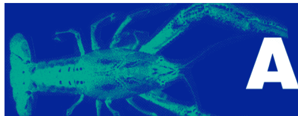
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Growth and stress factors in ballan wrasse (*Labrus bergylta*) larval development

Chiara Carla Piccinetti[1], Lorenzo Grasso[1], Francesca Maradonna[1], Giuseppe Radaelli[2], Cristina Ballarin[2], Giulia Chemello[1], Jan Ove Evjemo[3,4], Oliana Carnevali[1] & Ike Olivotto[1]

[1]Dipartimento di Scienze della Vita e dell'Ambiente, Università Politecnica delle Marche, Ancona, Italy

[2]Dipartimento di Biomedicina Comparata e Alimentazione, Università degli Studi di Padova, Agripolis, Legnaro, Italy

[3]Department of Biology, NTNU Norwegian University of Science and Technology, Trondheim, Norway

[4]SINTEF Fisheries and Aquaculture, Trondheim, Norway

Correspondence: I Olivotto, Dipartimento di Scienze della Vita e dell'Ambiente, Università Politecnica delle Marche, via Brecce Bianche, 60131 Ancona, Italy. E-mail: i.olivotto@univpm.it

Abstract

Ballan wrasse (*Labrus bergylta*) is a specialized cleaner fish used in salmon farms as a biological treatment against sea lice. Its commercial rearing is at present mostly experimental. A number of key aspects, including the molecular and physiological mechanisms that promote its growth and development, are still largely unexplored. In this study histological, biometric, biochemical and molecular approaches are combined for the first time to investigate the changes in growth (insulin-like growth factor 1 and 2 and myostatin) and stress (heat shock protein 70 and cortisol) markers that occur during ballan wrasse larval development by relating them to larval stages and feed changes. The real-time PCR data demonstrated that *igf1* transcripts rose from 1 day post-hatching (dph) and were no longer detectable 38 dph, whereas *igf2* and myostatin transcripts were low and stable until 28 dph, then rose in late larval stages. The biometric and histological data matched the molecular findings, documenting rapid growth and development of the larval digestive tract and assimilation ability. Cortisol was lowest at hatching, it rose slightly at first feeding, and then increased during larval development; a similar trend was detected for *hsp70* gene expression. The low cortisol levels found at the earliest larval stages reflect a poor stress-coping ability, a feature that may actually protect larvae from the elevated metabolic demands involved by stress responses and promote faster growth and survival. The present data can be applied to improve the rearing performances of this important cleaner species and reduce captures from the wild.

Keywords: growth factors, stress response, aquaculture, lipids, larval fish development

Introduction

Ever since its introduction, salmon farming has been afflicted by the ectoparasitic sea louse, *Lepeoptherius salmonis*. Sea lice live on salmon, feeding on mucus, skin and blood, inducing high mortality in farmed fish (Pike 1989) and costing farmers from €0.1 to €0.2 kg^{-1} of fish (Grant 2002). Wild cleaner fish (wrasse, Labridae) were first used as a commercial biological treatment against sea lice in the late 1980s, and their preventive and curative effect has been demonstrated (Rae 2002). However, drugs have remained the sea louse treatment of choice. Recently, reduced sea louse susceptibility to the available veterinary compounds has boosted the capture and deployment of wild wrasse, raising concern over overexploitation and depletion of wild stocks (Ottesen, Dunaevskaya & D'Arcy 2012).

Ballan wrasse (*Labrus bergylta* Ascanius), the fastest growing, most robust and effective louse cleaner fish available in Europe (Kvenseth, Andreassen & Solgaard 2003), is the subject of intense aquaculture efforts in Northern European countries, and farmed specimens have recently been reported to be as effective as their wild counterparts (Skiftesvik, Bjelland, Durif, Johansen & Browman 2013). However, commercial production

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is largely experimental, also because some major aspects, like early larval nutrition and the molecular and physiological mechanisms underpinning growth, development and stress response, are still poorly explored. Their investigation would allow improving the welfare of this new aquaculture species, thus enhancing commercial performances.

