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Effects of live feed quality on cod (Gadus morhua L.) larval hepatocyte and enterocyte development

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Abstract

Production of high quality juveniles is still a bottleneck in the intensive culture of cod (*Gadus morhua* L.), and suboptimal nutrition at the larvae's early life stages is one of the decisive factors for this. The cod larva hatches from small pelagic eggs, and has to start exogenous feeding shortly after hatching when it still lacks a functional stomach. Optimal feeding conditions are therefore crucial for the vulnerable larva at this early developmental stage. Copepod nauplii as live feed may provide nutritional benefits in terms of containing essential fatty acids in the phospholipid fraction that may be incorporated directly into membrane phospholipids, and also containing high amounts of free amino acids compared to rotifers and *Artemia* sp., which is applied as live feed in intensive production of cod today.

The aim of the present study was to examine the effects of feeding with the intensively reared copepod *Acartia tonsa* compared to both enriched and unenriched rotifers (*Brachionus ibericus)*, in terms of growth, survival, and functional development of the liver and gut. The liver and gut from larvae at 4 and 19 days post hatching (dph) was examined by performing quantitative measurements by stereology, and qualitative histological measurements on hepatocyte and enterocyte appearance. Mitochondrial development was given special emphasise to evaluate if there are tendencies implying a development in morphology of these energy producing organelles, and also whether they are affected by the nutritional composition of the live feed organisms.

Feeding with copepod nauplii instead of enriched rotifers resulted in higher growth, survival, and increased pigmentation of the cod larvae. In addition it was found that feeding with rotifers, and especially with the unenriched type, showed signs of mitochondrial dysfunction in terms of cristae interdigitation within both hepatocytes and enterocytes. This was not found in the copepod-fed larvae, which might be attributed to providing phospholipid in the diet that may enhance normal development of biomembranes. Glycogen deposits were found in the hepatocytes in larvae at 19 dph, although to a much greater extent in the larvae fed rotifers, which might be due to differences in carbohydrate content in the different live feed organisms. The liver tissue was generally more evidently reflecting developmental processes between 4 dph and 19 dph and the effect of the live feeds nutritional value on the metabolic energy state of the larvae, than what the gut tissue did.

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1 Introduction

1.1 Challenges regarding cod as aquaculture species

1.1.1 Background

During the last 30 years there has been a steady decline in the landings of Atlantic cod, and this trend is still continuing (FAO, 2010). This, together with the success of farming of Atlantic salmon (*Salmo salar*) created an interest in commercial farming of cod (Rosenlund, Halldórsson, 2007). The first breakthrough in large scale cod rearing in Norway happened in 1983 at the Institute of Marine Research Austevoll Biological Station (Øiestad *et al.*, 1985). Since then, there has been varying success in the cod production in terms of variable growth, development, and survival of juveniles (Kjesbu *et al.*, 2006). However, the Atlantic cod was recently rated as the third most important aquaculture specie after the Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*) (Fjelldal *et al.*, 2009). In 2009 the landings of wild caught cod from Norwegian fisheries was 242 000 metric tons (SSB, 2010a), and the total production and sale of cod from Norwegian aquaculture industry was 20 683 metric tons (SSB, 2010b). Thus, the amount of cultured cod is much lower than the wild caught cod, although it is clear that an increase in consumption must be supplied from aquaculture to prevent a collapse in the wild stocks in the North Atlantic (Brown *et al.*, 2003). Cod farming companies are currently having economical deficiencies in addition to the fisheries increased quotas of wild caught cod, which lowers the price and reduces the demand for cultured cod (Björnsdottir *et al.*, 2010).

Production of high quality juveniles is still a bottleneck in the intensive cultivation of cod, and suboptimal nutrition in the early developmental stages is one of the main reasons of varying and unpredictable production. Over the past few years there has been great progress in developing systems and technology for producing cod larvae, mainly in high-intensive systems, with enriched rotifers and *Artemia* sp. as start feed (Olsen *et al.*, 2004; Hamre, 2006). Nevertheless, there is an increasing need for better survival and development at the larval stage in cod production. In intensive production this developmental stage is often characterised by poor growth, low survival, and malformations (Hamre, 2006). Deformities of the skeleton is a problem that often occurs in intensively reared cod (Helland *et al.*, 2009; Lein *et al.*, 2009), which increase

production costs and the deformities constitute an ethical problem with regards to animal welfare. This problem is less often seen in extensive systems where copepods are used as live feed (Hamre, 2006). Deformities seen in later life stages may be the result of sub-optimal nutrition at the larval stage in high-intensive systems (Cahu *et al.*, 2003a; Kjørsvik *et al.*, 2009), since a higher fraction of larvae reared extensively with natural zooplankton as feed develops normally (Hamre, 2006; Imsland *et al.*, 2006).

Since the nutritional status in the earliest stages of development of the marine fish larvae is decisive for the quality of the fish at later stages (Galloway *et al.*, 1999; Hamre, 2006), it is therefore essential that the feed provided in the early life stages is of high quality and provided in sufficient amounts. In addition to this, the cod larvae seem to prioritise rapid growth rather than storing energy in the liver, which makes them vulnerable to starvation and malnutrition (Kjørsvik *et al.*, Submitted). Considerably increase in knowledge about the development of the digestive system of marine fish larvae has developed since the start of aquaculture production of cod (Govoni *et al.*, 1986; Kjørsvik *et al.*, 1991; Kjørsvik *et al.*, 2004; Rønnestad *et al.*, 2007; Wold *et al.*, 2008; Wold *et al.*, 2009; Kamisaka, Rønnestad, 2011), which has contributed to a better understanding of the nutritional demands of cod larvae and the ontogenetic changes during the important early life stages of fish. There are still many challenges to overcome regarding nutrition and the larvae's capability to utilize the feed. This highly relies on the quality of the feed, but also on the functional status of the larva's digestive system. Knowledge about fish larvae's digestive development and nutritional requirements will be indispensable when establishing optimal rearing conditions and feeding regimes during the early life stages.

1.1.2 Nutritional value of live feed organisms

Unlike fish larvae with precocial development of the digestive tract, like the wolffish (*Anarhichadidae* sp.), the cod larvae have altricial development and are less capable of digesting formulated diets immediately after hatching (Waagbø *et al.*, 2001; Støttrup, McEvoy, 2003). Intensive aquaculture of cod larvae commonly relies on enriched rotifers (*Brachionus* sp.) at start-feeding until 20-30 dph, after which they are fed enriched *Artemia* sp. nauplii with an overlapping phase in between (Olsen *et al.*, 2004). It is also common to add microalgae to the culture water (Reitan *et al.*, 1993) and this procedure may vary in duration. This is a feeding-regime that has been applied as a

control in several studies on cod (Kjørsvik *et al.*, 1991; Wold *et al.*, 2008; Kjørsvik *et al.*, Submitted), and it provides a stable nutrition source because of the availability of these organisms all year round.

It is well known that the marine fish larvae requires certain essential fatty acids and sufficient levels of them in their diet, and that these fatty acids are those that are needed to contribute to optimal growth and development (Rainuzzo *et al.*, 1997; Sargent *et al.*, 1999). Fatty acids that can be synthesised *de novo* includes saturated and monounsaturated fatty acids, but the polyunsaturated fatty acids (PUFA) cannot be synthesised by animals (Gurr *et al.*, 2002; Olsen *et al.*, 2004). The most important essential fatty acids for marine fish are the highly unsaturated fatty acids (HUFAs) eicosapentaenoic acid (20:5*n-3*; EPA), arachidonic acid (20:4*n-6*; ARA) and docosahexaenoic acid (22:6*n-3*; DHA), and the fish larvae cannot synthesise these fatty acids *de novo* because of the absence of the enzyme Δ5-desaturase (Waagbø *et al.*, 2001; Støttrup, McEvoy, 2003). Neither do the marine fish larvae have the capacity to elongate C18 fatty acids because of the absence of the enzyme C18-C20 elongase. It is therefore essential that the EPA, ARA and DHA is provided through the feed (Waagbø *et al.*, 2001; Støttrup, McEvoy, 2003). Furthermore, studies show that providing these fatty acids in the polar lipid fraction (phospholipid; PL) rather than in the neutral lipid fraction makes them more readily digested. Fatty acids in the polar lipid fraction may be incorporated directly into cell membranes, and has shown to be beneficial for larval growth, development, and digestive capacity (Coutteau *et al.*, 1997; Cahu *et al.*, 2003b; Wold *et al.*, 2007; Kjørsvik *et al.*, 2009). Rotifers and *Artemia* sp. do not naturally contain all the required nutrients for marine fish larvae, and the general challenge mostly regards the content and nutritional value of lipids and *n-3* HUFAs, in particular the low DHA content (Olsen *et al.*, 2004).

The methods for *n-3* HUFA enrichment of the rotifers are well established and the level of *n-3* HUFAs post enrichment is relatively stable. The most efficient way of achieving high lipid levels in the rotifers is by using a short-term enrichment method (Rainuzzo *et al.*, 1994). Rotifers are well suited for fatty acid manipulation because its fatty acid composition becomes almost equal to that of their dietary lipids post enrichment. This is because the rotifer requires very little *n-3* fatty acids for their own consumption, and dietary lipids will affect the composition of its stored triacylglycerides (TAG) (Olsen,

2004). The lipid level of rotifers depends on the level of lipid in their diet, and may vary between 10 % and 25 % of dry weight (Olsen, 2004). The absolute level of PL in rotifers is not in a high extent affected of enrichment method, and is quite stable until 6 days post enrichment when the levels starts to decrease. Thus, the high lipid content of rotifers does mainly reflect the high levels of TAG (Rainuzzo *et al.*, 1994).

The lipid content in enriched *Artemia* sp. is usually between 20 % and 30 % of dry weight, and DHA may vary from 5-20 % of total fatty acids (Stoss *et al.*, 2004; Hamre, 2006). The general challenge when feeding *Artemia* sp. to marine fish larvae has been related to their unstable DHA content post enrichment because of its rapid catabolism of DHA (Evjemo *et al.*, 1997; Olsen, 2004). Even when enriched with oils of high DHA content the *Artemia* sp. may still have very low concentrations of DHA in the polar lipid fraction and also very low DHA/EPA ratios (Bell *et al.*, 2003). Even though the DHA content is low, the total lipid content is quite high in enriched *Artemia* sp*.* compared to the cod larvae's natural feed (van der Meeren, 2003). This implies that the lipid content might get too high in enriched *Artemia* sp. and may affect fish larval digestion negatively by accumulation of lipid within the enterocytes (Hamre, 2006).

The nutritional composition of live feed organisms applied in mariculture, such as rotifers, *Artemia* sp., and natural copepods, has been studied in detail, and it is generally believed that feeding with copepods provides the highest nutritional quality at the cod larval stage (Kjesbu *et al.*, 2006). Copepods are the natural food for many marine fish species and they contain high fractions of PLs. In general all of the life stages of copepods are suitable as a food source for marine fish, but the small fish larvae have difficulties in catching the copepodites because of their higher swimming velocity. It is therefore more suitable to apply the earlier life stages of copepods when feeding small fish larvae (Støttrup, McEvoy, 2003).

Copepods are found among the natural zooplankton provided as feed in extensive cultivation of cod. The obstacle in using extensive systems is the large seasonal variations in natural zooplankton production (Svåsand *et al.*, 2004), and it is therefore desirable to intensively cultivate copepods all year round for live feeding of marine fish larvae. Advances in cultivation techniques of copepods have been made for many copepod species (Støttrup, Norsker, 1997; Payne, Rippingale, 2001; Drillet *et al.*, 2011), and great progress has been made in cultivation procedures and storage of eggs for the

calanoid copepod *Acartia tonsa* (Støttrup *et al.*, 1986; Drillet *et al.*, 2006), where it successfully has been used as live feed in cod larval rearing (Engell-Sørensen *et al.*, 2006; Overrein *et al.*, Unpublished). *Acartia tonsa* has six naupliar stages from NI to NVI, and it is the first three naupliar stages that are true naupliar stages (Mauchline, 1998). The generation time can vary greatly within the calanoid species when reared at different temperatures, but at 25 ^{o}C the *A. tonsa* generally has a generation time of 7 days, which makes it suitable for intensive production (Støttrup, McEvoy, 2003).

Marine copepods does naturally have much higher contents of DHA than both rotifers and Artemia *sp*., they also usually have EPA/ARA ratios above 20 and DHA/EPA ratios above 2 (Conceicao *et al.*, 2010). Marine copepod nauplii are relatively lean (9-10 % lipid of dry weight), but >60 % of their total lipids are PL, while only 20-30 % of the total lipids in *Artemia* sp. constitutes of PL. Much of the HUFA found in copepods are predominantly located in the phospholipid fraction, while much of the HUFA in rotifers and *Artemia* sp. are found in TAG (Olsen *et al.*, 2004).

The copepods contain high amounts of protein (55-58 % of dry weight)(Olsen, 2004), and especially free amino acids (FAA) (van der Meeren *et al.*, 2008; Conceicao *et al.*, 2010), which are readily absorbed and digested by the larval gut (Rønnestad *et al.*, 1999). The protein and FAA content is generally much higher in copepods than in rotifers and *Artemia* sp. Dietary protein is important due to the ten indispensable amino acids for fish that the fish larvae cannot synthesize *de novo*; arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine and valine, (Jobling, 2004). Amino acids provide the fundamental component of proteins, and are important as precursors or nitrogen source for a wide range of biomolecules. Sequence and function of amino acids is in general under strict control of genes. Also, the amino acid composition shows little variability between species, and is only moderately affected by nutrition. Thus, the amino acid composition of the prey will be relatively close to that of the predator. Fatty acid composition, on the other hand, is far more variable in the feed. Therefore there is a greater probability that there will be a deficiency of essential fatty acids rather than of essential amino acids (Olsen *et al.*, 2004). However, it is essential that the undeveloped gut of fish larvae is able to absorb the proteins and amino acids, which is far more effective when supplying a higher fraction of FAA in the diet. Thus FAA in the diet may enhance the utilization of proteins and growth since growth primarily is

an increase in muscle mass by protein synthesis (Rønnestad *et al.*, 1999; Applebaum, Rønnestad, 2004).

Digestive processes of carbohydrates are not well expressed in marine fish larvae (Kjørsvik *et al.*, 2004), and the natural feed, the copepods, do not contain any significant amounts of carbohydrates (Stoss *et al.*, 2004). Nevertheless, there has been found that carbohydrate digestive enzymes are at their highest levels in fish larvae during the first days of start-feeding, and decreases during the development. This downward regulation is irrespective of the dietary glucide concentration, and may be genetically determined (Cahu, Zambonino-Infante, 2001; Kjørsvik *et al.*, 2004). This enzymatic activity might be of imprtance when adding microalgae to the culture water that the young larvae ingest by drinking. Digestive mechanisms are triggered by the microalgae, and they might also contribute to the establishment of an early gut flora (Reitan *et al.*, 1997).

Vitamins, minerals, and pigments also have important roles in marine fish larval nutrition. Insufficient skin pigmentation may be a result of deficiencies in vitamin A and it's precursors. Vitamin A may also have decisive effects on normal bone development. Vitamin A levels in copepods are generally low, but its physiological functions may be covered by astaxanthin, which is an important source of retinoids (van der Meeren *et al.*, 2008; Hamre *et al.*, 2010). Antioxidant properties are also essential, and are provided through nutrients like astaxanthin, vitamin C and vitamin E (van der Meeren *et al.*, 2008). Vitamin E levels are low in copepods compared to rotifers and *Artemia* sp., but high levels of other nutrients with antioxidant properties are found in copepods such as astaxanthin and vitamin C. Astaxanthin is generally low in both rotifers and *Artemia* sp., compared to copepods that contain significant amounts of this pigment. Reduction of bone loss is prevented by the action of vitamin D and vitamin K, and they contribute to maintaining calcium homeostasis (Hamre *et al.*, 2010). Vitamin C has also shown to be important for normal bone development, since it is important for collagen production. Very low levels of vitamin D have been detected in the live feed organisms, while high levels of vitamin C is found in copepods compared to rotifers (Hamre, 2006; van der Meeren *et al.*, 2008). Iodine is necessary for the production of thyroid hormone, which is a regulator of the metamorphosis in fish larvae. Copepods contain far more iodine than rotifers and *Artemia* sp., and copepods may thus be favourable for normal metamorphosis of fish larvae (Hamre, 2006; Moren *et al.* 2006).

1.2 The importance of lipid quality

The essential fatty acid DHA is an important constituent in cell membranes, and is one of the most abundant fatty acid in the brain and in the retina of the eye of the marine fish larvae, especially in the phosphatidyl-ethanolamine (PE) fraction (Waagbø *et al.*, 2001). Further, the DHA/EPA ratio >2:1 and the EPA/ARA ratio >5:1 can be related to the rate of pigmentation in marine fish larvae (Rainuzzo *et al.*, 1997; Bell *et al.*, 2003). ARA has an important function as a precursor for eicosanoids (Bell *et al.*, 1995). The fatty acids of the *n-3* family are in general important as they seem to have specific roles in vision, reproduction, nerve function and cell membrane permeability (Gurr *et al.*, 2002; Parrish, 2009). Mitochondrial membrane function and permeability is very responsive to dietary change, with regard to the essential fatty acids, and affects the organelles ability to exchange ADP and ATP and regulate the activity of the respiratory chain (Berdanier, 2000).

There have been done several studies on the PL requirements in fish larvae (Coutteau *et al.*, 1997; Fontagnè *et al.*, 1998; Cahu *et al.*, 2003b), where PLs seem to be beneficial on formation and transport mechanisms of lipoproteins from the gut epithelial cells to the blood (Izquierdo *et al.*, 2000; Tocher *et al.*, 2009). The PLs ability to form intra-luminal micelles along with bile-salts and dietary lipids may also facilitate *n-3* HUFA uptake and digestion (Tocher *et al.*, 2009). PLs from copepods have a higher percentage of *n-3* HUFA than TAG (Lahlou, Vitiello, 1991), and may therefore be nutritionally more beneficial for the fish larvae (Olsen *et al.*, 2004). It seems that marine fish larvae have high capacity to utilize PLs, which might be due to their higher ability to modulate phospholipase A_2 rather than lipase (Cahu *et al.*, 2003b). There has also been reported better growth when microdiets containing more of the marine PLs, rather than the neutral lipids, has been fed to sea bass (*Dicentrarchus labrax*) larvae (Cahu *et al.*, 2003b) and cod larvae (Wold *et al.*, 2007; Kjørsvik *et al.*, 2009).

The acyl chain in the *sn*-2 position (*sn* = stereo specific number denoting the position of acyl chain on the glycerol backbone) of TAG in the feed is preserved in fish storage lipids. When dietary lipids are digested, the pancreatic bile salt-dependent lipase is secreted into the gut where it removes the acyl chain from the *sn*-1 position in both triacylglycerides and phospholipids. This action allows absorption of *sn*-2 lyso(phospho)lipids into the gut lumen, and they are then reacylated at the *sn*-1 position

from a new fatty acid from acyl Co-A. This generates a phospholipid that still has the 2-acyl fatty acids intact (Gurr *et al.*, 2002; Bell *et al.*, 2003), which can be directly incorporated into cell membranes. It is therefore essential that the fatty acid in the *sn*-2 position is one of the essential PUFA´s (Olsen, 2004). In *Artemia* sp. the *sn*-2 position in PLs is predominantly alpha-linolenic acid (18:3*n-3*) or linoleic acid (18:2*n-6*), while DHA is less abundant. Copepods, on the other hand, contains PLs with high amounts of DHA and EPA (45-60 % of the total fatty acids) in the *sn-2* position, and the high *n-3* HUFA fraction is very stable since these fatty acids function as cell membrane components and are therefore selectively retained during starvation (Bell *et al.*, 2003; Evjemo *et al.*, 2003; Olsen, 2004).

When there is excess supply of PUFA they are esterified to the *sn*-2 position of the glycerol, whereas saturated and monounsaturated fatty acids are more frequently esterified to the *sn*-1 and *sn*-3 positions. This discrimination explains why one of two fatty acids in PL are polyunsaturated and one out of three fatty acids are polyunsaturated in TAG (Olsen *et al.*, 2004). In general ester bonds of fatty acids with chain lengths less than 12 carbon atoms are more rapidly cleaved than those of the normal chain length (14C-18C), while the ester bonds of very long chained polyenoic fatty acids (20:5 and 22:6) (Gurr *et al.*, 2002), which are essential for marine fish larvae (Waagbø *et al.*, 2001; Støttrup, McEvoy, 2003), are more slowly cleaved (Gurr *et al.*, 2002). As previously mentioned PL are better utilized rather than neutral lipids, such as TAG, which might be due to the marine fish larvae better ability to modulate phospholipase A_2 rather than lipase (Cahu *et al.*, 2003b). This shows that when long chained essential fatty acids are incorporated into PL rather than TAG, they may be assimilated more efficient.

The cell requires energy, in the form of adenosine triphosphate (ATP), to run energy demanding and necessary operations to grow and develop, like synthesis of proteins from amino acids, nucleic acids from nucleotides, transport of molecules against a concentration gradient by ATP-powered pumps, contraction of muscle and beating of cilia. ATP is primarily synthesised from ADP by aerobic oxidation (oxidative catabolism), a process which occurs in the mitochondria (Lodish *et al.*, 2008). When degrading a fatty acid by the action of mitochondrial β-oxidation there will be a release of ATP. The amount of ATP released by degradation of fatty acids is remarkably higher than what is

released from carbohydrate degradation, which in general makes fatty acids a high valued energy source (Waagbø *et al.*, 2001). The enterocyte of the larval gut is highly metabolic active and capable of active transport (Kjørsvik *et al.*, 1991), thus requiring ATP to run energy demanding processes such as re-esterification of absorbed fatty acids into triacylglycerols and phospholipids (Gurr *et al.*, 2002). High energy demanding tissue requires well-functioning cells with abundant mitochondria (Ghadially, 1997), and it might therefore be essential for the survival of the marine fish larvae that the mitochondria in the digestive tissues are well developed and abundant in the cells at the early life stages.

Recent studies by Wold *et al.* (2008) reported a maturation process for the mitochondria in the enterocytes based on gradual changes in the mitochondrial membrane and cristae in cod larvae from 12 dph to 30 dph, with a division into three different types of mitochondria separated by the density of cristae. The mitochondrial membrane consists of phospholipids (Berg *et al.*, 2007), thus phospholipids in the diet might be the decisive component of this maturation. Furthermore, different characteristics in appearance of the mitochondrion can reveal the cells energetic and nutritional state. Swollen mitochondria (Ghadially, 1997) has been found in the hepatocytes in malnourished turbot (*Scophthalmus maximus* L.) larvae (Segner, Witt, 1990), and in the enterocytes of the turbot when fed soy phospholipids (MacQueen Leifson *et al.*, 2003). It appears that damage and swelling of mitochondria leads to suppression in ATP production and interruption of the ATP-dependent sodium pump of the cell membrane (Ghadially, 1997).

