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Ballan wrasse (*Labrus bergylta*) Larvae and Live-Feed Quality; Effects on Growth and Expression of Genes related to Mitochondrial Functions

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Marine Coastal Development
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Abstract

Salmon production is threatened by sea louse *Lepoptheirus salmonis*, affecting both the environment and the industry's economy. Use of the cleaner fish ballan wrasse (*Labrus bergylta*) is a promising method of salmon delousing with good results. The individuals used in salmon farms have so far derived from wildfish catches, but there is now interest in ballan wrasse aquaculture, as a more sustainable and environmental-friendly solution.

During this experiment ballan wrasse larvae were fed with either enriched rotifers *Brachionus* sp., followed by enriched *Artemia franciscana* (Rot treatment) or with reared copepods *Acartia tonsa* (Cop treatment) until 45 day post hatch. This is probably the first study on ballan wrasse larvae with copepods as exclusive first feed. Larvae of the two treatments were compared for growth, survival and expression of seven genes (cyc1, cox5a, mnsod, fxn, crls1 and pla2g6[*]) whose encoding products are localized in the mitochondrion. All genes were related to oxidative phospholyration, with two of them being parts of the final complexes of the electron transport (cyc1, cox5a).

Cop larvae had a significantly higher growth during the whole experiment, while survival did not differ significantly. Gene expressions had good correlations with the larval standard length, implying that body size is more reliable than age for denoting the larval development. All genes except pla2g6 were higher expressed for the Cop treatment during the first 8 days post hatch, suggesting higher mitochondrial activity and energy (ATP) generation for the initial larval period. Specifically cox5a expression corresponded with larval dry weight increase, implying a strong molecular effect of the initial diet quality on cellular energy generation and growth.

The present study underlines the importance of diet quality during the early days of the ballan wrasse life and results confirm other studies stating that reared *A. tonsa* is an optimal fish larval live feed for this period. Results also imply that copepods have a positive impact on the mitochondrial respiration, especially for the early larval days.

[*] cytochrome *c1*, cytochrome oxidase subunit Va, manganese superoxidase dismutase, frataxin, cardiolipin synthase, phosholipase A_2 group VI

Contents

Abbreviations

ARA	arachidonic acid, 20:4(ω-6)
ATP	adenosine triphosphate
cDNA	complimentary deoxyribonucleic acid
CL	cardiolipin
COX	cytochrome oxidase
cox5a	cytochrome oxidase subunit Va encoding gene
crls1	cardiolipin synthase encoding gene
cyc1	cytochrome *c1* encoding gene
DHA	docosahexaenoic acid, 22:6(ω-3)
dph	days post hatch
EPA	eicosapentaenoic acid, 20:5(ω-3)
FA	fatty acid(s)
FXN	frataxin
fxn	frataxin encoding gene
HUFA	highly unsaturated (minimum 3 double bonds) fatty acid(s)
MH	myotome height, perpendicular to the skeleton axis from behind the anus
mRNA	messenger ribonucleic acid
MNE	mean normalized expression
MnSOD	manganese superoxidase dismutase
mnsod	manganese superoxidase dismutase encoding gene
OP	oxidative phosphorlyration
PL	phospholipid(s)
PLA_2	phospholipase(s) of the superfamily A_2
PLA_2G_6	phospholipase A_2 group VI
pla2g6	phosholipase A_2 group VI encoding gene
PMMA	polymethyl methacrylate
PUFA	polyunsaturated (minimum two double bonds) fatty acid(s)
qPCR	quantatitative (or real time) polymerase chain reaction
RNase	ribonuclease
ROS	reactive oxygen species
RT-PCR	reverse transcription polymerase chain reaction
SE	standard error of the mean
SL	standard length, upper lip to end of notochord
SOD	superoxidase dismutase
$sPLA_2$	secratory phospholipase A_2
VDAC1	voltage-dependent anion channel 1
vdac1	voltage-dependent anion channel 1 encoding gene

1. Introduction

1.1 Background

Norway is Europe's leader in aquaculture production and the top producer of Atlantic salmon (*Salmo salar*) worldwide (FAO, 2012). Salmon products are the fourth most exported goods in Norway (SSB), making it a very important source for the country's income. However cultured salmon production is negatively affected by the ectoparasitic copepod sea louse (*Lepoptheirus salmonis*). Infestations have been occurring since very early in the history of salmon farming (Brandal & Egidius, 1977) and plague the farmers until today. Sea louse gets attached on the fish' skin and consumes mucus, epidermis and blood, often as deeply as bearing the skull (Brandal & Egidius, 1977). Fish' osmoregulation gets affected, making them prone to secondary bacterial infections (Wootten *et al*., 1982; Grimnes & Jacobsen, 1996; Bjørn & Finstad, 1997; Wagner et al., 2003). The fact that lice-infested fish cannot be sold, as well as deaths in the farmed population damages the production severely; the economic loss for Norway was over 500 million NOK in 1997 and over 117 million NOK in 2008 (Pike & Wadsworth, 1999; Costello, 2009). Apart from the economical, environmental issues rose as well; salmon cultures serve as a continuous "lice reservoir" putting wild salmonid populations at potential risk (Morton *et al.*, 2004; Heuch *et al.*, 2005; Krkosek *et al*., 2005; Skilbrei *et al.*, 2013; Torrissen *et al.*, 2013).

Throughout time various methods have been used for treating the infestations. Today five chemotherapeutant types are being applied by salmon farmers: organophosphates, avermectins, pyrethrins/pyrethroids, benzoylphenyl ureas, and hydrogen peroxide (Roth, 2000). Negative effects of the pesticides have been noted, suggesting that they cause sea louse to eventually become less sensitive (Jones *et al.*, 1992; Treasurer *et al.*, 2000; Sevatdal *et al.*, 2002; Fallang *et al.*, 2004; Whyte, 2013). Moreover, chemotherapeutants' toxicity increases for smaller fish (Kumaraguru & Beamish, 1981) making it possible for small non-targeted species as well as invertebrates of the local natural environment to be affected.

Sea louse pesticides *per se*, as well as the labor needed for the application are expensive. Growth gets affected directly by the treatment-related stress that may appear and the fact that some medicines require a period of starvation (Treasurer, 2002).

Biological control techniques are now used as a partial solution to the sea lice issue. Goldsinny (*Ctenolabrus rupestris*), rock cook (*Centrolabrus exoletus*) and cuckoo wrasse

(*Labrus ossifagus*) were the first species to be tested as salmon lice cleaners (Bjordal, 1988). Wrasses' superiority as a delousing method lies in the fact that they are environmentally less intrusive than chemicals, and induce no resistance-related dangers. In addition to that, they are less costly to use compared to pesticides (Treasurer, 2002).

In Norway, Labridae have been harvested naturally and used as cleaner fish in salmon farms since 1988. Even though at some point wrasses were mostly replaced by chemical pesticides, from 2006 use of wrasses has been increasing. The main reason was the resistance that lice developed towards the chemicals, resulting to poor effectiveness. Harvests of wrasses doubled from 2009 to the following year, reaching approximately 11 million fish. Need for cleaner fish was estimated in 2011 as 15 million individuals per year for the whole of Norway (Hamre & Sæle, 2011); this number is probably larger today, as the salmonid production has increased. These facts indicate that there is need for intensive production of cleaner fish. Even though there is still not evidence that natural wrasse populations are in danger, it is possible that excessive fishing can harm small and local populations (Espeland *et al.*, 2010). Delousing effectiveness varies from species to species and fish age, but naturally harvested wrasses tend to be a mixture of species and ages. Apart from the high mortality of wrasses during transportation, harvests often have a seasonal variation. Intensive production of cleaner fish, and specifically wrasses, would overcome these barriers, securing a steady supply of one species of a controlled size throughout the year.

1.2 Ballan wrasse (*Labrus bergylta*) in aquaculture

The first ballan wrasse farm in Norway was established in 2009 by Marine Harvest (Espeland *et al.*, 2010) and since then more companies have followed. Ballan wrasse is the largest (reaching 60 cm in length) and the third most abundant of the wrasses in the Norwegian waters (Kvenseth & Mortensen, 2005; Havforskningsinstittutet, 2012) and it is considered the most effective cleaner species, due to its broad versatility (Sayer & Treasurer, 1996; Espeland *et al*., 2010). It has been observed to be an active cleaner fish even in very low temperatures (3.5 ^{o}C; Kvenseth *et al*., 2003), although it was recently suggested that sudden changes in water temperature can be critical for its cleaning activity (Lein & Helland, 2013). Ballan wrasse is highly effective; only 0.5 % (number of individuals to number of farmed fish per sea cage) is considered enough for salmon delousing, while other wrasse species are needed in larger amounts (Kvenseth *et al.*, 2003; Solheim, 2011). Unlike other wrasses, it prefers

plucking adult bearing eggs lice, controlling the population of the next generation (Kvenseth *et al.*, 2003). However juvenile production of ballan wrasse is rather challenging.

1.2.1 Larvae nutrition; requirements and live-feed debate.

Pelagic marine fish larvae hatch being very small and relatively little developed. Newly hatched ballan wrasse is no exception; it measures approximately between 3 and 3.5 mm standard length, while the organ development seems to be similar as in other marine larvae (Gagnat, 2012). Ballan wrasse farmers use traditional live feed for the marine species: enriched rotifers followed by enriched brine shrimp (*Artemia* sp.) nauplii. Rotifers and brine shrimp are simple to produce in both small and large scale cultures and culturing systems are improving until today (Conceição *et al.*, 2010; Kostopoulou *et al.*, 2012). Nevertheless, production of high quality marine fish and specifically ballan wrasse juveniles is often a bottleneck, as it is hampered by mortality, low growth and skeletal deformities and malnutrition is often the case (Hamre, 2006; Ottesen *et al.*, 2012a; Sørøy, 2012).

First feeding with copepods has immerged as a new possibility instead of rotifers and brine shrimp the late years. So far copepods and in particular *Calanus finmarchicus* have been harvested naturally and used as feed in larval aquaculture. When harvested naturally, copepod supply can be seasonal or unpredictable, while it is possible that parasites are transferred to the larvae under feeding. These problems can be minimized by farming, nevertheless copepod cultivation has so far been considered expensive and has remained in experimental level. SINTEF and NTNU (Norway) have been researching culturing systems for production of calanoid copepods for the past decade (Evjemo *et al.*, 2008). In particular *Acartia tonsa* is a well-studied species (Ismar *et al.*, 2008; Nesse, 2010; Skogstad, 2010; Hagemann, 2011; Thuy, 2011), which has resulted in better growth, physiological development, stress tolerance and survival of Atlantic cod (Eidsvik, 2010; Halseth, 2010; Hansen, 2011; Kortner *et al.*, 2011; Norheim, 2011) and on ballan wrasse (Almli, 2012; Berg, 2012; Gaganat, 2012; Sørøy, 2012). After the encouraging results, NTNU and SINTEF are developing methods for large scale production of copepod eggs and foresee a promising future.

Ballan wrasse does not possess a functional stomach neither as a larva, nor as an adult, and pyloric caeca are also absent (Hamre & Sæle, 2011). There is therefore need for easily digestible first feed, rich in short chained proteins and free amino acids (Rønnestad *et al.*, 1999, 2000). Total amino acids in copepods are 596 ± 59 mg g^{-1} of dry weight, of which a

notable part (12-13 %) represents free amino acids (Hamre *et al.*, 2013). Stoss *et al.* (2004) referred to this amount as 55-58 % of the dry weight. The corresponding total amount of amino acids in rotifers and brine shrimp has been measured as 396 ± 12 and 471-503 mg g^{-1} of dry weight, from which free amino acids represent only 5-7 % and 9-10 %, respectively (Srivastava *et al.*, 2006; van der Meeren *et al.*, 2008; Hamre *et al.*, 2013). Amino acids, apart for being the basic structural unit of protein biosynthesis, function also as N_2 suppliers and precursors for various biochemical reactions. Ten amino acids, namely arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine and valine, cannot be synthesized *de novo* by the fish larvae, and therefore are essential to be supplied with feed, in a digestible form (Jobling, 2004). Ratio of these ten amino acids:free amino acids do not show big differences between the three live-feed species (Hamre *et al.*, 2013). Interestingly, though, the always free amino acid taurine, which is almost absent in rotifers (0.08 ± 0.04 % of protein) (Srivastava *et al.*, 2006; Hamre *et al.*, 2013) improves marine fish larval growth and enhances metamorphosis (Chen *et al.*, 2004, 2005; Pinto *et al.*, 2010).

As well as proteins, phospholipids (PL) are crucial nutrients for the marine fish, and requirements are much higher in the larval than the adult period. (Kanazawa *et al.*, 1981; Coutteau *et al.*, 1997; Sargent *et al.*, 1999; Cahu *et al.*, 2003a, 2003b). Marine fish larvae cannot synthesize enough PL to cover their fast growth and development (Coutteau *et al.*, 1997; Tocher *et al.*, 2008) and therefore it is necessary to balance the PL deficiency through their feed. PL possess a hydrophobic and a hydrophilic structural units and therefore form bilayers in water; this is the paramount of the biomembrane formation of all eukaryotic cells and cellular organelles, including mitochondria. PL play an important constructive and functional role in the cellular membranes, especially on neural tissues such as eyes and brain (Bell *et al.*, 1995; Furuita *et al.*, 1998). PL are involved in the lipid transportation from the gut, and are also suggested to improve the diet palatability (Szich *et al.*, 2005; Berg, 2012). Finally PL serve as an important energy source during egg development and endogenous feeding (Izquierdo & Koven, 2011).

PL and generally lipids are the main source of energy (Sargent *et al.*, 2002) and provide larvae with fatty acids (FA), important nutrients for the marine larvae. FA and can be saturated or unsaturated, depending on the existence of double bonds in their structure. Three highly unsaturated FA (HUFA) are of particular importance; docosahexaenoic acid (22:6(ω-3); DHA), eicosapentaenoic acid (20:5(ω-3); EPA) and arachidonic acid (20:4(ω-6); ARA) are essential for providing larvae a good growth and tissues development. Ratios of

DHA:EPA and EPA:ARA are also important (Curé *et al.*, 1996; Rodríguez *et al.*, 1998, Tocher, 2010). Since enzymes Δ5-desaturase and C18-C20 elongase are absent in marine fish larvae, the prementioned HUFA cannot be synthesized *de novo* (Støttrup & McEvoy, 2003; Olsen *et al.*, 2004). HUFA can be supplied either as structure units of PL, or as FA incorporated in the molecular structure of triacyglycerols (TAG; fats and oils). While rotifers and brine shrimp meet the larval protein requirements, they are naturally insufficient in *ω-3* HUFA, especially DHA and EPA, therefore they are enriched before use as a feed to larvae (Lubzens & Zmora, 2003; Hamre *et al*., 2013). Enrichment mediums contain DHA, EPA and arachidonic acid (20:4(ω-6); ARA), as well as proteins, vitamins, lipids, and some minerals. However the provided HUFA get incorporated in the neutral lipid fraction of rotifers and brine shrimp. HUFA supplied as PL compounds are more effectively digested, and thus more beneficial than the HUFA deriving from the TAG. Several studies show improvement of the digestive tract development, growth, lipid digestion and vertebrae formation, as well as direct incorporation of PL in the cellular membranes (Olsen *et al*., 1991; Coutteau *et al*., 1997; Cahu *et al.*, 2003a; Gisbert *et al*., 2005; Kjørsvik *et al*., 2009; Wold *et al*., 2009). In addition, brine shrimp catabolise the DHA of their enrichment rapidly, ending up with a low DHA and DHA:EPA amount, when fed to the larvae (Evjemo *et al.*, 1997; Olsen, 2004).

On the other hand copepods' PL are rich in PUFA and HUFA, in particular DHA and EPA, and the content depends on the species and their food (Witt *et al.*, 1984; Evjemo & Olsen, 1997; Nanton & Castell, 1998; Anderson & Pond, 2000; Payne & Rippingale, 2000; Evjemo *et al.*, 2003). Their EPA:ARA ratio is also higher than the enriched rotifers and *Artemia* (Hamre *et al.*, 2013). They are therefore a more suitable live feed for various species of marine fish larvae (Evjemo *et al.*, 2003; Kjesbu *et al.*, 2006; Eidsvik, 2010; Halseth, 2010; Overrein, 2010; Hansen, 2011; Norheim, 2011).

1.2.2 Diet effects under the microscope: cells and mitochondria

As mentioned above, copepods as a first feed affect positively the development of the pelagic larvae. Diet effects are often the reflection of biochemical reactions occurring on the cellular level, and the cell's powerhouse: the mitochondrion. Mitochondria are unique semi-autonomous organelles of eukaryotic cells with own genome, which serve important functions as energy production (ATP), respiration and apoptosis programming. The mitochondrial inner

membrane folds into numerous cristae, offering a big surface for functions and separating space into the matrix and the intermembrane space.

Studies on marine fish larvae have shown that the dietary fatty acid profile can have an effect on the mitochondrial condition (Olivotto *et al.*, 2011). Swollen mitochondria have been associated to dysfunction of lipid metabolism at sea bream, sea bass and pike-perch larvae (Diaz *et al*., 1998), or lipid malnutrition (Segner & Möller, 1984; Leifson *et al*., 2003a, 2003b). In a recent study in cod hepatocytes mitochondria showed a different inner membrane structure and density for different lipid diets (Wold *et al*., 2009). These observations, together with the prementioned variation in FA content (Paragraph 1.2.1) of the different live feed, indicate that the type of live feed offered to the larvae can have a direct effect on mitochondrial membranes, and possibly the cellular overall well-being. Mitochondrial inner membrane is structured of a PL bilayer, proteins and lacks cholesterol, but the role of membrane flexibility is played by cardiolipin (CL; Filho, 2007), a crucial PL which is exclusively synthesized in the mitochondrial inner membrane (Hatch, 1996). CL, unlike other PL, possesses four acyl-chains, connected to a three-glycerol back bone; this results to a cone-shaped structure, which is responsible for the hexagonal phase of the inner membrane during fusion. The hexagonal structures are also important for the spatial connection between the inner and the outer membrane (Houtkooper & Vaz, 2008).