Unpredictable chemical–physical disturbances perceived as stressors by fish and eliciting a physiological response are often unavoidable in intensive aquaculture (Huntingford, Adams, Braithwaite, Kadri, Pottinger, Sandøe & Turnbull 2006). The metabolic cost of the response to excess stress may adversely affect immunocompetence and digestive processes (Moberg 2000), hence fish growth and development. Fast growth, early energy storage, proper nutrition and a suitable environment enhance the survival of fish larvae, since the size advantage allows access to limited resources and/or evasion and escape from predators (Leggett & Deblois 1994). The shift from endogenous to exogenous feeding, environmental and chemical–physical rearing conditions, and larval stage management are key survival and development factors. In particular, development and survival to the juvenile stage rely on the ability of fish to capture and digest prey having a suitable size and nutritional profile. Lipids, especially highly unsaturated fatty acids (HUFA), play a crucial role in early development stages, and lipid deficiency can impair larval health, inducing poor growth, low feeding efficiency and anaemia, resulting in high mortality (Sargent, McEvoy, Estevez, Bell, Bell, Henderson & Tocher 1999; Copeman, Parrish, Brown & Harel 2002; Olivotto, Rollo, Sulpizio, Avella, Tosti & Carnevali 2006).

Eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) are essential compounds for marine fish larvae, providing an important energy source for growth and development (Sargent *et al.* 1999; Izquierdo, Robaina, Juárez-Carrillo, Oliva, Hernández-Cruz & Afonso 2008; Olivotto, Di Stefano, Rosetti, Cossignani, Pugnaloni, Giantomassi & Carnevali 2011). Since growth, ontogeny, and the addition of new and improved physiological competences follow a strict, genetically programmed sequence, where gene transcription and hormone regulation play a crucial role, clinical and zootechnical parameters may be insufficient to monitor fish growth and development. Combining conventional approaches, like biochemical, histological, morphological and physiological measures, with molecular biomarkers has the potential to provide interesting new data and to relate biotic and abiotic conditions to growth performance and stress response.

In fish farming, larval growth and stress response management are of primary importance, since they affect production and farm income. In vertebrates, the growth hormone (GH)/insulin-like growth factor (IGF) axis is responsible for neuroendocrine growth regulation. Development and growth result from the interplay of positive (IGF) and negative (myostatin, MSTN) signals and environmental factors (Maccatrozzo, Bargelloni, Radaelli, Mascarello & Patarnello 2001; Bertotto, Poltronieri, Negrato, Richard, Pascoli, Simontacchi & Radaelli 2011). In many fish species, IGF and MSTN levels can be regulated by nutritional and environmental factors: for example, a HUFA-rich diet has been reported to modulate fish growth and *igf* and *mstn* transcripts in clownfish (Olivotto, Buttino, Borroni, Piccinetti, Malzone & Carnevali 2008; Olivotto *et al.* 2011). A similar result has been induced by salinity and temperature changes in freshwater fish larvae (*Lophiosilurus alexaxdri*) (dos Santos & Luz 2009).

Thus, growth is regulated by a variety of signals that interact with biological and physiological functions to maintain the energy balance (Piccinetti, Ricci, Tokle, Radaelli, Pascoli, Cossignani, Palermo, Mosconi, Nozzi, Raccanello & Olivotto 2012; Piccinetti, Donati, Radaelli, Caporale, Mosconi, Palermo & Olivotto 2015). It is well established that changes in homoeostasis and the energy balance affect the whole organism, acting from the cellular to the physiological level and impairing biological functions including survival and development. These changes are stressors, and the organism's reactions to restore the homoeostatic balance are stress responses (Schreck 2010). In teleosts, the stress response is provided by the hypothalamus–pituitary–interrenal (HPI) axis (Wendelaar Bonga 1997). Cortisol, the main corticosteroid in teleost fish, is considered as the principal stress indicator (Barton 2002), regulating metabolic energy, hydromineral balance, oxygen uptake, immune competence, development, hatching, growth and metamorphosis (Wendelaar Bonga 1997; Palermo, Mosconi, Avella, Carnevali, Verdenelli, Cecchini & Polzonetti-Magni 2011).

Studies of fish larvae show that *de novo* cortisol synthesis begins shortly after hatching, but a significant elevation in whole body cortisol in

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response to a stressor is detected days to weeks later, depending on the species (Barry, Malison, Held & Parrish 1995). Cortisol is usually lowest at hatching, it increases slightly at first feeding, and continues to rise through the flexion stage and all subsequent stages, indicating that in most fish larvae it begins to be produced around the shift from endogenous to exogenous feeding (Perez-Dominguez, Tagawa, Seikai, Hirai, Takahashi & Tanaka 1999; Szisch, Papandroulakis, Fanouraki & Pavlidis 2005; Bertotto *et al.* 2011). In fish and mammals, heat shock protein (HSP) acts at the cellular level as a general stress response system in addition to the HPI axis (Cara, Aluru, Moyano & Vijayan 2005; Poltronieri, Negrato, Bertotto, Majolini, Simontacchi & Radaelli 2008). Heat shock protein family proteins play critical roles in the stress response of aquatic organisms (Piccinetti *et al.* 2012), but their ontogeny in post-hatching development remains to be established for most species, including ballan wrasse. The 70 kDa HSP protein (HSP70) has been extensively investigated and used as a biomarker, because various stressors induce fast and significant increases (Piccinetti *et al.* 2012). HSP70 is constitutively expressed in unstressed cells and is also induced in response to stressors, particularly those affecting the protein machinery (Boone, Ducouret & Vijayan 2002). Heat shock proteins participate in a variety of protein folding processes, and their activation is induced by changes in phospholipid membrane fluidity (Samples, Pool & Lumb 1999).

