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
Submission date: August 2013
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Acknowledgements

This M.Sc Thesis was part of the research project “Produksjon av berggylte (900554), funded by the Fishery and Aquaculture Industry Research Fund (FHF), a collaboration of NTNU, SINTEF, Nofima, NIFES and the Institute of Marine Research, as well as ballan wrasse juvenile and salmon producers. Experimental phase was carried out at NTNU Centre of Fisheries and Aquaculture and SINTEF Fisheries and Aquaculture. Molecular analyses were performed at NIFES Laboratory for Molecular Biology.

The thesis was written at the Department of Biology at NTNU, under the supervision of Elin Kjørsvik (professor, NTNU), Øystein Sæle (researcher, NIFES) and Jan Ove Evjemo (researcher, NTNU).

First I would like to thank all my supervisors for the guidance and encouragement. Elin, I will miss our discussions over a piece of paper with the red corrections. Thanks for being so thorough (and patient!). Øystein, thank you for the inspiring ideas and that chocolate cake. Jan Ove, thank you for all the practical teaching on live-feed.

I am very grateful to Hui-shan Tung for guiding me during the molecular analyses and for being such a warm human being! Many thanks to Marte Romundstad, Oda Skognes Høyland, Maren Ranheim Gagnat, Stine Wiborg Dahle and Andreas Hagemann for the cooperation during the experiment, as well as Christophe Pelabon and Per-Arvid Wold for the statistical enlightening. To my classmate He Song, thank you so much for being a real friend when I needed it. I will never forget your kindness.

To all the LAKS students warm greetings and my best wishes for the future. Special thanks to my studies consultant Lisbeth Aune, for being always there with genuine interest and understanding when totally I freaked out or simply needed some advice.

To my fantastic father, Spyros Stavrakakis I owe a huge “thank you” for his ideas, for being so proud of me and for the very fact that he was always available when I needed him, despite the distance. Finally, I could never thank enough my soon-to-be husband Raymond Hansen for all his love and support during this tough period.

Maria Georgia Stavrakaki

Trondheim, 14 August 2013
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 phosphorylation, 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.