Norheim (2011) fed Atlantic cod larvae with rotifers, brine shrimp and copepods in different sequences and enrichments. Apart from the expected positive observations of copepods on growth and survival, she also examined digestive tissues and made a striking observation: in many mitochondria of larvae fed- especially unenriched- rotifers on 19 dph (days post hatch), inner membrane was not distinct and cristae seemed undefined. On the contrary, larvae fed copepods displayed clear mitochondrial cristae, even the ones that had been fed copepods for just until the 7 dph. Such findings were observed in both liver and gut tissue and a possible conclusion was low functionality, since inner membrane plays an active role in the oxidative phosphorylation (OP). The rotifer-fed larvae were measured to have high glycogen concentration stored in the liver, which indicated an inability of those cells to metabolize glycogen, probably because of the bad quality cristae. Olivotto *et al.* (2011) made a similar observation for the muscle tissue of false percula clown fish. Norheim (2011) assumed that copepods, being rich in PL, affected directly the inner membrane structure, by providing a high amount of PL and PUFA to the fish. Mitochondria were also smaller for yolk sac larvae (4 dph), which according to Ghadially (1997) means low metabolic activity; interestingly,

mitochondria of the cod larvae (19 dph) fed unenriched rotifers were found to be smaller than the ones of the larvae fed either enriched rotifers or copepods.

Various mitochondrial actions are space-specific, i.e. oxidative phosphorylation (OP) occurs in the intermembrane space, and inner membrane very often plays an important role. Other factors that can change membrane composition in fish are osmotic pressure and temperature and are studied for many decades (Richardson & Tapell, 1962; Wodtke, 1977; Bell *et al.*, 1985). Age and stage of development, as well as the metabolic activity of the tissue are known to affect the size and the density of the mitochondria (Ghadially, 1997; Høvde, 2006; Wold *et al.*, 2008). Diet can also have a direct effect on the mitochondrial membranes composition, actions, and hence alter their appearance.

Outside the mitochondrion, PL are hydrolyzed by phospholipases. Phospholipases of the superfamily A_2 (PLA_2) play an important role catalyzing the hydrolysis of the ester bond at the *sn-2* position of PL, producing a free fatty acid and a lysophospholipid (Izquierdo & Henderson, 1998; Izquierdo, 2000). PLA_2 are grouped according to their specificity, tissue of function and dependency on the presence of calcium cations (Ca^{2+}). A family of PLA_2, are Ca^{2+} independent ($iPLA_2$) and function within the cytoplasm. $iPLA_2$ are involved in important functions, such as cell proliferation, apoptosis, membrane transport and direct alteration of the membranes tubulation7 (Brown *et al.*, 2003; Morrison *et al.*, 2012). Another important family is secretory PLA_2 ($sPLA_2$), which is Ca^{2+} dependent; its activity is found to be affected by the diet of ballan wrasse larvae, showing increased activity for a rotifer diet in comparison to a more rich in phosphorus formulated feed (Hansen *et al.*, 2013). Because of its notable role on cellular PL metabolism, a coding gene of an $iPLA_2$ together with various genes involved in mitochondrial functions are being focused on in this study.

1.5 Aim of the study

In the present study ballan wrasse larvae are treated with two different feeding regimes. First treatment includes enriched rotifers of the *Brachionus sp.*, with enriched *Artemia franciscana* taking over at 40 dph, after a co-feeding period. Second treatment includes a HUFA-rich diet of exclusively cultivated *Acartia tonsa* fed cultured *Rhodomonas baltica*. This experiment is possibly the first to involve cultivated copepods as the exclusive feed for the whole start feeding period of ballan wrasse. To observe the direct dietary effect, larval growth (dry

weight, % daily dry weight increase, standard length and myotome height) and % survival were examined.

With the interest on ballan wrasse growing in Norway and inspired by Norheim's work (2011), the present study aims to observe effects on the mitochondria, when ballan wrasse is fed cultivated copepods as the only live feed throughout the larval phase. Expression of genes linked to mitochondrial functions (mitochondrial respiration, membrane permeability, PL metabolism) is measured. Selected genes and function summary are displayed on Table 1.

Expression of the specific genes is measured for the first time on ballan wrasse and some of them have never before tested on fish tissue. Finally, experimental hypothesis is that larvae fed copepods will show a better growth, higher or similar survival, and different expression of genes than the ones fed the traditional live-feed.

Table 1. Genes, encoding products, important functions and cellular mechanisms involved. Reference: Genecards® online database (genecards.org)

Mechanisms	**Gene**	**Encoding product**	**Product functions**
subunits in oxidative phospholyration chain	**cyc1**	cytochrome *c1*	Subunit of the *b-c1* complex (III complex), which accepts electrons from Rieske protein and transfers them to cytochrome *c* in the mitochondrial respiratory chain.
	cox5a	cytochrome oxidase subunit Va (COX 5a)	Subunit Va of cytochrome oxidase enzyme (COX). COX is the terminal enzyme of the respiratory chain (IV complex); it transfers electrons from cytochrome *c* to molecular oxygen.
membrane permeability, osmoregulation, Ca^{+}, ATP transfer, oxidative phosphorylation	**vdac1**	voltage-dependent anion channel 1 (VDAC1)	Protein which is a major component of the outer mitochondrial membrane, facilitating exchange of metabolites and ions.
ROS regulation, oxidative phosphorylation	**mnsod**	manganese superoxidase dismutase (MnSOD)	Enzyme that catalyzes the dismutation of superoxide into O_2 and H_2O_2, protecting the cell from oxidation.
multiple functions, oxidative phosphorylation	**fxn**	frataxin (FXN)	Protein which regulates iron transport and respiration and promotes the heme biosynthesis.
mitochondrial inner membrane structure, permeability, apoptosis, fusion, oxidative phosphorylation	**crls1**	cardiolipin synthase (CRLS1)	Enzyme that synthesizes cardiolipin (CL), transferring the phosphatidylglycerol group from one molecule to another.
cell proliferation, apoptosis, mitochondrial membrane transport, alteration of the membranes tubulation, inner membrane lipids protection	**pla2g6**	phospholipase A_2 group VI (PLA_2G_6)	Cytosolic, Ca^{2+} independent enzyme which metabolizes phospholipids. Encoded in mitochondria.

2. Materials and Methods

The experimental part of the present study, as well as growth and survival analyses took place at NTNU Centre of Fisheries and Aquaculture and SINTEF Fisheries and Aquaculture, (Trondheim, Norway) from 28 September to 9 November 2012. Molecular analyses were performed at NIFES Laboratory for Molecular Biology (Bergen, Norway) from 4 to 26 February 2013.

2.1 Rearing of ballan wrasse (*Labrus bergylta*) larvae

Ballan wrasse (*Labrus bergylta*, Ascanius 1767) larvae aged 2 days post hatch (dph) were purchased from Nofima (Sunndalsøra, Norway) and transported by car in plastic bags. Upon arrival oxygen and temperature were measured and larvae were acclimatized to temperature of the holding tank (12 ^{o}C; 250 L), before release. The holding tank was gently aerated through a central tube, to minimize turbulence. Larval density was measured the following day (3 dph), by taking several samples of 1 L and counting the larvae. Larvae were then transferred to six 100 L, flow-through, black-walled tanks at an estimated density of 8200 larvae per tank. Temperature and O_2-levels were measured daily (ProODO Optical Dissolved Oxygen Meter, YSI Inc., OH, USA), being kept at 12-16 ^{o}C (table 2.1) and at 80 % respectively. The aeration was kept at low levels.

Sea water was pumped from a distance of 800 m from the shore of Brattørkaia (Trondheim, Norway) in 70 m of depth. Water was first flowed through two sand filters where particles larger than 40 μm were removed. It was then matured as described by Skjermo *et al*. (1997). Matured water flowed through a heat exchanger which was bringing it to 14 ^{o}C, before entering a water degasser of low atmospheric pressure to avoid N_2 supersaturation. Water was then gathered in a 5000 L tank and went through the above cycle repeatedly. Tank held biofilter substrates of 1 m^3 volume and total surface of 800 m^2. Water finally flowed through a second heat exchanger where it got chilled to desired temperature (12 ^{o}C) before entering the rearing tanks. Maximum flow from the maturation tank was twice its volume per day (5.9 L $minute^{-1}$).

Table 2.1 Experimental setup for first feeding experiment of ballan wrasse larvae. Tank conditions, treatments and sampling dates.

Days post hatch	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16-21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32-35**	**36**	**37**	**38**	**39**	**40**	**41**	**42**	**43**	**44**	**45**
Day degrees	36	48	60	73	96	109	123	137	151	166	181	196	212	228-312	328	344	360	376	392	408	424	440	456	472	488-536	552	568	584	600	616	632	648	664	680	696
Temperature (°C)	12			13			14			15			16																						
Clay (g tank^{-1} day^{-1})	-	3.2									4.8				7.5			8			8.5		9												
Water exchange rate	2 times day^{-1}													4 times day^{-1}				6 times day^{-1}							8 times day^{-1}										
Feeding frequency	-	2 times day^{-1}												3 times day^{-1}																					

Rot - 3 tanks	-	**Enriched rotifers**																																	
(individuals L^{-1} feeding^{-1})	-	3500												5000											12000										
																							Enriched brine shrimp												
(individuals L^{-1} feeding^{-1})																							3000												
Cop - 3 tanks	-	**Copepods**																																	
(individuals L^{-1} feeding^{-1})	-	5000																										10000							
Stage	-	n												c		n		c		n		c				67 % c + 33 % a						67 % n + 33 % a		

Mortality													x	x	x		x			x		x		x	x	x		x			x		x		x
Sampling																																			
Gene expression	x					x							x								x					x								x	
DW, SL and MH	x					x							x								x					x									x

n, nauplii; c, copepodites; a, adults

Table 2.2 Copepod stages and age during Cop treatment. Dph = days post hatch.

Stage	**Age**	**Body length**
nauplii	1-5 dph	94-211µm
copepodites	6-10 dph	394-820 µm
adult	>11 dph	1-1.5 mm

Frequency of water exchanges was initially twice the volume of the larval tanks per day and gradually increased to eight times per day at the end of the experiment (Table 2.1). For visual predators, like ballan wrasse larvae, the contrast of the pray as well as its mobility effects increase the predation rate (Utne-Palm, 1999). Thus, ceramic clay (Vingerling K148, Sibelco, Germany) was added in the water to increase the visual contrast and reduce the bacterial load (Attramadal *et al*., 2012). To meet the increasing water exchange rate, increasing mass of clay was added, under the following pattern:

- 4-21 days post hatch (dph): 1.6 g per feeding
- 22-24 dph: 2.5 g per feeding
- 25-27 dph: 2.5 g with two feedings and 3 g with one feeding
- 28-29 dph: 2.5 g with one feeding and 3 g with two feedings
- 30-45 dph: 3 g per feeding

Upon arrival and for 24 hours larvae were kept in darkness; on 4 dph illumination was provided continuously through the whole experiment by daylight fluorescent tubes (MASTER TL-D 90 Graphica, 18W/965, Philips, Netherlands).

2.2 First feeding experiment

Larvae started feeding at 4 dph, with two feeding regimes (Table 2.1), having three replicates for each treatment. The experiment lasted until 45 dph, when fish were anesthetized and removed.

Feedings were done manually, twice per day until 12 dph, with a minimum of 7 hours between them and three times per day until the end of the experiment, with a minimum of 5 hours between them.

First treatment (Rot) included feeding with rotifers (*Brachionus* sp.), enriched with Multigain (Biomar, Norway) until 40 dph. Brine shrimp (*Artemia franciscana*) nauplii enriched with Multigain (Biomar) was offered at 30 dph. Co-feeding with both rotifers and brine shrimp was held from 30 to 40 dph; until 37 dph brine shrimp was fed to the larvae once a day, while until 40 dph it was fed twice a day. Co-feeding was followed by exclusive feeding with brine shrimp from 41 dph until the end of the experiment (table 2.1).

Second treatment (Cop) included feeding with cultivated *Acartia tonsa*: nauplii until 15 dph, copepodites until 30 dph and thereafter a combination of copepodites and adult copepods (2:1) until the end of the experiment (Table 2.2).

Copepod density fed to Cop larvae was 5000 individuals L^{-1} until 36 dph and 10000 L^{-1} until the end of the experiment. Rotifers were fed to Rot larvae at a density of 3500 individuals L^{-1} until 8 dph, 5000 individuals L^{-1} until 15 dph and 1200 individuals L-1 until 40 dph. Lorica length of the adult *Brachionus* is approximately 184 µm (Penglase *et al.*, 2010), and it is similar to the body length of *A. tonsa* at stage NIV (nauplius IV; 5 dph; Alver *et al.*, 2011). During 4-8 dph copepod nauplii fed to the larvae were of an earlier stage (NII), thus more individuals were offered to the Cop larvae than the Rot ones. At 24, 26 and 42-45 dph copepod nauplii of late stages (NV and NVI) were fed to the larvae, instead of copepodites, due to poor copepod hatching. Brine shrimp was fed at a fixed density of 3000 individuals L^{-1}. The frequency of the feedings was adjusted to appetite of the ballan wrasse larvae. When copepods and rotifers were fed, this was done by skipping a feeding, if the density of the live feed seemed too high in the larvae tank. The estimation was done by sampling water from the surface and examining it under stereoscope. *Artemia* loses nutritional value with starvation (Evjemo *et al*., 1997b, 2001; Figueiredo *et al*., 2009) and hence excessive un-predated brine shrimp groups found in the larvae tanks were removed with use of a ladle, right before the next feeding.

2.3 Production of live feed

2.3.1 Production of microalgae *Rhodomonas baltica*

R. baltica (Clone NIVA 5/91 Cryptophycea: Pyrenomonadales) was cultured continuously in polymethyl methacrylate (PMMA) cylinders of 160 and 200 L volume and of 40 cm diameter. During periods of high production, two 300 L plastic bags fastened on metallic grid were used in addition.

Each cylinder was illuminated by 6 fluorescent tubes (GE Polylux XL 830 F58W) and the temperature was kept at 20 ^{o}C. Medium aeration was provided with 1-2 % CO_2. pH was kept 7.5 - 8.5, by adjusting the CO_2 flow.

Sea water of 34 ppt was filtered through a sand filter, heated to 20 ^{o}C and filtered through a 1 μm mesh. Thereafter it was chlorinated (10-15 % NaOCl 1:4000) without aeration for at least 5 hours and dechlorinated ($Na_2S_2O_3$ 3:100) under heavy aeration for at least 5 hours before entering the cultivation cylinders.

A percentage of 40-50 % of the cylinders volume was harvested daily. Following harvesting cylinders were filled up with sea water and 1 ml of Conwy medium L^{-1} sea water was added as nutrient medium (Appendix I).

Every day 1-3 cylinders were harvested entirely and the emptied cylinders were cleaned with warm water under pressure and a plastic brush dipped in chlorinated water. Each cleaned cylinder was then filled with 50 % of the culture volume of another cylinder, 50 % sea water and 1 ml of Conwy medium L^{-1} sea water as normal.

2.3.2 Production of copepods *Acartia tonsa*

Copepods were cultivated in 3 black tanks of 1700 L. Temperature, O_2- levels, pH and salinity were measured daily (ProODO Optical Dissolved Oxygen Meter, YSI Inc., OH, USA; LH-T28, Lohand, China) and kept at 19-22 ^{o}C, over 4 mg L^{-1} dissolved O_2, 7.6-8 and 34-35 ppt respectively. All tanks were under continuous indoor illumination.

Sea water (34 ppt) was flowing through the tanks after it was sand filtered, heated to 20 ^{o}C and treated by a 1 μm filter. Filters covering the water outlets were washed daily by fresh water under pressure.

Harvested microalgae (paragraph 2.3.1) was pumped into a 1000 L reservoir and fed continuously to copepods by an Electromagnetic Dosing Pump (AXS602, Seko, Italy).

Sediments (eggs and waste) were gathered by a collecting arm and siphoned out of the tanks daily. Eggs were filtered through two sieves of 120 and 100 μm and collected by a 64 μm sieve under continuous rinsing with sea water. Afterwards they were rinsed by sea water and stored in NUNC EasyFlasks™ (Nunc A/S, Denmark) containing sea water, at 2 ^{o}C (Pharmaceutical Refrigerator MPR-311D (H), Sanyo, Japan).

Flasks containing copepod eggs (500 000 eggs ml^{-1}) were emptied in 100 L white tanks under moderate aeration for hatching. After 24 hours and at least 3 times per day *Rhodomonas* was fed to nauplii / copepodites until harvesting.

For estimating the density, a small amount of water was collected using a transparent pipe, after mild stirring; 7 drops of 50 µl of the sampled water were fixed with Lugol's solution and individuals were counted under stereoscope. The highest and lowest counts were canceled and density in the tanks was estimated as the mean of the 5 remaining. The desired number of individuals was harvested, concentrated by a 64 µm sieve and stored in cold room (8 ^{o}C) under moderate aeration upon feeding to the larvae (< 12 hours).

2.3.3 Production of rotifers *Brachionus* sp.

Brachionus sp. was purchased from Profunda AS (Norway) and cultured in three tanks of 250 L with conical bottoms in densities less than 750 ml^{-1}.

Sediments were flushed for 5 s daily from an outlet of the tank bottom. Baker's yeast (2.6 g million of $individuals^{-1}$ day^{-1}) dissolved in sea water was fed to rotifers continuously by a pump. Multigain (0.14 g per million of individuals; Biomar) was mixed in sea water with an immersion blender and added twice a day in the culture tanks as enrichment.