In this study histological, biometric, biochemical and molecular approaches are combined for the first time to investigate the changes in growth and stress markers that occur during ballan wrasse larval development. These data provide key insights that can improve fish health and farm performances, thus reducing collection from the wild.

Materials and Methods

Ethics

All procedures involving animals were conducted in accordance with the European law on experimental animals and were revised and approved by the Aquaexcel Ethical Committee for Animal Experiments (Project No. 0037/03/10/25 approved 12/02/2013). All efforts were made to minimize suffering; larvae were killed using an excess of anaesthetics (MS222; Sigma-Aldrich, Milan, Italy).

Larval rearing

All experiments were carried out at the 'Cod Tech Laboratory', Norwegian University of Science and Technology (NTNU), Trondheim, Norway.

Seawater (34 ppt) was treated with a sand filter and filtered through a 1-μm mesh before heating and microbial maturation. During maturation it was continuously treated with a degasser and passed through a 1-μm filter before use in the rearing tanks.

One day post-hatching (dph), *L. bergylta* larvae were placed in nine 100-L conical bottom tanks at a density of 100 larvae L^{-1} per tank.

The water exchange rate was gradually increased, from twice daily 2 dph to eight times daily 30 dph. O_2 concentration and temperature were measured daily (Traceable® VWR® Digital Thermometer; VWR, Foster, CA, USA; pH/mV-meter; WTW ph 315i, Leipzig, Germany); O_2 was maintained >80% and temperature was gradually increased from 12°C (1 dph) to 16°C (5 dph). Larvae were reared under continuous illumination (daylight fluorescent tubes, Philips MASTER TL-D 90 Graphica, 18W/965, Amsterdam, The Netherlands).

Dead larvae and debris were removed by siphoning the bottom every day at 14:00 hours. Tanks were aerated from the bottom of the cone, and from day 8 dph were equipped with a surface skimmer. The sieve of the water outlet, placed in the middle of the tank, was cleaned daily. Sieve mesh size was increased from 200 μm at the beginning of the experiment, to 750 μm at the end of the experiment.

All analyses were performed on larvae randomly collected from all nine tanks.

Live feed production

Culture, harvesting and enrichment of rotifers (Brachionus plicatilis)

Brachionus plicatilis rotifers were grown in four tanks with conical bottoms (250 L) in seawater (34 ppt) at a temperature of 19–23°C. The water exchange rate was 1–1.5 times a day; the oxygen level was kept >80%. Once a week cultures were washed and moved to clean tanks. Rotifers were continuously fed DHA *Chlorella* (ratio, 2.5 mL per

million rotifers per day^{-1}; Chlorella Industry, Minato-ku, Tokyo, Japan). Cultures were diluted (25–40%) at densities >750 mL^{-1}, and debris was removed daily by flushing for 5 s from an outlet at the bottom of the cone. Culture density and egg ratio was measured daily in 12 samples (each 50 μL) discarding the highest and lowest values. Before administration to the fish larvae, rotifers underwent short-time enrichment [0.15 g Multigain (BioMar, Brande, Denmark) per million rotifers] for 2 h, with a maximum density of 400 mL^{-1}. Enriched rotifers were stored at 8°C prior to use (max. 24 h). Before being fed to the fish larvae, rotifers were concentrated and washed using a sieve. Loss during transfer and cleaning was estimated to be 20%.

Culture of Artemia salina

Artemia salina cysts (EG® INVE Aquaculture, Dendermonde, Belgium) were hydrated in freshwater (4.9 L of water for 450–500 g cysts, 15–25°C) with heavy aeration for 1 h. Cysts were then decapsulated according to the company's instructions, weighed and stored in a refrigerator (max. 6 days). Two days prior to being fed to the larvae, the necessary amount of decapsulated cysts was placed in seawater (25–28°C, pH 8–8.5) for hatching at a maximum density of 2 g cysts L^{-1}. Heavy aeration was provided at all times to ensure O_2 levels >2.5 mg L^{-1}. After 24 h, the hatched *Artemia* were washed and concentrated using an *Artemia* washer and then placed in new tanks (100–300 nauplii mL^{-1}). Over the next 24 h *Artemia* were enriched twice [10 g Multigain (BioMar) per 60 L], then washed, concentrated (200 mL^{-1}), and stored in a cool area (8°C, max. 20 h). The required amount of *Artemia* was estimated before each feeding and concentrated in the smallest possible volume.

