue, indicating that the liver is more sensitive to subpar nutritional quality and could be interesting to look closer at in another study with a larger sample size.

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Appendix 1

Embedding ballan wrasse larvae in EPON

The larvae were washed in 0.11 M hepes buffer 3 x 10 minutes. Then they were post-fixated in 3% KFC: 4% osmium 1:1 (final: 1.5%:2%) for 1 hour at room temperature protected against light. The larvae were then washed 5 x 2 minutes in distilled water.

The larvae were bulk colored for 1.5 hours in room temperature with 1.5% uranylacetat in distilled water while being protected against light. Then they were washed 5 x 2 minutes in distilled water.

The next step was dehydrating the larvae in ethanol. First 10 minutes in 70% ethanol, 10 minutes in 90% ethanol, 2 x 10 minutes in 96 % ethanol, and finally 2 x 15 minutes in absolute ethanol.

The larvae were transferred to individual glass vials and propylenoxid was added to the vials for 2 x 15 minutes (with a lid on the vials). Then a mixture of prop.ox and EPON was added in a 3:1 ratio for 30 minutes, then a 1:1 ratio for 30 minutes and finally in a 1:3 ratio for 30 minutes. Finally clean EPON was added to the vials and they were set for spinning overnight (without lid).The next day the EPON was replaced by new EPON and was set to polymerize in 60°C for 24 hours.