Small amounts of water from the rotifer culture were collected using a transparent pipe; 12 drops of 25 µl of the sampled water were fixed with Lugol's solution and density was estimated as with *A. tonsa* (Paragraph 2.3.2). Egg ratio was calculated (number of eggs/ number of individuals) simultaneously, as a reflection of the culture growth (Øie & Olsen, 1997).

The desired number of rotifers was harvested, concentrated by a 64 µm sieve, washed by sea water to reduce the bacterial load and transferred in a 100 L tank of sea water (24 ^{o}C, moderate aeration) where it was short-term enriched with Multigain (Biomar) for 2 hours (0.14 g per million of individuals). Density was estimated *de novo*, and the enriched rotifers were concentrated by a 64 µm sieve, rinsed by sea water and stored in cold room (8 ^{o}C) under moderate aeration upon feeding to the larvae (< 12 hours).

2.3.4 Production of brine shrimp *Artemia franciscana*

A. franciscana dry cysts were purchased from INVE Aquaculture (EG®, Belgium) and decapsulated before incubation. Cysts were first hydrated for 1 hour in 4.9 L fresh water (for 450-500 g dry cysts), under heavy aeration and at controlled temperature of 10-25 ^{o}C. NaOH 59.4 g was dissolved in 150 ml of water and NaOCl 1.44 L was added to the hydrated cysts (Sorgeloos *et al.*, 1977). With use of a transparent pipe, color of cysts was checked constantly, and the process was stopped when the color had turned from brown to orange, as the chorion was removed. Decapsulated cysts were then filtered through a 125 µm mesh net, and rinsed under running sea water of temperature 15-25 ^{o}C for 5-7 minutes. $Na_2S_2O_3$ 0.1% was added directly to the concentrator for 5-7 minutes, and cysts were re-rinsed under running sea water of temperature 15-25 ^{o}C for 5-7 minutes. Cysts were then gathered and stored at 5 ^{o}C for use within 6 days.

The desired mass of decapsulated *Artemia* cysts was weighed and transferred into a tank filled with 60 L sea water for hatching under heavy aeration. The O_2 levels were 4.5 mg L^{-1} (minimum 2.5 mg L^{-1}; Hoff & Snell, 1987), temperature of 25-28 ^{o}C, and illumination ~1000 lux on the tanks surface. After 24 hours the tank was flushed for debris by opening an outlet on the bottom for 2 s. The brine shrimp nauplii were then gathered and washed in an *Artemia*-washer for 10 minutes. After washing, nauplii were transferred into a similar tank to the hatching one, where they were enriched twice a day with Multigain (10 g for 60 L; Biomar), before being harvested, rinsed, concentrated and fed to ballan wrasse larvae.

2.4 Sampling and analyses

Ballan wrasse larvae individuals were collected manually and randomly from each tank using a ladle. The ladle was rinsed with hot and immediately after cold fresh water between tanks, to avoid bacterial transmissions. Sampling at the beginning of the experiment (3 dph) was performed before the larvae were distributed from the holding to the treatment tanks. Equal number of individuals (*n*) was thereafter sampled from each tank. Collected larvae were anaesthetized by tricaine methanesulfonate (MS-222, Finquel®, Agent Chemical Laboratories Inc., USA) overdose and rinsed in distilled water.

2.4.1. Survival

From 15 dph and until the end of the experiment all six tanks' bottoms were siphoned every second or third day and the collected waste was counted for dead larvae. At the end of the experiment (45 dph) living fish were counted.

A mortality factor was calculated from the number of larvae that survived every day and this factor was used to estimate the proportional realistic number of larvae that was sampled out of the tanks. % Survival was estimated after the initial larvae number was corrected by deducting this realistic number of larvae that was sampled out. Living larvae accidentally siphoned out were considered dead and mortality up to 15 dph was considered to show a negative linear regression with time (dph).

2.4.2 Dry weight and daily weight increase

Larvae were sampled with a ladle for dry weight measurement at 3, 8, 15, 28, 36 and 45 dph (Appendix II).

Samples were analyzed for carbon content (C_{mass}; µg) by Marte Schei (SINTEF) using an Elemental Combustion Analyzer (Costech Analytical Technologies Inc., CA, USA). Acetanilide ($C_6H_5NH(COCH_3)$) was used as standard. Larval dry weight was then calculated using the equation

$DW = C_{mass} * 2.34$ (Reitan *et al*., 1993).

Daily weight increase (%DWI) was calculated using the equation

$\%DWI = (e^{SGR} - 1) * 100$ (Ricker, 1958), where

$SGR = (\ln DW_2 - \ln DW_1) / (t_2 - t_1)$

DW = *dry weight at time t*.

2.4.3 Standard length and myotome height

Ballan wrasse larvae were sampled at 3, 8, 15, 28, 36 and 45 dph for standard length (SL; mm) and myotome height (MH; mm) measuring (Appendix IV). Individuals were anaesthetized and photographed by a Zoom Stereomicroscope SMZ1000 (Nikon Instruments Inc., NY, USA). The pictured larva was then measured for SL (distance between the upper lip

tip and either the end of vertebrae for preflexion larvae, or the peduncle for postflexion larvae) and MH (distance from the anus to the upper body outer skin, perpendicular to the skeleton axis) by a digital camera (Infinity 1-3, Lumanera Co., ON, USA), using Infinity Analyze software (Lumanera Co.; Fig. 2.1), as described by Galloway *et al.* (1999). SL and MH measurements were performed by Stine Wiborg Dahle (SINTEF).

Figure 2.1 Representation of standard length (upper lip to end of notochord) and myotome height (perpendicular to the skeleton axis from behind the anus) measurements on a 15 day old ballan wrasse larva. Adapted by Gagnat, 2012.

2.4.4 Molecular analysis

Larvae were sampled with a ladle at 3, 8 and 15 dph (*n* = 30 per tank), 28 dph (*n* = 25 per tank), 36 and 44 dph (*n* = 20 per tank) to be analyzed molecularly (Appendix V). All individuals from each tank were then transferred to 2 ml tubes, from where excess water was removed using a glass suction pipette. Tubes were immediately frozen in a liquid N_2, before storage in -80 ^{o}C.

Cleaning agent for removing RNase (RNase ZAPTM, Sigma-Aldrich Co., MO, USA) was applied on all surfaces and equipment before every step of molecular analysis.

2.4.4.1 RNA extraction

Using a stainless steel laboratory spatula, frozen tissue samples (whole larvae) were transferred into Precellys® (Bertin Technologies, France) tubes, together with 750 µl QIAzol Lysis Reagent (Qiagen) and five 1.4 mm ceramic (zirconium oxide) beads (Precellys®) for each tube, and then run by an homogenizer (Precellys® 24) under 6000 rpm, 3 x 15 s. After 5 minutes incubation, 150 µl chloroform ($CHCl_3$) was added in each tube; samples were shaken

manually for 15 s and centrifuged under 12000 rpm x 15 s at 4 ^{o}C (Centrifuge 5415R, Eppendorf AG, Germany). Using a pipette, 340 µl of the upper, aqueous phase were transferred into 2 ml EZ1® tubes and run by an EZ1 BioRobot® according to manufacturer's instructions*. RNase-free lyophilized DNase (Qiagen) 10 µl was used.

The extracted total RNA concentration was measured by a NanoDrop® ND-1000 UV–Vis Spectrophotometer (NanoDrop Technologies, DE, USA). Optical density ratio at 260/230 and at 260/280 nm ranged from 1.92 to 2.31 and from 1.93 to 2.17, respectively.

The total RNA quality was then assessed by an Agilent 2100 Bioanalyzer using a RNA 6000 Nano LabChip® kit (Agilent Technologies, CA, USA) according to manufacturer's instructions†. The RNA Integrity Numbers (RIN; Schroeder *et al.*, 2006) were found between 9.0 and 9.8.

2.4.4.2 Reverse transcription polymerase chain reaction (RT-PCR reaction)

Table 2.2 lists the reagents of the RT reaction mix. All reagents were purchased by Applied Biosystems (CA, USA), except Oligo d(T)16 Primers and RNase Inhibitor, which were purchased by Roche Applied Sciences (Switzerland).

RT-PCR was performed in duplicates on a 96-well-plate. A twofold serial dilution of total RNA mixture from all samples (pool) was run in triplicates, ranging from 31 to 1000 µg ml^{-1}. An amount of 500 µg total RNA from each sample was used for the reaction synthesis. The plate was made accepting deviation ≤ 5 %; measured concentrations show in table 2.3. The last two wells were loaded with a no-template control (ntc) and a non-amplification control (nac; a reaction without the Reverse Transcriptase). The plate was run on a GeneAmp® PCR System 9700 (Applied Biosystems); incubation lasted 10 minutes at 25 ^{o}C, while reverse transcription was performed at 48 ^{o}C for 60 minutes, before inactivation for 5 minutes at 95 ^{o}C. The product plate (cDNA) was covered with a sealing tape and stored at -20 ^{o}C.

* http://www.qiagen.com/Products/Catalog/Automated-Solutions/Sample-Prep/EZ1-RNA-Universal-Tissue-Kit#resources

† http://www.chem.agilent.com/Library/usermanuals/Public/G2938-90034_KitRNA6000Nano_ebook.pdf

Table 2.2 RT reaction mix for a 30µl reaction. Reagents, volumes (µl) and final concentrations.

	Reagents	**30µl r x n**	**Final concentration**
Non-enzymatic reagents	RNase-free H2O	1.3	-
	10xTaqMan RT reagent	3.0	1x
	25mM MgCl2	6.6	5.5mM
	10mM deoxyNTPs mix	6.0	500µM per dNTP
	Oligo d(T)16 primers	1.5	2.5µM
Enzymes	RNase inhibitor (20U/µl)	0.6	0.4U/µl
	Multiscribe Reverse Transcriptase (50U/µl)	1.0	1.67U/µl

Table 2.3 RT plate with the measured total RNA concentrations (ng) of samples in duplicates, a twofold serial dilution of RNA mixture from all samples in triplicates and two controls

	1	2	3	4	5	6	7	8	9	10	11	12
A	997 pool	997 pool	997 pool	486 pool	486 pool	486 pool	254 pool	254 pool	254 pool	119 pool	119 pool	119 pool
B	62 pool	62 pool	62 pool	28 pool	28 pool	28 pool	503 S1	503 S1	493 S2	493 S2	495 S3	495 S3
C	513 S4	513 S4	512 S5	512 S5	492 S6	492 S6	499 S7	499 S7	501 S8	501 S8	503 S9	503 S9
D	507 S10	507 S10	507 S11	507 S11	489 S12	489 S12	498 S13	498 S13	493 S14	493 S14	502 S15	502 S15
E	506 S16	506 S16	512 S17	512 S17	513 S18	513 S18	515 S19	515 S19	494 S20	494 S20	510 S21	510 S21
F	513 S22	513 S22	492 S23	492 S23	505 S24	505 S24	506 S25	506 S25	492 S26	492 S26	491 S27	491 S27
G	507 S28	507 S28	500 S29	500 S29	502 S30	502 S30	511 S0	511 S0			nac	ntc
H												

pool, pool of all samples; S0-S30, sample 0 – sample 30; nac: non amplification control; ntc, non template control

2.4.4.3 One Step RT PCR

Primers (table 2.4) were designed with use of the Primer-BLAST tool in the PubMed website. "Primer Pair Specifity Checking Parameters" were set to Gasterosteidae (taxid:69291) and primers were finally purchased from Life Technologies AS (UK) in desalted form. Primers were spun down (Centrifuge 5415D, Eppendorf), diluted with TE buffer 1x pH 8

(AppliChems GmbH, Germany) to final concentration 50 mM and left for 2 minutes, before getting mixed on a vortex mixer (Lab Dancer S40, VWR®, Germany) for 15 s.

Table 2.4 Gene abbreviations, PCR primer sequences, primer amplicon sizes and RT-qPCR efficiencies for target and analysed reference genes.

Gene	Forward primer (5'- 3')	Reverse primer (5'- 3')	Amplicon size (bp)	PCR eff.
cyc1	AACCTTTCCCCAACGTGGTT	CAGTCAGAGAGTTGTGGCGT	177	1.94
cox5a	CCGAGGTCCTCAGGTGTAGA	CTGAAAGCCTGTCGGAGGTT	161	2.07
pla2g6	TGATGGTGACCAGCGTTCTC	GTCGAAAGTAGGTTGGGGCA	192	1.96
vdac1	GGAATTCCAAGCCGAGACCA	GCATCCTTCTCTGCCAAGGT	154	2.08
fxn	TGTCAACCTGCGCACAAATG	GTCCAACGTCTCATCTGCCA	159	2.01
mnsod	TAAACTTCAGAGCGGGCTGG	TCCCCGACCTGACTTACGAT	189	2.00
crls1	CTGCTGCTGGTGTGGTTTTC	ATCCAGCTCTTCCTGGTTGC	192	2.00
uba52	GGCCAGCTGTCTGAGAGAAG	GTCAAGGCCAAGATCCAAGA	183	1.91
rpl37	ATGATCAGAGCGGGCATAAC	CGCCTCCAGCTCATCTTAAA	159	2.03
bactin	CGTAACCCTCGTAGATGGGC	ACTGAACCCCAAAGCCAACA	182	2.03

cyc1, cytochrome c1; cox5a, cytochrome c oxidase subunit Va; pla2g6, phospholipase A_2 group VI; vdac1, voltage-dependent anion channel 1; fxn, frataxin; mnsod, manganese superoxide dismutase; crls1, cardiolipin synthase 1; uba52, ubiquitin A-52; rpl37, ribosomal protein l37; bactin, beta actin.

Primers were tested using total RNA from sample 0 (3 dph), as RNA template and a Qiagen® OneStep RT-PCR Kit (Qiagen; table 2.7.2). All components were kept on ice and were mixed on a vortex mixer (Lab Dancer S40, VWR) except the Enzyme Mix and the RNase inhibitor, which were kept in a -20 ^{o}C block and were removed right before use. A master mix (common reagents) of 10 % greater volume than needed was made and shared into PCR tubes. A mixture of 900 µl distilled H_2O as long as 25 µl of each 100 mM dATP, dTTP, dGTP and dCTP was used as dNTP mix. The exclusive reagents (forward and reverse primers) were then added into each of the PCR tubes (table 2.5). RT-PCR (GeneAmp® PCR System 9700, Applied Biosystems) was performed on the temperature profile displayed on table 2.6.

PCR products were tested by electrophoresis. A gel was made by diluting 0.76 g agarose (UltraPure™ Agarose, Invitrogen Life Technologies) in 50 ml of 1x TAE buffer (1:50) and heated in a microwave oven (DES-System M050, Whirlpool, MI, USA). Fluorescent nucleic acid dye 5 µl (GelRed™ Nucleic Acid Gel Stain, 10,000x in water, Biotium Inc., CA, USA) was added and the mixture was purred into a gel tray and left to fasten for 30 minutes. The PCR products were buffered with BlueJuice™ Gel Loading Buffer (Invitrogen Life Technologies) and loaded in the gel wells, together with a molecular weight marker (GelPilot 50 bp Ladder (100), Qiagen). The gel was electrophorised under 80 V for 1 hour (POWER

PAC 200, Bio-Rad Laboratories Inc., CA, USA). The electrophoresis product was imaged with a gel doc (G:BOX, Syngene, UK).

Table 2.5 One Step RT-PCR reaction mix. Components, 1 x volumes (μl) and final concentrations.

Components	Volume 1 x (μl)	Final conc. (μM)
5x QIAGEN One Step RT-PCR buffer	5	1x; 2.5mM Mg2+
dNTP mix (10mM each)	1	400 of each dNTP
Primer forward	0.3	0.6
Primer reverse	0.3	0.6
RNase-free H2O	57	-
QIAGEN One Step RT-PCR Enzyme Mix	1	-
5x Q-solution	5	1x
Template RNA (sample 0)	1	
RNase inhibitor		

All components were included in Qiagen® OneStep RT-PCR Kit, except for RNase inhibitor, which was purchased by Ambion® (CA, USA).

Table 2.6 RT reaction: duration and temperature profile. Source: Qiagen One-Step RT-PCR Handbook

Process	Duration	Temperature (^{o}C)
Reverse transcription	30 min	50
PCR activation	15 min	95
35 three-level cycles:		
Denaturation	45 sec (>30, <60)	94
Annealing	45 sec (>30, <60)	60 (>50, <68)
Extension	1 min	72
Final extension	10 min	72

2.4.4.4 Real time PCR or quantitative PCR (qPCR)

Template of the real time reaction is the cDNA product from the RT reaction (paragraph 2.6). H_2O 30 μl (Milli-Q Integral Water Purification System, Millipore, CA, USA) was added in each of the cDNA plate's wells and the plate was mixed for 5 minutes at 1500 rpm (MixMate®, Eppendorf).

LightCycler® 480 SYBR Green I Master (Roche Applied Sciences) containing Taq DNA polymerase and DNA double-strand-specific SYBR Green I Master dye was used to run the

reaction. For each real time reaction a mixture containing 2.8 μl of H_2O (Milli-Q Integral Water Purification System), 0.2 μl of each of the 10 primers (table 2.5; 0.1 μl of forward and 0.1 μl of reverse) as well as 2 μl of cDNA were pipetted in each well if totally three 384-well-plates.

Pipetting was performed automatically by a Biomek® 3000 Workstation (Beckman Coulter, CA, USA) and real time PCR was performed by a LightCycler® 480 Real-Time PCR System (Roche Applied Sciences). Real time reaction started with activation and denaturing of 5 minutes at 95 ^{o}C, followed by forty 15-s-denaturing cycles at 95 ^{o}C. A 60-second-annealing step at 60 ^{o}C and a 30-second-synthesis step followed.