1.3 Development of cod larval digestive capacity

1.3.1 Gut development

The cod larval digestive system has an altricial development like many other pelagic fish larvae, meaning that the digestive system is a simple and closed straight tube at hatching, and starts to differentiate morphologically, histologically and physiologically towards its adult mode after the first exogenous feeding (Govoni *et al.*, 1986; Waagbø *et al.*, 2001). At this developmental stage there is no functional stomach present. The mouth and anus will develop during the yolk-sac phase until the larvae is ready to start exogenous feeding (Kjørsvik *et al.*, 1991). The lack of a functional stomach means that

the larva is unable to secrete pepsinogen and hydrochloric acid (HCl) for gastric digestion of the feed (Waagbø *et al.*, 2001), and is one of the main reasons why start-feeding has been a bottleneck in marine pelagic fish larval rearing (Kjørsvik *et al.*, 2004).

Before the onset of exogenous feeding there is a rapid change in sequencing of the gut; it is differentiated into the foregut, midgut and hindgut (Figure 1.1). The foregut, and especially the midgut, seems to be active in absorbing lipids from the diet, while hindgut is characterized by pinocytotic activity (Figure 1.2) (Kjørsvik *et al.*, 1991; Kjørsvik *et al.*, 2004). At this stage the gut is often less than 50 % of the body length (Waagbø *et al.*, 2001). The gut will increase in width and length during the first feeding period, thus increasing its ability to absorb nutrients. The foregut will later, during metamorphosis, develop into the oesophagus and stomach, the midgut into the anterior intestine, and the hindgut into the posterior intestine. Posterior to the oesophagus there may appear a small expansion or swelling, and this is the site where the stomach later will develop (Kjørsvik *et al.*, 2004).

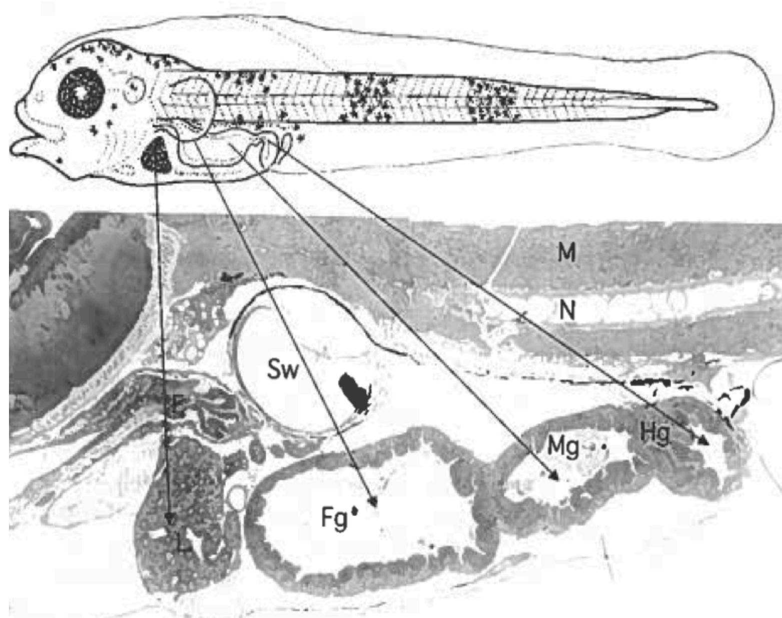

Figure 1.1: Gut differentiation in cod larvae. The drawing (Galloway *et al.*, 1999) illustrates a larva at the onset of feeding, and the picture (Kjørsvik *et al.*, 2004) is a longitudinal histological section that displays the compartments of the gut of a 17-day-old larva. L=liver; Fg=foregut; Mg=midgut; Hg=hindgut; Sw=swimbladder; N=notochord, M=muscle.

Generally, the gut wall (the mucosae) is a thin layer of smooth muscle, connective tissue and squamous epithelium (enterocytes), lined with numerous of absorptive microvilli (the brush border). Mitochondria are abundant within the enterocytes, which reflects the high energy demanding processes of these cells (Young *et al.*, 2006; Wold *et al.*,

2008). Free ribosomes and membranous vesicles containing lipoprotein droplets are also common characteristics of enterocytes. The cells are tightly bound near their luminal surface by tight junctions which prevents leakage of luminal content and provides mechanical support (Young *et al.*, 2006). There are found no digestive gland structures in the cod larval digestive system, thus there are no pepsinogen present (Kjørsvik *et al.*, 2004). However, the mucosa is involved in nervous and hormonal activation of enzyme and bile synthesis and secretion from the pancreas and the liver. The pancreatic cells start to synthesise enzymes before the onset of exogenous feeding, and all the pancreatic digestive enzymes seems to be present in pelagic marine fish larvae at this stage (Hoehne-Reitan *et al.*, 2001a; Hoehne-Reitan *et al.*, 2003; Kjørsvik *et al.*, 2004), thus the functions and tissues that are essential in nutrient digestion, absorption and metabolism are present at the beginning of exogenous feeding.

The gut will continue to grow in volume and length throughout the whole larval phase, making the intestinal folding more abundant and an increasing gut passage with increasing growth (Kjørsvik *et al.*, 2004; Wold *et al.*, 2008). The establishment of an efficient brush border membrane with its associated transport proteins represents a development towards a more adult mode of digestion in the enterocytes (Zambonino-Infante, Cahu, 2001; Gurr *et al.*, 2002; Kvåle *et al.*, 2007), and is affected by nutrient composition of the diets (Zambonino-Infante, Cahu, 2001).

1.3.2 Digestive capacity

The most important digestive enzymes in pelagic marine fish larvae are proteolytic trypsin-like enzymes and lipolytic enzymes such as lipases. These enzymes are produced in the pancreas, which secrets them into the gut where they become active because of the alkaline environment that the bile creates (Kjørsvik *et al.*, 2004). Studies on marine fish larvae digestion have shown that trypsinogen, which is an inactive precursor of trypsin (Pedersen *et al.*, 1990), and bile-salt dependent lipase is synthesised by the pancreatic cells before the onset of exogenous feeding (Hoehne-Reitan *et al.*, 2001b).

Bile and digestive lipases emulsify and brake down neutral lipids and PLs by hydrolysis mainly to polar monoglycerides and free fatty acids after entering the gut lumen. Monoglycerides and free fatty acids are ready to be absorbed by the brush border membrane of the enterocytes in the mucosa layer in the midgut (Figure 1.2). Inside the

enterocytes of the midgut, the fatty acids are re-esterified into triglycerides in the cells agranular (smooth) endoplasmic reticulum. After re-esterification the triglycerides becomes temporarily deposited and stored as large lipid droplets in the cells (Waagbø *et al.*, 2001; Kjørsvik *et al.*, 2004; Young *et al.*, 2006). Feeding larvae with fats that contain mainly TAG leads to problems regarding fat assimilation and accumulation of large lipid droplets in the enterocytes and in the liver occurs. This can lead to problems in digestion when the lipid accumulation is too large to be transported into the blood and further to the liver. This accumulation can however be prevented by adding PLs to the diet (Fontagnè *et al.*, 1998) since the may enhance lipoprotein synthesis (Tocher *et al.*, 2008).

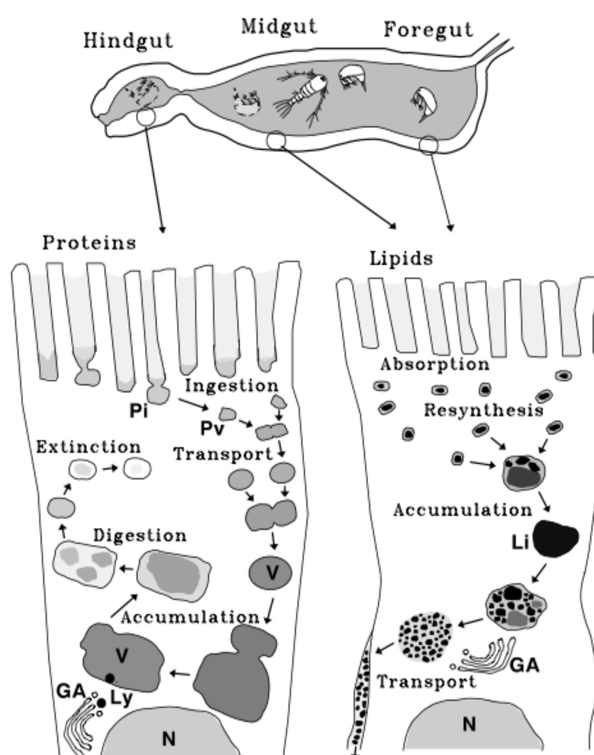

Figure 1.2: Schematic drawing of enterocytes and the nutrient absorption in the different parts of the larval gut (van der Meeren, 1993).

Other important nutrient assimilating processes involves hydrolysing polypeptide fragments, which is facilitated by the pancreatic enzymes including trypsin, chymotrypsin, elastase and carboxypeptidase (Young *et al.*, 2006). The low pH of a functional stomach denatures proteins, so that the peptide bonds become available for the pepsin. Cod larvae lack a functional stomach, thus the first step in protein digestion is omitted (Hamre, 2006). Proteins are digested relatively slow in the stomach-less fish larvae, and are partly broken down to peptides and amino acids in the gut lumen by pancreatic proteases. It is therefore essential that amino acids are provided through live

feed organisms where more than 50 % of the nitrogen is found in free amino acids, peptides and the water soluble proteins (Carvalho *et al.*, 2003). The digestive products are absorbed directly by the hindguts pinocytotic activity (Watanabe, 1982; Kjørsvik *et al.*, 1991) where it is further digested in several intracellular steps (Figure 1.2). The action of pinocytosis is characteristic for an undeveloped digestive system in some species (Kjørsvik *et al.*, 2004).

1.3.3 Liver development

The liver develops during late embryogenesis and the yolk-sac stage as a glandular outgrowth of the primitive gut, and plays an essential role in digestive and metabolic processes (Hoehne-Reitan, Kjørsvik, 2004; Kjørsvik *et al.*, 2004; Young *et al.*, 2006). The liver does not only catabolise fat, carbohydrate and protein, but also functions as storage of energy in the form of glycogen and lipids (Lie *et al.*, 1986; Young *et al.*, 2006). The secretory product of the liver is bile, which acts as an emulsifier on ingested lipids in the gut lumen (Hoehne-Reitan, Kjørsvik, 2004; Young *et al.*, 2006). Thus an optimal functioning liver implies good capacity for lipid digestion and nutrient processing. The liver structure reflects any physiological unbalance due to nutritional problems or starvation, and is thus a good marker for nutritional effects of dietary composition and feeding regimes (Hoehne-Reitan, Kjørsvik, 2004).

The hepatocytes are in general large polyhedral cells (Young *et al.*, 2006) arranged in cords between the sinusoids and bile canaliculi in yolk sac larvae (Morrison, 1993). The hepatocytes have round nuclei that can vary greatly in size (Young *et al.*, 2006), and the hepatonuclear size decreases due to starvation and malnutrition (Hoehne-Reitan, Kjørsvik, 2004). Well-functioning hepatocytes contain large quantities of stored glycogen and lipids (Young *et al.*, 2006), and may function as an indicator of the nutritional status of the larva. The size of the hepatocytes may also vary with the amount of stored glycogen and fat, as found in turbot larvae (Segner *et al.*, 1994) and cod larvae (Kjørsvik *et al.*, Submitted). Hepatocytes executes a wide range of vital biosynthetic and degradative activities, which is reflected by their especially high content of rough endoplasmic reticulum, smooth endoplasmic reticulum, Golgi stacks, free ribosomoes, mitochondria, lysosomes and peroxisomes (Young *et al.*, 2006), thus requiring high amounts of energy. An increase in nucleus surface area coverage might be

a sign of high transport between nucleus and cytoplasm, and implies high metabolic activity (Ghadially, 1997).

The cod larval liver grows allometrically and the growth of the liver seems to be strongly correlated to larval size. Glycogen is the most occurring storage form of energy in the cod larval liver, and lipid droplets are not that commonly observed in larvae that has not yet reached metamorphosis (Kjørsvik *et al.*, Submitted). There is no glycogen present during the early yolk sac stage in Atlantic cod (Morrison, 1993), and has not been found until 15-17 dph in previous studies (Kjørsvik *et al.*, 1991; Kjørsvik *et al.*, Submitted). The mitochondria in hepatocytes can vary in appearance and reflect the nutritional status of the larvae. Kjørsvik *et al.* (Submitted) found that cod larvae without visible glycogen stored in the liver had mitochondria that appeared somewhat enlarged and had more empty inner space. They also found that these larvae had unusual cup-shaped and more irregularly shaped mitochondria, which was suggested to be a sign of cellular stress due to lower nutritional status in these larvae. Another observation from these studies was that glycogen levels were positively correlated to hepatocyte size and nuclei size, and suggests that hepatonuclear size can be used as a measurement on nutritional status in developing cod larvae.

1.4 Aim of the study

The aim of this study is to evaluate the effect of feeding cod larvae with intensively cultivated nauplii of the copepod *Acartia tonsa* compared to rotifers of different nutritional value, in terms of larval growth, survival, and functional development of enterocytes and hepatocytes from 4 dph to 19 dph. The study will also examine if there are any effects of feeding with copepods for only a short period in the first days of development. The nutritional effects of copepods will be evaluated against feeding with enriched and unenriched rotifers, to examine if the nutritional differences in the live feed can be distinguished in the tissue of the digestive system. To evaluate this the following objectives will be studied for the larval gut and liver:

- area fraction of nuclei, mitochondria and glycogen
- area size of cells, nuclei and mitochondria
- general membrane structure appearance within the cells
- cristae appearance within the mitochondria

With stereological methods it is possible to determine area size of cells and surface area fractions of organelles, which can provide results indicating the development over time. Histological observations will also be a helpful tool in determining the appearance of different characteristics in organelles, with special emphasis to the mitochondria structures and maturation of both enterocytes and hepatocytes. One section through the liver, and two sections of the gut will be examined, respectively the foregut and the midgut, which is especially active in lipid absorption.

2 Materials and methods

2.1 Start-feeding of cod larvae

2.1.1 Larval rearing

Atlantic cod eggs were supplied from a breeding company (Nasjonal avlsstasjon for torsk, Havbrukssenteret i Tromsø) and were disinfected with 400 ppm glutaraldehyd for 8 minutes according to Salvesen and Vadstein (1995) before incubation in darkness in a cone-bottomed cylindrical tank with 270 L sweater (34 ppt). Temperature in the incubator was 6 ^{o}C, the seawater was moderately aerated, the water exchange was 3 litre hour^{-1}, and the waste was flushed out by opening the vent in the bottom each day.

Two days prior to hatching the eggs were transferred to 100 L cone-bottomed cylindrical rearing tanks with a density of 100 eggs L^{-1}. The volume of eggs in the rearing tanks was estimated from a measurement of the eggs diameter according to Holm *et al.*, (1991). The seawater in the tanks was sand filtered, filtered through a 1 µm mesh, and microbially matured (Skjermo *et al.*, 1997) before entering the tanks. Each tank had a moderate aeration, and they were all equipped with surface skimmers. The water exchange was gradually increased from 2 times day^{-1} to 8 times day^{-1} until 31 dph, and temperature was gradually increased from 6 to 12 ^{o}C (Table 2.1). Each larval rearing tank was illuminated 24 hours a day by two fluorescent tubes á 18W/965.

The sampling of larvae was carried out by randomly selecting larvae from each tank. All of the larvae that were sampled for the different purposes were anesthetized with tricaine methanesulfonate (MS-222, Finquel®, Argent Chemical Laboratories Inc., USA) before further treatment.

2.1.2 Larval feeding regimes

Four different feeding regimes were established, and each treatment had three replicates (Table 2.1). The feeding regimes differed in type of live feed in the first 20-28 dph. The density of the feed organisms in the tanks was 1.2 million 100 L^{-1}. The larvae that received the Copepod treatment were fed *Acartia tonsa* nauplii at stage NIV, which is equivalent to the size of the rotifers (*Brachionus ibericus*) (Cayman type) (Penglase *et al.*, 2010; Alver *et al.*, 2011) that were fed to larvae receiving the RotMG and RotChl treatment. The larvae that received the RotMG treatment were fed rotifers that were

enriched with Multigain (BioMar AS, Norway). Larvae that received the RotChl treatment were fed unenriched rotifers. The Cop 7 treatment was a co-feeding regime of copepods and enriched rotifers. This treatment received variable fractions of each live feed organism due to the variable hatching success of *A. tonsa* nauplii (Table 2.2).

The copepods and rotifers were distributed manually in the cod rearing tanks three times a day. *Artemia* sp. and formulated feed (Gemma Micro 300, Skretting AS, Norway) were distributed into the cod rearing tanks by a feeding robot (Storvik aqua AS, Norway). The cod larvae were fed *Artemia* sp. four times a day, and the dry feed (10 g $tank^{-1}$ day^{-1}) was fed to the fish four times an hour for 24 hours a day. A paste of the green algae *Nannochloropsis occulata* (Reed Mariculture Inc., USA) was added to the tanks 3 times a day (2 mg C L^{-1}) (Reitan *et al.*, 1993) during the live feed period. There was a weaning period between each transition in feed type (Table 2.1).

Table 2.1: Experimental layout for the start-feeding of cod, including the four different feeding regimes. The table includes when sampling and cleaning was executed, and displays the gradual increase in water exchange and temperature.

	May																			
Date	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
D°	6	12	18	24	31	38	46	54	63	72	82	92	103	114	126	138	150	162	174	186
Dph	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Sampling	X	X		X	X			X						X					X	
Cleaning	X									X		X		X		X		X		X
Water exchange day^{-1}	2	2	2	2	2	2	3	3	3	3	4	4	4	4	4	4	4	6	6	6
Temperature (°C)	6	6	6	6	7	7	8	8	9	9	10	10	11	11	12	12	12	12	12	12

	May									June											July
Date	23	24	25	26	27	28	29	30	31	1	2	3	4	5	6	7	8	9	10	11	1
D°	198	210	222	234	246	258	270	282	294	306	318	330	342	354	366	378	390	402	414	426	
Dph	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	60
Sampling													X							X	X
Cleaning		X		X		X		X		X		X	X	X	X	X	X	X	X	X	X
Water exchange day^{-1}	6	6	6	6	6	6	6	6	6	6	8	8	8	8	8	8	8	8	8	8	8
Temperature (°C)	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12

Treatment

- 3 x Copepod: *Artemia sp. (Multigain)*; *Acartia tonsa (Rhodomonas baltica)*; Dry feed (Gemma micro)
- 3 x RotMG: *Artemia sp. (Multigain)*; *Brachionus ibericus* (*Chlorella* sp., Multigain); Dry feed (Gemma micro)
- 3 x RotChl: *Artemia sp. (Multigain)*; *Brachionus ibericus* (*Chlorella* sp.); Dry feed (Gemma micro)
- 3 x Cop 7: *Artemia sp. (Multigain)*; *Acartia tonsa (R.baltica)*; *Brachionus ibericus* (*Chlorella* sp., Multigain); Dry feed (Gemma micro)

Table 2.2: The fraction of rotifers and copepods supplied in the Cop 7 treatment.

Dph	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Copepod (*A. tonsa*) (%)	100	100	100	100	57	40	54
Rotifer (*B. ibericus*) (%)	0	0	0	0	43	60	46

2.2 Live feed production

2.2.1 Microalgae

The microalgae *Rhodomonas baltica* (clone NIVA 5/91 crypthophycea: pyrenomonadales) was cultivated, and used as feed for the copepod culture (*Acartia tonsa*). *Rhodomonas baltica* was cultivated semi-continuously at a temperature of 20 ^oC in 300 L plastic bags and in 160 L and 200 L polycarbonate plastic cylinders (40 cm in diameter) with seawater (34 ppt), which was disinfected by adding sodium hypochlorite (NaClO) and after 5 hours neutralised by adding sodium thiosulphate ($Na_2S_2O_3$) (Hoff, Snell, 1987). The dilution rate of the algae culture was 40-50 % day^{-1}, and 1.5 mL Conwy medium (Walne, 1974) per litre of cultivation volume was added to the culture after dilution for nutrient enrichment. The culture was held at a density above 1 million cells/litre (counted with Multisizer™ 3 Coulter Counter (capillary diameter 100 μm), Beckman Coulter Inc., USA), pH values (measured with a pH/mV-meter, WTW pH 315i, Germany) were held at 7.5-8.3 (from diluted to concentrated culture) and the culture was aerated with 1-2 % CO_2. The cultures were grown with continuous light, and each cylinder was illuminated by six fluorescent tubes á 58 W/840 from three sides of each cylinder.

2.2.2 Copepods

Eggs of the copepod *Acartia tonsa* Dana (clone DFH.AT1) were collected for hatching of nauplii that was fed to the cod larvae. The *Acartia tonsa* was cultivated in seawater (34 ppt) in one 1000 L tank and one 1600 L tank at 20^oC, and were fed *Rhodomonas baltica ad libitum* (Nesse, 2010). The minimum density of available algae was 30 000 cells mL^{-1} (Skogstad, 2010). The water exchange was 100 % day^{-1}, pH was 7-8 (measured with a pH/mV-meter, WTW pH 315i, Germany), the oxygen (O_2) level was held above 60 % saturation (measured with OxyGurad Handy Polaris Portable DO Meter, WMT, USA), and the culture was continuously illuminated. Eggs produced by the culture sedimented to the bottom of the tanks, and were collected by swiping the bottom so that the eggs could be collected in a bucket by siphoning. The eggs were sieved through a 120 μm mesh (to get rid of waste) and then through a 64 μm mesh where they were concentrated and washed until the colour turned dark blue as an indicator of a clean sample. They were transferred to a 55 mL EasyFlasks™ (Nunc A/S, Denmark) with seawater (34 ppt) and

stored at 2 ^{o}C with water exchange every 2-3 weeks. In each batch of eggs the hatching success was tested (Appendix 2).

The stored copepods eggs were hatched, and the nauplii were used as live feed for the cod larvae. The copepod eggs where hatched with a density of 150 eggs mL^{-1} in 100 L tanks with a flat bottom, and in the same conditions as the adult individuals, except there were no continuous flow through and slightly more aeration. 1-2 L of *Rhodomonas baltica* was added to the tanks to trigger hatching. From 3 to 6 dph the nauplii were transferred and further cultivated in 300 L tanks with a cone bottom, continuous flow through (100 % exchange day^{-1}), and aeration from the bottom. The nauplii were fed *Rhodomonas baltica* (30 000 cells mL^{-1}) until they had reached a length of 180 μm (NIV).

On the day before feeding the nauplii to the fish larvae they were counted with an automatic counter (Alver *et al.*, 2007) to ensure correct harvesting volume. The required amount of nauplii was pumped out of the tank and concentrated with a 64 μm sieve before being fed to the fish larvae.