Experimental design

Ballan wrasse larvae, reared in nine 100-L tanks (100 larvae L^{-1}), were fed three times a day according to the protocol described in Table 1. At each feeding time, Instant Algae(R) paste Isochrysis 1800 (Reed Mariculture, Campbell, CA, USA) was added to the tanks to obtain a concentration of 40 cells mL^{-1}.

Larvae were sampled before feeding, at 20:00 hours, at six time points: at hatching (1 dph); during the rotifer feeding stage (10 and 18 dph); during the rotifer/*Artemia* stage (28 dph); during the *Artemia* stage (38 dph), and during administration of early weaning feed (48 dph). Larvae and juveniles were gently collected and placed into a 1-L beaker with a lethal MS222 dose (1 g L^{-1}); those for morphometrics were immediately processed; and those for molecular, biochemical and histological analyses were placed in RNAlater® (Life Technologies, Milan, Italy), frozen at −80°C, or fixed in 4% paraformaldehyde, depending on the analysis to be performed.

Biometrics

Dry weight and daily weight increase

Twelve larvae per time point were placed in a 1-L beaker with a lethal MS222 dose (1 g L^{-1}), rinsed with fresh distilled water, placed in individual, preweighed capsules, dried at 60°C for at least 24 h and finally weighed (Mettler-Toledo microgram balance UMX2 automated-s ultra-microbalance, (Columbus, OH, USA) and UM3 precision single-pan balance, Switzerland). The per cent daily weight increase (% DWI) was calculated using the equation $\% \text{ DWI} = (\exp^{g-1}) \times 100$, where the growth coefficient g is: $g = (\ln W_2 - \ln W_1)/(t_2 - t_1)$. W_1 and W_2 are dry weights at t_1 and t_2.

Standard length

Standard length (SL) was measured in 12 fish larvae per time point. Measurements were taken on pictures of the larvae obtained with a stereo microscope (Leica M205C; Leica Microsystems, Wetzlar, Germany; Nikon digital sight DS-SM; Nikon Corporation, Tokyo, Japan) using software CAST 2 (Olympus, Tempovej, Denmark). Standard length was measured from the tip of the upper lip to the end of the vertebrae pre-flexion and to the peduncle (root of the caudal fin) post flexion.

Table 1 Ballan wrasse feeding protocol used in the study

Live preys	3–24 dph	25–28 dph	29–38 dph	39–48 dph
Rotifers	12 ind mL^{-1}	6 ind mL^{-1}	–	–
Artemia salina nauplii	–	3 ind mL^{-1}	6 ind mL^{-1}	3 ind mL^{-1} + early weaning feed (BioMar, ProStart, 300) (2% fish weight)

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Histology

For general morphological investigations, 30 larvae per sampling point were placed in a 1-L beaker with a lethal MS222 dose (1 g L^{-1}), fixed by immersion in neutral 4% paraformaldehyde prepared in phosphate-buffered saline (PBS, 0.1 M, pH 7.4) at 4°C overnight, washed in PBS, dehydrated through a graded ethanol series, and embedded in paraffin. Then, 4-μm thick consecutive sections were cut using a microtome, stained with Mayer's haematoxylin-eosin, dehydrated, mounted in Eukitt, and examined under an Olympus Vanox photomicroscope (New York Microscope Company, Hicksville, NY, USA). General gut/intestine and liver morphology was evaluated. The section area occupied by fat vacuoles was estimated (visually and semiquantitatively) to assess liver steatosis. Results were calculated according to American Gastroenterological Association criteria as 'percentage of hepatocytes in the tissue involved' and expressed on a 4-point scale as follows: 0 (no steatosis), 1 up to 33% (mild steatosis), 2 up to 33–66% (moderate steatosis) and 3 >66% (severe steatosis).

Analysis of fatty acids

Total fatty acids (FA) were extracted from triplicate rotifers and *A. salina* nauplii samples (0.5 g each) and from triplicate larval fish samples (approximately 50 larvae) with chloroform/methanol (2:1 v/v) (Folch, Lees & Stanley 1957). All determinations were performed in triplicate.