A normalization factor (Vandesompele *et al*., 2002) was calculated by use of geNorm VBA applet for Microsoft Excel and bactin, rlp37 and uba52 as reference genes (table 2.4). The normalization factor was then used for further calculation of the mean normalized expression (MNE) for the target genes. Finally, MNE was examined according to larval age and SL.

2.5 Software and methods

Microsoft Office Excel 2007 was used for the initial processing of data. All graphs were made on SigmaPlot™ 12.5 (Cranes Software International).

Molecular data were analyzed statistically on SPSS Statistics 20 (IBM, NY, USA) using the Kruskal-Wallis non-parametric test for independent samples. Data of DW, % DWI, SL, MH as well as % Survival were tested for homogeneity of variance using the Levene test, followed by One-way ANOVA. If the variance was proven homogenous, post-Hoc test Student-Newman-Keuls was performed; if not, Dunett's T3 test was used instead. Genes MNE versus SL (paragraph 3.2.2) were transformed to logarithmic scale and linear regressions for the two treatments were tested for equality using ANCOVA on the R-2.14.2 programming language (R Foundation for Statistical Computing, Austria). Significance level of 0.05 was used as standard in all statistical analyses.

3. Results

3.1 Growth and Survival

3.1.1 Dry weight and daily weight increase

Mean dry weight (DW) of the larvae was 50.54 ± 2.07 µg at 3 dph and reached 4.12 ± 0.20 mg for the Rot treatment and 5.23 ± 0.30 mg for the Cop treatment at 45 dph. DW of the Cop fish was significantly higher than the Rot ones from 8 dph and throughout the whole experiment (Fig. 3.1). Introduction of Rot larvae to brine shrimp at 30 dph accelerated their growth, following the exponential curving of the Cop larvae.

From 3 to 8 dph Rot larvae showed a negative mean percentage of daily weight increase (% DWI; Fig. 3.2), while Cop larvae showed a significantly higher, positive % DWI for the same period. Both treatments resulted in a much higher % DWI period (8 - 15 dph), where the % DWI was still significantly higher for the Cop larvae, as well as for 36 to 45 dph. For the rest of the periods, as well as for the whole experiment (3 - 45 dph) % DWI were not significantly different for both treatments. Rot larvae % DWI increased significantly for the period 36 to 45 dph, indicating the brine shrimp addition to the Rot diet.

Cop larvae had their maximum % DWI during the period from 8 to 15 dph ((19.55 ± 1.46) %), followed by a significant drop. Rot larvae reached their maximum ((15.51 ± 1.77) %) during the period from 36 to 45 dph, after brine shrimp was offered (from 30 dph). Regardless of treatment, % DWI for the whole experiment was approximately 11 %.

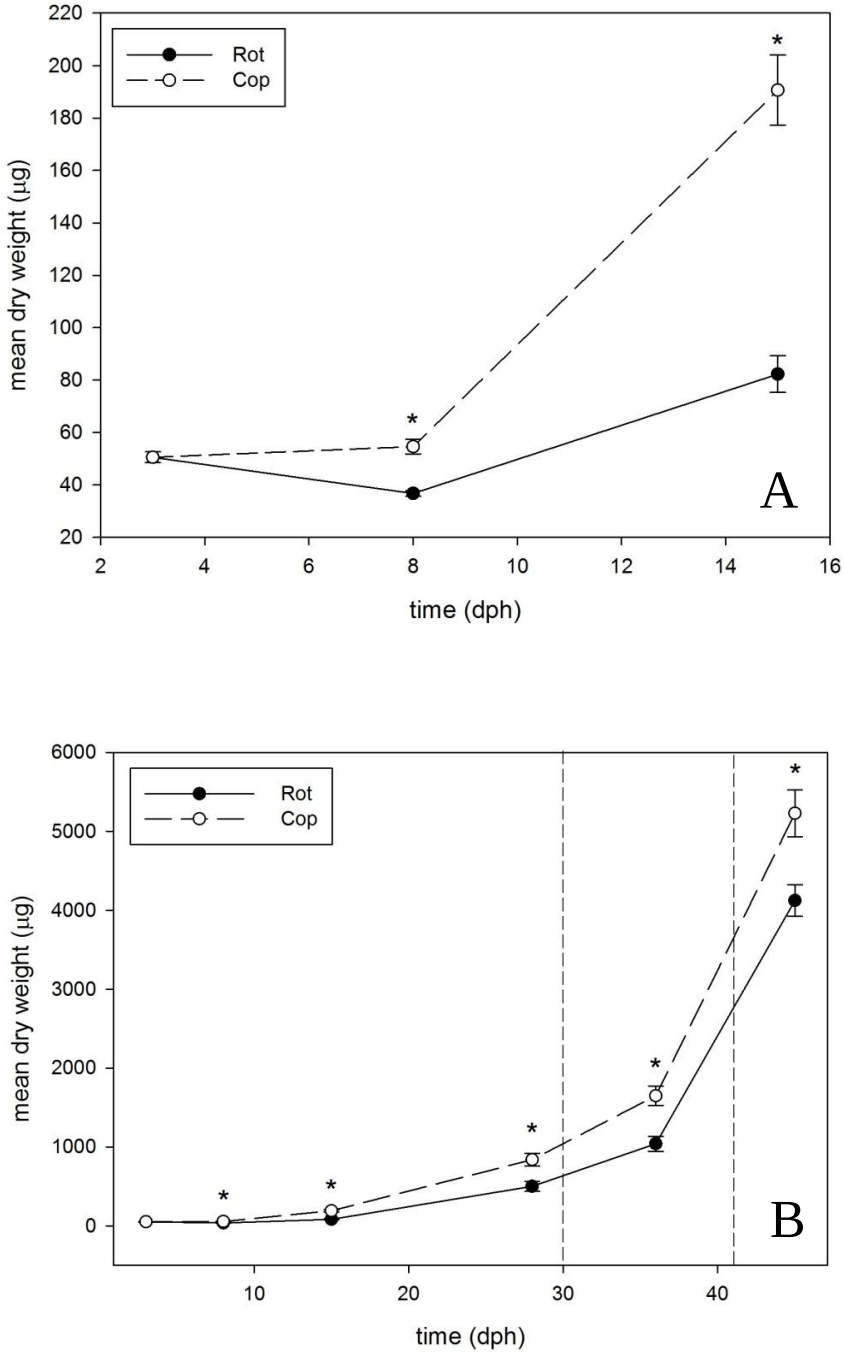


Figure 3.1 Mean larval dry weight (µg) of ballan wrasse larvae from 3 to 15 dph (graph A) and from 3 to 45 dph (graph B) when fed either rotifers and brine shrimp (Rot), or copepods of continuously increasing size (Cop). The area marked between two dashed lines (graph B) denotes the time period when both brine shrimp and rotifers were fed. On 41 dph and up to 45 dph brine shrimp was fed exclusively. Data are mean ± SE (n = 20 individuals replicate^{-1} for 3 dph; n = 35 individuals replicate^{-1} for 8, 15 and 28 dph; n = 45 individuals replicate^{-1} for 36 and 45 dph). * denotes statistically significant differences between treatments (one-way ANOVA; p <0.05).

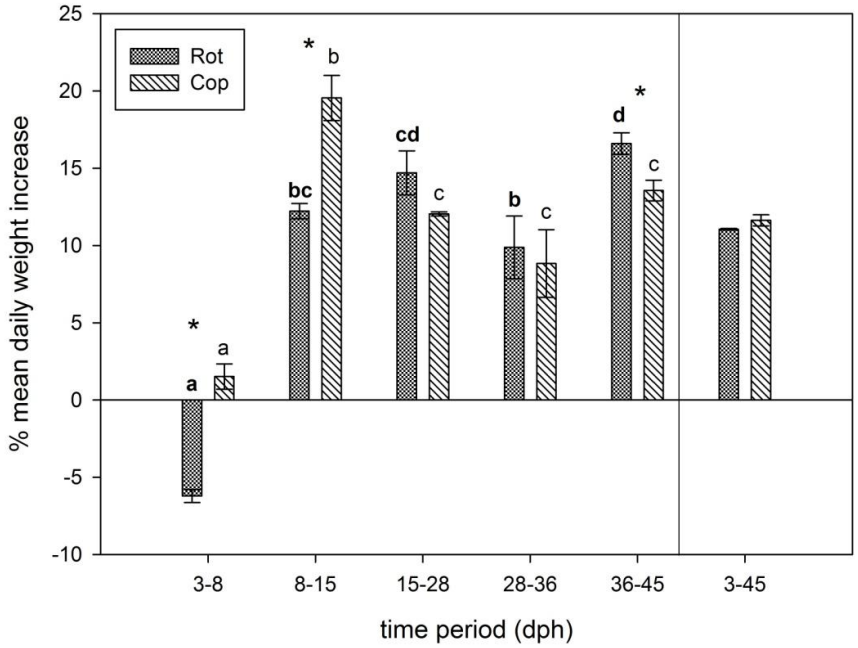


Figure 3.2 Comparison of % daily weight increase for ballan wrasse larvae. Rotifers were fed to the Rot larvae up to 40 dph and brine shrimp was offered from 30 dph until 45 dph. Cop larvae received exclusively copepods of continuously increasing size. Data are mean ± SE (n = 3 replicates). Statistically significant differences within each treatment are denoted by letters (bold for Rot; Student-Newman-Keuls; p <0.05). Statistically significant differences between the treatments are denoted by * (one-way ANOVA; p <0.05).

3.1.2 Standard length and myotome height

Ballan wrasse larvae had a mean standard length (SL) of 3.33 ± 0.09 mm at 3 dph, while at the 45 dph reached 9.93 ± 0.16 mm (Rot) and 11.40 ± 0.23 mm (Cop). SL between the two treatments did not differ significantly before the 15 dph (Fig. 3.3), while it continuously increased significantly for both treatments.

Mean larval myotome height (MH) differed significantly between the two treatments from 8 dph and on to the end of the experiment. Rot larvae did not increase significantly in MH before the 15 dph, unlike Cop larvae which continuously increased (Fig. 3.4). The initial MH was 0.24 ± 0.01 mm (3 dph) and increased to 1.94 ± 0.05 mm for Rot larvae and 2.44 ± 0.6 mm for Cop ones.

SL versus MH showed a linear regression of high fitness: 95.5 % and 97 % for Rot and Cop treatments respectively (Fig. 3.5). The points of Rot larvae seemed more scattered than the points of the Cop larvae, especially for SL between 4.5 and 8 mm. These SL values correspond roughly to time period between 8 and 28 dph, when exclusively rotifers were fed.

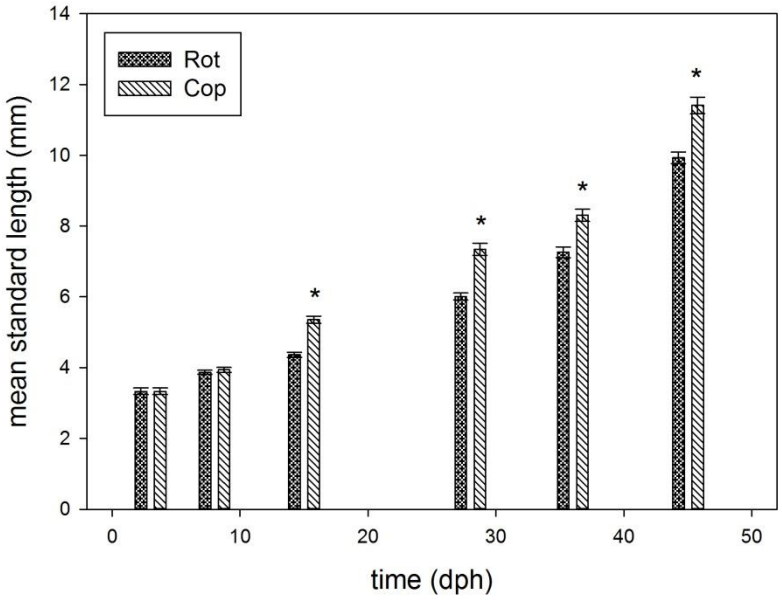


Figure 3.3 Comparison of standard length of ballan wrasse larvae at 3, 8, 15, 28, 36 and 45 dph. Rotifers were fed to the Rot larvae up to 40 dph, while brine shrimp was offered from 30 dph and until 45 dph. Cop larvae received exclusively copepods of continuously increasing size. Data are mean ± SE (n = 12 individuals replicate^{-1} for 3 and 8 dph; n = 20 individuals replicate^{-1} for 15, 28, 36 and 45 dph). * denotes statistically significant differences between treatments (one-way ANOVA; p <0.05).

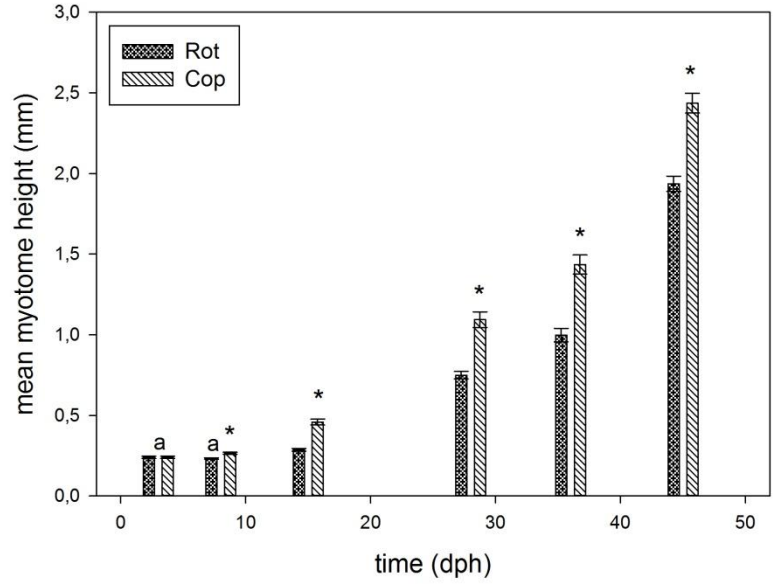


Figure 3.4 Comparison of myotome height of ballan wrasse larvae at 3, 8, 15, 28, 36 and 45 dph. Rotifers were fed to the Rot larvae up to 40 dph, while brine shrimp was offered from 30 dph and until 45 dph. Cop larvae received exclusively copepods of continuously increasing size. Data are mean ± SE (n = 12 individuals replicate^{-1}, for 3 and 8 dph; n = 20 individuals replicate^{-1} for 15, 28, 36 and 45 dph). * denotes statistically significant differences between treatments, while the same letter denotes the existence of no significant difference between bars (one-way ANOVA; p <0.05).

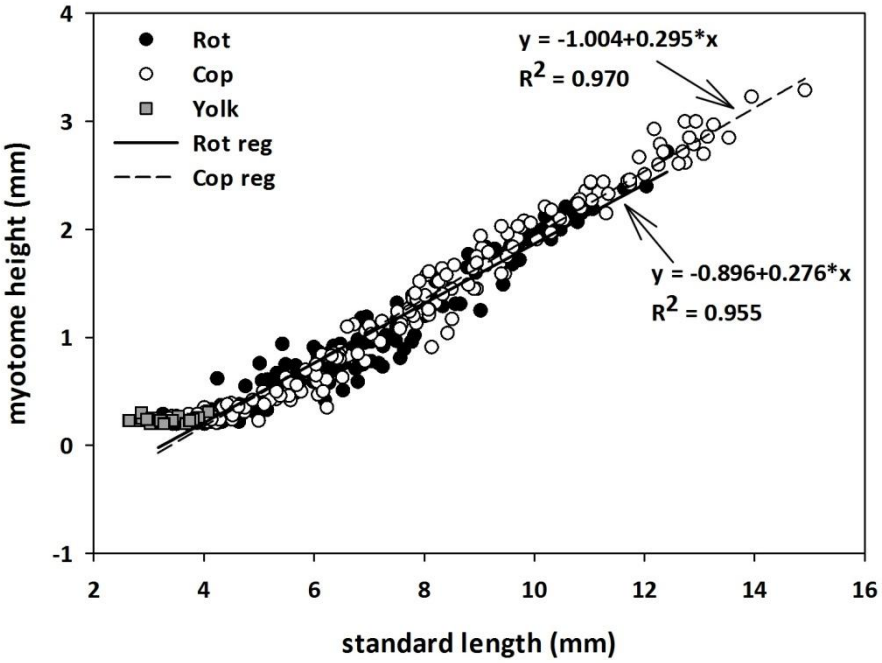


Figure 3.5 Myotome height (mm) versus standard length (mm) of ballan wrasse larvae when fed either rotifers and brine shrimp (Rot; closed circles), or copepods of continuously increasing size (Cop; open circles). Measurements before treatment (3 dph) are marked as "Yolk" (grey squares). Data are 564 individual measurements (n = 12 individuals replicate^{-1}, for 3 and 8 dph; n = 20 individuals replicate^{-1} for 15, 28, 36 and 45 dph). Solid and dashed lines are best fit linear models for Rot and Cop treatments respectively. Plot equations and R^2 are displayed.

3.1.3 Survival

The highest mortality of the ballan wrasse larvae occurred before 15 dph. At 15 dph the mean % Survival of the larvae did not differ significantly for the two treatments. At the end of the experiment Cop larvae had a higher % Survival; the difference was however very small and not significant.

	% Survival	
dph	Rot	Cop
15	15 ± 1	15 ± 1
22	13 ± 0	13 ± 2
29	11 ± 1	12 ± 2
37	9 ± 1	12 ± 2
45	9 ± 1	11 ± 2

Table 3.1 % Survival of ballan wrasse larvae fed either rotifers up to 40 dph and brine shrimp from 30 dph (Rot) or copepods of continuously increasing size (Cop). Data are mean ± SE (n = 3 replicates). No significant difference was observed between treatments (one-way ANOVA; $p \geq 0.05$). Complete results are displayed in Appendix III.