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*cytochrome c1, cytochrome oxidase subunit Va, manganese superoxidase dismutase, frataxin, cardiolipin synthase, phosholipase A2 group VI*
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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)
PLA2 phospholipase(s) of the superfamily A2
PLA2G6 phospholipase A2 group VI
pla2g6 phospholipase A2 group VI encoding gene
PMMA polymethyl methacrylate
PUFA polyunsaturated (minimum two double bonds) fatty acid(s)
qPCR quantitative (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
sPLA2 secretory phospholipase A2
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 (Lepophtheirus 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’s 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; Havforskningsinstituttet, 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 °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; Gagnat, 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 $596 \pm 59 \text{ mg g}^{-1}$ 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 N₂ 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 triacylglycerols (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 A2 (PLA2) 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). PLA2 are grouped according to their specificity, tissue of function and dependency on the presence of calcium cations (Ca2+). A family of PLA2, are Ca2+ independent (iPLA2) and function within the cytoplasm. iPLA2 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 PLA2 (sPLA2), which is Ca2+ 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 iPLA2 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>
<thead>
<tr>
<th>Mechanisms</th>
<th>Gene</th>
<th>Encoding product</th>
<th>Product functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>subunits in oxidative phospholyration chain</td>
<td>cyc1</td>
<td>cytochrome <em>c</em>1</td>
<td>Subunit of the <em>b-c1</em> complex (III complex), which accepts electrons from Rieske protein and transfers them to cytochrome <em>c</em> in the mitochondrial respiratory chain.</td>
</tr>
<tr>
<td></td>
<td>cox5a</td>
<td>cytochrome oxidase subunit Va (COX 5a)</td>
<td>Subunit Va of cytochrome oxidase enzyme (COX). COX is the terminal enzyme of the respiratory chain (IV complex); it transfers electrons from cytochrome <em>c</em> to molecular oxygen.</td>
</tr>
<tr>
<td>membrane permeability, osmoregulation, Ca²⁺, ATP transfer, oxidative phosphorylation</td>
<td>vdac1</td>
<td>voltage-dependent anion channel 1 (VDAC1)</td>
<td>Protein which is a major component of the outer mitochondrial membrane, facilitating exchange of metabolites and ions.</td>
</tr>
<tr>
<td>ROS regulation, oxidative phosphorylation</td>
<td>mnsod</td>
<td>manganese superoxidase dismutase (MnSOD)</td>
<td>Enzyme that catalyzes the dismutation of superoxide into O₂ and H₂O₂, protecting the cell from oxidation.</td>
</tr>
<tr>
<td>multiple functions, oxidative phosphorylation</td>
<td>fxn</td>
<td>frataxin (FXN)</td>
<td>Protein which regulates iron transport and respiration and promotes the heme biosynthesis.</td>
</tr>
<tr>
<td>mitochondrial inner membrane structure, permeability, apoptosis, fusion, oxidative phosphorylation</td>
<td>crls1</td>
<td>cardiolipin synthase (CRLS1)</td>
<td>Enzyme that synthesizes cardiolipin (CL), transferring the phosphatidyglycerol group from one molecule to another.</td>
</tr>
<tr>
<td>cell proliferation, apoptosis, mitochondrial membrane transport, alteration of the membranes tubulation, inner membrane lipids protection</td>
<td>pla2g6</td>
<td>phospholipase A₂ group VI (PLA₂G₆)</td>
<td>Cytosolic, Ca²⁺ independent enzyme which metabolizes phospholipids. Encoded in mitochondria.</td>
</tr>
</tbody>
</table>
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 °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 O₂-levels were measured daily (ProODO Optical Dissolved Oxygen Meter, YSI Inc., OH, USA), being kept at 12-16 °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 °C, before entering a water degasser of low atmospheric pressure to avoid N₂ supersaturation. Water was then gathered in a 5000 L tank and went through the above cycle repeatedly. Tank held biofilter substrates of 1 m³ volume and total surface of 800 m². Water finally flowed through a second heat exchanger where it got chilled to desired temperature (12 °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 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 | 41 | 42 | 43 | 44 | 45 |
| Clay (g tank⁻¹ day⁻¹) | - | 3.2 | 4.8 | 7.5 | 8 | 8.5 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 |
| 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
(individuals L⁻¹ feeding⁻¹)
- | 3500 | 5000 |
Enriched rotifers
(individuals L⁻¹ feeding⁻¹)
- | 12000 |
Enriched brine shrimp
Cop - 3 tanks
(individuals L⁻¹ feeding⁻¹)
- | 5000 |
Copepods
Stage
- | n | c | n | c | n |
| Mortality | x | x | x | x | x | x | x | x | x | x | x | x | x | x |
Sampling
Gene expression
- | x | x | x | x |
DW, SL and MH
- | x | x | x | x |

n, nauplii; c, copepodites; a, adults

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

<table>
<thead>
<tr>
<th>Stage</th>
<th>Age</th>
<th>Body length</th>
</tr>
</thead>
<tbody>
<tr>
<td>nauplii</td>
<td>1-5 dph</td>
<td>94-211μm</td>
</tr>
<tr>
<td>copepodites</td>
<td>6-10 dph</td>
<td>394-820 μm</td>
</tr>
<tr>
<td>adult</td>
<td>&gt;11 dph</td>
<td>1-1.5 mm</td>
</tr>
</tbody>
</table>
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 prey 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 Cryptophyceae: 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 °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 °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 (Na₂S₂O₃ 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, O₂- 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 °C, over 4 mg L⁻¹ dissolved O₂, 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 °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 °C (Pharmaceutical Refrigerator MPR-311D (H), Sanyo, Japan).
Flasks containing copepod eggs (500 000 eggs ml<sup>-1</sup>) 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 °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<sup>-1</sup>. Sediments were flushed for 5 s daily from an outlet of the tank bottom. Baker’s yeast (2.6 g million of individuals<sup>-1</sup> day<sup>-1</sup>) 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 °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 °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 °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 °C for 5-7 minutes. Na₂S₂O₃ 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 °C for 5-7 minutes. Cysts were then gathered and stored at 5 °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 O₂ levels were 4.5 mg L⁻¹ (minimum 2.5 mg L⁻¹; Hoff & Snell, 1987), temperature of 25-28 °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$_{\text{mass}}$; µg) by Marte Schei (SINTEF) using an Elemental Combustion Analyzer (Costech Analytical Technologies Inc., CA, USA). Acetanilide (C$_6$H$_5$NH(COCH$_3$)) was used as standard. Larval dry weight was then calculated using the equation

\[ DW = C_{\text{mass}} \times 2.34 \]

(Reitan et al., 1993).