2.2.3 Rotifers

Rotifers (*Brachionus ibericus*, Cayman type) were cultured semi-continuously in 250 L cone bottomed tanks with seawater (34 ppt). The temperature was maintained at 25 ^{o}C, water exchange was 100 % day^{-1}, and O_2 level was held above 75 % saturation. The density of the rotifer culture was held under 800 rotifers mL^{-1} to maintain a proper environment for further growth of the culture. The rotifers were continuously fed a paste of the green algae *Chlorella* sp. (Chlorella Industry Co., Ltd., Japan), and the amount of feed (2.4 mL *Chlorella* sp. million $rotifer^{-1}$) added to the feeding containers was calculated from the density of rotifers in the tank.

The rotifers used for feeding the cod larvae that received the RotChl treatment was collected directly from the cultivation tanks, rinsed, and distributed in the cod larval rearing tanks. The rotifers used for feeding of the cod larvae receiving the RotMG treatment and the Cop 7 treatment were short-time enriched with Multigain (BioMar AS, Norway) (0.2 g million $rotifer^{-1}$) in a separate tank with a density less than 400 rotifers mL^{-1}, for two hours. The rotifers were rinsed before being distributed in the cod larval rearing tanks.

2.2.4 *Artemia* sp.

Artemia franciscana (EG® INVE Aquaculture, Belgium) cysts were decapsulated according to Sorgeloos *et al.*, (1977) before set to hatch in a cylindrical cone bottomed 60 L tank with seawater (34 ppt) and aeration. The hatched *Artemia* sp. were washed and transferred to an enrichment tank with 60 L of sweater with heavy aeration. The density in the enrichment tank was 100-300 nauplii mL^{-1}. The temperature was maintained around 25-28 ^{o}C. The *Artemia* sp. was enriched with 10 g 60 L^{-1} Multigain (BioMar AS, Norway) twice in 24 hours. The *Artemia* sp. were rinsed before being distributed in the cod larval rearing tanks.

2.3 Larval growth

2.3.1 Dry weight

For dry weight measurements, 12 larvae were sampled from each tank at 2, 5, 8, 14 and 19 dph, 20 larvae were sampled at 33 dph, and 50 larvae were sampled at 40 and 60 dph (Table 2.1). The larvae were rinsed in distilled water before transfer to individual pre-weighted tin capsules, dried at 60 ^{o}C for 24 hours before being analysed. Larvae sampled at 2, 5, 8, 14, and 19 dph was analysed for carbon and nitrogen content in an Elemental combustion analyser (Costech Analytical Technologies Inc., USA) using acetanilide as standard (performed by Marte Schei, SINTEF). The weighing of larvae sampled at 33, 40, and 60 dph was performed with two micro balances (UM3 and UMX2, Mettler-Toledo Inc., USA).

The carbon content (μg C $larva^{-1}$) measured on larvae sampled at 2, 5, 8, 14, and 19 dph was multiplied with a factor of 2.34, based on the average carbon content of fish larvae, to obtain the cod larval dry weight (Reitan *et al.*, 1993). Dry weight of larvae sampled at 33, 40 and 60 dph was obtained directly by weighing. The specific growth rate (SGR) was calculated by the equation according to Kjørsvik *et al.* (2004):

$$SGR = ln\ (W_t/W_0)/t \qquad (2.1)$$

where W_0 is the initial dry weight and W_t is the dry weight after time t. Further, the daily weight increase (%) (DWI) was calculated for each dry weight sampling interval, according to Kjørsvik *et al.* (2004):

$$\%\ DWI = (e^{SGR}-1)x100\% \qquad (2.2)$$

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

Standard length of fixed larvae was measured from the tip of the snout to the end of the notochord, and myotome height was measured perpendicular to the axial skeleton behind the anus (Figure 2.1). The measurements were performed on 5 larvae from 4 dph and from 19 dph, and on 30, 45 and 45 larvae from each treatment at respectively 19, 33, 38 and 60 dph. The measurements were performed with a stereomicroscope (Leica MZ7.5, Leica Microsystems, Germany), at magnifications 1.25, 2.00 and 5.00 adjusted to the size of the larvae. The values were set at the nearest 0.05 mm.

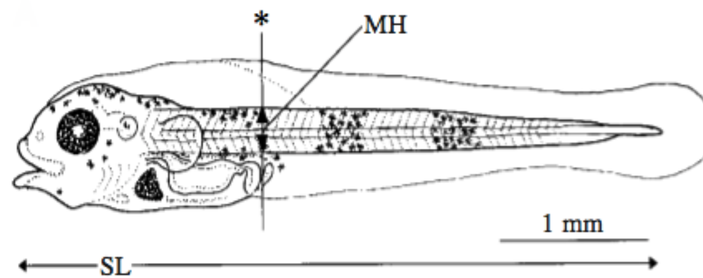

Figure 2.1: Schematic drawing of a cod larvae at the onset of exogenous feeding, showing where the points of measuring standard length (SL) and myotome height (MH) are set (Galloway *et al.*, 1999).

2.3.3 Survival

Survival was measured when cleaning the tanks from 38 dph to 60 dph (Table 2.1), and dead fish larvae in the wastewater was counted and registered. Larvae survival was calculated from the initial number of hatched eggs and from the number of larvae at 60 dph, and was corrected for sampling.

2.4 Histological analyses

Five larvae from 4 dph and five larvae from each treatment at 19 dph were fixed in 2.5 % paraformaldehyde and 2.5 % glutardialdehyde in 0.08M cacodylic buffer (Galloway *et al.*, 1998) (Appendix 1) and stored in glass vials in a cold room. The larvae were embedded in EPON (Appendix 3), and then cut in 1 μm thick sections for further analyses in light microscope, and in 70 nm thick sections for further analyses in transmission electron microscope, with an Leica Richert Ultracut microtome (Leica Microsystems, Germany) (Bozzola, Russel, 1992) (sectioning performed by Tora Bardal, NTNU). The larvae were cut in three different sections (Figure 2.2); one for analyses of the liver (liver section), one for analyses of the foregut (T1 section), and one for analyses of the midgut (T2 section).

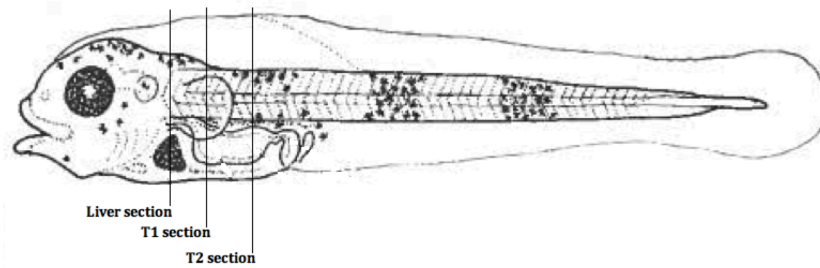

Figure 2.2: Schematic drawing (modified from original) of a cod larvae at the onset of exogenous feeding (Galloway *et al.*, 1999), showing where the sections where cut for histological analyses on the liver and gut.

2.4.1 Light microscope

The sections were stained with Toluidine Blue and Basic Fuchsin (Appendix 4) and studied with a Zeiss Axioskop 2 plus microscope (Zeiss Inc., Germany) equipped with a JVC TK-C1381 color video camera (JVC, Japan). The sections where analysed consecutive with the stereological analysis program CAST 2 (Olympus Inc., Denmark).

The whole cross section of the liver and gut was used to quantitatively measure surface area of these sections, hepatocyte area size and enterocyte area size in CAST 2. The cell area sizes were calculated directly from the number of nuclei divided on the area of the organ.

2.4.2 Transmission electron microscope

The section were contrasted with lead citrate (Appendix 5) and observed in a Jeol JEM-1011 transmission electron microscope (Jeol LTD., Japan) at an accelerating voltage at 80 kV. The photos taken from the transmission electron microscope was further analysed with the stereological analysis program CAST 2 (Olympus Inc., Denmark) for quantitative measurements. In CAST 2 a point grid was located on all of the photos (x5 000 magnification) taken from the liver and intestine. On each photo there were added six counting frames ensuring that there was 72 point per counting frame to cover an adequate area (based on previous tests (Wold, NTNU, pers. com.). Each point measured a given area, and the total area covered by mitochondria, nucleus and glycogen was measured. The measurements were based on the amount of organelles displayed in the randomly selected photos from the sections. This gave a two-dimensional quantification, and fractions of area were calculated (Gundersen *et al.*, 1988). Qualitative studies of the

cell structure, and especially the mitochondrial structure and appearance, were done with electron microscopic photos taken with x10 000 magnification.

2.5 Statistical analysis

All data presented as percentage were transformed to arc sine values before statistical testing was performed. Normality was tested with a Shapiro-Wilk-test ($P<0.05$). The data were further tested for homogeneity of variance using a Levene test ($P<0.05$). To compare means the data was tested using a one-way ANOVA ($P<0.05$) followed by the post-hoc-tests Dunnett T3 test and the Student-Newman-Keuls-Test for comparison of the different treatments. Correlations were tested by using linear regression analyses, and with the Pearson correlation test ($P<0.05$ and $P<0.01$). For the data that was not normally distributed, the non-parametric Kruskal-Wallis-test was applied ($P<0.05$). Standard error (SE) was calculated for all data that had replicates, and is illustrated by error bars in figures. All figures, tables and statistical analyses that are presented as results were performed with the software PASW Statistics 18.0 for Mac OS X (SPSS Inc. USA).

3 Results

3.1 Larval growth, survival and characteristics

3.1.1 Dry weight

Already at 8 dph the larvae fed copepods had significantly higher dry weight (71.6±2.7 μg $larva^{-1}$ for the Copepod treatment and 70.7±2.5 μg $larva^{-1}$ for the Cop 7 treatment) than the larvae fed rotifers (Figure 3.1). The difference between the Copepod treatment and the other treatments continued in the same trend throughout the whole start-feeding period (Figure 3.2). However, from 14 dph the larvae from the Cop 7 treatment were significantly lower in dry weight than the larvae from Copepod treatment. At this point the larvae from the Cop 7 treatment were no longer receiving *A. tonsa* nauplii and were only fed enriched rotifers.

From 33 dph to 40 dph the larvae were weaned from *Artemia* sp. to formulated feed, and in this period there was a slowdown in dry weight increase for the larvae from the Cop 7, RotMG and the RotChl treatment. However, the larvae from the Copepod treatment did not have such a slowdown, and continued to increase its weight thorough this period.

At 60 dph the larvae from the copepod treatment (22.27±1.44 mg $larva^{-1}$) had significantly higher dry weight than the other larvae, and the larvae from the RotChl treatment (11.98±0.67 mg $larva^{-1}$) had significantly lower dry weight than the copepod-fed larvae. Dry weight of larvae from the RotMG treatment did not differ significantly from the RotChl treatment or the Cop 7 treatment at 60 dph, and the dry weight of the larvae from the Cop 7 treatment were somewhat in between the RotChl and the Copepod treatment (Figure 3.2).

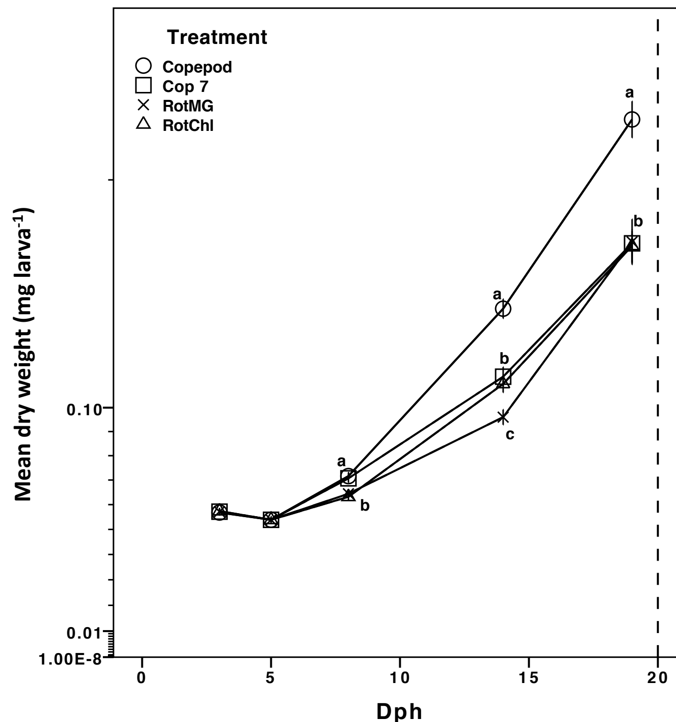

Figure 3.1: Mean dry weight (mg larva^{-1}) for the four different treatments in the rotifer/copepod live feed period of 20 days (n=9-12) (y-axis presented in logarithmic scale). The dashed line denotes the start of feeding with *Artemia* sp. The letters indicates significant differences at 8, 14 and 19 dph. Error bars indicate ± SE. The dry weight data with standard error are presented in Table A.1 Appendix 6.

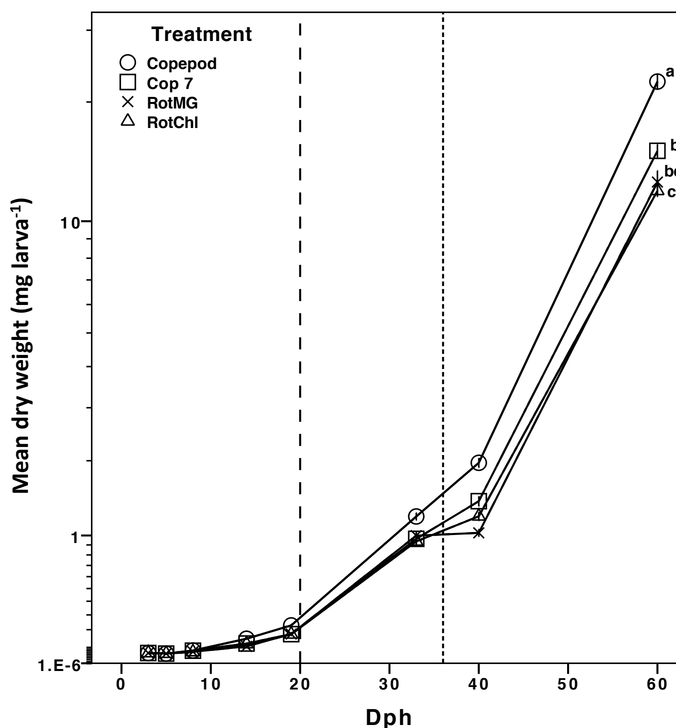

Figure 3.2: Mean dry weight (mg larva^{-1}) for the whole start-feeding period of 60 days (n=9-50) (y-axis presented in logarithmic scale). The dashed line denotes the start of feeding with *Artemia* sp. The dotted line denotes the start of the dry-feed period. The letters indicates significant differences at 60 dph. Error bars indicate ± SE. The dry weight data with standard error are presented in Table A.1 Appendix 6.

3.1.2 Daily weight increase (DWI)

DWI was negative during the first days prior to exogenous feeding due to absorption of yolk. Significant differences in DWI between the treatments were found in the rotifer/copepod period (5-19 dph) and in the weaning period from live- to dry-feed (33-40 dph), with the copepod treatment (10.8±0.1 % at 5-19 dph, and 10.8±0.5 % at 33-40 dph (Table A.2 Appendix 7)) having a significantly higher DWI than the other three groups in these periods (Figure 3.3). The DWI showed a decrease at 33-40 dph (weaning from *Artemia* sp. to dry feed) for the larvae from the RotChl, RotMG and Cop 7 treatments, when compared to the other DWI intervals. This was not the case for the larvae from the Copepod treatment, which had maintained its DWI at this point. The DWI for the whole start-feeding period (2-60 dph) was 11.0±0.2 % for the Copepod treatment, 10.3±0.2 % for the Cop 7 treatment, 9.8±0.2 % for the RotMG and for the RotChl treatment (Table A.1 Appendix 7), where the Copepod treatment had significantly higher total DWI than the three other treatments (Figure 3.3). This demonstrates that the larvae had higher weight increase when being fed copepods exclusively for the first 20 dph, and that this advance continued throughout the whole start-feeding period.

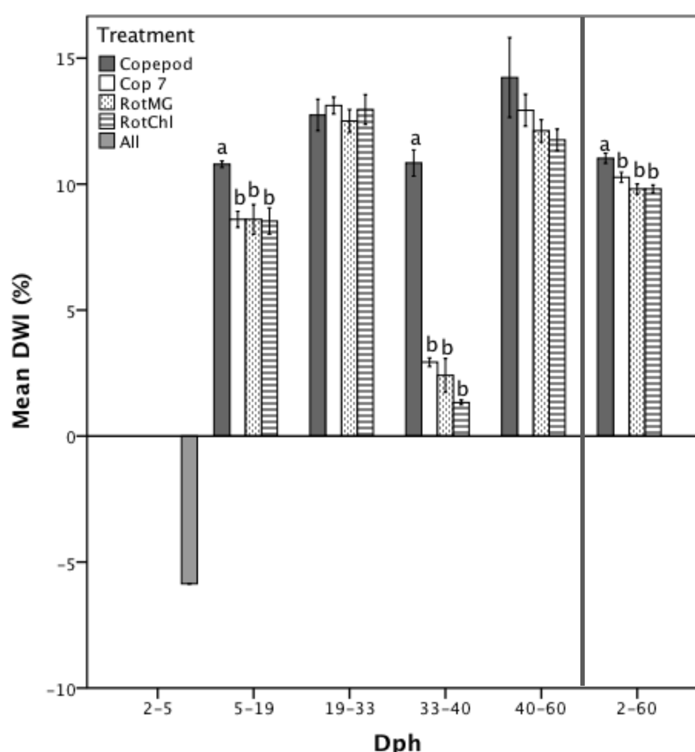

Figure 3.3: Daily weight increase (DWI (%)) for the cod larvae during the whole start-feeding period of 60 days split into five intervals according to sampling days (n=9-50), and one for the whole period. Letters indicate significant differences between treatments. Error bars indicate ± SE. Dataset of DWI with standard error is presented in Table A.2 Appendix 7.

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

There were significant differences in both SL and MH between the treatments at all sampling days (Figure 3.4). The larvae from the Copepod treatment showed significantly higher SL and MH than the larvae that received the other treatments at all days, except on day 33 where SL of larvae from the Cop 7 treatment (9.45±0.14 mm) did not differ significantly from SL of larvae from the Copepod treatment (9.94±0.17 mm). The MH values were significantly higher at all days for the larvae from the Copepod treatment. At the end of the experiment the larvae from the Cop 7 treatment had significantly higher SL than the larvae from the RotMG and the RotChl treatment, but still had significantly lower SL than the larvae from the Copepod treatment. The two rotifer treatments were among those treatments with lowest SL and MH values through the whole start-feeding period.

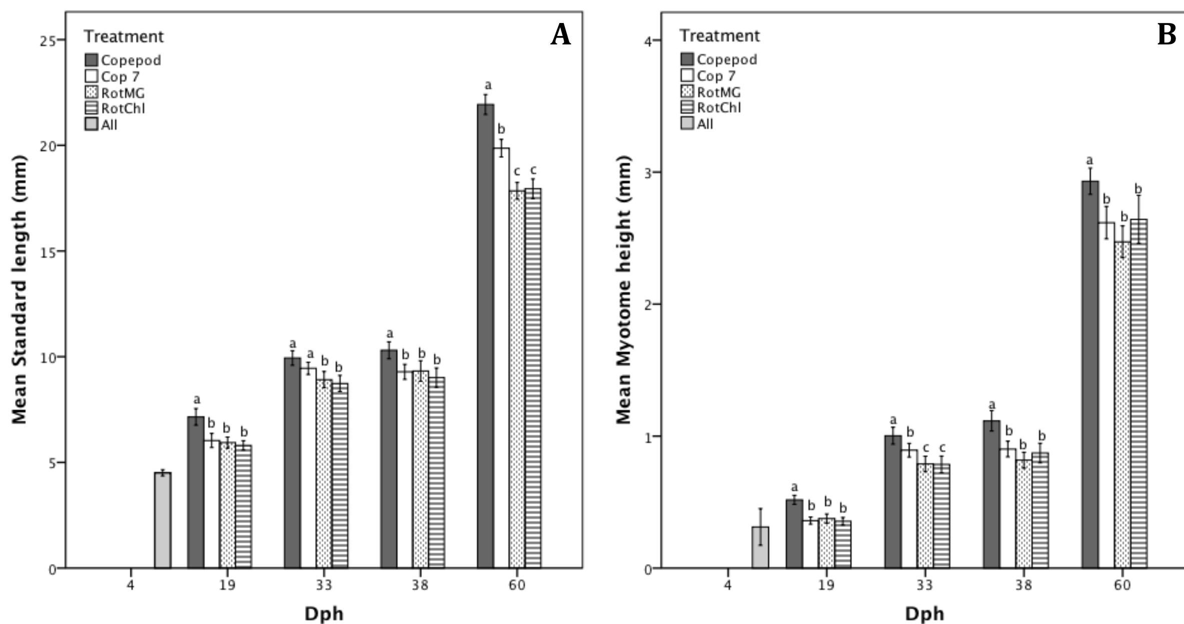

Figure 3.4: A) Measured standard length (SL) at 4 dph, 19 dph, 33 dph, 38 dph and 60 dph (n= 5-45). Letters indicate significant differences between treatments on each day. B) Measured myotome height (MH) at 4 dph, 19 dph, 33 dph, 38 dph and 60 dph (n=5-45). Letters indicate significant differences between treatments on each day. Error bars indicate ± SE.

3.1.4 Pigmentation

Differences in pigmentation were observed between the treatments during the start-feeding experiment. The larvae from the Copepod treatment were generally more pigmented than the larvae from the other treatments, and the copepod-fed larvae also had a more yellow tint. The larvae from the RotChl treatment were, compared to larvae from the other treatments, less pigmented.

3.1.5 Survival

At 38 dph the Copepod treatment had significantly more survivors (32±1 %) than the other three treatments. However, this difference was no longer significant at 45 dph, but the difference had increased at 60 dph where the Copepod treatment (20±2 %) again had significantly more survivors than the other treatments (Table 3.1). The RotMG treatment and RotChl treatment showed quite similar numbers and trends in survival throughout the whole experiment, with respectively 11±1 % for RotMG and 10±2 % for RotChl at 60 dph. The Cop 7 treatment had a survival of 14±3 % at 60 dph.

Table 3.1: Mean survival (%) ± SE of each treatment at 38, 45 and 60 dph (n=3). Letters denote significant differences. Data for daily measured survival from 38 to 60 dph is presented in Table A.3 Appendix 8.