3.2 General Observations

Differences between the ballan wrasse larvae of the two treatments could be observed by naked eye from approximately 25 dph. Cop larvae were larger, more vivid and of more intense pigmentation than the Rot ones. Yellow tint appeared on the Cop larvae and on some of the Rot larvae after being introduced to brine shrimp (Fig. 3.6). At 45 dph Cop larvae were visibly orange colored; differences in eyes were also observed, with the Cop larvae's eyes displaying deep orange coloration.

Larvae tended to stay towards the walls of the tanks, regardless of diet, while no group formation was observed. Very little bacterial growth was observed in the tanks, except for a pink-colored bacterial colony on the bottom of one of the Rot tanks at 27 dph.

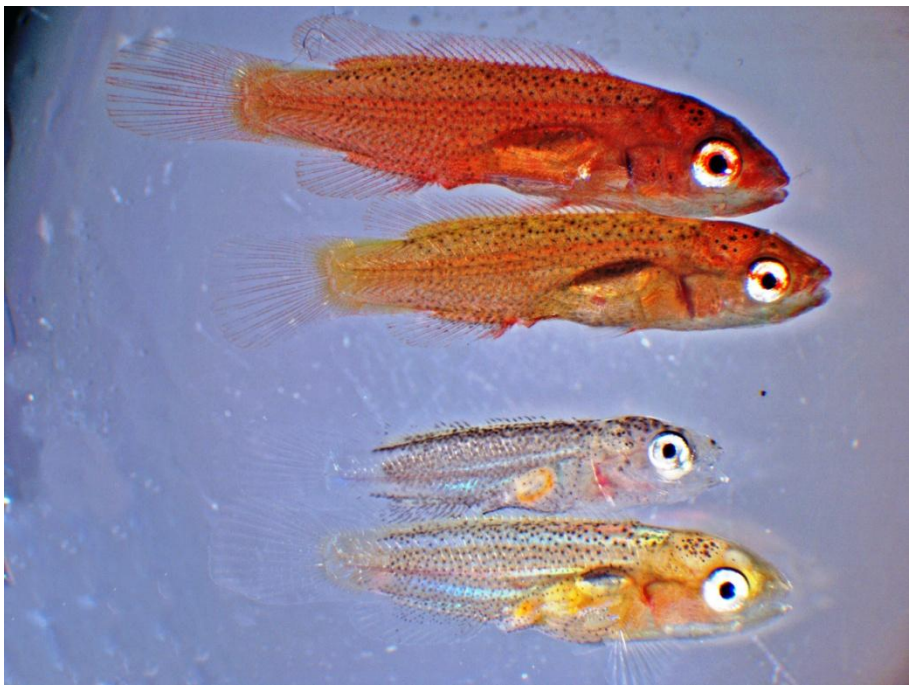


Figure 3.6 Ballan wrasse larvae at 45 dph, when fed either rotifers and brine shrimp (two lower individuals), or copepods of continuously increasing size (two upper individuals). Size and pigmentation differences are displayed.
Photo: Maren Ranheim Gagnat. Magnification 3.6x.

3.3 Gene Expression

3.3.1 Gene expression versus larval age

Mean mRNA expression of cytochrome *c1* gene (cyc1) at 3 dph was 0.528. Rot larvae showed down-regulation at 8 dph, making the difference between treatments significant. By 15 dph it had slightly increased, and slowly dropped again until 45 dph. The difference was significant at 36 dph as well. Cop larvae showed a slight up-regulation, dropped to the same level as Rot ones at 15 dph, remained stable and dropped again at 44 dph. Both treatments ended similar levels, 0.296 ± 0.030 and 0.308 ± 0.016 for Rot and Cop treatments, respectively (Fig. 3.7 A).

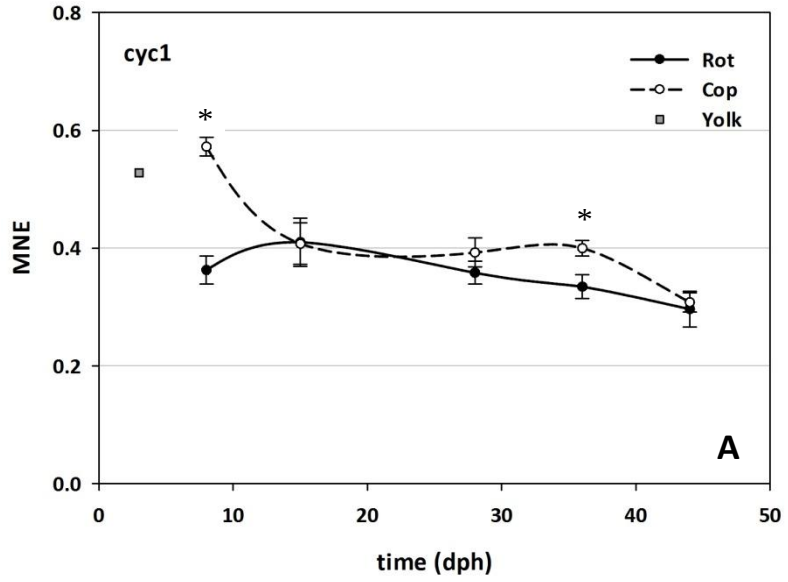
Cytochrome oxidase Va encoding gene (cox5a) expression at 3 dph was 0.558. Cop larvae expression values up-regulated slightly, before dropping continuously until 44 dph. Rot larvae down-regulated at 8dph, creating a significant difference between treatments at 8 and 15 dph, and remained approximately stable until 44 dph. At approximately 32 dph treatments crossed (Fig. 3.7 B).

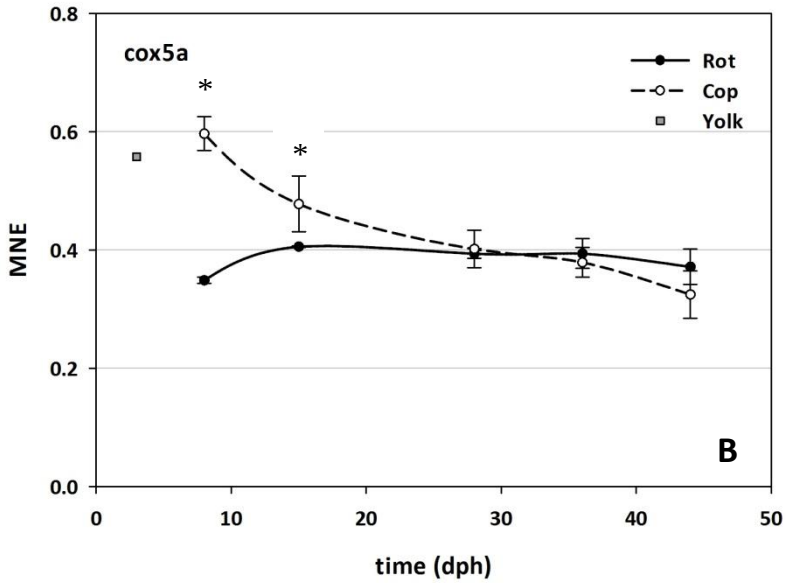
Expression of the voltage-dependent anion channel 1gene (vdac1) started 0.49 for the yolk sac larvae (3 dph). Rot treatment showed a possible (as the mean standard error was relatively high) down-regulation and remained quite stable until 28 dph, when it started dropping until 44 dph. Cop lavrae up-regulated at 8 dph, and declined smoothly until 44. Cop larvae showed a significantly higher expression for vdac1 at 8 and 44 dph.

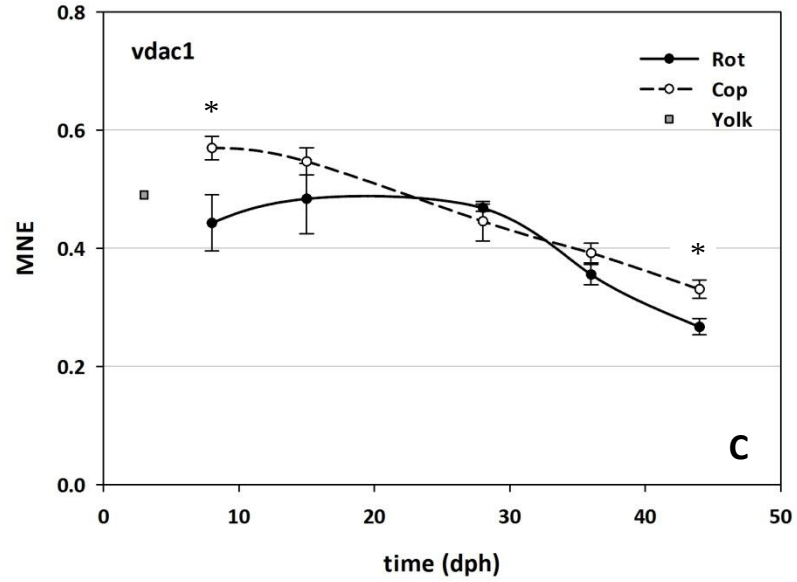
Manganese hyperoxidase gene (mnsod) expression was 0.551 for the yolk sac larvae. After a down regulation at 8 dph larvae of the Rot treatment remained steady at the same levels as in their yolk sac phase. Cop larvae showed a similar mnsod expression, but without the down-regulation of the 8 dph. Expression between the two treatments was significantly different at 28 dph, where Cop treatment picked before returning back to its previous values.

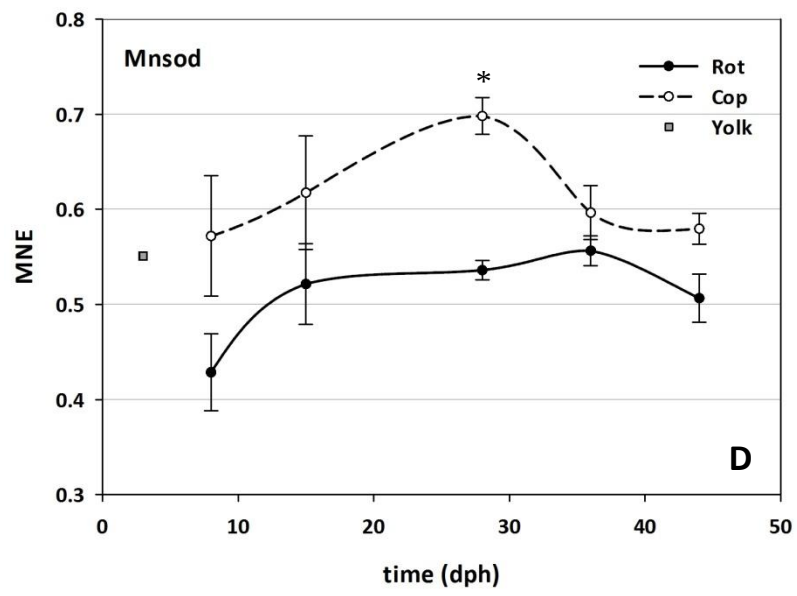
Yolk sac larvae had mean mRNA expression of frataxin gene (fxn) at 0.608. Expression of the Cop larvae at 8dph was approximately the same and had an approximately exponential decline ever since, and up to 44 dph. Rot larvae had a big down-regulation at 8 dph, up-regulated at 15 dph, and went on to an almost linear-appearing decline until 44 dph (Fig. 3.7 E). Treated larvae were significantly different for fxn expression at 8, 28, 26 and 44 dph, with Cop larvae having a higher expression at 8 dph and lower for the rest three time points.

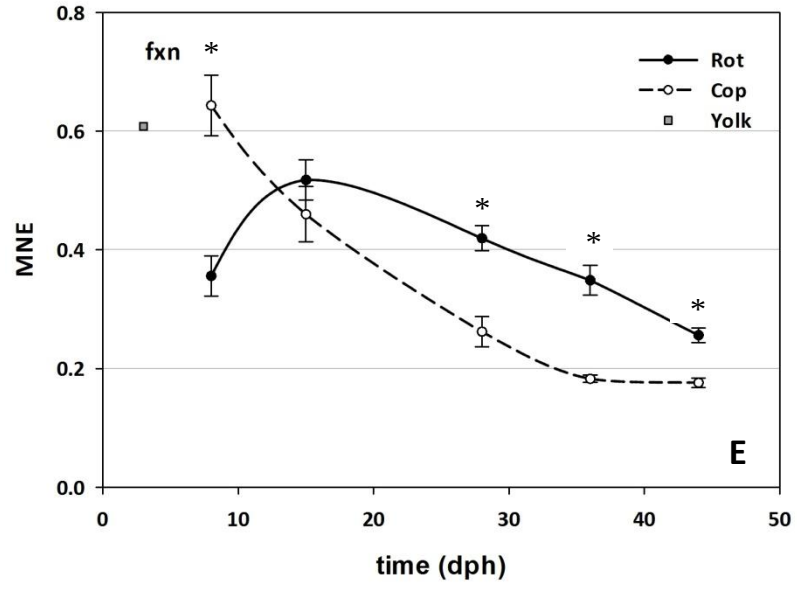
According to figure 3.7, the two treatments had equal expressions at sometime between 8 and 15 dph. Expression of all genes is listed analytically for each tank in Appendix V.






vdac1
Rot
Cop
Yolk
MNE
time (dph)
C


Mnsod
Rot
Cop
Yolk
MNE
time (dph)
D


fxn
Rot
Cop
Yolk
MNE
time (dph)
E

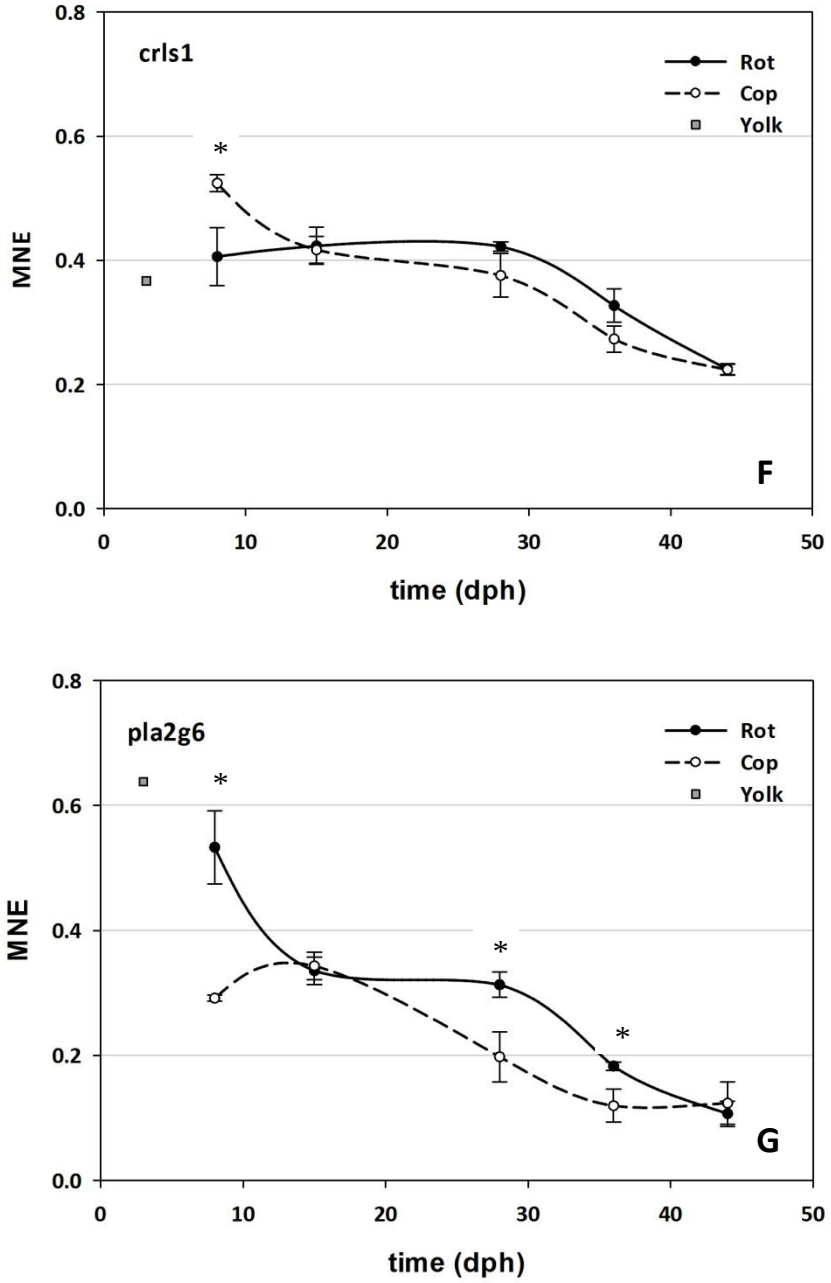


Figure 3.7 Whole fish mRNA gene expression (MNE; mean normalized expression) of cytochrome *c1* (cyc1), cytochrome oxidase Va (cox5a), voltage-dependent anion channel 1 (vdac1), manganese superoxidase dismutase (mnsod), frataxin (fxn), cardiolipin synthase (crls1), and phospholipase A_2 group VI (pla2g6) of ballan wrasse larvae from 3 to 44 dph when fed either rotifers and brine shrimp (Rot), or copepods of continuously increasing size (Cop). Expression before treatment (3 dph) is marked as "Yolk". Data are mean ± SE (n = 3 replicates), except for 3 dph where n = 1. * denotes significant differences between treatments (Kruskal-Wallis non-parametric ANOVA; $p < 0.05$)

Mean mRNA expression of cardiolipin synthase (crls1) at 3 dph was 0.367 and remained similar for the Rot larvae at 8 dph. Crls1 expression of the Cop larvae was up-regulated to its maximum at 8 dph, making the difference between the two treatments significant. Crls1

expression for the two treatments crossed sometime between 8 and 15 dph and thereafter Cop larvae had a lower expression, although the difference was not significant.