Methanol transesterification of lipids was carried out using 2 N methanolic KOH and hexane; the FAME obtained were analysed by HRGC using a DANI 1000DPC gas chromatograph (Norwalk, CT, USA) equipped with a split-splitless injector and a flame ionization detector (FID). Separation was obtained using a fused silica WCOT capillary column CP-Select CB for FAME (50 × 0.25 mm i.d., 0.25 μm f.t.; Varian, Superchrom, Milan, Italy). Chromatograms were acquired and processed using Clarity integration software (Data Apex, Prague, Czech Republic). Oven temperature was 180°C for 6 min, it was then increased by 3°C min^{-1} at 225°C and held for 10 min; injector and FID temperature was set at 250°C. The carrier gas (Helium; SOL, Ancona, Italy) flow rate was 1 mL min^{-1}. Fatty acids were identified by comparing the retention times of their methyl esters with standard FAME mixtures. Analytical precision was tested by repeated injection of standard solutions. The relevant standard deviations were <5% for all FA both in terms of within-day precision, calculated on six successive injections, and between-day precision, evaluated over 6 days. All solvents and reagents were of analytical grade and were purchased from Carlo Erba Reagents (Milan, Italy). Fatty acid compositions are expressed as per cent weight (area normalization).

Cortisol assay

Cortisol was extracted from whole larvae (three larval pools per sampling time). Samples were collected and processed according to Piccinetti *et al.* (2012). Briefly, samples from different groups were weighed, homogenized and extracted with 4 vol. of dichloromethylene for 30 s. The dichloromethylene fraction was collected; the remaining fraction was extracted again using the same procedure. This step was performed three times. The collected fractions were pooled, dried and re-dissolved in 1 vol. EIA buffer. Cortisol levels were analysed with Cortisol EIA kit (Cayman Chemical Company, Arcore, Italy) using a standard curve in the 2.5–1000 pg mL^{-1} range.

Assay sensitivity was 2 ng per tube; inter- and intra-assay coefficients of variation were 6.3% and 4.4% respectively. To validate the cortisol assay, parallelism between the calibration standard curve and serially diluted extracts was assessed.

Molecular analysis

RNA extraction and cDNA synthesis

Total RNA extraction from whole larvae was optimized using RNAzol® RT (Sigma-Aldrich) following the manufacturer's protocol. Total RNA was extracted from five pools of 10 larvae per sampling point. Final RNA concentrations were determined by Nanophotometer™ P-Class (Implem GmbH, Munich, Germany). RNA integrity was established by ethidium bromide staining of 28S and 18S ribosomal RNA bands on 1% agarose gel. RNA was stored at −80°C until use. Total RNA was treated with DNAse (10 IU at 37°C for 10 min; MBI Fermentas, Milan, Italy) and 5 μg of RNA was used for cDNA synthesis with iScript cDNA Synthesis Kit (Bio-Rad, Milan, Italy).

Primer design

Ubiquitin (*ubq*) and ribosomal protein 37 (*rpl37*) were selected as reference genes; their primers were taken from Hansen, Folkvord, Grøtan and

Sæle (2013). Primer sequences for *igf1*, *igf 2*, *mstn* and *hsp70* were chosen from high-homology regions among the sequences available in GenBank. All primers were used at a final concentration of 10 pmol μL^{-1}. Primers amplified a single PCR product. Sequences showed high homology to *igf1*, *igf2*, *mstn* and *hsp70* as follows:

igf1: 94% homology with *Dicentrarchus labrax* [accession number GQ924783.1].

igf2: 93% homology with *Mugil cephalus* [accession number AY427955.1].

mstn: 96% homology with *Sparus aurata* [accession number AF258448.1].

hsp70: 97% homology with *D. labrax* [accession number AY423555.2].

All primers are reported in Table 2.

Real-time PCR

Polymerase chain reactions were performed using SYBR Green in an iQ5 iCycler thermal cycler (Bio-Rad). All reactions were run in triplicate. The reactions were set on a 96-well plate. A mixture of 1 μL of diluted (1/20) cDNA, and 5 μL of 2× concentrated iQ TM SYBR Green Supermix (Bio-Rad) containing SYBR Green as a fluorescent intercalating agent, 0.3 μM of the forward primer and 0.3 μM of the reverse primer were used for each sample. For all reactions the thermal profile was 3 min at 95°C, followed by 45 cycles of 20 s at 95°C, 20 s at 60°C and 20 s at 72°C. Fluorescence was monitored at the end of each cycle. Dissociation curve analysis showed a single peak in all cases. *ubq* and *rpl37* were used as the housekeeping genes to standardize results by removing variation in mRNA and cDNA quantity and quality. No amplification product was observed in negative controls and no primer-dimer formation was detected in control templates. Data were analysed using the iQ5 optical system software version 2.0 (Bio-Rad) including Genex Macro iQ5 Conversion and Genex Macro iQ5 files.