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

\[ \%DWI = (e^{SGR} - 1) \times 100 \]

(Ricker, 1958), where

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

\[ DW = \text{dry weight at time } t. \]

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 N₂, before storage in -80 °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 (CHCl₃) was added in each tube; samples were shaken
manually for 15 s and centrifuged under 12000 rpm x 15 s at 4 °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 °C, while reverse transcription was performed at 48 °C for 60 minutes, before inactivation for 5 minutes at 95 °C. The product plate (cDNA) was covered with a sealing tape and stored at -20 °C.

* http://www.qiagen.com/Products/Catalog/Automated-Solutions/Sample-Prep/EZ1-RNA-Universal-Tissue-Kit#resources
Table 2.2 RT reaction mix for a 30µl reaction. Reagents, volumes (µl) and final concentrations.

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

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

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
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</table>

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 Specificity 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.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5’- 3’)</th>
<th>Reverse primer (5’- 3’)</th>
<th>Amplicon size (bp)</th>
<th>PCR eff.</th>
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</thead>
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<td>cyc1</td>
<td>AACCTTTCCCCAACCCTTTTT</td>
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<td>1.96</td>
</tr>
<tr>
<td>vdac1</td>
<td>GGAATTCCAAGCCGAGACCA</td>
<td>GCATCTTTCTCTGCAAGTT</td>
<td>154</td>
<td>2.08</td>
</tr>
<tr>
<td>fxn</td>
<td>TGGTCAACCTGCGACAAATG</td>
<td>GTCACACGCTCATCTGGCA</td>
<td>159</td>
<td>2.01</td>
</tr>
<tr>
<td>mnsod</td>
<td>TAAACCTAGAGCCGGCTGG</td>
<td>TCCCCGACCTGACTTACAGT</td>
<td>189</td>
<td>2.00</td>
</tr>
<tr>
<td>crls1</td>
<td>CTGCTGCTGTTGGTTTTTTC</td>
<td>ATCCAGCTCTTTGTTGTC</td>
<td>192</td>
<td>2.00</td>
</tr>
<tr>
<td>uba52</td>
<td>GGGCAGCTGCTGAGAGAAG</td>
<td>GTCAAGGCGGATACAGAAG</td>
<td>183</td>
<td>1.91</td>
</tr>
<tr>
<td>rpl37</td>
<td>ATGATAGAGAGGGCGAATAAC</td>
<td>CGCTCGACCTGTACTTAAA</td>
<td>159</td>
<td>2.03</td>
</tr>
<tr>
<td>bactin</td>
<td>CGTAACCTCGATAGGCGCGC</td>
<td>ACTGAACTCCGAGCAAGCA</td>
<td>182</td>
<td>2.03</td>
</tr>
</tbody>
</table>

cyc1, cytochrome c1; cox5a, cytochrome c oxidase subunit Va; pla2g6, phospholipase A2 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 °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 H2O 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 purged 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.

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

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

<table>
<thead>
<tr>
<th>Process</th>
<th>Duration</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse transcription</td>
<td>30 min</td>
<td>50</td>
</tr>
<tr>
<td>PCR activation</td>
<td>15 min</td>
<td>95</td>
</tr>
<tr>
<td>35 three-level cycles:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>45 sec</td>
<td>(&gt;30, &lt;60) 94</td>
</tr>
<tr>
<td>Annealing</td>
<td>45 sec</td>
<td>(&gt;30, &lt;60) 60</td>
</tr>
<tr>
<td>Extension</td>
<td>1 min</td>
<td>72</td>
</tr>
<tr>
<td>Final extension</td>
<td>10 min</td>
<td>72</td>
</tr>
</tbody>
</table>

**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). H2O 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 H₂O (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 °C, followed by forty 15-s-denaturing cycles at 95 °C. A 60-second-annealing step at 60 °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.