Phospholipase 2 group VI gene (pla2g6) expression was 0.639 at 3 dph, making it the highest expressed gene for the yolk sac larvae of those examined. Larvae of both treatments were down-regulated at 8 dph, with Cop treatment more severely. Pla2g6 expressions of the two treatments were equal at approximately 15 and 41 dph (Fig. 3.7 G), and significantly different at 8, 28 and 36 dph, with Rot larvae being higher expressed.

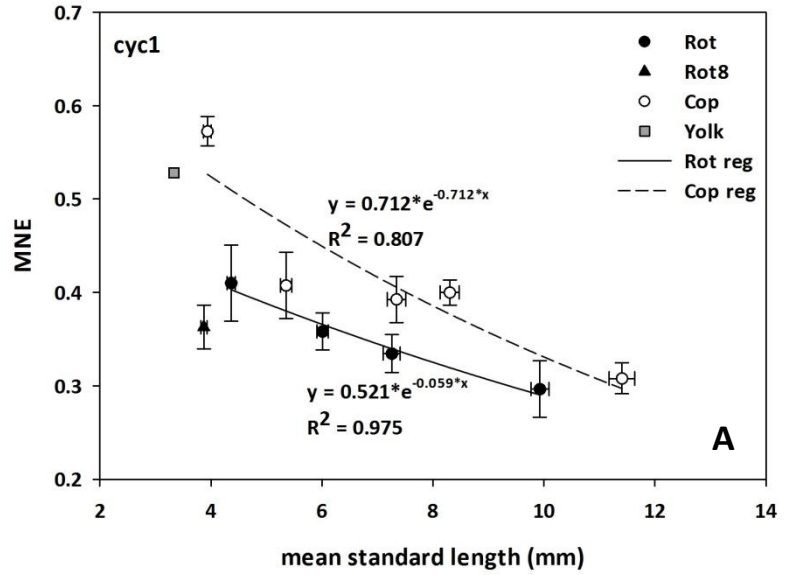
3.3.2 Gene expression versus larval standard length

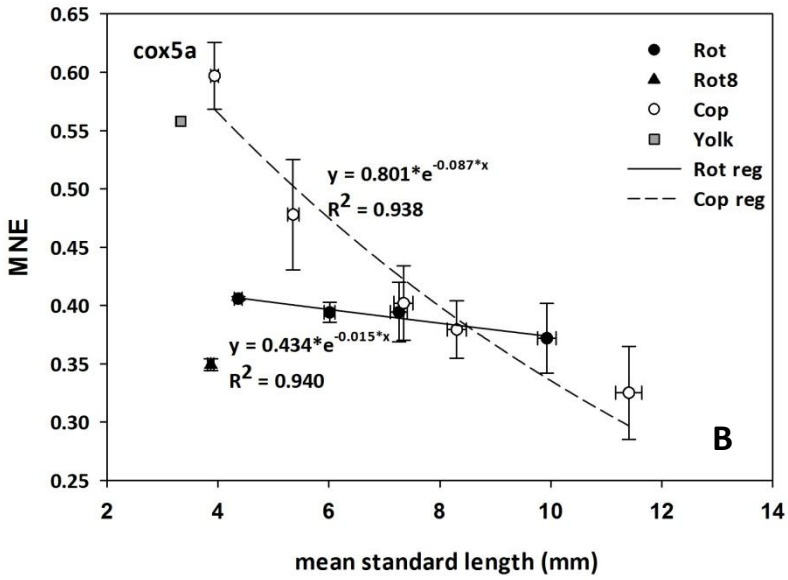
MNE of the selected genes was examined for correlation with larval SL. Expression data for Rot larvae of all genes -except pla2g6- at 8 dph (Rot8; Fig. 3.8 A-F) are not included in the Rot curve, as they changed severely the coefficient of determination value (R^2). The same was done for pla2g6 with Cop data at 8 dph (Cop8; Fig. 3.8 G). Rot8 and Cop8 points were always below the Rot correlation lines. Rot8 and Cop8 were nevertheless included in Rot and Cop variables when tested for correlation equality (ANCOVA). All correlation observed between MNE and SL was quite high, and the majority displayed $R^2 \geq 0.9$.

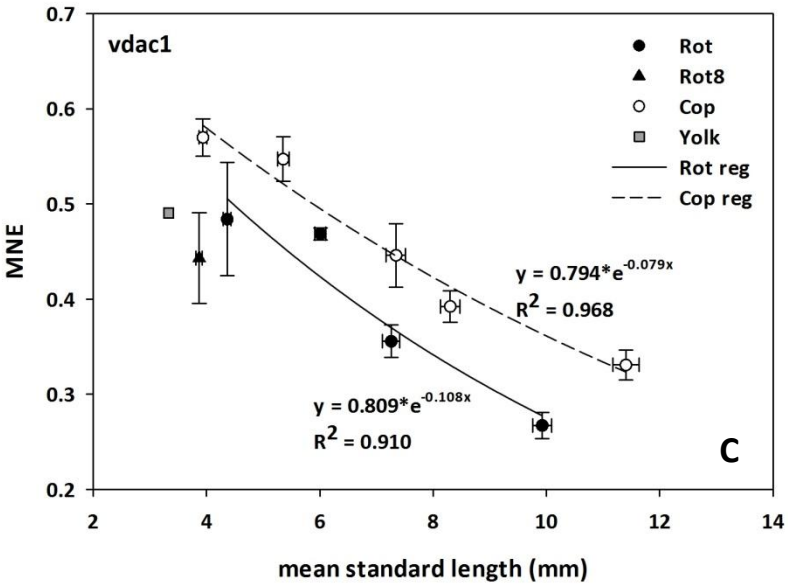
In relation to SL, cyc1 was significantly higher expressed for the Cop larvae than the Rot ones, but slopes did not differ significantly. Expression for Rot larvae down-regulated at 8 dph and both treatments correlated exponentially with SL. Yolk sac larvae expression was closer to the Cop exponential model (Fig. 3.8A).

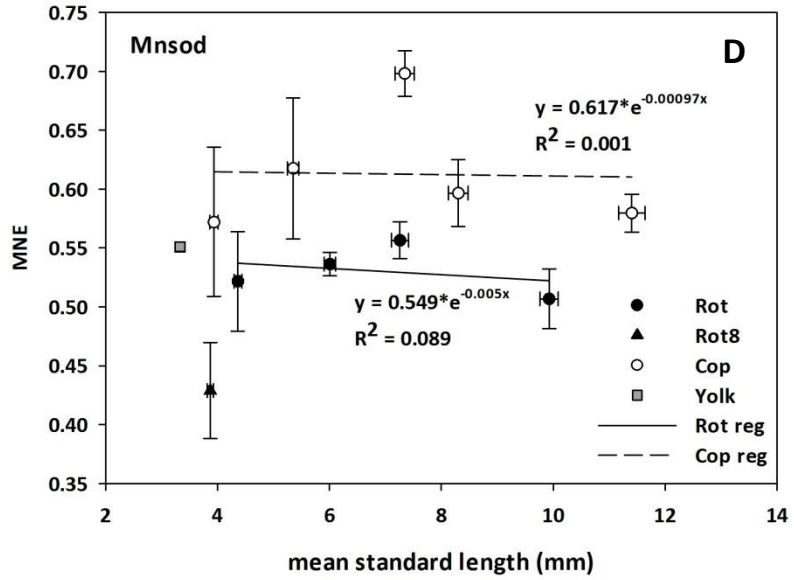
Expression of cox5a in Rot larvae down-regulated from the yolk sac levels at 8 dph. Both treatments showed an exponential correlation with SL, but correlations were significantly different in terms of both slope and elevation. Expression started higher for the Cop larvae end ended up higher for the Rot ones (Fig. 3.8B), with a crossing point for larval SL at approximately 8.5 mm.

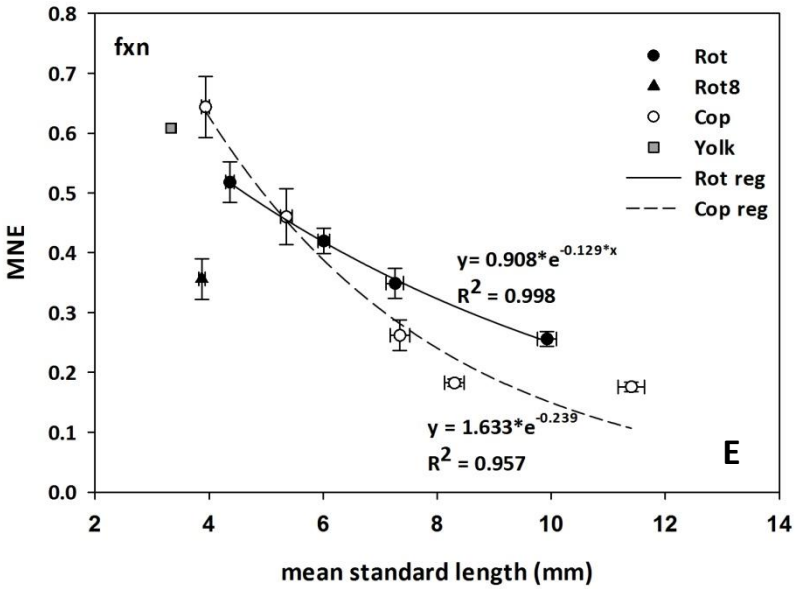
Correlation lines of vdac1 expression for the two treatments appeared almost parallel, but Cop larvae had significantly higher vdac1expression for these SL values. At 8 dph Cop up-regulated from the yolk sac larvae MNE (Fig. 3.8C).

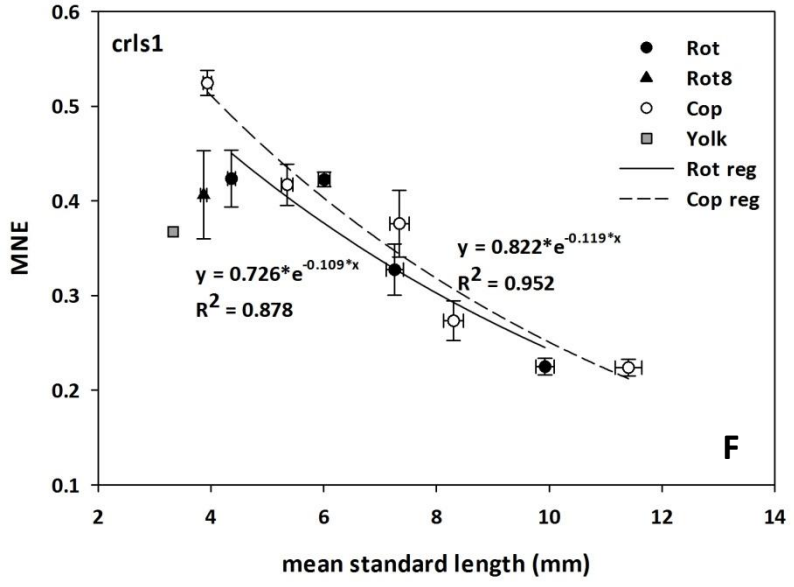

cyc1
Rot
Rot8
Cop
Yolk
Rot reg
Cop reg
y = 0.712*e-0.712*x
R2 = 0.807
y = 0.521*e-0.059*x
R2 = 0.975
A
MNE
mean standard length (mm)


cox5a
Rot
Rot8
Cop
Yolk
Rot reg
Cop reg
y = 0.801*e-0.087*x
R2 = 0.938
y = 0.434*e-0.015*x
R2 = 0.940
B
MNE
mean standard length (mm)


vdac1
Rot
Rot8
Cop
Yolk
Rot reg
Cop reg
y = 0.794*e-0.079x
R2 = 0.968
y = 0.809*e-0.108x
R2 = 0.910
C
MNE
mean standard length (mm)


Mnsod
D
y = 0.617*e-0.00097x
R2 = 0.001
y = 0.549*e-0.005x
R2 = 0.089
Rot
Rot8
Cop
Yolk
Rot reg
Cop reg
MNE
mean standard length (mm)


fxn
Rot
Rot8
Cop
Yolk
Rot reg
Cop reg
y= 0.908*e-0.129*x
R2 = 0.998
y = 1.633*e-0.239
R2 = 0.957
E
MNE
mean standard length (mm)


crls1
Rot
Rot8
Cop
Yolk
Rot reg
Cop reg
y = 0.822*e-0.119*x
R2 = 0.952
y = 0.726*e-0.109*x
R2 = 0.878
F
MNE
mean standard length (mm)

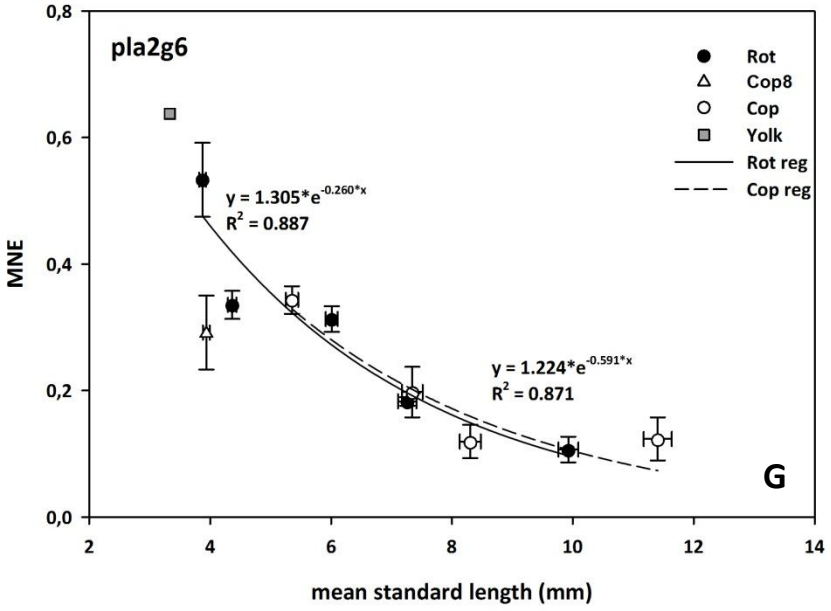


Figure 3.8 Whole larvae mRNA expression (MNE; mean normalized expression) of cytochrome *c1* (cyc1), cytochrome oxidase Va (cox5a), voltage-dependent anion channel 1 (vdac1), manganese superoxidase dismutase (mnsod), frataxin (fxn), cardiolipin synthase (crls1), and phospholipase A_2 group VI (pla2g6) of ballan wrasse from 3 to 44 dph versus their mean standard length (mm) for the same time, except 45 dph instead 44 dph. Larvae were fed either rotifers and *Artemia* (Rot), or copepods of continuously increasing size (Cop). Expression before treatment (3 dph) is marked as "Yolk". Expression of Rot larvae at dph 8 is marked as "Rot8" (cyc1, cox5a, vdac1, mnsod, fxn and crls1) and expression of Cop larvae at dph 8 is marked as "Cop8" (pla2g6). Data are mean ± SE (n = 3 replicates, except for 3 dph where n = 1, for perpendicular error bars; n = 12 individuals replicate^{-1} for 3 and 8 dph and n = 20 individuals replicate^{-1} for 15, 28, 36 and 45 dph for horizontal error bars). Solid and dashed lines are best fit exponential models for Rot and Cop treatments, respectively. Plot equations and R^2 are given.

MNE regression of mnsod was rather linear and displayed relatively stable values throughout the larval development for both treatments (Fig. 3.8D). Mnsod was again higher expressed for Cop larvae, and the difference was significant. MNE of the yolk sac larvae was followed by a down-regulation for the Rot larvae at 8 dph.

Fxn expression in Rot larvae had the strongest correlation (R^2 = 0.998) with SL observed in this study, after a down-regulation at 8 dph. For SL < 5.3 Cop treatment had higher fxn expression, but was lower expresser after this SL value (Fig. 3.8 E). The two treatments' regressions differ significantly both in terms of elevation and slope.

Crls1 in Cop larvae displayed an up-regulation at 8 dph. MNE correlation to SL showed no significant difference for the two treatments (Fig. 3.8 F).

When correlations of pla2g6 MNE with SL were tested statistically, they differed significantly for the two treatments (with Cop8 included in the Cop treatment). However differences were

marginal (p = 0.0831, equivalent to p = 0.05 according to R™), with Rot larvae being higher expressied until dph 15, and lower thereafter. With Cop8 excluded, the two exponential models were rather identical, except for 8 dph, where Cop larvae expression down-regulated (Fig. 3.8 G). Finally expression of pla2g6 in the yolk sac larvae seemed to be in line with the Rot expression.

4. Discussion

4.1 Diet and Larval Development

In the present study, first feeding with *A. tonsa* resulted in a significantly better growth of the ballan wrasse larvae than a diet with *Brachionus* sp. and *A. franciscana*, throughout the first 45 dph. This was clear for all three growth parameters, DW, SL and MH.

DW increased continuously in an exponential pattern (Fig. 3.2 B) and the two curves developed almost parallel to each other. This is a typical growth pattern for marine fish larvae (Kjørsvik *et al*., 1991; Galloway *et al*., 1999; Gamboa-Delgado *et al*., 2008; Wold *et al*., 2008; Penglase *et al*., 2010; Hansen, 2011; Garrido *et al*., 2012; Penglase *et al*., 2013; Srichanun *et al*., 2013).

Larvae of the two treatments had a relatively big difference in DW and MH at 8 dph. DW loss was observed at 8 dph for the Rot larvae, which possibly affected the further growth pattern. In a previous experiment (Gagnat, 2012) ballan wrasse displayed growth rate changes when copepods were replaced with a different live feed of lower value (either rotifers or brine shrimp). Here, Cop treatment resulted to a fairly steady growth after 8 dph throughout the experiment, implying that exclusive feeding with copepods is an optimal treatment of ballan wrasse larvae.