Data analysis

Biometric, biochemical and molecular data are expressed as mean ± standard deviation (SD). Significance was determined by one-way ANOVA with Tukey's multiple comparisons test using the statistical software package SIGMA STAT 3.1 (Systat Software, Chicago, IL, USA). A P value <0.05 was regarded as statistically significant.

Results

Morphometric data

Dry weight

From 1 to 48 dph, mean dry weight increased significantly from 0.045 ± 0.003 to 4.00 ± 0.26 mg. From 1 to 28 dph values rose, but differences among sampling points were not significant (Fig. 1). However, a significantly stronger and faster weight increase ($P < 0.05$) was noted from 38 dph, during the *Artemia* feeding stage (Fig. 1).

Daily weight increase (% DWI)

Variations in % DWI were analysed in the intervals between feed changes (Fig. 2). The first significant difference ($P < 0.05$) was found between 10 and 18 dph (rotifer feeding stage); % DWI then remained almost constant until 18–28 dph. Another significant increase was detected from 28 to 38 dph (*Artemia* phase) and was followed by a significant reduction from 38 to 48 dph (early weaning feed).

Standard length

Standard length was measured 1, 10, 18, 28, 38 and 48 dph. From 1 to 48 dph mean SL increased from 4.42 ± 0.09 to 13.45 ± 1.00 mm (Fig. 3). As in the case of % DWI, SL increased significantly between 38 and 48 dph. Differences between 1 and 28 dph were not significant.

Table 2 List of primers used in this study

Gene	Primer for	Primer rew
ubq	GGCCAGCTGTCTGAGAGAAG	GTCAAGGCCAAGATCCAAGA
rpl37	CCTCCGTGTTTCACTGGGCAGAC	ACCCAGACGTGCTGCAGTGG
igf1	AGCCCAGAGACCCTGTGC	CAGCTCACAGCTTTGGAAGCA
igf2	AATACGAGGTGTGGCAGAGG	TTGATCTTCTCCGCCTGTCT
mstn	GGCCTGGACTGTGATGAGAA	GCATGTTGATGGGTGACATC
hsp70	GTACGGTCTGGACAAAGGCA	GGTTCTCTTGGCCCTCTCAC

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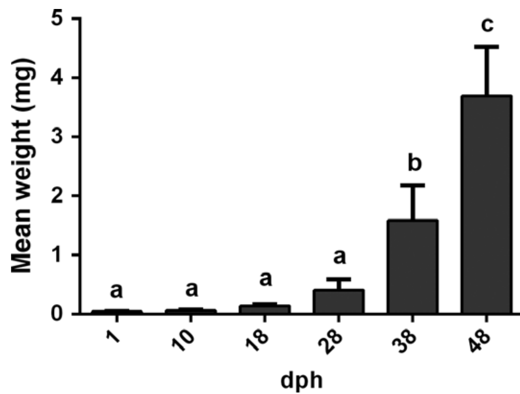

Figure 1 Mean dry weight (mg) of ballan wrasse larvae sampled 1, 10, 18, 28, 38 and 48 dph. Error bars indicate mean ± SD; (a–c) indicate significant differences among sampling points ($P < 0.05$) as analysed by one-way ANOVA followed by Tukey's multiple comparison test.

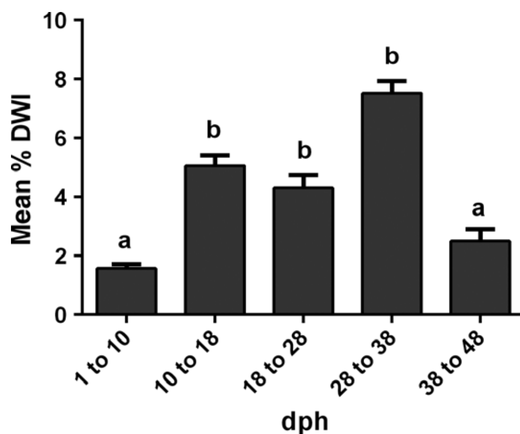

Figure 2 Mean per cent dry weight increase (% DWI) of ballan wrasse larvae sampled at 1, 10, 18, 28, 38 and 48 dph. Error bars indicate mean ± SD; (a–c) indicate significant differences among sampling points as analysed by one-way ANOVA followed by Tukey's multi-ple comparison test.