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

3.2 Gut morphology

The larval intestinal tissue was lined by columnar epithelium with numerous microvilli. Very little feed was observed in the larval gut lumen at 19 dph. There was observed differences between larvae at 4 dph and 19 dph where the organelle membrane structures generally were more sharply defined and distinct at 19 dph (tissue from T1 and T2 sections at 4 dph (Figure 3.5 A) and 19 dph (Figure 3.5 B-E)). Mitochondria and other organelles were not as distinguishable from the cytoplasm at 4 dph, as they were at 19 dph. Also endoplasmic reticulum (ER) was more abundant and distinct at 19 dph than at 4 dph. At 19 dph incidents of undefined and interdigitated cristae were found in the T2 sections in larvae from the RotChl treatment (Figure 3.5 E). Very few lipid droplets were observed in the intestinal tissue, and were not quantified since no lipid droplets were covered by the counting frame in CAST 2.

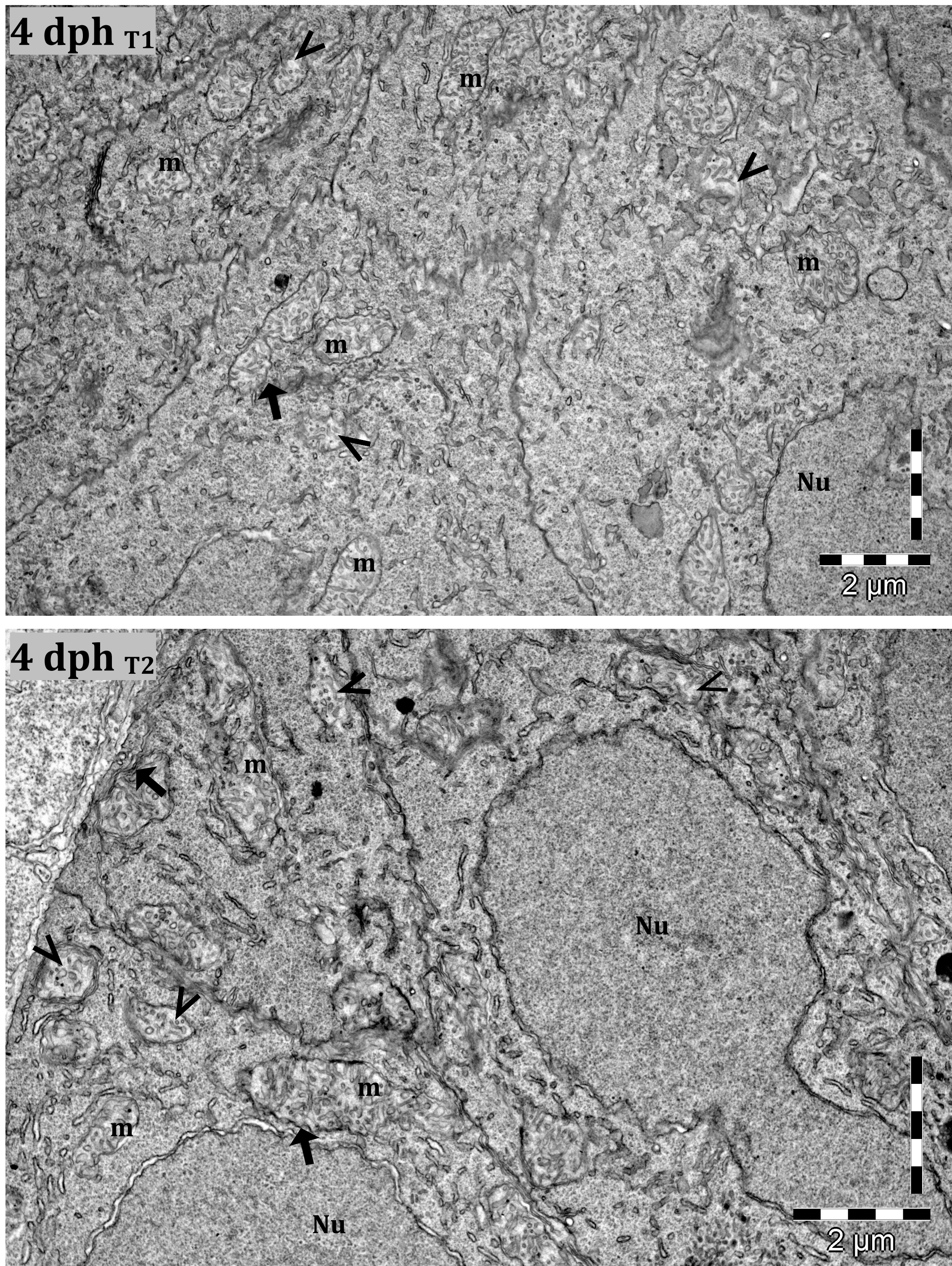

Figure 3.5 A: Transmission electron microscopy photos that show the general appearance of the intestinal tissue in the T1 and the T2 sections from larvae at 4 dph. Nuclei (Nu) and mitochondria (m) are displayed on the photos. No lipid droplets were observed within the enterocytes at 4 dph. Figure continues on pp. 32-35. The photos illustrates that there was a difference in organelle membrane distinctness over time. The larvae had rugged and less distinct outer mitochondrial membranes at 4 dph (A) (arrows) than it generally had at 19 dph (B, C, D, E). Incidents of less dense cristae structures were found at 4 dph (A) (arrowheads).

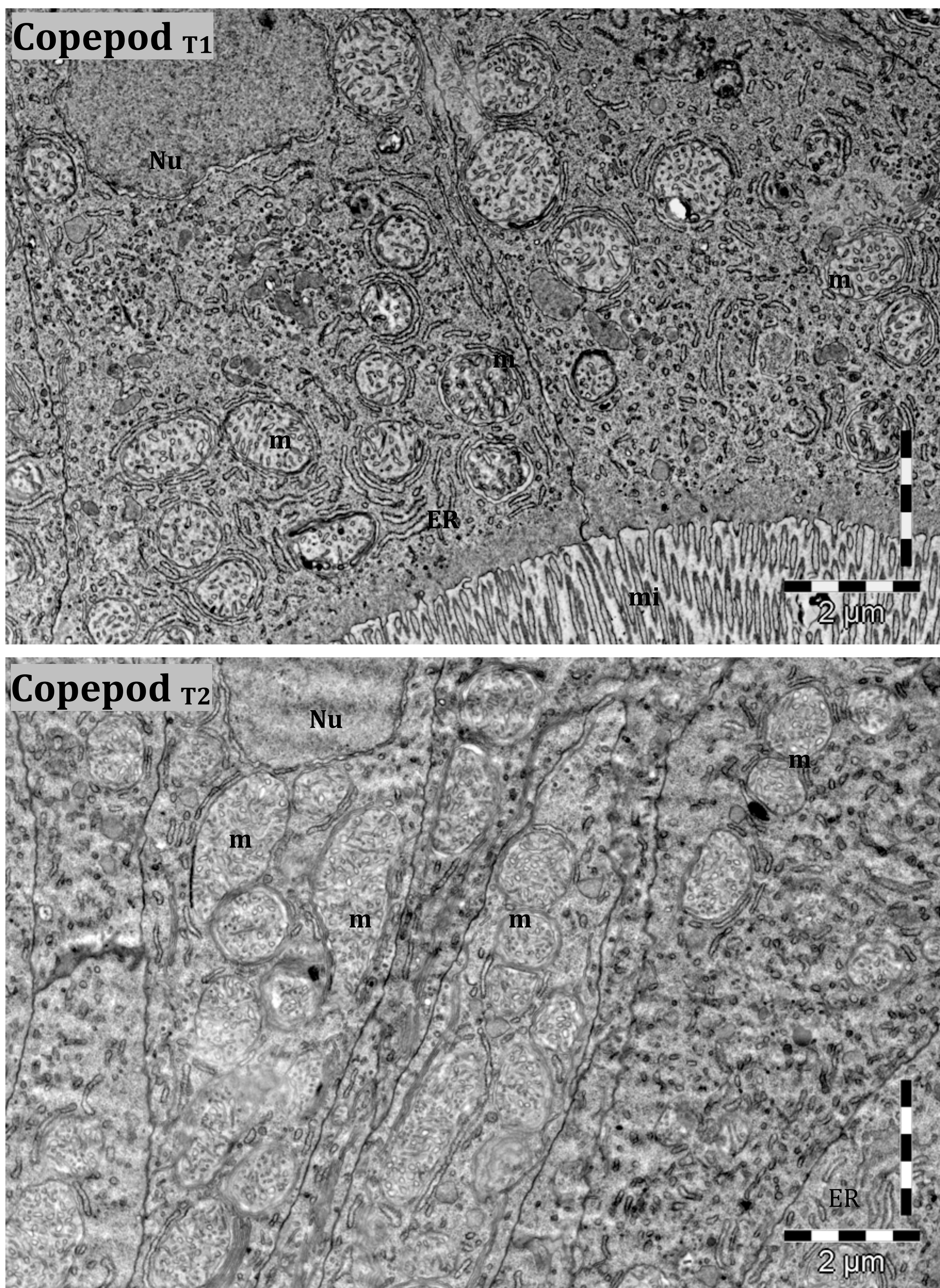

Figure 3.5 B: Transmission electron microscopy photos that show the general appearance of the intestinal tissue in the T1 and the T2 sections from larvae from the Copepod treatment at 19 dph. Nuclei (Nu), mitochondria (m), endoplasmic reticulum (ER) and microvilli (mi) are displayed on the photos. Figure continues on pp. 33-35.

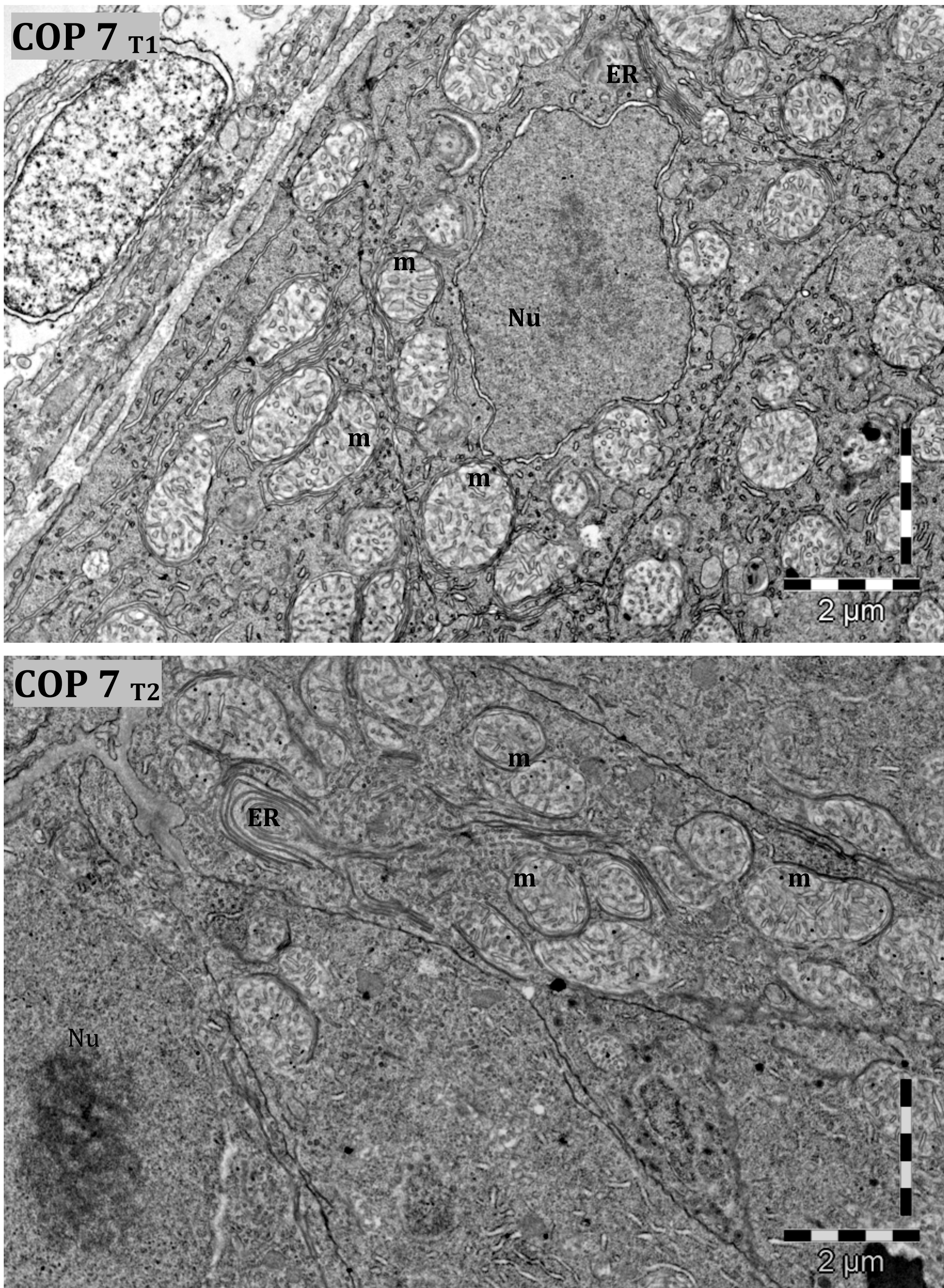

Figure 3.5 C: Transmission electron microscopy photos that show the general appearance of the intestinal tissue in the T1 and the T2 sections from larvae from the Cop7 treatment at 19 dph. Nuclei (Nu), mitochondria (m), and endoplasmic reticulum (ER) are displayed on the photos. Figure continues on pp. 34-35.

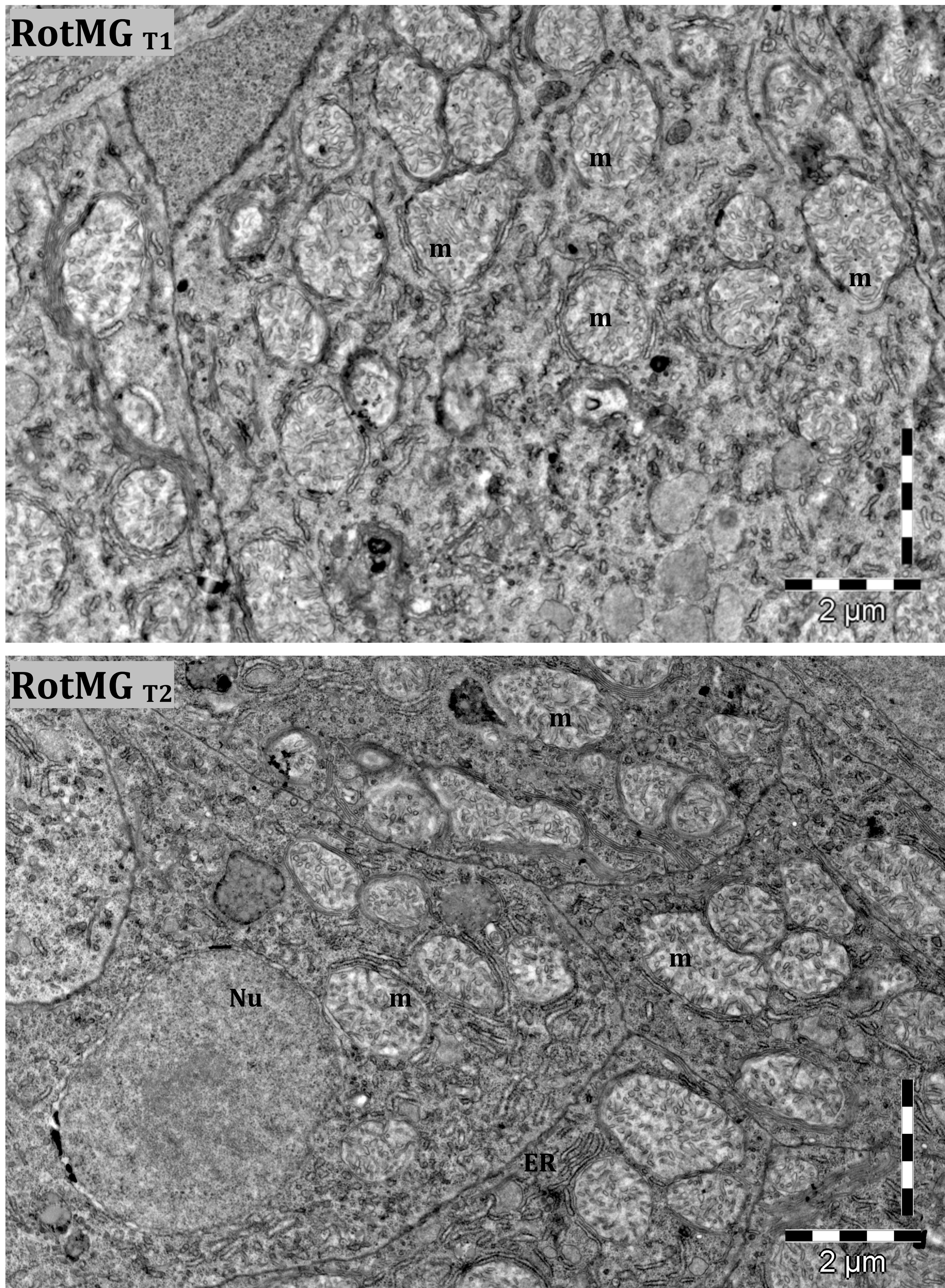

Figure 3.5 D: Transmission electron microscopy photos that show the general appearance of the intestinal tissue in the T1 and the T2 sections from larvae from the RotMG treatment at 19 dph. Nuclei (Nu), mitochondria (m), and endoplasmic reticulum (ER) are displayed on the photos. Figure continues on p. 35.

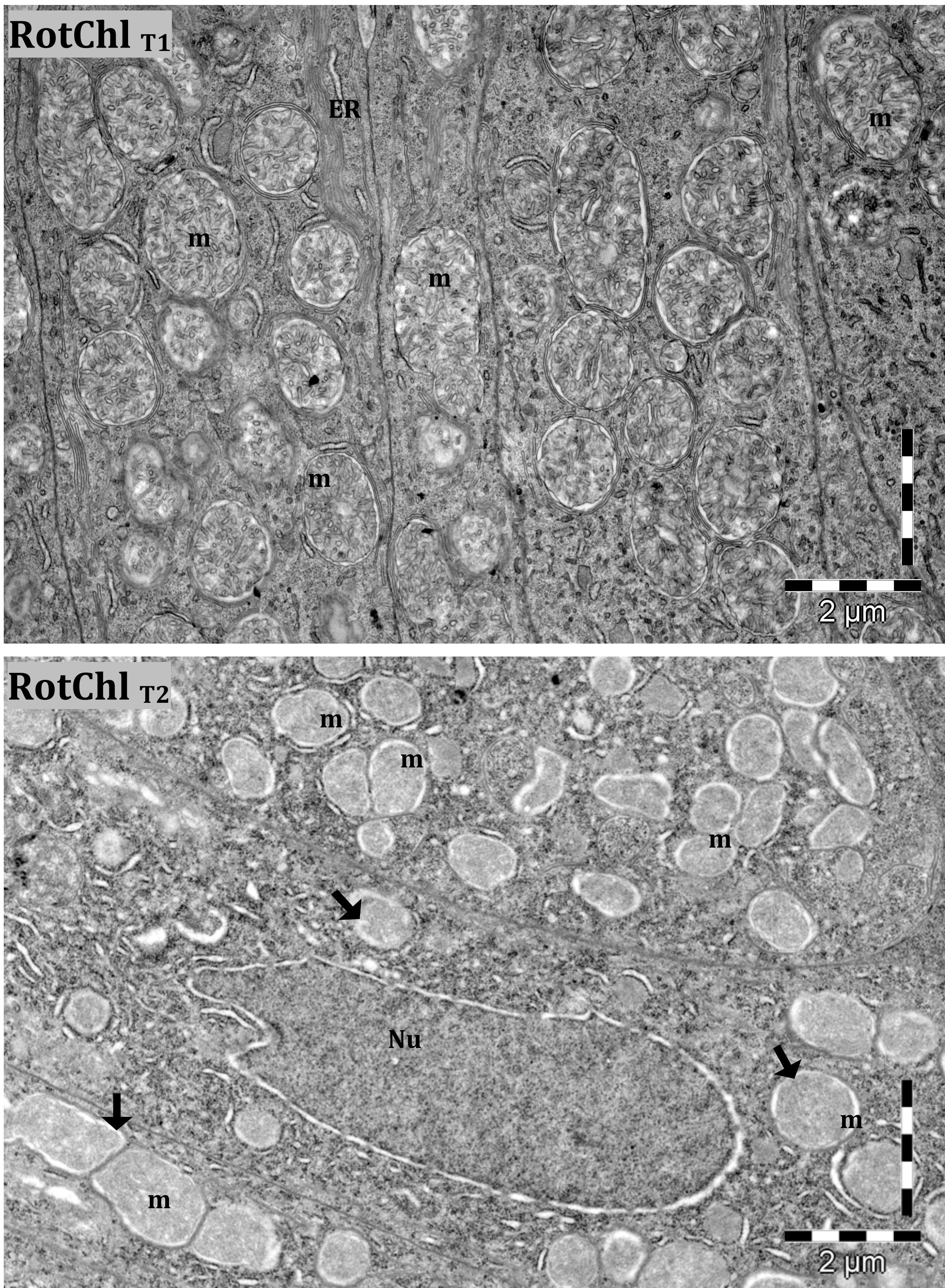

Figure 3.5 E: Transmission electron microscopy photos that show the general appearance of the intestinal tissue in the T1 and the T2 sections from larvae from the RotChl treatment. Nuclei (Nu), mitochondria (m), and endoplasmic reticulum (ER) are displayed on the photos. Photo RotChl $_{T2}$ illustrates the appearance of undefined and interdigitated cristae (arrows) within the enterocytes mitochondria of a larva from the RotChl treatment.

3.2.1 Mitochondrial appearance

Figure 3.6 illustrates the quantification of the observed appearance of the outer mitochondrial membrane in enterocytes in the T1 sections and in the T2 sections, both from larvae at 4 dph and larvae from the four different treatments at 19 dph. The membrane appearance was either smooth or rugged, and the photos in Figure 3.5 show examples of rugged outer mitochondrial membrane structure in enterocytes in the larvae at 4 dph (Figure 3.5 A) and a smooth outer membrane structure at 19 dph from the Copepod treatment (Figure 3.5 B). The outer mitochondrial membrane was generally much less defined at 4 dph than the smooth and easily distinguishable membrane structure that is found surrounding the mitochondria in larval enterocytes at 19 dph.

In both gut sections the larvae at 4 dph had significantly more rugged outer mitochondrial membrane structures compared to all the larvae at 19 dph (Figure 3.6). None of the treatments at 19 dph was significantly different from one another in outer mitochondrial membrane appearance in any of the sections, and the sections showed a quite similar trend in outer mitochondrial membrane smoothness.