Yolk sac larvae of the present study were larger than in Gagnat's (2012) experiment, but their growth and % Survival were lower. Possible reasons for variations in growth and/ or survival are differences in egg and larval quality (Lavens & Sorgeloos, 1991; Kjørsvik *et al.*, 2003; Giménez *et al.*, 2006), related to genetics and spawning season. Differences in the rearing environment is also a possibility, although experiments were held in the same facilities (NTNU and SINTEF), environmental conditions were similar. Finally, cultivated rotifers were fed with baker's yeast while in Gagnat's experiment rotifers were fed with *Chlorella* sp. There is a possibility that baker's yeast results in a higher bacterial load in the water, which could affect the larvae growth. However growth results of the Rot larvae were quite similar to results of Dunaevskaya *et al.* (2012), who also fed rotifers with *Chlorella*.

DW measurements are possibly the most accurate and reliable growth parameter for the fish larvae, as volume embodies both measurements of length and height. Here SL solely would have been misleading for making conclusions about larval growth, as at 8 dph it was similar

for both treatments, where DW and MH showed that Cop larvae were significantly thicker and heavier.

Harvested copepods as an early larval diet have result in a better somatic growth in larvae of several fish species (Shields *et al*., 1999; Copeman *et al.*, 2002; Bell *et al.*, 2003; Skalli & Robin, 2004; Hansen, 2011). It is commonly accepted that copepods' superiority as a first feed lies in their high content of HUFA in the PL, and particularly DHA, EPA, and ARA, as well as their ratios DHA: EPA: ARA (Watanabe, 1993; Evjemo *et al.*, 2003; Molejon & Alvarez-Lajonchere, 2003) and higher levels of protein. In addition, PL are considered to improve palatability (Szich *et al.*, 2005; Berg, 2012), which possibly makes larvae more willing to prey on copepods.

Ballan wrasse flexion larvae have a mean SL of approximately 6 mm, while post-flexion period occurs for larvae of approximately 6-11 mm (Dunaevskaya *et al.*, 2012; Sørøy, 2012). In this experiment SL of 6-11 mm corresponded to approximately 21-45 dph for the Cop larvae. For Rot larvae post-flexion period started 7 days later, at approximately 28 dph and would probably finish after 45 dph (they never reached 11mm). Until the point of 6 mm major organs have already developed, fin separates and forms (Dunaevskaya *et al.*, 2012; Gagnat, 2012) and a big increase of DW follows for both treatments. As Galloway *et al.* (1999) suggested for cod, diet quality during early days in marine pelagic larval growth is crucial. The impact of copepods as a start-feed in was displayed clearly in % DWI results, as copepods resulted in a much more rapid larval growth during the early days of the start feeding (3-8 and 8-15 dph).

Pigmentation differences were observed between the ballan wrasse larvae of the two treatments (Fig. 3.6). Rotifers and/or brine shrimp as start feed cause malpigmentation on turbot, Atlantic halibut and less pigmentation on Atlantic cod larvae (Reitan *et al.*, 1994; Næss *et al.*, 1995; Shields *et al.*, 1999; Eidsvik, 2010; Norheim, 2011). The pigmentation variations have been attributed to differences in HUFA-levels of the live feeds (Reitan *et al.*, 1994; Evjemo & Olsen, 1997). The yellow tint observed is probably irrelevant to FA quality or levels and is due to high carotenoids content of copepods and *Artemia* when compared with rotifers (Hamre *et al.*, 2013). Also, *R. baltica* is rich in xanthophyll carotenoids (Tanaka & Katayama, 1975), which, through the copepods, were finally taken up by the ballan wrasse larvae. At 45 dph, eyes of some Cop larvae displayed partially the deep orange/red coloration of the adult ballan wrasse, while all sampled Rot larvae still had silver-colored eyes (Fig. 3.6).

Cop larvae seemed quicker to respond to stimulations of the environment and were notably more difficult to collect for sampling than the Rot larvae. Sørøy (2012) observed that ballan wrasse larvae fed copepods were much more efficient to capture their prey, possibly because of visual problems caused by DHA deficiency (Bell *et al.*, 1995).

As in Gagnat's (2012) experiment larval % Survival had no significant difference for the two treatments. The fact that the vast majority of larvae had died before 15 dph (85 %; Table 3.1) underlines the great sensitivity of the ballan wrasse larvae during their yolk sack and preflexion periods. It is possible that environmental factors for the early days of the ballan wrasse larviculture are still suboptimal.

Based on the growth results of the present study, as well as Gagnat's (2012), and especially on the steady, uninterrupted growth that copepods provide, it can be concluded that copepods is probably the optimum feed for ballan wrasse larvae. Copepods live-feed should be used as the exclusive first feeding at least for the first 30 dph, to secure the availability of PL-included HUFA as well as protein in the crucial stages of larval development.

4.2 Gene Expression: the Importance of Size

Even given its weaknesses,
size still matters, at any age
(Sæle & Pittman, 2010)

In this study, gene expression was first seen versus larval age. Results were rather discouraging, in terms of understandability; there were clear differences between treatments, especially during the early dph (up to 8 or 15 dph), but expression lines did not have an obvious pattern. Also, similarities between genes that participate in a related or even the same pathway could not be pinpointed. Seeing the genes versus larval length instead, made fairly clear correlations. The whole situation resembled to an English text written with Greek characters: one can see that this is a text which could make sense, if only seen in some other way. The other way, the "English characters for the English text" is "gene expression in relation to larval development", and in this case larval development is size.

Existence of right criteria is important for understanding the larval development and a notable part of literature argues that age is a non-reliable way to distinguish life stages of fish. Body size (length or thickness), as well as ontogenic criteria, such as cranial ossification and hormonal development, seem more preferable (Schreiber & Specker, 1998; Adriaens & Verraes, 2002; Sæle & Pittman, 2010). Coefficient of developmental variation in age is much higher than that in size for some marine species, including two wrasses (bluehead wrasse and California speedhead wrasse; Amara & Lagandere, 1995; Sæle & Pittman, 2010). Even for the post larval stages, fish development is often size-related: Chen & Ge (2013) recently found that gonad differentiation and sexual maturation on zebrafish is not a matter of age, but of body size. Balon (1999) criticizes ichthyologists for still using age as a measurement to denote "stage", as there are big variations (within species, batch and individual) on the developmental stage that a fish is hatched in. Apart from genetic and environmental conditions such as temperature, diet is a determining factor for the larval body size, and thus, development.

Such findings and arguments imply that body size is a more reliable measurement than age to denote "development". Development can have several aspects, besides the obvious ontogeny-related ones: cellular and molecular functions. The present study confirmed that expression of cyc1, cox5a, vdac1, Mnsod, fxn, crls1 and pla2g6 correlated strongly to larval SL, but not to larval age.

4.2.1 The enigmatic 8 dph

The expression for the Rot larvae at 8 dph was far out of the Rot regression model for all selected genes, except pla2g6; here it was Cop treatment which gave a lower expression and was "out of line" at 8 dph. This finding implies that there may be one or more dietary factors which deter the normal expression of the specific genes. When seen compared to the yolk sac larvae, this 8 dph- "out of line" effect occurred by either down-regulation of the Rot larvae expression (cyc1, cox5a, mnsod, fxn), or by up-regulation of the Cop expression (vdac1, crls1). In the case of pla2g6, Cop expression was down-regulated, while Rot was never up-regulated. These earliest up- or down-regulations seemed to dictate further expression of genes, except for crls1 and pla2g6 (Rot and Cop expressions were not significantly different after 8 dph); it can be therefore assumed that a "8 dph effect" is of great importance.

Both cyc1 and cox5a encode for parts of the two electron transport subunits (III and IV, respectively; Fig. 4), suggesting that rotifers and brine shrimp weaken the oxidative phosphorylation (OP) process on the very first days of exogenous feeding, generating less amounts of energy (ATP). Encoding products of vdac1, mnsod, fxn and crls1 are also closely related to OP. Regardless of being caused by Rot down-regulation or by Cop up-regulation, the higher expression of these six genes, which occurred for the Cop treatment, would imply higher mitochondrial activity, energy generation and release. This extra energy was possibly what facilitating the relatively high growth of Cop larvae at the early days. Future studies are encouraged to observe closely the period 0-15 dph, with frequent samplings, so that initiation and duration of this pattern is localized accurately. The overall differences that the two diets issued, may affect the fish development, not only at this specific period but also further (Royle *et al.*, 2005; Geurden *et al.*, 2007; Vagner *et al.*, 2007).

Large variations (high SE) of the gene expressions at 8 dph were noticed, especially for mnsod, declining at later days. This could indicate the variation in the preying ability of the larvae (some larvae fed better than others) or it can be due to the larger number of individuals that were sampled for the early measurements.

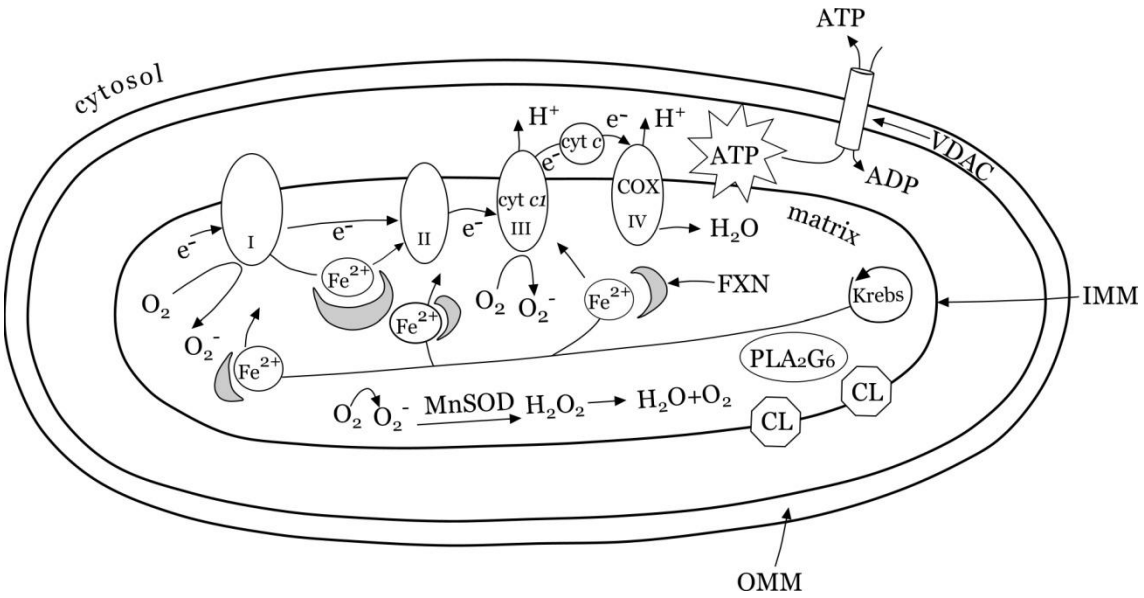


Figure 4. Schematic diagram of oxidative phosphorylation in the mitochondrion, including the encoding products of the studied genes (Seleznev *et al.*, 2006; Madamanchi & Runge, 2007; Pandolfo, 2008; Arnarez *et al.*, 2013). OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane; VDAC, voltage-dependent anion channel; cyt *c*, cytochrome *c*; cyt *c1*, cytochrome *c1*; COX, cytochrome oxidase; FXN, frataxin; MnSOD; manganese superoxidase dismutase; CL, cardiolipin; PLA2G6, phospholipase A_2 group VI.

4.2.2 Expression of the selected Genes

Encoding products of all selected genes are localized in the mitochondrion and they are- directly or indirectly- related to the OP (Fig. 4). According to the results, gene expression responses can be grouped after the pattern of the 8 dph effect; down-regulation of Rot, up-regulation of Cop or down-regulation of Cop expression.

Down-regulated expression for Rot larvae

Cytochrome oxidase Va gene Correlations of cox5a expression with SL differed significantly for the two treatments. Cox5a expression in Rot larvae was approximately constant for the SL values, implying strongly that a dietary factor keeps the cox5a levels stable when ballan wrasse larvae are fed rotifers and *Artemia*, instead to the normal decline. It is therefore suggested further investigation on whether cox5a expression continues as stable during the formulated feeding also.

COX forms the last complex (subunit IV) of the electron transport chain of OP and is responsible for ATPase activation and ATP generation (Fig. 4). The fact that cox MNE had an almost stable correlation with SL implies that ATP generation was constant for Rot larvae, while cox5a expression for Cop was very high in the beginning and dropped thereafter. The difference on energy generation that this may denote was actually reflected on % DWI results. Even though % DWI it is not a very sensitive measurement, it displays a high growth difference for 3-15 dph between treatments. Ballan wrasse larvae are expected to need a rather high amount of energy during those periods for development and new tissue formation. Gagnat (2012) states that when ballan wrasse larvae were fed copepods, intestinal walls were thicker, with longer folds at 8 dph compared with rotifer-fed ones.

Larval % DWI did not differ significantly during the following periods, until 36-45 dph, where Cop larvae had significantly lower % DWI, corresponding with cox5a expression (Fig. 3.8 B). It could be therefore suggested that cox5a gene expression reflects growth rates for ballan wrasse fish larvae. Finally, Rot larvae having a rather stable cox5a expression may have led to a continuous production of ATP from the mitochondrion.

Martin *et al.* (2013) recently found that COX activity increased with juvenile trout body mass and had no interaction with dietary ω-3 HUFA, while concentration of cytochrome *c1* was affected by the interaction of diet and body mass. The importance of a diet rich in HUFA in the PL, as well as short-chained protein, is much greater for the larval development than the

later stages. In addition, the pathways of lipid accumulation, as well as the developmental stages and the dietary needs *per se* are quite different between marine pelagic fish and salmonids (Kjørsvik *et al.*, 2004). On the other hand another study on rats had shown that diets rich in ω-3 reduced activity of COX (Yamaoka *et al.*, 1988). Without ignoring the fact that enzyme activity and gene expression are not always equivalent, the present findings correspond more with the latter experiment.

Norheim's (2011) findings (morphological and functional changes in mitochondria; paragraph 1.2.3) are similar to the symptoms of the renal oncocytoma in humans, which up-regulates cox5a expression (Yusenko *et al.*, 2010). Ballan wrasse and humans, as well as non-optimal nutrition and oncocytoma are not comparable; nevertheless the very symptom of alterations in mitochondrial morphology/functions is perhaps related to OP and thus would be affected by subunit IV (COX).

Expression of cox5a is also reported to up-regulate with the presence of heme (Trueblood *et al.*, 1988) and regulate with oxygen *per se* (Burke *et al.*, 1997) possibly could relate to FXN (see below).

Cytochrome *c1* gene MNE of cyc1 correlated similarly with SL for the two treatments, but Cop larvae displayed a significantly higher size related expression. Hulbert *et al*. (2006) reported that activity of cytochromes in three different species (laboratory rat, bearded dragon lizard and cane toad) correlated positively with the level of polyunsaturation of the PL in the mitochondrial membrane. In spite the fact that no fish was examined, the wide variety of these three species could imply a broad-taxa application. On the other hand, FA levels of the mitochondrial PL reflected the dietary FA in rainbow trout juveniles (Martin *et al*., 2013). Cop larvae had a higher expression of cyc1 throughout the whole experiment (Fig. 3.8 A), which accorded well with Martin *et al.* (2013) results. Moreover, the same team showed an interaction of cytochrome *c1* activity between diet and body mass, while diet alone did not have an important effect on it. Cytochrome *c1* is a fundamental protein, as it is a subunit of the complex III of the electron transport chain during OP (Fig. 4). Results thus suggested a lower respiration activity and ATP generation for larvae consuming rotifers and brine shrimp, compared to those consuming copepods.

Manganese superoxide dismutase gene Mnsod was the only of the examined genes whose expression remained approximately stable for both treatments for the whole experiment, with Cop larvae displaying a significantly higher expression.

MnSOD is a mitochondrial antioxidant enzyme (Weisiger & Fridovic, 1973; Fridovich, 1975) which catalyzes superoxide (O_2^-) conversion to hydrogen peroxide (H_2O_2) during the OP (Fig. 4), protecting the cell from superoxide accumulation, which causes oxidative stress and apoptosis. O_2^- attacks mitochondrial membrane PL, such as CL, altering the membrane permeability (Seleznev *et al.*, 2006). SODs are present already at fish embryos; for turbot larvae SOD activity reduced with age (Peters & Livingstone, 1996), while the reverse was observed for trout (Aceto *et al.*, 1994; Fontagné *et al.*, 2008). Expression of SOD genes during the embryo stages would be an interesting future study on ballan wrasse. Although Fontagné *et al.* (2006, 2008) observed higher SOD activity for higher oxidization degree in the feed, it is unlikely that copepods of the present study provided larvae with oxidized lipids. Live feed may have affected mnsod levels both through the provided nutrients and indirectly, by increasing the OP activity, and therefore increasing the need for removal of cytotoxic oxygen products, as it was earlier suggested for fxn. The higher mnsod MNE that was observed for the Cop larvae could imply better cellular protection and functionality. Rot larvae were possibly less capable of handling cellular stress than the Cop ones.

MnSOD expression responds to viral and bacterial infections (Liu *et al.*, 2013) but this case would be rather unlikely here, as expression was quite stable.

Frataxin gene Frataxin (FXN) is a mitochondrial protein whose role is not well understood yet, but it seems to be necessary for the cellular control of the iron homeostasis and iron metabolism (Pandolfo & Pastore, 2009; Shan & Cortopassi, 2011). FXN deficiency is responsible for the human Friedreich ataxia and it has been suggested to participate in OP and energy conversion in the mitochondrion (Ristow *et al.*, 2000; González-Cabo *et al.*, 2005). It is also proposed to be an iron-storage protein and an iron chaperone on heme biosynthesis (Becker *et al.*, 2002; Schoenfeld *et al.*, 2005; Zhang *et al.*, 2005) and Fe-S formation (Zhang *et al.*, 2006; Lill, 2009; Stemmler et al., 2010; Shan & Cortopassi, 2011). Some studies (Bulteau *et al.*, 2004; Rouault & Tong, 2008) imply that FXN protects and repairs damaged aconitase Fe-S clusters in the mitochondrial matrix and also regulates reactive oxygen species

(ROS) concentration and thus preventing cellular oxidative stress (Shoichet *et al.*, 2002; Gakh *et al*., 2006).