Histology

One dph the liver parenchyma (L) was normal without signs of steatosis (Fig. 4a–l), and no villi were detected on the intestinal mucosa (I) (Fig. 4a–i). At this time the yolk sac was still visible (YS in the inset). Ten dph the liver parenchyma (L) showed mild steatosis, the intestinal tract (I) was differentiated and the mucosa was organized into villi and microvilli (Fig. 4b). Eighteen dph the liver parenchyma was normal

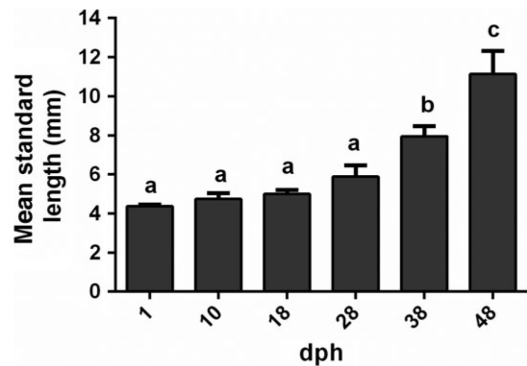

Figure 3 Mean standard length (mm) of ballan wrasse larvae sampled 1, 10, 18, 28, 38 and 48 dph. Error bars indicate mean ± SD; (a–c) indicate significant differences among sampling points as analysed by one-way ANOVA followed by Tukey's multiple comparison test.

without steatosis (Fig. 4c), the intestinal tract was quite well differentiated and the mucosa showed organized villi (Fig. 4d); the microvilli developed a brush border on the surface of the columnar cells of the mucosal epithelium; no goblet cells were detected. Twenty-eight dph the liver parenchyma showed moderate steatosis (Fig. 4e), and the intestine exhibited long villi covered with a thick brush border (Fig. 4f). Severe steatosis was detected 38 dph (Fig. 4g), but the intestine showed a similar appearance compared with the previous sampling point (data not shown). Finally, an increased number of goblet cells was detected 48 dph (Fig. 4h); the liver parenchyma exhibited no signs of steatosis (inset in H).

Fatty acid content

Based on HRGC data, the FA content of live preys and larvae expressed as relative per cent area of total FA, is reported in Table 3.

The relative EPA per cent content in rotifers and *A. salina*, both enriched with Multigain, ranged from 4.48 ± 0.03 to 4.36 ± 0.08 respectively; DHA content was 24.37 ± 0.01 in rotifers and 10.95 ± 0.11 in *A. salina*. Arachidonic acid (AA) and total lipids/mg prey were 1.88 ± 0.01 and 81.55 ± 2.38 in rotifers and 2.51 ± 0.02 and 129.7 ± 1.56 in *A. salina* nauplii (Table 3).

The present findings demonstrate that both development stage and diet affect FA composition and content in ballan wrasse larvae. In particular,

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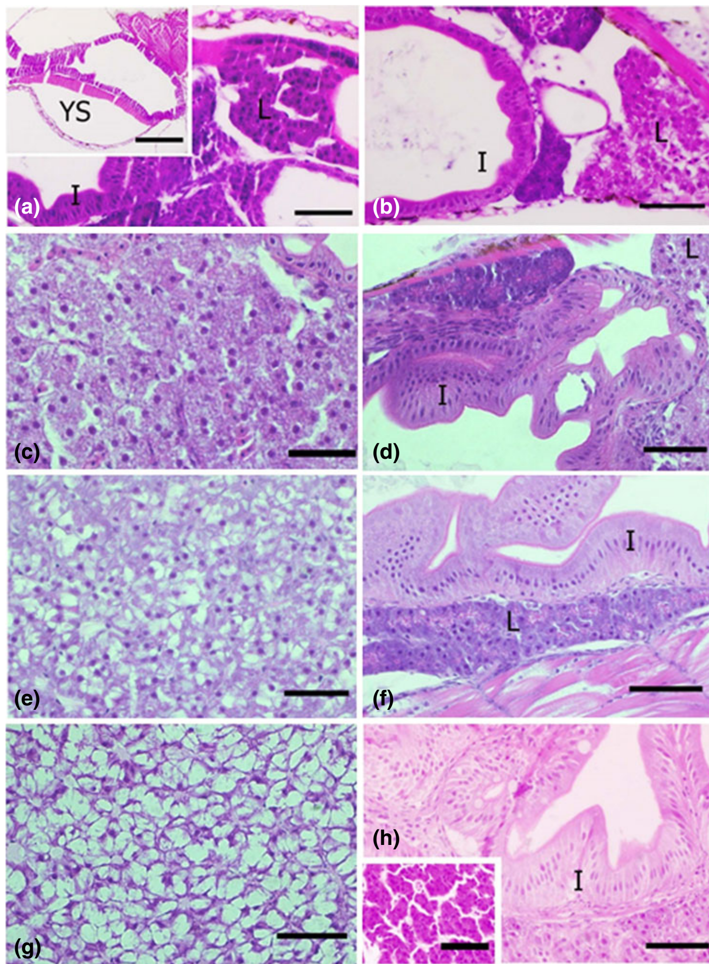

Figure 4 (a–h) Histological sections of ballan wrasse larvae sampled from 1 to 48 dph. Evaluation of liver (L) steatosis and intestine (I) morphology.