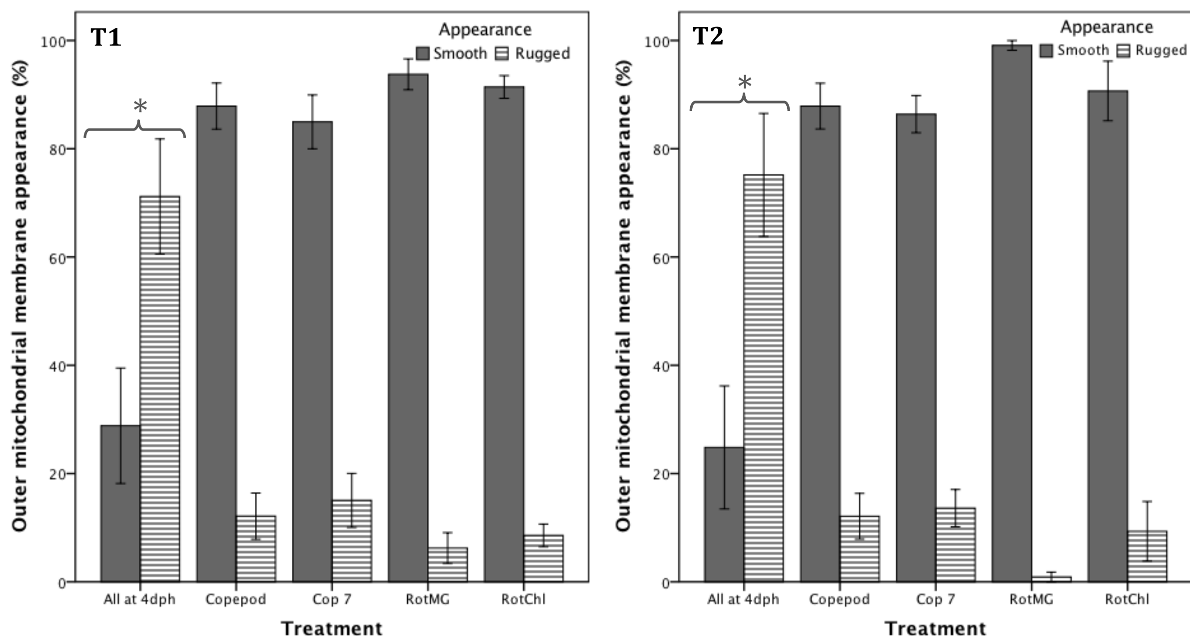

Figure 3.6: Mean outer mitochondrial membrane appearance (%) of larvae at 4 dph and from each treatment at 19 dph in the T1 sections (n=2-5) and in the T2 sections (n=4-5). Significant differences (*) in outer mitochondrial membrane structure were found between larvae at 4 dph and the larvae at 19 dph in both sections. Error bars indicate ± SE.

Figure 3.7 illustrates the quantification of the differences observed in cristae density and appearance (%) in the mitochondria from enterocytes in the T1 sections and in the T2 sections. The approach discriminates between less dense cristae and denser cristae, where the denser appearance is equivalent to the Type 2 mitochondria found in enterocytes by Wold *et al.* (2008). The other type had less dense cristae than the Type 2 mitochondria. No significant differences in cristae density were found between the treatments at 19 dph in any of the sections, however there was a trend implying that there was somewhat more incidents of the dense type at 19 dph than at 4 dph, but this was uncertain because of high standard error. At 4 dph there was found that some of the cristae was difficult to recognize, and was thereby classified as diffuse. This only occurred in the sections from the larvae at 4 dph, and was found in 4 out of five larvae.

The T2 sections showed some occurrence of an interdigitated cristae type at 19 dph, where the mitochondrial content appeared grey and undistinguishable. Figure 3.5 E T_2 shows a photo from the intestinal tissue where interdigitated cristae were found, and no defined membrane boundaries could be recognized. In the cases where interdigitated cristae were found in the T2 sections, the amount were not significant because of the high standard error of these measurements since the interdigitated cristae type were only found in one of the larvae from the RotChl treatment (26.44±24.46 %). In the T1 sections the interdigitated type of cristae were not found.

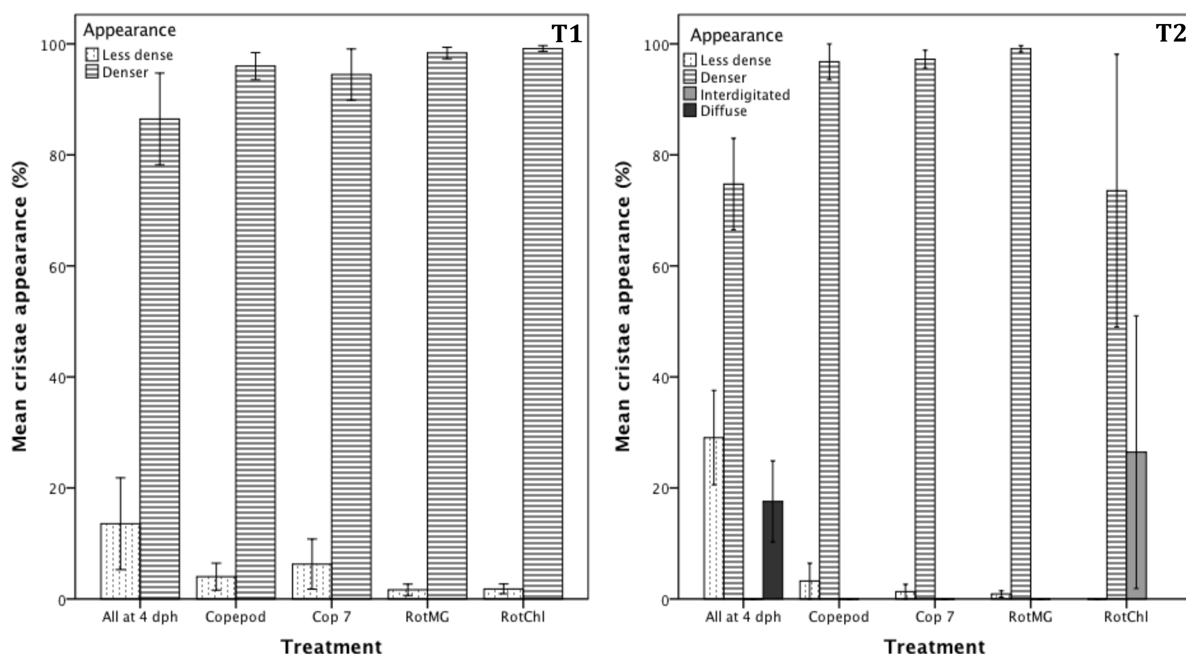

Figure 3.7: Mean cristae appearance (%) in enterocytes of larvae at 4 dph and of each treatment at 19 dph, in T1 sections (n=2-5) and T2 sections (n=4-5). No significant differences were found in cristae density. The interdigitated cristae type was only found in the T2 sections at 19 dph. Error bars indicate ± SE.

3.2.2 Surface area fractions of enterocyte components

The surface area fractions of mitochondria were not significantly different between the treatments at 19 dph in any of the sections (Figure 3.8). Neither was there any difference in surface area fraction of mitochondria between the larvae at 4 dph and the larvae from all the treatments at 19 dph. The highest values of nuclei area fraction, although not significant, in the T1 sections at 19 dph were found in the larvae that received the Cop 7 treatment (15.7±2.0 %) and the Copepod treatment (14.6±3.2 %), and the larvae from the RotMG treatment had the lowest surface area fraction of nuclei (10.2±1.7 %) (Figure 3.8 T1). In the T2 sections however, the surface area fraction of nuclei was significantly higher in the larvae from the RotChl treatment (11.0±0.9 %) than it was in the larvae from the Copepod treatment (5.5±1.0 %) (Figure 3.8 T2). At 4 dph the nuclei area fraction (17.0±2.7 %) found in the T2 sections was significantly higher than from any of the larvae from the different treatments at 19 dph. This is the greatest difference between the T1 and the T2 sections, and Figure 3.9 illustrates that the relationship between surface area fractions of mitochondria and nuclei is opposite for some treatments (Copepod, Cop 7 and RotMG) in the two sections.

No correlation was found between SL and surface area fraction of mitochondria or nuclei in the T1 sections. In the T2 sections there was no correlation between SL and surface area fraction of mitochondria, but there was significant correlation between the SL and surface area fraction of nuclei ($P<0.05$). The correlation was not linear ($R^2= 0.212$), but showed a negative relationship between the standard length of the larvae and the surface area fraction of nuclei in the enterocytes. This means that the surface area fractions of nuclei get smaller as the larvae and cells grow.

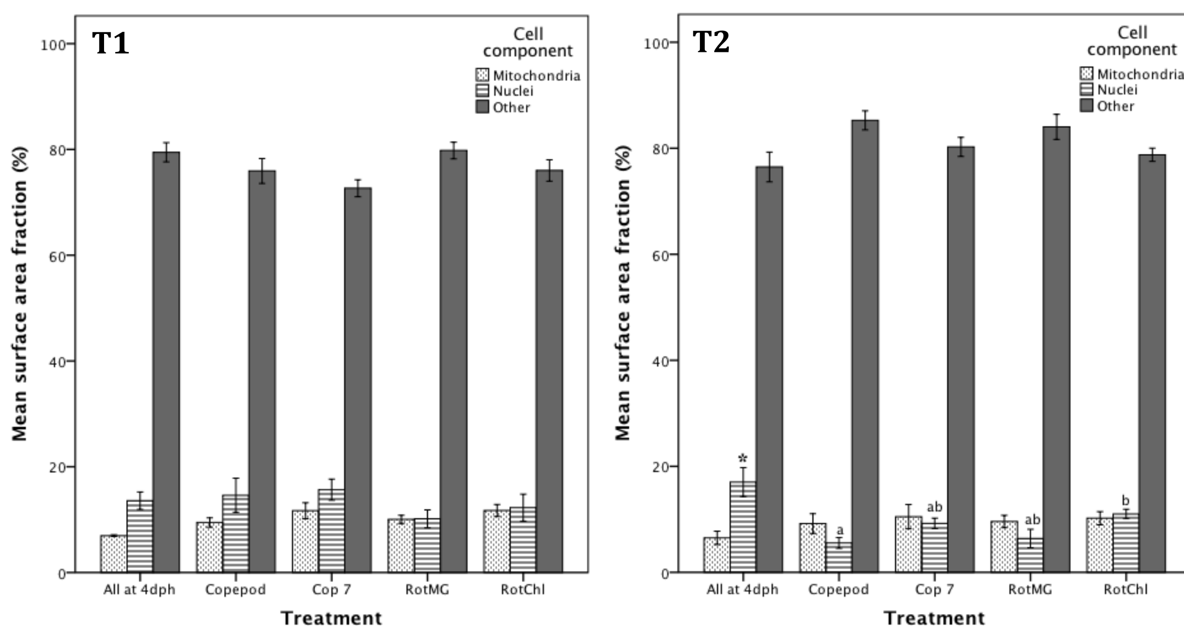

Figure 3.8: Mean surface area fractions (%) of mitochondria and nuclei in the larvae's enterocytes at 4 dph and 19 dph in both T1 and T2 sections (n=5). Error bars indicate ± SE. **T1)** No significant differences were found between the treatments, or between the larvae at 4 dph and 19 dph. Data with standard error are presented in Table A.4 Appendix 9. **T2)** Significant differences in surface area fraction of nuclei were found, where the larvae from the RotChl treatment had significantly higher values than the larvae from the Copepod treatment at 19 dph (letters denotes significant differences). Larvae at 4 dph had significantly (*) larger surface area fraction of nuclei than all the treatments at 19 dph. Data with standard error are presented in Table A.5 Appendix 9.

3.2.3 Area size of enterocytes, nuclei and mitochondria

Enterocyte area size in the T1 sections did not differ significantly in any of the groups (Table 3.2). However, the numbers indicate that the larvae at 4 dph (243.1±35.6 μm^2) had smaller cells than the larvae at 19 dph. The measurements of nucleus area size in the T1 sections showed significant differences from 4 dph to 19 dph, where the larvae at 4 dph actually had the largest nuclei (21.5±1.4 μm^2). Mitochondrial area size was not significantly different in any of the groups, and ranged from 0.8±0.0 μm^2 (RotChl) to 1.0±0.0 μm^2 (Cop 7) (Table 3.2).

A Pearson correlation test were done to compare enterocyte area size in the T1 sections to SL of the larvae from 4 dph and 19 dph, and showed that there was a positive correlation between SL and enterocyte area size ($P<0.05$). However, a linear regression analysis did not give any linear relationship between the two variables (R^2= 0.17). No significant correlation was found between nucleus area size and SL. No correlation was found between enterocyte area size and area fraction (%) of nuclei or mitochondria.

In the T2 sections the larvae at 4 dph (290.5±38.4 μm^2) had smaller enterocyte area size than the treatments at 19 dph (Table 3.3), although not significantly. Enterocyte area size was not very different between the treatments at 19 dph, and ranged from 375.5±42.6 μm^2 (RotChl) to 429.6±59.4 μm^2 (RotMG). Unlike the T1 sections, the nucleus area size in the T2 sections was not significantly different between the groups, or between the larvae at 4 dph and 19 dph (Table 3.3). However, there were significant differences in mitochondrial area size in the T2 sections, where the Cop 7 treatment (1.2±0.0 μm^2) had the largest mitochondrial area size. The Copepod treatment (0.7±0.1 μm^2) and the RotMG treatment (0.7±0.1 μm^2) had the smallest mitochondrial area size in the T2 sections (Table 3.3).

A Pearson correlation test were done to compare enterocyte area size in the T2 sections to SL of the larvae at 4 dph and 19 dph, and showed that there was a positive correlation between SL and enterocyte area size ($P<0.05$). However, linear regression analysis did not give any linear relationship between the two variables (R^2= 0.216). No significant correlation was found between nucleus area size and SL. No correlation was found between enterocyte area size and area fraction (%) of nuclei or mitochondria.

Table 3.2: Enterocyte area size, mitochondrial area size, and nucleus area size ± SE, in the T1 sections from all larvae at 4 dph and from the four different treatments at 19 dph (n=5). Letters in superscript denotes significant differences between the groups.

Dph	Treatment	Enterocyte area size (μm^2)	Nucleus area size (μm^2)	Mitochondrial area size (μm^2)
4	All	243.1 ± 35.6	21.5 ± 1.4^{a}	1.0 ± 0.0
19	Copepod	412.6 ± 61.3	19.5 ± 0.8^{ab}	0.8 ± 0.1
	Cop 7	369.7 ± 30.2	16.0 ± 1.2^{b}	1.0 ± 0.1
	RotMG	379.5 ± 14.8	15.8 ± 1.0^{b}	0.9 ± 0.1
	RotChl	426.3 ± 62.0	17.1 ± 1.0^{b}	0.8 ± 0.0

Table 3.3: Enterocyte area size, mitochondrial area size, and nucleus area size ± SE, in the T2 sections from all larvae at 4 dph and from the four different treatments at 19 dph (n=5). Letters in superscript denotes significant differences between the groups.

Dph	Treatment	Enterocyte area size (μm^2)	Nucleus area size (μm^2)	Mitochondrial area size (μm^2)
4	All	290.5 ± 38.4	18.6 ± 0.2	1.0 ± 0.1^{ab}
19	Copepod	427.8 ± 49.9	18.0 ± 1.1	0.7 ± 0.1^{b}
	Cop 7	376.8 ± 28.7	15.9 ± 1.6	1.2 ± 0.0^{a}
	RotMG	429.5 ± 59.4	19.4 ± 1.1	0.7 ± 0.1^{b}
	RotChl	375.5 ± 42.6	18.6 ± 0.6	0.9 ± 0.1^{ab}

3.3 Liver morphology

The liver sections showed polyhedral cells with round nuclei and abundant mitochondria (Figure 3.9). Bile canaliculi and ducts were visible between the cells. At 19 dph the larval liver hepatocytes had organelles that were more distinct and sharply defined (Figure 3.9 B-E) than at 4 dph where the membrane structures appeared rugged. Outer mitochondrial membranes were smoother at 19 dph and made the mitochondria appear rounder, than at 4 dph. In addition glycogen was found within the hepatocytes at 19 dph, but not at 4 dph. Also, it was observed that endoplasmic reticulum seemed more abundant at 19 dph. Very few lipid droplets were observed within the hepatocytes. The outer mitochondrial membrane was irregularly shaped in larvae at 4 dph (Figure 3.9 A). Interdigitated cristae were found in larval hepatocyte mitochondria from both rotifer treatments, but especially in the RotChl treatment (Figure 3.9 E).

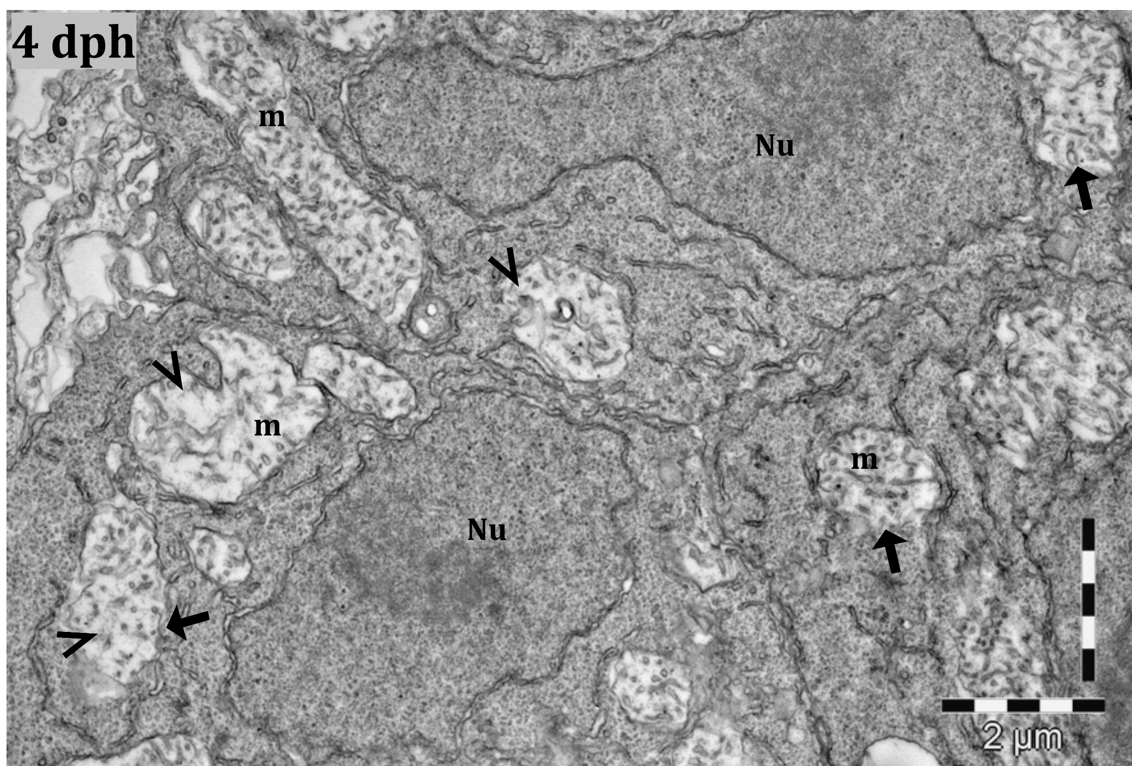

Figure 3.9 A: Transmission electron microscopy photo that shows the general appearance of the liver tissue from larvae at 4 dph. Nuclei (Nu), mitochondria (m) are displayed on the photos. The figure continues on pp. 42-43. The photos illustrates that there was a difference in organelle membrane distinctness over time. The larvae had rugged outer mitochondrial membranes at 4 dph (A) (arrows) and smoother outer mitochondrial membranes at 19 dph (B, C, D, E). Cristae structures were less dense at 4 dph (A) (arrowheads) than it generally was at 19 dph.

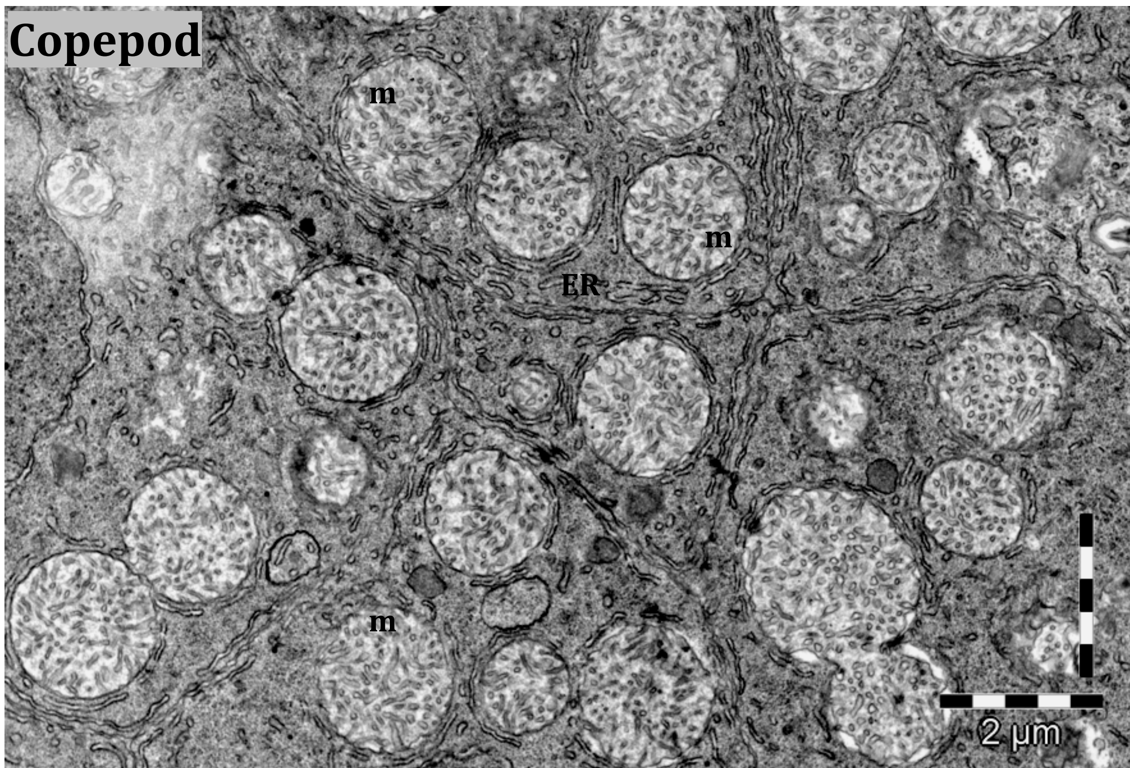

Figure 3.9 B: Transmission electron microscopy photo that shows the general appearance of the liver tissue in larvae from the Copepod treatment at 19 dph. Nuclei (Nu), mitochondria (m) and endoplasmic reticulum (ER) are displayed on the photos. The figure continues on p. 43.