In the present study fxn was higher expressed in Cop larvae shorter than approximately 5.5 mm (Fig. 3.8 E) and higher expressed in Rot larvae longer than that. Very few experiments on FXN expression versus diet have been made so far. Hansen *et al.* (2010) studied how an iron-rich diet affected the fxn expression on young pigs and findings suggested that FXN depends on the age *x* iron status more than iron alone. Also, diet rich in iron resulted in an initially lower fxn expression, but at some point it reversed to a higher expression. Data on iron content of copepods, brine shrimp and rotifers are generally quite broad, depending on the food, and hence are difficult to compare, but it seems that copepods can reach much higher content than the two other species (Hamre *et al.*, 2013). Nevertheless, iron concentration in the fish larvae does not seem to reflect the live feed concentration (Yamamoto *et al.*, 2013), something that explains the high activity of proteins that control the iron homeostasis, such as FXN.

Blood cells in ballan wrasse larvae fed rotifers were observed at 7 dph by Dunaevskaya *et al.* (2012), but they could have appeared much earlier. Appearance of blood cells is species-specific and in some species occurs before hatching (zebrafish; Pelster & Burggren, 1996). Hemoglobin appears before hatching, while it does not transfer O_2 during embryonic and early developmental stages (Pelster & Burggren, 1996). Results could suggest that FXN promoted early heme synthesis- first in Cop and later in Rot larvae. Further studying with hemoglobin manipulation and fxn expression would answer if there is a connection, or they are simply irrelevant and the fxn results are triggered by something else. Another scenario is that the fxn MNE drop denotes a reduced need for protection from oxidative shock, possibly because of reduced OP rates, as suggested above (cox5a).

Up-regulated expression for Cop larvae

Voltage dependent anion channel 1 gene MNE of vdac1 didn't have significant different correlation between the two treatments, but Cop larvae displayed a significantly higher size related expression, after their up-regulation at 8 dph.

VDAC is an ion-channel protein, located only in the outer membrane of mitochondria (Rostovtseva *et al.*, 2005) where it regulates cell apoptosis (Okada *et al.*, 2004). Its central

role is to regulate the Ca^{2+} crossing the outer membrane (Gincel *et al.*, 2001; Shoshan-Barmatz *et al.*, 2006), and hence possibly coordinates energy metabolism (Fig. 4). Abu-Hamad *et al.* (2006) showed that low expression of human vdac1 resulted in decreased energy production (ATP synthesis), low ATP and ADP levels, suggesting decreased metabolite crossing between mitochondria and cytosol. Interestingly experiments in seahorses and mice have shown that vdac1 expression is related to neurons synaptic plasticity, and is impaired in learning process and cued fear, unlike vdac3 which is related to contextual fear conditioning (Weeber *et al.*, 2002; Levy *et al.*, 2003). The neuron-involved nature of VDAC could explain the difference observed between the two expressions of vdac1 for Rot and Cop treatments.

Strong relation between cytosolic calcium levels and vdac genes expression has been demonstrated, focusing on the binding properties of VDAC with Ca^{2+} (Gincel *et al.*, 2001; Shoshan-Barmatz *et al.*, 2006; Israelson *et al.*, 2008; Keinan *et al.*, 2013). Besides on humans and other mammals, expression of vdac isoforms has been measured on olive flounder, largemouth bass and black-chinned tilapia (Lü *et al*., 2007; Doperalski *et al.*, 2011; Tine *et al.*, 2011). Tine *et al.* (2011) suggested a relation of osmoregulation and vdac expression, affected by water salinity. There were no salinity alterations in the present experiment, but higher MNE of vdac1 in the Cop larvae could suggest more functional osmoregulation responses or more rapid development of the membranes functionality.

Cardiolipin Synthase gene Crls1 expression was higher expressed in the Cop larvae at 8 dph; expression thereafter was similar.

Enzyme CL synthase is involved in the glycerophospholipid metabolism pathway catalyzing the CL synthesis by phosphatidylglycerol (PG) and cytidine diphosphate-diacylglycerol (CDP-DAG) in the mitochondria inner membrane (Houtkooper *et al.*, 2006; Lu *et al.*, 2006).

Reduced CL levels are related to apoptotic or aging cells (Paradies *et al.*, 1997). CL modulates the calyzing activity of major respiratory-involved proteins, including COX to which it has several binding sites (Arnarez *et al.*, 2013). It also binds to cytochrome *c*, making it membrane-attachable. Interestingly, Gohil *et al.* (2004) found that CL biosynthesis is interdependent with the mitochondrial respiratory chain, as CL is also regulated by it. These observations were made on yeast, but Gohil *et al.* state that this function possibly applies to all eukaryotes. Here copepods resulted to a higher crls1 expression for the early larval days,

which may have enhanced the OP process. It is therefore possible that mitochondrial activity and OP were targeted by dietary factors in both treatments, with rotifers and brine shrimp weakening them, and copepods boosting them.

The same study (Gohil *et al.*, 2004) indicated that pH in the mitochondrial matrix affects CL synthase, but not the expression of clrs1 gene. Consequently, even though crls1 gene expression does not show big differences between the two treatments- except at 8 dph-, a finding of deformed mitochondria, similar to Norheim's (2011) could still point out to CL synthase. Further study, with measurement of CL activity is therefore suggested, as such a finding could answer- or reject- several questions.

Finally, crls1 MNE dropped continuously during larval development for both treatments. Jiang *et al.* (1993) showed that Crls1 is derepressed when yeast reached stationary growth phase, while it was repressed during the exponential growth phase. Wold *et al.* (2009) observed less dense mitochondrial membrane structures in cod larvae fed a diet rich in neutral lipids, which could be related to crls1 lower activity.

Down-regulated expression for Cop larvae

Phospholipase A_2 group VI gene Pla2g6 was the only gene where the Cop expression down-regulated from the yolk sac larvae levels. After 8 dph, expression of pla2g6 for the two treatments seemed identical (Fig. 3.8 G).

PLA_2G_6 is a Ca^{2+} independent, non-specific enzyme. It is found in mitochondria (Williams & Gottlieb, 2002; Seleznev *et al.*, 2006) and is suggested to protect the inner membrane, and especially CL, from reactive oxygen species (ROS), such as O_2^- and OH^- (Fig. 4; Seleznev *et al.*, 2006). PLA_2G_6 binds directly to acidic phospholipids such as CL, phosphatidic acid, phosphatidylglycerol and phosphatidylinositol, whose actual presence increases PLA_2G_6 activity (Morrison *et al.*, 2012). Cruzado *et al.* (2012) found that phosphatidylnositol and phosphatidylethanolamine are the only lipids that increase quantatively during yolk sac larvae phase in brill and this perhaps explains the higher MNE of pla2g6 at the beginning of this experiment.

Studies on mice, trout juveniles and adult Atlantic salmon have shown that some PLA_2 are involved in generating inflammatory responses (Sapirstein & Bonventre, 2000; Barry & Yang, 2008; Martinez-Rubio *et al.*, 2013). In the experiment of Martinez-Rubio *et al.* (2013)

pla2g6 was less expressed for the diet rich in ω-3, and the difference stopped being significant with time. EPA and DHA are accounted as anti-inflammatory FA (Calder, 2009; Calder *et al.*, 2009). This could explain the elevated expression for the Rot treatment at 8 dph: as Gagnat (2012) observed, and was prementioned, intestinal walls were less thick and developed for rotifer-fed ballan wrasse larvae at 8 dph, and thus digestion would be less effective for those larvae. In addition to that, differences in digestibility of feed types could lead to inflammatory responses, and elevated pla2g6 expression would be needed. Apart from the nutrients of each diet *per se*, other factors, for example oxidation of the rotifers enrichement medium could trigger bad digestion and inflammatory responses. PLA_2G_6 activity is also found to increase with the endoplasmic reticulum stress (Lei *et al.*, 2012).

Finally, PLA_2G_6 is directly involved to membrane turbulence, permeability and possibly fluidity (Brown *et al.*, 2003; Seleznev *et al.*, 2006). However, if differences in mitochondrial membranes morphology are observed in ballan wrasse larvae after 8 dph, another reason should be targeted, since MNE pla2g6 was similar for the two treatments.

5. Conclusions

Cultivated *Acartia tonsa* as the exclusive first feed for ballan wrasse larvae resulted in significantly heavier, longer and thicker larvae at 45 dph with visibly more intense pigmentation. Hence, this study confirmed earlier findings on superiority of copepods as a live feed. Larvae fed copepods had however equivalent survival with those fed rotifers.

Gene expression findings imply that reared copepods as an early live feed during the first two weeks, may result to more active mitochondria and higher generation of ATP. Particularly big differences on gene expression at 8 dph point out that there is one or more dietary factors which play an important role in the larval mitochondria during the very early days. This coincided with the big differences that were observed in the dry weight increase. Especially cox5a expression is possibly linked to the growth difference and may therefore be a trustworthy molecular indicator for energy generation and growth for the ballan wrasse larvae.

The present study underlines the importance body size of the marine fish larvae in relation to molecular responses. Ballan wrasse larval functional development can be seen more realistically in relation to growth than to age. Also, the biggest impact of the diet occurred in the early days of the larval life; this encourages future studies to investigate closely the first 15 dph.

It was demonstrated that diet influences energy metabolism of the ballan wrasse larva immediately after the feeding start. Even though nutritional differences of copepods versus rotifers and brine shrimp have various effects on the larval physiology, it is likely that the early molecular differences described here are important for mitochondrial formation and function, as well as growth differences observed later in life of the marine fish larvae.

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Appendix I

Conwy medium recipe (Walne, 1974)

$NaNO_3$ (Sodium Nitrate)	100.0 g
Na-EDTA (EDTA disodium salt)	45.0 g
H_3BO_3 (Boric Acid)	33.6 g
$NaH_2PO_4 \cdot 2H_2O$ (Sodium Phosphate. monobasic)	20.0 g
$FeCl_3 \cdot 6H_2O$ (Ferric Chloride. 6-hydrate)	1.3 g
$MnCl_2 \cdot 4H_2O$ (Manganous Chloride. 4-hydrate)	0.136 g*
Vitamin B_1 (Thiamin HCl)	0.1 g
Vitamin B_{12} (Cyanocobalamin)	0.05 g
Trace Metal Solution†	1 ml
Distilled water	1000 ml

Use 1 ml Conwy medium per liter of seawater

*SMALLER AMOUNT OF MNCL2 THAN IN THE ORIGINAL CONWY MEDIUM RECIPE (WALNE. 1974)

† Trace Metal Solution

ZnCl2 (Zinc Chloride)	2.1 g
CoCl2•6H2O (Cobalt Chloride. 6-hydrate)	2.1 g
(NH4)6Mo7O24•6H2O (Ammonium Molybdate. 4-hydrate)	2.1 g
CuSO4•5H2O (Copper Sulfate)	2.0 g
Distilled water	100 ml

Acidify with 1 M HCl until solution becomes clear

Appendix II

Table A.1 Mean dry weight (DW) of each treatment tank of ballan wrasse larvae fed either rotifers and brine shrimp (Rot) or copepods (Cop). Sample size (n), age (dph) and standard error of the mean (SE).

Dph	Treatment	Tank	DW (µg)	SE	n
3	All	All	50.5391	2.0704	20
8	Rot	1	36.2677	2.1248	12
		2	35.5302	1.2994	12
		3	38.3413	1.8148	11
	Cop	1	56.4361	4.5613	10
		2	57.0664	5.6041	11
		3	50.2618	4.4626	12
15	Rot	1	82.2038	8.0193	12
		2	83.4593	19.5508	11
		3	81.0541	7.4645	12
	Cop	1	204.0888	23.4732	9
		2	168.9913	20.4129	12
		3	198.8440	25.5533	12
28	Rot	1	359.6511	41.9369	10
		2	526.2658	131.9035	11
		3	615.3768	111.9855	11
	Cop	1	901.6525	160.0503	10
		2	720.0814	137.9045	9
		3	891.4774	122.0577	12
36	Rot	1	1018.7004	130.6942	14
		2	934.5086	102.9270	15
		3	1164.9965	115.4549	15
	Cop	1	1507.8280	211.5113	14
		2	1944.0270	304.3053	14
		3	1491.1924	154.7034	14
45	Rot	1	4234.6793	316.6909	15
		2	3964.4210	444.3044	15
		3	4171.1311	215.9231	14
	Cop	1	4325.6657	277.4251	15
		2	6701.2468	437.9861	15
		3	4661.2978	537.3116	15

Appendix III

Table A.2 % Survival of each treatment tank of ballan wrasse larvae fed either rotifers and brine shrimp (Rot) or copepods (Cop) on measurement days. Living larvae accidentally removed are considered dead.

Treatment	Tank	% Survival														
		Dph														
		2	15	17	20	23	24	27	29	31	34	36	38	41	43	45
Rot	1	100	15.6	14.5	13.2	11.6	10.8	10.1	9.7	9.1	8.2	7.7	7.6	7.4	7.3	7.3
	2	100	14.3	13.6	12.7	11.9	11.8	11.6	11.5	11.3	10.8	10.5	10.1	10.0	9.9	9.8
	3	100	13.6	12.6	12.3	11.8	11.4	11.0	10.8	10.6	10.4	10.1	9.9	9.8	9.8	9.7
Cop	1	100	17.3	17.0	16.6	16.1	15.8	15.6	15.5	15.4	15.4	15.2	14.9	14.9	14.9	14.8
	2	100	14.3	13.8	12.4	11.9	11.7	11.4	11.4	11.2	11.1	10.8	10.6	10.6	10.5	10.5
	3	100	12.8	12.4	10.8	10.2	9.9	9.6	9.6	9.5	9.4	9.2	9.2	9.2	9.2	9.1

Appendix IV

Table A.3 Mean standard length (SL) of each treatment tank of ballan wrasse larvae fed either rotifers and brine shrimp (Rot) or copepods (Cop). Sample size (n), age (dph) and standard error of the mean (SE).

Dph	Treatment	Tank	SL (mm)	SE	n
3	All	All	3.33	0.09	20
8	Rot	1	3.90	0.09	12
		2	3.86	0.10	12
		3	3.86	0.11	12
	Cop	1	3.95	0.14	11
		2	4.00	0.09	12
		3	3.86	0.13	12
15	Rot	1	4.49	0.11	12
		2	4.13	0.14	12
		3	4.47	0.08	12
	Cop	1	5.30	0.21	11
		2	5.35	0.14	12
		3	5.40	0.19	12
28	Rot	1	5.91	0.11	11
		2	6.12	0.22	11
		3	6.00	0.18	11
	Cop	1	7.40	0.33	10
		2	6.91	0.31	11
		3	7.70	0.23	12
36	Rot	1	6.81	0.30	14
		2	7.14	0.15	15
		3	7.80	0.28	15
	Cop	1	8.07	0.22	15
		2	8.64	0.42	14
		3	8.22	0.26	15
45	Rot	1	8.33	0.24	15
		2	9.75	0.32	15
		3	9.90	0.31	15
	Cop	1	11.00	0.28	15
		2	12.40	0.30	15
		3	10.81	0.47	15

Appendix V

Table A.4 Mean normalized expression (MNE) of cyc1, cox5a, vdac1, fxn, crls1, pla2g6 in ballan wrasse larvae of each treatment tank fed either rotifers and brine shrimp (Rot) or copepods (Cop), sample size (n) and age (dph).

Dph	Treatment	Tank	MNE							
			cyc1	cox5a	vdac1	mnsod	fxn	crls1	pla2g6	n
3	All	All	.528	.558	.490	.608	.551	.367	.639	30
8	Rot	1	.367	.352	.475	.322	.362	.475	.627	30
		2	.401	.356	.505	.424	.502	.427	.548	30
		3	.320	.339	.350	.322	.422	.317	.426	30
	Cop	1	.541	.541	.541	.541	.446	.541	.301	30
		2	.589	.635	.561	.691	.647	.498	.289	30
		3	.587	.615	.608	.699	.624	.533	.286	30
15	Rot	1	.400	.408	.485	.552	.488	.416	.313	30
		2	.345	.407	.381	.450	.471	.376	.314	30
		3	.485	.403	.586	.552	.606	.479	.380	30
	Cop	1	.478	.571	.502	.552	.735	.457	.302	30
		2	.378	.447	.578	.429	.577	.412	.352	30
		3	.367	.417	.561	.400	.541	.382	.376	30
28	Rot	1	.366	.411	.458	.407	.541	.435	.321	25
		2	.388	.386	.467	.391	.517	.408	.343	25
		3	.321	.385	.480	.461	.551	.424	.275	25
	Cop	1	.441	.417	.498	.314	.731	.445	.253	25
		2	.377	.341	.457	.239	.701	.353	.220	25
		3	.360	.449	.384	.234	.664	.329	.119	25
36	Rot	1	.333	.382	.322	.356	.530	.312	.196	20
		2	.371	.443	.367	.388	.584	.380	.176	20
		3	.300	.358	.378	.303	.556	.290	.176	20
	Cop	1	.414	.333	.364	.171	.546	.308	.076	20
		2	.412	.418	.392	.193	.645	.276	.114	20
		3	.373	.387	.421	.183	.599	.235	.168	20
44	Rot	1	.316	.428	.245	.235	.502	.213	.147	20
		2	.337	.361	.265	.257	.465	.242	.088	20
		3	.237	.326	.292	.277	.553	.219	.085	20
	Cop	1	.316	.298	.331	.160	.579	.224	.059	20
		2	.331	.404	.304	.182	.552	.239	.136	20
		3	.276	.274	.358	.186	.608	.209	.175	20