<table>
<thead>
<tr>
<th>% Survival</th>
<th>Rot</th>
<th>Cop</th>
</tr>
</thead>
<tbody>
<tr>
<td>dph</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>15 ± 1</td>
<td>15 ± 1</td>
</tr>
<tr>
<td>22</td>
<td>13 ± 0</td>
<td>13 ± 2</td>
</tr>
<tr>
<td>29</td>
<td>11 ± 1</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>37</td>
<td>9 ± 1</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>45</td>
<td>9 ± 1</td>
<td>11 ± 2</td>
</tr>
</tbody>
</table>

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 1 gene (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 larvae 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.
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 superoxide 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 and 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 vdac1 expression for these SL values. At 8 dph Cop up-regulated from the yolk sac larvae MNE (Fig. 3.8C).
D

\[ y = 0.617e^{-0.00097x} \]
\[ R^2 = 0.001 \]

Mean standard length (mm)

MNE

E

\[ y = 0.908e^{-0.129x} \]
\[ R^2 = 0.998 \]

\[ y = 1.633e^{-0.239} \]
\[ R^2 = 0.957 \]

Mean standard length (mm)

MNE

F

\[ y = 0.726e^{-0.109x} \]
\[ R^2 = 0.878 \]

\[ y = 0.822e^{-0.119x} \]
\[ R^2 = 0.952 \]

Mean standard length (mm)

MNE
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 superoxide dismutase (mnsod), frataxin (fxn), cardiolipin synthase (crls1), and phospholipase A2 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 replicate1 for 3 and 8 dph and n = 20 individuals replicate1 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 expressed 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*  
*(Sale & 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 A2 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 of 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$_2$O$_2$) 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 O2 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\textsuperscript{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\textsuperscript{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 crls1 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 A2 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).

\[ \text{PLA}_2\text{G}_6 \] 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 \( \text{O}_2^- \) and OH\(^-\) (Fig. 4; Seleznev et al., 2006). \( \text{PLA}_2\text{G}_6 \) binds directly to acidic phospholipids such as CL, phosphatidic acid, phosphatidylglycerol and phosphatidylinositol, whose actual presence increases \( \text{PLA}_2\text{G}_6 \) activity (Morrison et al., 2012). Cruzado et al. (2012) found that phosphatidylinositol and phosphatidylethanolamine are the only lipids that increase quantitatively 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 \( \text{PLA}_2 \) 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 enrichment medium could trigger bad digestion and inflammatory responses. PLA2G6 activity is also found to increase with the endoplasmic reticulum stress (Lei et al., 2012).

Finally, PLA2G6 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.
References


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Appendix I

Conwy medium recipe (Walne, 1974)

NaNO₃ (Sodium Nitrate) 100.0 g
Na-EDTA (EDTA disodium salt) 45.0 g
H₃BO₃ (Boric Acid) 33.6 g
NaH₂PO₄•2H₂O (Sodium Phosphate. monobasic) 20.0 g
FeCl₃•6H₂O (Ferric Chloride. 6-hydrate) 1.3 g
MnCl₂•4H₂O (Manganese Chloride. 4-hydrate) 0.136 g*
Vitamin B₁ (Thiamin HCl) 0.1 g
Vitamin B₁₂ (Cyanocobalamin) 0.05 g
Trace Metal Solution† 1 ml
Distilled water 1000 ml

Use 1 ml Conwy medium per liter of seawater

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* 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).

<table>
<thead>
<tr>
<th>Dph</th>
<th>Treatment</th>
<th>Tank</th>
<th>DW (μg)</th>
<th>SE</th>
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<tr>
<td>3</td>
<td>All</td>
<td>All</td>
<td>50.5391</td>
<td>2.0704</td>
<td>20</td>
</tr>
<tr>
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<td>36.2677</td>
<td>2.1248</td>
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<td></td>
<td>2</td>
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<td>1.2994</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>38.3413</td>
<td>1.8148</td>
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</tr>
<tr>
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<td>4.5613</td>
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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.

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### 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).

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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).

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