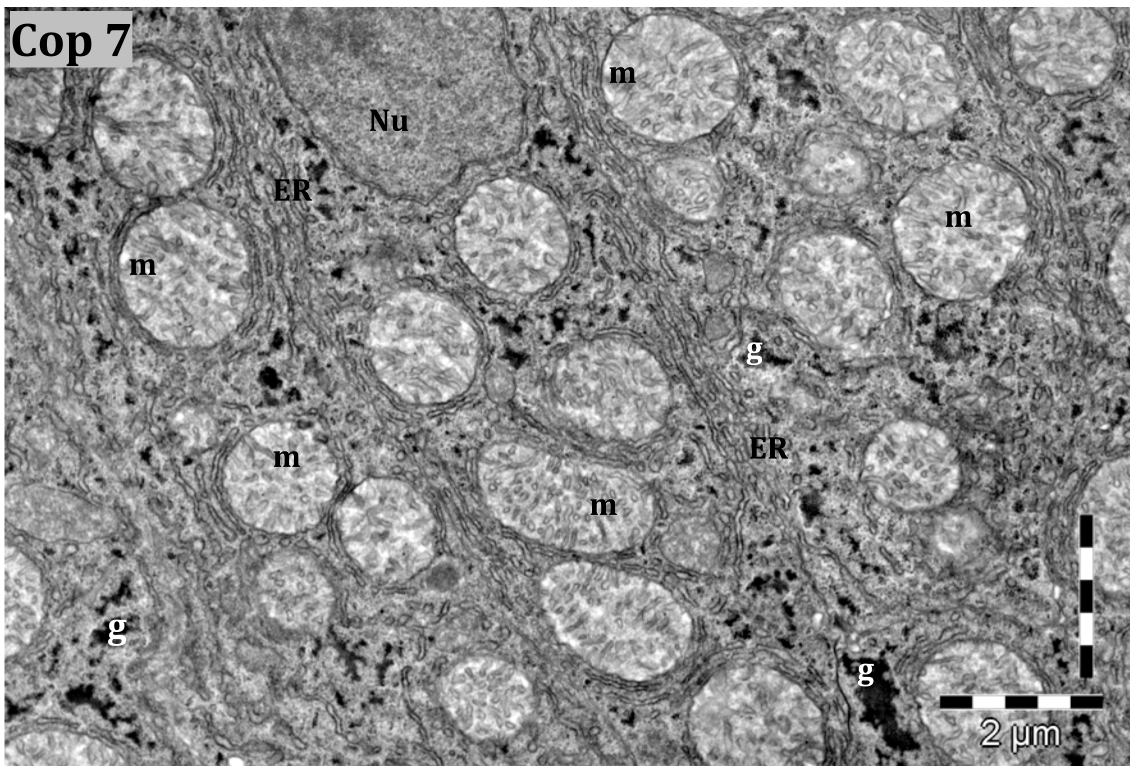

Figure 3.9 C: Transmission electron microscopy photo that shows the general appearance of the liver tissue in larvae from the Cop7 treatment at 19 dph. Nuclei (Nu), mitochondria (m), endoplasmic reticulum (ER) and glycogen (g) are displayed on the photos. The figure continues on p. 43.

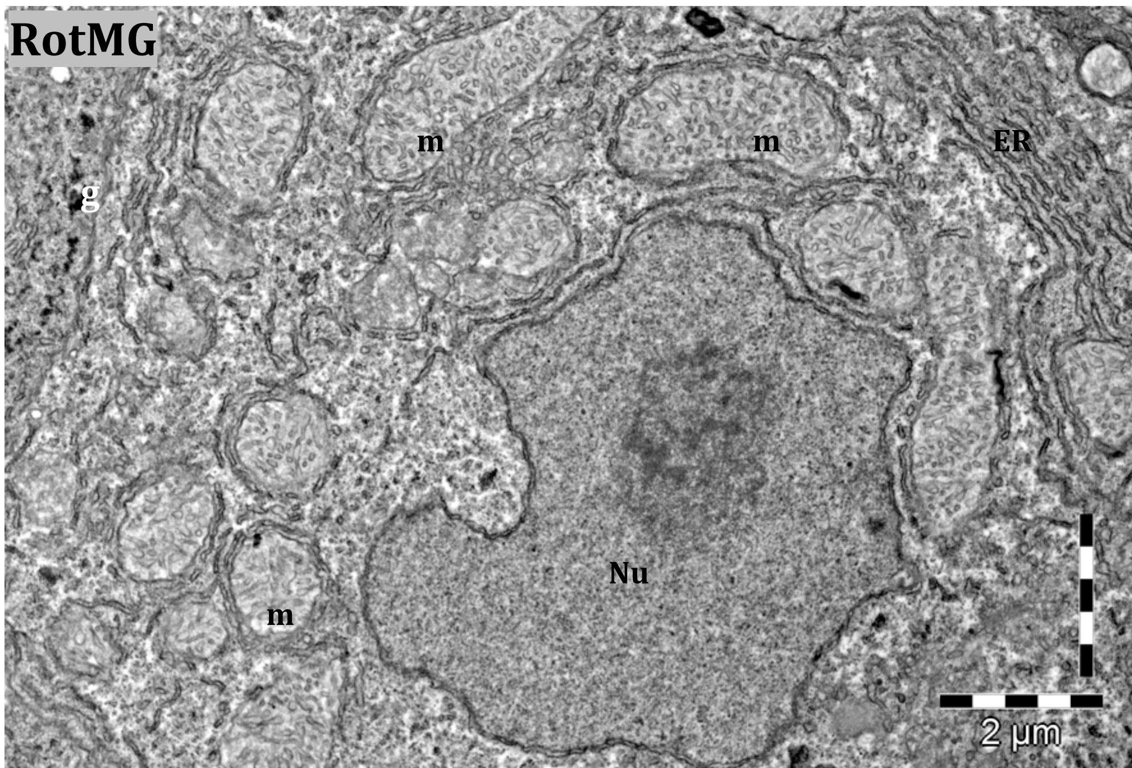

Figure 3.9 D: Transmission electron microscopy photo that shows the general appearance of the liver tissue in larvae from the RotMG treatment at 19 dph. Nuclei (Nu), mitochondria (m), endoplasmic reticulum (ER) and glycogen (g) are displayed on the photos.

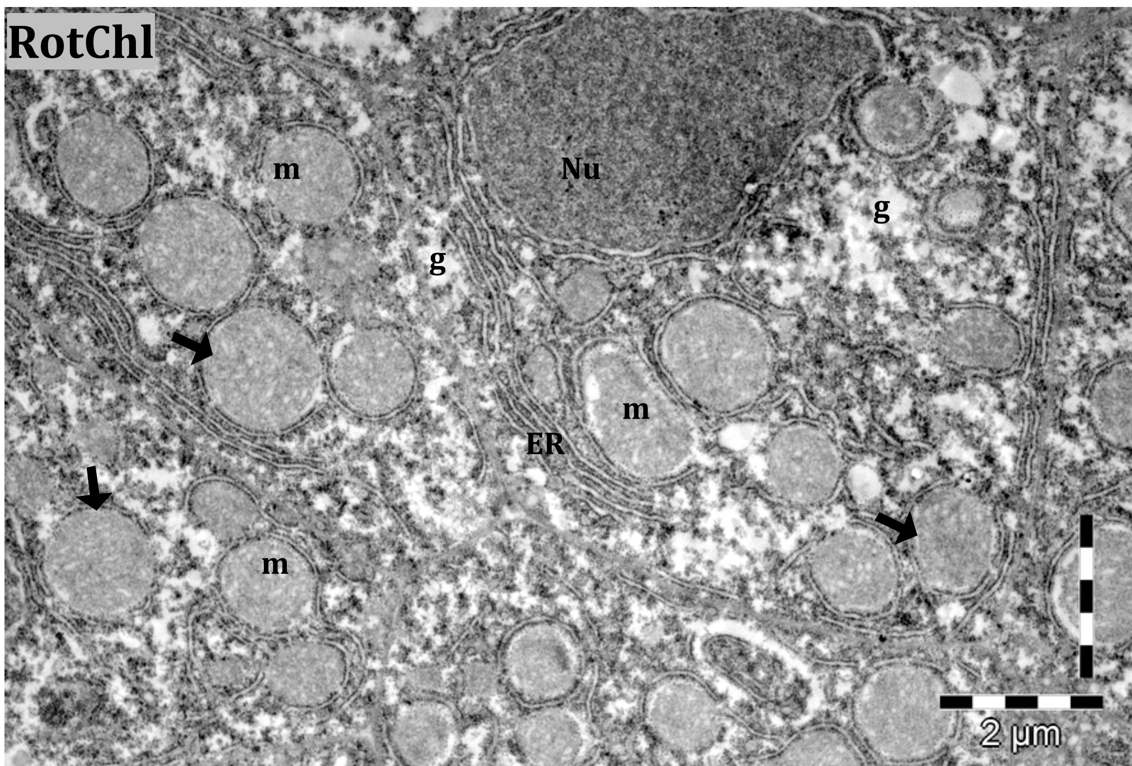

Figure 3.9 E: Transmission electron microscopy photos that shows the general appearance of the liver tissue in larvae from the RotChl treatment at 19 dph. Nuclei (Nu), mitochondria (m), endoplasmic reticulum (ER), and glycogen (g) are displayed on the photos. The photo illustrates the appearance of interdigitated cristae (arrows) within the enterocytes mitochondria of larvae from the RotChl treatment.

3.3.1 Mitochondrial appearance

Figure 3.10 illustrates the quantification of the observed appearance of the outer mitochondrial membrane in hepatocytes, both from larvae at 4 dph and from larvae of the four different treatments at 19 dph. The outer mitochondrial membrane was either classified as smooth or rugged. Figure 3.9 demonstrates the difference of rugged outer mitochondrial membrane structures in hepatocytes in the larvae at 4 dph (Figure 3.9 A), and smooth outer membrane structures from the Copepod treatment at 19 dph (Figure 3.9 B). The outer mitochondrial membrane at 4 dph was significantly less defined than the distinct and sharply defined membrane structure that was found surrounding the mitochondria from the day 19 larvae. There was no significant difference in outer mitochondrial membrane smoothness between the larvae from the different treatments at 19 dph (Figure 3.10).

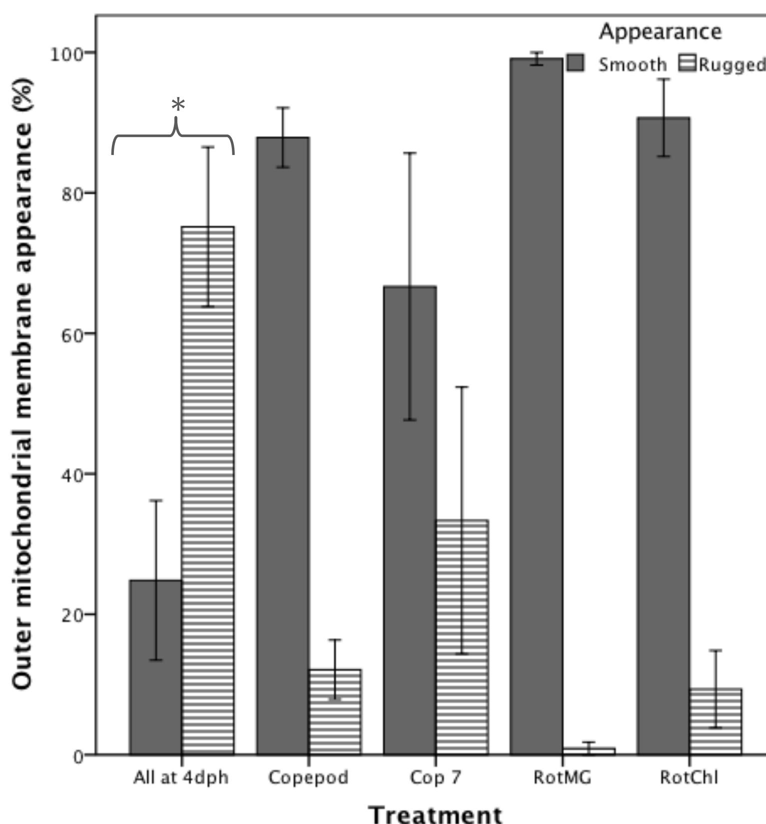

Figure 3.10: Mean outer mitochondrial membrane appearance (%) in liver of larvae at 4 dph and from each treatment at 19 dph (n=4-5). Significant differences (*) in membrane structure were found between larvae at 4 dph and all the larvae at 19 dph. Error bars are set at ± SE.

Figure 3.11 and Figure 3.9 demonstrates the differences in cristae density and appearance (%) inside the hepatocytes mitochondria. The approach discriminates between two kinds of density, dense or less dense, where the dense appearance is equivalent to the Type 2 mitochondria found in enterocytes in Wold *et al.* (2008). There were no significant differences between the treatments at 19 dph in cristae density. The cristae were significantly less dense at 4 dph than at 19 dph.

There was significant difference in appearance of interdigitated cristae between the treatments at 19 dph, which were only found in the RotChl (44.22±20.96 %) and in the RotMG (10.00±10.00 %) treatments, although to a greater extent in the RotChl treatment. In the liver sections 4 out of 5 RotChl larvae had interdigitated cristae. However, in only one larva there was found exclusively interdigitated cristae, which was the same larva that had grey cristae in the T2 section. Figure 3.10 E demonstrates the occurrence of interdigitated cristae within the mitochondria, where membrane boundaries could not easily be distinguished.

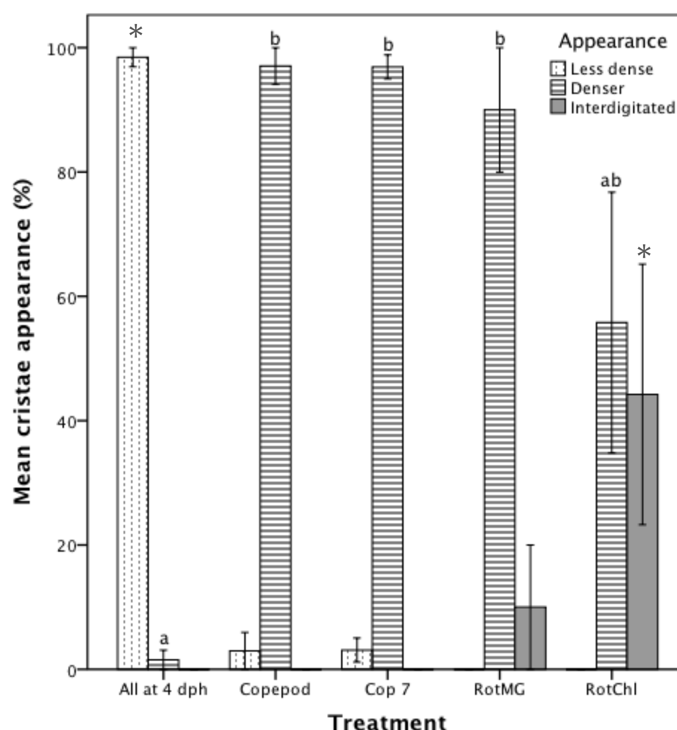

Figure 3.11: Mean cristae appearance (%) in hepatocytes of larvae at 4 dph and of each treatment at 19 dph (n=4-5). There were no significant differences in cristae density in the mitochondria between the treatments at 19 dph, but at 4 dph the larvae had significant less dense cristae (*). Letters denotes significant differences in density of cristae. The interdigitated cristae type was only found in the RotChl treatment and in the RotMG treatment, which was significantly higher in the RotChl treatment (*). Error bars illustrates ± SE.

3.3.2 Surface area fractions of hepatocyte components

Glycogen was found in the liver of the cod larvae at 19 dph, and there were significant differences in surface area fraction of glycogen between some of the treatments (Figure 3.12). The larvae from the Copepod treatment had significantly lower levels (2.2±1.4 %) than larvae from the RotMG treatment (17.4±1.8 %) and RotChl treatment (25.3±4.8 %). The glycogen storage in the larval liver in the Copepod treatment was so low that it did not differ significantly from the larvae at 4 dph where there was no glycogen present. The surface area fraction of mitochondria was not significantly different in any of the treatments, or between larvae at 4 dph compared to all larvae at 19 dph. Neither was there any difference between the treatments in surface area fraction of the nuclei within the hepatocytes (Figure 3.12).

Significant correlation was found between larval standard length and surface area fraction of glycogen ($P<0.01$) although not a linear one ($R^2=0.345$). There was a negative relationship between the two variables, which implies that the smaller larvae had more glycogen stored in the liver than the larger larvae had, where the larger larvae belonged to the Copepod treatment and had <10% surface area fraction of glycogen. No correlation was found between standard length and surface area fraction of mitochondria or nuclei.

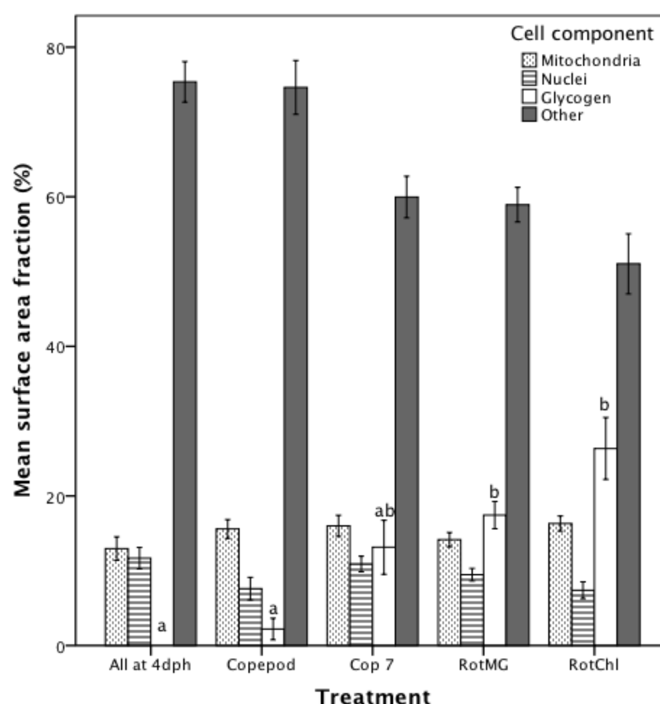

Figure 3.12: Mean surface area fractions (%) of mitochondria, nuclei and glycogen in the larvae's hepatocytes of each treatment at 4 dph and 19 dph (n=4-5). Letters indicate significant differences in surface area fraction of glycogen. No glycogen was found at 4 dph. Data with standard error are presented in Table A.6 Appendix 9. Error bars indicate ± SE.

3.3.3 Area size of hepatocytes, nuclei and mitochondria

The hepatocyte area size was significantly smallest at 4 dph (319.2±16.4 μm^2), and at 19 dph the larvae from the Copepod treatment (429.0±17.6 μm^2) and RotChl treatment (442.1±22.3 μm^2) had the largest hepatocyte area size (Table 3.4). The larvae from the RotMG (378.9±21.5 μm^2) and Cop 7 treatment (394.5±38.0 μm^2) did not differ from any of the other groups at 19 dph, or from the larvae at 4 dph, in hepatocyte area size. The nucleus area size did not show any significant differences, but the trend indicates that the larvae from the Copepod treatment (21.7±0.9 μm^2) had the largest nuclei (Table 3.4). The larvae at 4 dph (1.3±0.1 μm^2) and the larvae from the RotChl treatment (1.4±0.1 μm^2) at 19 dph had the smallest mitochondria. The larvae from the Cop 7 treatment (2.0±0.1 μm^2) had the largest mitochondria at 19 dph, while the other two treatments (Rot MG and Copepod) at 19 dph did not differ from any of the other treatments (Table 3.4).

A positive correlation was found between SL and hepatocyte area size ($P<0.01$) in larvae from 4 dph and 19 dph. However, linear regression analysis did not give any linear relationship between the two variables ($R^2=0.274$). There were also found significant positive correlations between SL and nucleus area size ($P<0.05$), although a linear relationship was neither found here ($R^2=0.193$). No correlation was found between hepatocyte area size and area fraction (%) of nuclei or mitochondria.

Table 3.4: Hepatocyte area size, mitochondrion area size, and nucleus area size ± SE, in the liver sections from all larvae at 4 dph and from the four different treatments at 19 dph (n=4-5). Letters in superscript denotes significant differences between the groups.

Dph	Treatment	Hepatocyte area size (μm^2)	Nucleus area size (μm^2)	Mitochondrial area size (μm^2)
4	**All**	319.2 ± 16.4^{b}	17.9 ± 1.5	1.3 ± 0.1^{a}
19	**Copepod**	429.0 ± 17.6^{a}	21.7 ± 0.9	1.7 ± 0.2^{ab}
	Cop 7	394.5 ± 38.0^{ab}	20.2 ± 0.8	2.0 ± 0.1^{b}
	RotMG	378.9 ± 21.5^{ab}	20.1 ± 0.8	1.7 ± 0.1^{ab}
	RotChl	442.1 ± 22.3^{a}	19.9 ± 0.6	1.4 ± 0.1^{a}

4 Discussion

4.1 Effects of different live feed organisms on larval growth, development and survival

The present study demonstrated that the intensively cultivated copepod *Acartia tonsa* for use as live feed organism improved growth, development, and survival of the cod larvae compared to larvae fed the enriched rotifer *Brachionus ibericus.* This implies that the dietary composition of cultivated copepods in the present study was more suitable for larval start-feeding than that of enriched rotifers.

The cod larvae fed copepods exclusively for the first 20 dph showed improved growth compared to the larvae from the other three treatments, in terms of dry weight, standard length and myotome height. Already at 8 dph the larvae from both the Copepod and Cop7 treatment showed a significantly higher dry weight than the other treatments, which is an early difference compared to other studies on start-feeding of cod (Galloway *et al.*, 1999). This significantly higher increase in dry weight continued through the whole start-feeding period for the larvae from the Copepod treatment. A parallel study done on the cod larvae from the present study showed that some larvae from the two rotifer treatments were the only ones where the vertebrae were not fully ossified at 60 dph (Hansen, 2011), and indicates that they had reached a lower developmental stage than the copepod-fed larvae. However, the larvae from the Cop7 treatment did not keep up with the high growth of the larvae from the Copepod treatment after being fed only enriched rotifers, and at 60 dph their growth were found somewhere in between the Copepod and the two rotifer treatments. This was also found in survival of this treatment, and indicated that feeding with copepods for only a short interval in the first days of start-feeding was beneficial for survival and growth of the cod larvae. In addition, Hansen (2011) found that larvae from both the Copepod treatment and the Cop7 treatment were significantly more efficient in catching prey compared to the larvae from the two rotifer treatments, implying that there might be a positive correlation between the copepod-fed larvae’s efficient prey catching and improved growth.

The superior nutritional value of copepods compared with rotifers is mostly attributed to their high fraction of essential fatty acids in phospholipids (Olsen *et al.*, 2004). However, the copepod nauplii contain 0.2 fold higher amounts of protein and 4.2 fold higher amounts of free amino acids than rotifers (van der Meeren *et al.*, 2008; Conceicao *et al.*, 2010). Other studies done on protein and free amino acid content of copepods and *Artemia fransiscana* reports higher free amino acid content in copepods, and also higher content of certain essential amino acids in the calanoid copepod *Temora longicornis* (Helland, 2003). Considering the immature digestive system of cod larvae, high free amino acid content in the live feed is essential for optimal amino acid absorption. Higher availability of amino acids may improve protein utilization and growth performance of the fish larvae, since growth primarily is an increase in muscle mass by protein synthesis. This became evident when higher muscle growth was observed in larvae from the Copepod treatment at 19 dph (Halseth, pers. com., Master´s thesis in prep.). Thus, the present study demonstrated that the cod larvae being fed copepods utilized their growth potential best, and this may be attributed to the nutritional value of essential fatty acids in the phospholipid fraction, but also to the higher content of proteins and free amino acids.

Differences in pigmentation of the cod larvae were observed, where the larvae being fed copepods were more pigmented than the larvae from the other treatments, and had more of a yellow tint. This yellow tint was also reported in a previous study done on cod larvae being fed *Rhodomonas baltica*-fed copepods (Eidsvik, 2010) and was linked to the carotenoid monadoxanthin, which has previously been found in the *Rhodomonas* strain D3 comprising 15 % of the total carotenoids (Chapman, 1966). Improved pigmentation has also been reported in flatfish being fed copepods (Shields *et al.*, 1999), where it is suggested to be due to the copepods higher content of iodine, which is a precursor for thyroid hormones that initiates the metamorphic processes. Iodine limitation is thus a possible cause of malpigmentation and abnormal metamorphosis in flatfish larvae (Solbakken *et al.*, 2002; Moren *et al.*, 2006; Conceicao *et al.*, 2010). Copepods are generally rich in pigment, and particularly the carotenoid astaxanthin, which may be an important source of retinoids that may have detrimental effects on normal bone development in fish larvae (Moren *et al.*, 2005; van der Meeren *et al.*, 2008) and is involved in light reception in the eye (Kjørsvik *et al.*, 2004). Astaxanthin also has antioxidant properties as a co-antioxidant with vitamin E in suppressing lipid

peroxidation (Bell *et al.*, 2000). This suggests that a higher degree of pigmentation in the copepod-fed larvae in the present study may function as an indicator of normal development.

According to the dry weight measurements, DWI calculations and standard length measurements, larvae from all treatments showed a positive increase in growth during the whole start-feeding experiment, although the growth did slow down somewhat during the weaning from *Artemia* sp. to dry feed at 33-40 dph. Except for this period, the dry weight showed similar trends as several other studies on cod reviewed by Folkvord (2005), and standard length measurements in this study showed similar larval sizes as reported in previous start-feeding experiments done at similar temperature regimes (Busch *et al.*, 2010). The slowdown in growth between 33 and 40 dph might be due to difficulties for these larvae to handle the weaning from *Artemia* sp. to formulated feed, which seems to be common for pre-metamorphic cod larvae (Stoss *et al.*, 2004). The larvae from the Copepod treatment did not have such a slowdown in growth, and seemed to be better prepared for adaption to dry feed. However, the significant higher survival that was found for the larvae from the Copepod treatment started to decrease after the weaning period, and at 46 dph the higher survival of these larvae compared to the other treatments was no longer significant. The combination of maintained high growth rate but high mortality in this period indicates that there was the fastest growing and largest larvae from the Copepod treatment that survived the weaning period and was rapidly adapted to dry feed. At the end of the experiment the larvae from the Copepod treatment still had most survivors, in spite of the high mortality in the weaning period. Compared to other studies that also used copepods as live feed (Busch *et al.*, 2010), the survival of the larvae from the Copepod treatment in the present study was quite higher. Even the larvae from the RotChl treatment, that had the lowest survival in this study, had higher survival than previously reported for larvae fed copepods extensively (Busch *et al.*, 2010).

Enrichment of rotifers is considered necessary since they do not contain sufficient amounts of essential fatty acids (Olsen, 2004). The survival and growth patterns for the two rotifer treatments in the present study were very similar, and this raises the question whether the enriched rotifers has better nutritional value than the rotifers that were only fed *Chlorella* sp. Studies on *Brachionus plicatilis* fed different types of algae

have shown that *Chlorella*-fed rotifers were nutritionally the least adequate for marine fish larvae (Segner *et al.*, 1984). Thus, the RotChl treatment was expected to show poor results in larval development, survival, and growth because of low values of PUFA, and was not expected to be just as good as the enriched RotMG treatment. However, other results from the present study and from Hansen (2011) imply that the inadequate nutritional value of the unenriched rotifers was more evidently affecting other measured criteria negatively such as stress tolerance, ossification, and mitochondrial development.

The growth of fish larvae is highly dependent on temperature (Otterlei *et al.*, 1999; Folkvord, 2005). However, comparison to other studies is intricate since most authors relates to temperature range instead of day degrees. Otterlei *et al.* (1999) found an optimal growth rate of 25% day^{-1} for Norwegian coastal cod larvae at temperatures from 14 to 16°C, 15 days after the onset of first feeding in an extensive production system feeding with natural zooplankton. There has also been reported a daily weight increase of 13.7-21.7 % in extensive production of cod larvae with temperatures of 8.2-11°C during the first 16 dph (van der Meeren, Næss, 1993). The results from the present study shows that the daily growth rate was 9.61±0.53 % between 14 and 19 dph with temperatures from 11 to 12°C, and that daily growth rates obtained for the Copepod treatment was at its maximum at 40-60 dph (14.23±1.58 %) with a temperature of 12°C. This shows that growth rates in the present study were lower than reported in extensive systems feeding with copepods. However, fluctuations in temperature are something that often occurs in extensive systems (Busch *et al.*, 2010), thus growth rates of the larvae varies. In intensive production the temperature is controlled, thus providing a more predictable growth rate.

In extensive production the zooplankton specie, prey biomass, and supply may vary and be quite high at times, making the growth of the fish larvae vary as well. It is also recommended to feed to satiation and to constantly adjust the supply of prey after the appetite of the fish larvae in intensive production of cod (Helland *et al.*, 2009). In the present study the feed supply was maintained at a constant level, and prey size and biomass was highly controlled to maintain similar feeding criteria for the copepod-fed larvae and the rotifer-fed larvae, making sure that potential differences were only due to

the nutritional composition of the prey. Thus, higher growth would be obtainable in the present study if the cod larvae were fed to satiation.

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

The present study demonstrated that the cod larval hepatocytes and enterocytes showed signs of an immature state at the start of exogenous feeding in terms of cell size and organelle appearance. Mitochondria from both liver and gut cells were affected by the nutritional value of the live feed organisms in terms of negative effects of feeding with unenriched rotifers.

4.2.1 Growth of cells and nuclei

Tissue grows most commonly by hyperplasia (increase in cell numbers), but may also grow by hypertrophy (increase in cell volume) (Wolpert *et al.*, 1998). In the present study hypertrophy was observed in both gut and liver. The hepatocyte area size at 4 dph was significantly smaller compared to the larger hepatocyte sizes at 19 dph. Allometric growth has previously been reported for the cod larval liver (Kjørsvik *et al.*, Submitted), and is typical for larval growth of the most vital organs (Osse, Van den Boogaart, 2004). This difference in cell size between developmental stages was also found in the two sections of the gut, whereas enterocyte area size was smaller at 4 dph than it generally was at 19 dph, although not significant. This has also been reported previously for cod larval gut development and has been positively related to larval standard length (Wold *et al.*, 2008).

In both sections of the gut the larvae that had received copepods exclusively for the first 20 days were among those that had the largest enterocytes at 19 dph, although not significantly larger. Reduction in gut epithelium thickness has been reported in fish larvae that has been starved (Kjørsvik *et al.*, 1991; McFadzen *et al.*, 1994), and a reduction in enterocyte diameter has also been reported in starved European eel (*Anguilla anguilla* L.) (Rodríguez *et al.*, 2005). In the present study none of the treatments showed epithelial defects with any sign of starvation, but the fact that the larvae fed copepods had among the largest enterocytes gave an indication that the feed was beneficial for growth of the gut, and that the larvae were well-fed.

In the present study the larvae from the Copepod treatment and the RotChl treatment had the significantly largest hepatocytes at 19 dph. There has been suggested that large sizes of turbot (*Psetta maxima*) hepatocytes is related to high energy storages (Segner *et al.*, 1994), which is supported by the results from this study where RotChl larvae had the largest hepatocytes and the greatest surface area fraction of glycogen. On the other hand, this explanation does not fit with the large hepatocyte size of the larvae from the Copepod treatment where very little glycogen was observed. Studies on common carp (*Cyprinus carpio*) larvae show that larvae fed diets low in phospholipids had smaller liver volume and smaller liver nuclei than larva fed diets with higher amounts of phospholipid (Fontagnè *et al.*, 1998). This correlates well with what was found in the present study for larvae being fed copepods that contain high levels of phospholipid. This indicates that the copepods' high content of DHA and EPA in polar lipid fraction could be beneficial for cell growth since the *n-3* HUFA from phospholipids can be incorporated more efficiently into larval tissue than fatty acids from neutral lipids (Izquierdo *et al.*, 2000), thus contributing to growth of cell membranes.

Wold *et al*. (2008) found positive correlations between standard length and enterocyte volume (μm^3) during larval development by linear regression, and in the present study there was a positive correlation between standard lengths and enterocyte area size (μm^2) in both T1 sections and in T2 sections, although not a linear relationship. This correlation indicates that the enterocytes gets larger as the larvae grow in size. Kjørsvik *et al.* (Submitted) also found positive correlations between larval standard length and liver volume (μm^3), and between standard length and number of hepatocytes per liver. In the present study there was also found positive correlation between standard length and hepatocyte area size (μm^2). However, even if the correlation tests showed significant correlation, the relationship between the variable were not linear. This might be due to the fact that the present observations were based on area sizes and not volume estimations, which may give different impressions. The small sample sizes gave little differences in standard lengths since the samples only were gathered from two days during the experiment, respectively 4 and 19 dph. The correlations are thus not clear on a scatter plot when the intervals on the x-axis are quite small.

The measurements of nucleus area size in the hepatocytes indicated that the larvae from the Copepod treatment had the largest nuclei, though not significantly larger than the

other treatments or the larvae at 4 dph. However, previous studies has found that hepatocyte nuclei were larger before mouth-opening in cod larvae (Kjørsvik *et al.*, Submitted). Wold *et al.* (2009) found that larvae fed diets with high amounts of neutral lipids rather than phospholipids had smaller nuclei sizes. Large nuclei can imply a high metabolic activity and development of hepatocytes, since nucleus area of contact between the nucleus and cytoplasm is decisive for exchange of macromolecules essential for DNA replication and protein synthesis between the nuclei and the cytoplasm (Ghadially, 1997). In the present study the combination of large hepatocyte area size and large nucleus area size probably makes the larvae fed copepods the ones with the most developed, and probably the most active liver. This also correlates well with the fact that the Copepod treatment had the larvae with the highest growth rate (DWI (%)), and was at all times the larvae that were largest in both dry weight and length measurements.

In the T1 sections the size of the nuclei decreased from 4 dph to 19 dph, but at 19 dph the larvae fed copepods had the largest nuclei compared to the other three treatments. The observation of larger nuclei at an early developmental stage has previously been reported for cod larval hepatocytes (Kjørsvik *et al.*, Submitted), and could imply a high metabolic activity and development of the cells at an early stage. The large nuclei and high growth of the copepod-fed larvae at 19 dph was the same pattern as seen in the liver, and gives the impression that the larvae fed copepods exclusively had the most beneficial nutritional status, since an increase in nucleus size generally is associated with an increase in metabolic capacity (Ghadially, 1997). The T2 sections did not show any noteworthy differences in nuclei area sizes between treatments at 19 dph, or between 4 dph and 19 dph. However, the surface area fractions of nuclei in the T2 sections were significantly higher at 4 dph and in the RotChl treatment at 19 dph, meaning that the nuclei occupied a significantly larger space within the cells at 4 dph and in the RotChl treatment at 19 dph.

Wold (2008) found no correlation between larval standard length and nucleus volume in the enterocytes. In the present study there was found negative correlation between larval standard length and surface area fraction of nuclei in the T2 sections. This relationship expresses that a significantly smaller area within the enterocytes are covered by nuclei in the midgut of the larger larvae. This might be due to the larger

larvae's increase in enterocyte size but at the same time modest increase in nuclei size, compared to the other treatments which have larger nuclei per cell area. As discussed earlier, the standard length of the larvae from the Copepod treatment was the highest at all times. At 19 dph these larvae were significantly larger than larvae from the other treatments that showed no significant differences in standard lengths. Since the larger larvae belonged to the Copepod treatment, the negative correlation between standard length and area fraction of nuclei therefore implies that growth is not negatively affected by the lower coverage of nuclei within the enterocytes.

4.2.2 Mitochondrial development

There is a positive correlation between the metabolic activity of a tissue and the number and size of the mitochondria and also the number, size, surface area and concentration of cristae (Ghadially, 1997). The observations of the mitochondrial appearance in both liver and gut tissue in the present study showed that there were significant differences in both cristae density and outer mitochondrial membrane smoothness between larvae at 4 dph and larvae at 19 dph. Kjørsvik *et al.* (Submitted) found that mitochondria in cod larval hepatocytes were smallest before mouth-opening and that they were less developed than for older larvae. In the present study the double-layered outer mitochondrial membrane was rugged and less distinct at 4 dph, which gave the impression of an immature state of mitochondria in both liver and gut cells. This immaturity was also reflected by the appearance of the other membrane structures within the cells. The cristae of mitochondria in the hepatocytes were evidently less dense at 4 dph in than at 19 dph. This indicates that there might be a maturation process of the cells and mitochondria going on during the start-feeding both in the liver and gut. A similar state of immaturity based on cristae density has also been described in a previous study on cod larval enterocytes (Wold *et al.*, 2008), and is supported by the results from the cod larval liver in the present observations. However, there were not found any incidents of the densest type of mitochondria (Type 3) as described by Wold *et al.* (2008) in the present study, and the difference in cristae density were not as evident in the enterocytes as it were in the hepatocytes.

Kjørsvik *et al.* (Submitted) found that the mitochondria occupied a smaller part of the hepatocyte volume in the cod larvae before mouth-opening, than they did for older larvae, thus indicating a lower energy metabolism in cod larvae at the early larval stage.

In the present study there were no differences in surface area fraction of mitochondria within the hepatocytes between larvae at 4 dph and 19 dph. However, there were significant differences in mitochondrial area size, where larvae at 4 dph and larvae from the RotChl treatment at 19 dph had significantly smaller mitochondrial area size than the other treatments. Thus indication a possibly lower metabolic activity in these cells, since size of mitochondria generally is positively correlated to metabolic activity (Ghadially, 1997).

Previous studies have shown that mitochondria in the intestinal tissue of turbot larvae may have compensated for metabolic abnormalities, that lead to reduced function, by expanding in size and swelling (MacQueen Leifson *et al.*, 2003). Also, swelling of mitochondria and cellular shrinkage has been reported in hepatocytes of milkfish (*Chanos chanos*) supplied *Chlorella*-fed *B. plicatilis* (Segner *et al.*, 1984). Another observation that was registered in the present study was the appearance of cristae that had no clearly defined membrane boundaries and made the inside of the mitochondria look grey and interdigitated. This observation was similar to what was found in mitochondria in muscle tissue of false percula clownfish (*Amphiprion ocellaris*, Pomacentridae) larvae 11 dph fed unenriched *B. plicatilis* (Olivotto *et al.*, 2011). Within the hepatocytes in the present study this was an observation only found in the larvae from the RotMG and RotChl treatment, and to a higher degree in the RotChl treatment. This appearance gave the indication that there was something abnormal about these mitochondria, and it also is striking that this only appears in the larvae fed rotifers exclusively. The larvae from the RotChl treatment also were the ones with the significantly smallest mitochondrial area size in the liver at 19 dph. Comparing this to the measurements of glycogen surface area fraction in the liver, we can see that these larvae also are the ones with highest amount of glycogen storages. The present study suggests that these larvae may have poor growth due to a combination of inability to utilize glycogen and to perform energy demanding processes because of their interdigitated cristae. This observation was also found in Olivotto *et al.,* (2011), where several glycogen deposits occupying the cytoplasm of muscle tissue were associated with poorly developed mitochondria that had cristae membrane interdigitation found in larvae fed unenriched rotifers.

In the T1 sections there were found no incidents of the undefined and interdigitated cristae type that were found in the liver sections, but the T2 sections on the other hand, had incidents of this phenomenon in one larva fed unenriched rotifers. As found in the liver sections, the undefined and interdigitated cristae had no clear membrane structures separating the cristae from one another. This is suggested to negatively affect the membrane transport system that is crucial for oxidative phosphorylation (Berg *et al.*, 2007), and witness of less metabolic active mitochondria. These findings were also similar to those found in muscle tissue of false percula clownfish larvae fed unenriched rotifers in Olivotto *et al.,* (2011), which also suggests indications of a lower activity grade of mitochondrial metabolism. Also, analysis done on the larvae's efficiency in catch of prey showed that the larvae fed rotifers spent significantly more time swimming before catching a prey than the larvae fed copepods (Hansen, 2011) This, in relation to the observation of membrane interdigitation of the cristae implies that these larvae might be in danger of exhaustion and malnutrition if using more energy than they can produce.

The fact that the grey and interdigitated types of mitochondrial cristae membranes now has been reported both in liver and gut, indicates that implications in the crucial mechanism of nutrient assimilation may occur in these larvae, which again affects their survival and growth negatively. Copepods generally contain high fractions of phospholipids, and much of the EPA and DHA found in copepods is incorporated in the phospholipids (45-60 % of the total fatty acids) (Olsen, 2004). The cod larvae fed copepods did not show any signs of membrane interdigitation in the cristae structures. This may imply that supplying PUFA in the phospholipid fraction is beneficial for structuring of cell membranes, since cell biomembranes are constructed by a phospholipid bilayer (Lodish *et al.*, 2008).

4.2.3 Lipid and glycogen storages

There were very few observations of lipid droplets in the hepatocytes and enterocytes, which have been found more frequent in previous studies both in the liver (Lie *et al.*, 1986; Segner *et al.*, 1994) and the gut cells (Kjørsvik *et al.*, 1991; Caballero *et al.*, 2003; MacQueen Leifson *et al.*, 2003; Elbal *et al.*, 2004; Wold *et al.*, 2008) of marine fish larvae. The deposition of lipid in the hepatocytes functions as energy storage, and lipid droplets in enterocytes function as lipid storage before being transported via the blood to the

liver as large lipoprotein complexes. Wold *et al.,* (2008) found that lipid droplets disappeared at 30 dph, and suggested that this might be a sign of a more mature digestive system due to increased digestive capacity. However, very few lipid droplets within the enterocytes at 19 dph in the present study might be due to suboptimal feeding concentrations in the rearing tanks at this stage where the transition to *Artemia* sp. was just about to start. Thus, the larvae were relying on larger prey size or higher feed ratios to continue its growth and meet its metabolic demands.

Kjørsvik *et al.* (Submitted) found no stored glycogen in cod larvae before exogenous feeding, and in the present study the cod larvae did neither store glycogen in the hepatocytes at 4 dph. In contrast to these findings, the turbot and sea bream (S*parus aurata*) larvae had glycogen storages already at hatching (Segner *et al.*, 1994; Guyot *et al.*, 1995), which indicates that there are great differences in glycogen storage strategies between species. Previous studies of cod larvae fed rotifers showed that larvae with the highest growth rates (DWI≥12 %) and larger nuclei had the highest quantities of glycogen deposited in their liver as energy storage (Kjørsvik *et al.*, Submitted). Fish larvae have high ability to quickly mobilize energy stored as glycogen in order to act instantaneously (Waagbø *et al.*, 2001; Berg *et al.*, 2007; Kjørsvik *et al.*, Submitted), for instance when being attacked (Fuiman *et al.*, 2006). In the present study the surface area fraction of glycogen was significantly higher in those larvae that had the lowest survival and growth (DWI of 9.8 ± 0.2 %), respectively larvae from the RotChl treatment. The larvae that had been fed copepods (DWI of 11.0 ± 0.2 %) had significantly lower, and in some cases, no stored glycogen in the liver. If we compare these results with the theory that storage of glycogen is equivalent to high growth rates and well-developed larvae, this does not correlate well with the fact that the larva fed copepods in this study where the ones that seemed to be best fitted in terms of survival, growth and pigmentation.

However, the difference in storage of glycogen might be due to difference in content of nutrients in the live-prey itself. Since glycogen is a readily mobilized storage form of glucose (Berg *et al.*, 2007), it is essential that the food contain this carbohydrate. The size of the glycogen storages will vary with the amount of carbohydrate that is provided through the feed (Waagbø *et al.*, 2001). The literature gives very little concern to the carbohydrate content of feeds for marine fish larvae, since it is not regarded as an

essential nutritious substance. In rotifers, 10.5 to 27 % of the dry weight is carbohydrate and it is composed of 61-80 % glucose, 9-18 % ribose and 0.8-7.0 % galactose, mannose, deoxyglucose, fucose and xylose (Støttrup, McEvoy, 2003). The carbohydrate content in copepods ranges from 0.2 to 5.1 % of dry weight, excluding chitin that ranges from 2.1 to 9.3 % of dry weight (Corner, O´Hara, 1986). The lower carbohydrate content is suggested to be due to the use of glucosamine to produce chitin in these organisms (Perumal *et al.*, 2009). This implies that the copepods contain less carbohydrate than the rotifers, thus making the larva less able to store glycogen when fed copepods. It has previously been reported that Atlantic halibut juveniles lowered their growth and showed accumulation of glycogen in the liver when weaned to diets containing carbohydrate levels above 5% (Stoss *et al.*, 2004). In the present study a similar pattern was found in cod larvae fed rotifers, which had the highest glycogen accumulation in the liver and lower growth than the larvae from the other treatments.

Studies have shown that adult cod fed formulated feed containing high amounts of carbohydrate had three-folded the amount of blood glucose in one hour when being exposed to stress, and used four days to recover. On the other hand, cod fed diets containing low carbohydrate content showed a moderate increase in blood glucose response, and recovered after two days (Waagbø *et al.*, 2001). This may imply that also cod larvae being fed carbohydrate-rich feed will be poorly fit to cope with stress. In a parallel study Hansen (2011) found that the cod larvae copepods had significantly lower mortality than larvae fed unenriched rotifers after being exposed to stress, implying that the rotifer-fed larvae handled the stress badly. Whether if this is due to the high carbohydrate content of rotifers that elevates the larvae's blood glucose levels, or the low content of PUFA is not possible to elude from this study.

The lower glycogen levels in the copepod-fed larvae might also be explained by their higher growth rate and that these larvae seem to prioritize rapid growth, rather than storing the glycogen in the liver. Also, this implies that these larvae have better ability to utilize the glycogen. However, it remains uncertain if the larvae fed copepods are better at utilizing glycogen from the liver than the other treatments, since there are both hormonal control and several enzymatic steps involved in glycogen breakdown before being released into the blood (Berg *et al.*, 2007), which has not been analysed in this

study. However, it is noteworthy that trout (*Salmo trutta*) hepatocytes with glycogen levels above 15 % of dry weight are considered inactive (Waagbø *et al.*, 2001).

4.3 Optimisation of live feed production procedures and histological methods to evaluate cod larval nutritional status

The intensive start-feeding of cod performed in the present study was highly controlled, and was similar to what has been reported as the best management practice in cod larval rearing (Helland *et al.*, 2009). However, the supply of copepod nauplii for the larvae being fed copepods was a challenging matter, in terms of nauplii supply. The supply of copepod eggs was predictable, but the hatching success of the eggs was variable. This resulted in a somewhat varying supply of nauplii to the larvae from the Cop7 treatment, where co-feeding with enriched rotifers was performed as a solution. The present start-feeding experiment of cod larvae was successful, in terms of that the larvae from the Copepod treatment had a continued supply of nauplii. However, high concern should be taken into the production, storage, and hatching of copepod eggs to establish a highly predictable supply of feed for the larvae when feeding with *A. tonsa*.

In the present study cod larvae were analysed on 4 dph and 19 dph, where other studies performing histological and stereological analysis usually have sample sizes that cover more of the development going on during start-feeding (Segner, Witt, 1990; Kjørsvik *et al.*, 1991; Elbal *et al.*, 2004; Wold *et al.*, 2008; Wold *et al.*, 2009; Kjørsvik *et al.*, Submitted). A larger sample size over several days can provide more clearly defined results on developmental features. Also a larger sample size per day may give a statistically more reliable result, since normality tests on small sample sizes (<12) do not contain enough information to discriminate between Gaussian and non-Gaussian populations, since they do not contain enough information. However, sectioning and analysis of a larger sample size would be very time consuming and would not have been feasible in the present study. Incidents of low quality TEM photos contributed to lowering the sample size even more in some cases. The relatively low sample size should therefore be taken into consideration when interpreting the results from both qualitative and quantitative analyses on liver and gut.

Many previous stereological studies provide volume measurements and volume fractions of tissue components (Segner, Witt, 1990; Wold *et al.*, 2008; Wold *et al.*, 2009; Kjørsvik *et al.*, Submitted), where several subsequent sections must be made through the tissue of interest. Surface area measurements in a cell may sometimes give deceptive results since the angle and position of the section may be decisive in how the cells and organelles appear on the section. However, in the present study it was desirable to use few sections and still cover an adequate selection of the organs of interest. Comparison of volume measurements from previous studies and surface area measurements from the present study may be intricate. However, trends in size measurements from different studies were comparable with the present studies and gave somewhat similar implications.

High densities of mitochondria in the midgut epithelia cells in cod larvae demonstrated that these cells are energetically active and capable of active transport (Kjørsvik *et al.*, 1991). The intestinal tissue from the T2 sections showed to be somewhat more sensitive to the different nutritional compositions in the live feed than the T1 sections did. The relationship between surface area fractions of mitochondria and nuclei showed an opposite trend in the two gut sections. In the T1 sections, the surface area fraction of nuclei was generally larger than the area fraction of mitochondria, and opposite in the T2 sections. This may imply a difference in energy metabolism between the two different parts of the gut, thus the midgut might reflect the energy state of the larvae more evidently. This may be related to that there has been reported that lipid vacuoles and bodies are more frequent found in the midgut, thus the midgut has a higher absorption of lipids (Kjørsvik *et al.*, 1991) and having a higher metabolic activity. Also, the mitochondria in the T2 sections showed indications of negative effect due to suboptimal nutritional values in the live feed, whereas the mitochondria in the T1 sections did not seem to have any abnormalities due to this. Abnormalities in mitochondria structures may thus imply that the digestive activity of the midgut was reduced.

It has previously been reported that cod larval hepatocytes are more sensitive to dietary induced effects than the intestine, and it has been recommended to focus on liver structure for further analysis of digestive systems in cod larvae (Wold *et al.*, 2009).

Correlations of standard length and nucleus size during development of cod larvae have also shown to be more evident in liver (Kjørsvik *et al.*, Submitted) than it has been in gut (Wold *et al.*, 2008). Compared to the findings in the present study, where cristae structure were observed to be more evidently maturing in the hepatocytes and higher fraction of cristae interdigitation was found, it indicates that maturation features and dietary induced effects might be more evident reflected in the hepatocytes by histological and stereological analyses.

Conclusion

In the present study there has been demonstrated that intensively produced copepods may successfully be used as live feed in the intensive start-feeding of cod larvae. The results show great differences in growth, development, and survival between larvae fed copepods and those fed rotifers, thus indicating nutritional benefits of feeding with copepods instead of rotifers. Since copepods are in fact the natural prey for marine fish larvae, it is likely that there are specific larval adaptations to the copepods biochemical composition.

The results in the present study suggest that the high mortality that occurs during the larval stage of cod may be influenced by the energy status of the larvae, where ability to utilize energy rich essential fatty acids for growth is crucial for survival. Cristae interdigitation was found within mitochondria in the gut and liver of larvae fed rotifers, implying mitochondrial dysfunction. However, this was not found in the copepod-fed larvae, and therefore suggests that feeding cod larvae with copepods is beneficial for normal development of mitochondria, and might be due to the copepods high content of phospholipids that may be directly incorporated into biomembranes.

The glycogen content found in the cod larvae from the present study argues that the size of glycogen storages does not correlate positively with growth and survival of the cod larvae. Copepods, which are considered as the most nutritional favourable live feed for marine fish larvae, does not contain as high amounts of carbohydrates as rotifers. Thus, they may provide less stored glycogen in the liver. Since glycogen is the stored form of glucose, and carbohydrates are not considered as an essential nutritious substance, it is suggested that glycogen storage in cod larvae is does not necessarily give implications of a well-nourished larva.

The liver tissue did more evidently reflect the energy state of the larvae, than the tissue from the gut. The liver did also show a more evident maturation of mitochondria in terms of cristae density. In the analyses of the gut it was the midgut that was more evidently reflecting the live feeds nutritional value. It is therefore suggested that the midgut and especially the liver may be more favourable when analyzing for the nutritional effect on functional development of digestive organs at an early larval stage.

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Appendix 1

Fixation of cod (*Gadus morhua* L.) larvae with glutardialdehyde

The larvae were transferred with a glass pipette to a small sieve (Cell stainor, BD Falcon™, USA) placed in a well of a Tissue culture test plate (TPP®, Switzerland). The first well contained tricaine methanesulfonate (MS-222, Finquel®, Argent Chemical Laboratories Inc., USA) that anesthetized the larvae. The larvae were transferred to another well containing formaldehyde and phosphate buffer for rinsing. The last step was to transfer the larvae into the glutardialdehyde fixation medium consisting of 4.5 mL n-fix stock solution and 500 μL glutardialdehyde. The stock solution consisted of 0.08 M cacodylic buffer, 0.5 % sucrose and 2.5 % paraformaldehyde (PFA). The larvae with the fixation medium were transferred into glass vials for storage in a cold room.

Appendix 2

Hatching test of copepod (*Acartia tonsa*) eggs

The bottle of seawater and eggs were shaken for random distribution of the eggs. 50 μL were diluted to 1 mL of seawater. From this dilution 50 μL was transferred to a petri dish. The droplet was photographed with a stereo microscope (MZ-12.5, Leica Microsystems, Germany) equipped with a colour digital camera (DFW-SX900, Sony, Japan), and the eggs were counted. 10 mL of seawater was added to the dish, it was covered with a lid, tightened and was set to hatch for 48 hours.

The hatched nauplii were fixated with fytofix. A stereo microscope (MZ-12.5, Leica Microsystems, Germany) was applied to count the nauplii, and each counted nauplii were removed from the dish by suction (peristaltic pump, sx-mini Watson-Marlow Alitea Inc., Sweden). The hatching percent was thereby calculated from the initial numbers of eggs.

Appendix 3

Embedding cod (*Gadus morhua* L.) larvae in EPON 812

The plastic medium that the larvae were embedded in consiststed of EPON 812 (25.5 g), DDSA (12.25 g), NMA (12.25 g) and DMP 30 (0.5 g). This solution was contained in 20 mL disposable plastic syringes and stored at -20 ^{o}C.

The larvae were washed in 0.08 M cacodylic buffer 3 x 10 minutes. The they were postfixated in 3 % potassium hexacyanoferrate(II) : 4 % osmium 1:1 (final: 1.5 % : 2 %) in one hour at room temperature protected against light. The larvae were then washed 5x3minutes in distilled water.

The larvae were bulk coloured for 1.5 hours in room temperature with 1.5 % uranylacetat in distilled water while being protected against light. Then they were washed 5x2 minutes in distilled water.

The next step was dehydrating the larvae in ethanol. First 10 minutes in 70 % ethanol, 10 minutes in 90 % ethanol, 2x10 minutes in 96 % ethanol, and finally 3x15 minutes in absolute ethanol.

The embedding in EPON was done gradually by mixing absolute ethanol and EPON and exposing the larvae gradually for a higher concentration of EPON. The larvae were first exposed to a mixture of 3 parts absolute ethanol and 1 part EPON in 30 minutes, then 1 part ethanol and 1 part EPON in 30 minutes, then 1 part ethanol and 3 parts of EPON, and finally only EPON. The EPON and larvae were put to stir over night, and the EPON was replaced by new EPON the next morning. This was then set to polymerize in 60 ^{o}C for 24 hours.

Appendix 4

Staining of histological sections for light microscopy

The sections that were analysed in light microscope were stained with Toluidine Blue and Basic Fuchsin.

Toluidine Blue:

Stock solution was made from 1.0 g Toluidin Blue and 1.0 g sodium borate in 100 mL distilled water. This solution was filtrated two times and stored at room temperature (pH 8-8.5).

Basic Fuchsin:

Stock solution was made from 0.1 g Basic Fuchsin that was added to 100 mL distilled water. This was dissolved by heating it up to a 100 ^{o}C. Then it was filtrated and stored at room temperature (pH 5.5-6.0). The sodium borate solution was prepared by adding 0.65 g sodium borate to 100 mL distilled water. The mix of the stock solution and sodium borate solution (1:1) was made during the staining process immediately after mixing.

Staining procedure:

The sections were gathered from a water droplet on an object glass and heated at 60 ^{o}C until the water evaporated. Then the object glass was heated to 75 ^{o}C to make sure that the sections were fastened properly to the glass. The sections were covered with Toluidin Blue in 2 minutes, and rinsed with distilled water. The object glass was again heated at 75 ^{o}C before the sections were covered with Basic Fuchsin solution for 30 seconds. The sections were first rinsed with distilled water, then with 70 % ethanol, and with distilled water once more. They were dried before a cover glass was mounted on each object glass with Cytoseal™XYL (Richard-Allan Scientific, USA).

Appendix 5

Contrasting histological sections for transmission electron microscopy

The sections that were analysed in electron microscope were contrasted with lead citrate to get a proper image contrast.

Lead citrate:

Lead citrate was made beforehand by dissolving 0,1g of lead citrate in 50 mL 0.1 sodium hydroxide (0.2 g sodium hydroxide to 50 mL distilled water). This was contained on a plastic tube over night.

Contrasting procedure:

Each section was placed on a copper grid covered with a carbon film, with mesh sizes 75 (Jed Pela Inc., USA) and 100 (EMS Inc., USA). The section was bulk coloured with uranyl acetate beforehand.

Three beakers were filled with distilled water and one with lead citrate. The grid with the section was put into the beaker with the lead citrate for 30 seconds by holding it with a pair of tweezers. The grid was rinsed consecutive in the three beakers of distilled water. The moist was removed from the grid by gently pushing a filter paper where there were remains of water droplets.

Appendix 6

Table A. 1: Mean dry weight (mg larva^{-1}) data from each tank of each treatment, divided into each sampling day. Standard error (SE) of mean is presented for each tank, and the total number of larvae weighted (Total N) is presented for each treatment on every sampling day.

			Dry weight (mg larva^{-1})		
Dph	**Treatment**	**Tank**	**Mean**	**SE**	**Total N**
3	Copepod	1	.0558	.0020	12
		2	.0571	.0023	12
		3	.0575	.0025	11
	Cop 7	1	.0579	.0020	9
		2	.0571	.0023	12
		3	.0567	.0023	15
	RotMG	1	.0571	.0023	12
		2	.0571	.0023	12
		3	.0571	.0023	12
	RotChl	1	.0578	.0022	12
		2	.0571	.0023	12
		3	.0571	.0023	12
5	Copepod	1	.0544	.0022	12
		2	.0539	.0021	12
		3	.0533	.0023	11
	Cop 7	1	.0533	.0025	10
		2	.0539	.0021	12
		3	.0538	.0020	13
	RotMG	1	.0539	.0021	12
		2	.0539	.0021	12
		3	.0539	.0021	12
	RotChl	1	.0539	.0021	12
		2	.0539	.0021	12
		3	.0539	.0021	12
8	Copepod	1	.0742	.0028	12
		2	.0705	.0024	12
		3	.0700	.0029	11
	Cop 7	1	.0639	.0022	12
		2	.0754	.0029	12
		3	.0729	.0024	11
	RotMG	1	.0654	.0015	12
		2	.0578	.0040	12
		3	.0700	.0022	12
	RotChl	1	.0660	.0024	12
		2	.0613	.0023	12
		3	.0622	.0026	12
14	Copepod	1	.1369	.0061	12
		2	.1397	.0088	12
		3	.1512	.0068	11
	Cop 7	1	.1218	.0058	12
		2	.1122	.0062	11
		3	.1052	.0086	12
	RotMG	1	.0884	.0046	12
		2	.1082	.0038	12
		3	.0915	.0063	12
	RotChl	1	.1078	.0055	12
		2	.1095	.0084	12
		3	.1127	.0049	12

19	**Copepod**	**1**	.2217	.0122	12
		2	.2276	.0164	12
		3	.2355	.0169	11
	Cop 7	**1**	.1793	.0177	12
		2	.1592	.0104	12
		3	.1753	.0172	12
	RotMG	**1**	.1836	.0081	12
		2	.1506	.0199	12
		3	.1816	.0215	12
	RotChl	**1**	.1872	.0041	12
		2	.1728	.0113	12
		3	.1486	.0180	11
33	**Copepod**	**1**	1.0881	.0840	20
		2	1.3692	.0707	20
		3	1.1937	.0649	19
	Cop 7	**1**	1.0593	.0744	20
		2	.8534	.0886	20
		3	.9796	.0853	20
	RotMG	**1**	.9007	.0735	20
		2	1.1003	.0924	20
		3	.9950	.0562	20
	RotChl	**1**	1.0603	.0603	20
		2	.8359	.0585	20
		3	.9160	.0769	20
40	**Copepod**	**1**	1.9436	.1417	40
		2	2.5393	.1493	40
		3	2.0920	.1529	39
	Cop 7	**1**	1.3485	.0977	40
		2	1.5911	.1345	40
		3	1.2880	.0911	40
	RotMG	**1**	1.0163	.0655	40
		2	.9846	.0711	40
		3	1.0975	.0752	39
	RotChl	**1**	1.4450	.1115	40
		2	1.0187	.0691	39
		3	1.1900	.0715	39
60	**Copepod**	**1**	23.0411	1.4971	48
		2	18.1025	1.2519	46
		3	25.6804	1.5650	50
	Cop 7	**1**	16.6316	1.0472	49
		2	16.9039	.9974	50
		3	12.1357	.8001	55
	RotMG	**1**	10.9420	.7068	50
		2	10.3994	.8053	49
		3	14.4905	1.2500	50
	RotChl	**1**	12.2723	.7200	48
		2	10.1739	.5445	50
		3	13.4890	.7593	50

Appendix 7

Table A. 2: Mean DWI (%) for each treatment in intervals, and for the whole start-feeding period of 60 days (n=9-50). Standard error (SE) of mean is presented for each interval.

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

Appendix 8

Table A. 3: Mean survival (%) of each treatment from 38-60 dph (n=3). Standard error of mean (SE) for each treatment is calculated for each day. The total N is three tanks for each treatment.

		Survival (%)	
Dph	**Treatment**	**Mean**	**SE**
38	**Copepod**	31.81	1.34
	Cop 7	21.84	3.79
	RotMG	15.60	.34
	RotChl	15.15	2.29
39	**Copepod**	31.02	1.07
	Cop 7	21.66	3.72
	RotMG	15.47	.32
	RotChl	15.09	2.31
40	**Copepod**	29.70	1.93
	Cop 7	21.48	3.70
	RotMG	15.40	.32
	RotChl	14.98	2.32
41	**Copepod**	29.40	1.99
	Cop 7	21.13	3.71
	RotMG	14.96	.64
	RotChl	14.84	2.35
42	**Copepod**	26.51	2.08
	Cop 7	19.92	3.59
	RotMG	14.52	.55
	RotChl	14.57	2.35
43	**Copepod**	24.32	2.22
	Cop 7	18.82	3.56
	RotMG	13.94	.48
	RotChl	14.14	2.27
44	**Copepod**	23.55	2.14
	Cop 7	18.20	3.61
	RotMG	13.52	.56

44	**RotChl**	13.62	2.29
45	**Copepod**	22.80	2.03
	Cop 7	17.62	3.65
	RotMG	12.96	.55
	RotChl	12.79	2.14
46	**Copepod**	22.45	1.97
	Cop 7	17.22	3.73
	RotMG	12.51	.49
	RotChl	12.37	2.06
47	**Copepod**	22.26	1.96
	Cop 7	16.92	3.69
	RotMG	12.13	.41
	RotChl	12.00	2.02
48	**Copepod**	22.08	1.98
	Cop 7	16.71	3.63
	RotMG	11.91	.38
	RotChl	11.73	2.02
49	**Copepod**	21.82	1.97
	Cop 7	16.50	3.51
	RotMG	11.72	.42
	RotChl	11.48	1.91
50	**Copepod**	21.51	2.01
	Cop 7	16.28	3.53
	RotMG	11.55	.42
	RotChl	11.35	1.88
51	**Copepod**	21.31	2.03
	Cop 7	16.09	3.42
	RotMG	11.39	.42
	RotChl	11.18	1.80
52	**Copepod**	21.24	2.03
	Cop 7	16.03	3.41
	RotMG	11.21	.51
	RotChl	11.09	1.76

53	**Copepod**	21.20	2.01
	Cop 7	15.95	3.41
	RotMG	11.11	.54
	RotChl	10.98	1.70
54	**Copepod**	21.17	2.00
	Cop 7	15.81	3.39
	RotMG	11.03	.56
	RotChl	10.91	1.68
55	**Copepod**	21.03	2.01
	Cop 7	15.66	3.42
	RotMG	10.95	.58
	RotChl	10.71	1.75
56	**Copepod**	20.77	1.99
	Cop 7	15.38	3.45
	RotMG	10.85	.58
	RotChl	10.48	1.81
57	**Copepod**	20.53	2.02
	Cop 7	15.18	3.45
	RotMG	10.76	.58
	RotChl	10.40	1.81
58	**Copepod**	20.38	2.02
	Cop 7	15.02	3.42
	RotMG	10.70	.60
	RotChl	10.28	1.78
59	**Copepod**	19.98	1.99
	Cop 7	14.60	3.29
	RotMG	10.61	.60
	RotChl	10.16	1.80
60	**Copepod**	19.53	2.04
	Cop 7	14.28	3.31
	RotMG	10.52	.61
	RotChl	9.95	1.73

Appendix 9

Table A. 4: Mean surface area fractions (%) of mitochondria, nuclei and other cell components in enterocytes from the T1 sections, presented with standard error (SE) of mean and total number of analysed larvae for each treatment (Total N).

Treatment	Surface area fraction of mitochondria (%)			Surface area fraction of nuclei (%)			Surface area fraction of other cell components (%)		
	Mean	Standard Error of Mean	Total N	Mean	Standard Error of Mean	Total N	Mean	Standard Error of Mean	Total N
4 dph	7.0	.2	2	13.6	1.7	2	79.5	1.8	2
RotChl	11.7	1.1	5	12.3	2.6	5	76.0	2.0	5
RotMG	10.0	.8	5	10.2	1.7	5	79.8	1.6	5
Cop 7	11.7	1.5	5	15.7	2.0	5	72.7	1.6	5
Copepod	9.5	.9	5	14.6	3.2	5	75.9	2.4	5

Table A. 5: Mean surface area fractions (%) of mitochondria, nuclei and other cell components in enterocytes from the T2 sections, presented with standard error (SE) of mean and total number of analysed larvae for each treatment (Total N).

Treatment	Surface area fraction of mitochondria (%)			Surface area fraction of nuclei (%)			Surface area fraction of other cell components (%)		
	Mean	Standard Error of Mean	Total N	Mean	Standard Error of Mean	Total N	Mean	Standard Error of Mean	Total N
4 dph	6.5	1.3	5	17.0	2.7	5	76.5	2.8	5
RotChl	10.2	1.2	5	11.0	.9	5	78.8	1.2	5
RotMG	9.6	1.2	5	6.4	1.7	5	84.0	2.4	5
Cop 7	10.5	2.3	5	9.2	1.0	5	80.3	1.8	5
Copepod	9.2	1.9	5	5.5	1.0	5	85.3	1.8	5

Table A. 6: Mean surface area fractions (%) of glycogen, mitochondria, nuclei and other cell components in hepatocytes with standard error (SE) of mean and total number of analysed larvae for each treatment (Total N).

Treatment	Sufrace area fraction of glycogen (%)			Surface area fraction of mitochondria (%)			Surface area fraction of nuclei (%)			Surface area fraction of other cell components (%)		
	Mean	Standard Error of Mean	Total N	Mean	Standard Error of Mean	Total N	Mean	Standard Error of Mean	Total N	Mean	Standard Error of Mean	Total N
4 dph	0	0	5	13.0	1.6	5	11.7	1.4	5	75.4	2.7	5
RotChl	26.3	4.1	5	16.3	1.0	5	7.4	1.1	5	51.0	4.0	5
RotMG	17.4	1.8	5	14.2	1.0	5	9.5	.8	5	58.9	2.3	5
Cop 7	13.1	3.6	5	16.0	1.4	5	10.9	1.0	5	60.0	2.8	5
Copepod	2.2	1.4	5	15.6	1.2	5	7.6	1.5	5	74.6	3.6	5