Table 3 Lipid content in live preys. EPA, DHA, AA content and DHA/EPA, AA/EPA ratio and total lipids/mg prey in rotifers and *Artemia salina* nauplii. Values are mean ± SD

Fatty acid	Rotifers	*A. salina* nauplii
EPA (C20:5n3)	4.48 ± 0.03	4.36 ± 0.08
DHA (C22:6n3)	24.37 ± 0.01	10.95 ± 0.11
AA (C20:4n6)	1.88 ± 0.01	2.17 ± 0.03
DHA/EPA	5.43 ± 0.03	2.51 ± 0.02
AA/EPA	0.42 ± 0.02	0.52 ± 0.01
Total lipids (mg mg^{-1} prey)	81.55 ± 2.38	129.71 ± 1.56

EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

total lipid content per mg decreased from 1 to 18 dph (shift from endogenous to exogenous feeding), it increased during the *Artemia* feeding stage (28–38 dph), and declined again during the weaning stage (Table 4). The DHA/EPA ratio at 1 dph was 2.42 ± 0.02, it then increased during the rotifer feeding stage (4.24 ± 0.22 and 5.39 ± 0.11, respectively) and fell during the *Artemia* and weaning phases (2.24 ± 0.08, 2.30 ± 0.15, 3.51 ± 0.21 respectively). Finally, AA/EPA increased from 1 to 48 dph from 0.25 ± 0.03 to 0.99 ± 0.12 (Table 4).

Cortisol content

Cortisol showed a general rising trend from 1 to 48 dph, from 3.97 ± 1.21 to 16.24 ± 1.80 pg mg^{-1} larvae (Fig. 5). There were no significant differences between 1 and 10 dph (3.97 ± 1.22 and 6.00 ± 2.30 pg mg^{-1} respectively), whereas levels were significantly higher 18, 28, 38 and 48 dph compared with 1 and 10 dph (11.10 ± 2.11, 13.63 ± 2.00, 13.42 ± 1.13 and 16.21 ± 1.84 pg mg^{-1} respectively) (Fig. 5).

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Molecular data

Larval gene expression was evaluated by real-time PCR 1, 10, 18, 28, 38 and 48 dph.

As regards the growth factors, *igf1* transcript was expressed only during early larval development, from 1 to 28 dph, and no signal was detected between 38 and 48 dph (Fig. 6a). In contrast, *igf2* and *mstn* were predominantly expressed at the end of the larval stage, 38 and 48 dph (Fig. 6b,c).

With reference to the stress response, *hsp70* showed a similar trend to *mstn*. In particular, transcript levels were very low in the early larval stages (1–28 dph) and much higher ($P > 0.05$) on 38 and 48 dph (Fig. 7).

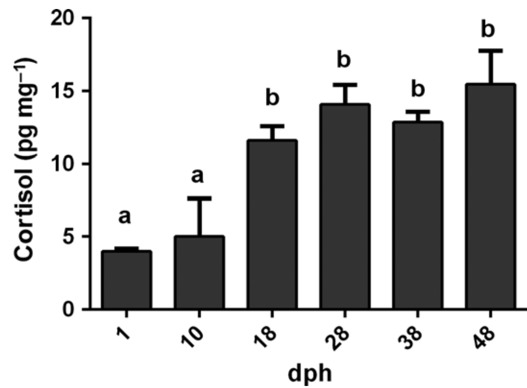

Figure 5 Cortisol content in ballan wrasse larvae sampled 1, 10, 18, 28, 38 and 48 dph. Error bars indicate mean ± SD; (a–c) indicate significant differences among sampling points analysed with one-way ANOVA followed by Tukey's multiple comparison test.

Discussion

The larval stage is a critical period in the fish life cycle, involving several biological, physiological and molecular changes (Pérez-Domínguez & Holt 2006). Larval growth and larval stage duration are affected by feeding and environmental conditions (Pavlidis, Karantzali, Fanouraki, Barsakis, Kollias & Papandroulakis 2011; Piccinetti *et al.* 2012). As a consequence captive rearing can have a profound impact on the number of individuals that successfully reach the juvenile stage (Sargent *et al.* 1999; Jenkins & King 2006). Size advantages and the development of new anatomical features are essential for access to limited resources and/or to evade and escape predators (Leggett & Deblois 1994; Olivotto *et al.* 2011).

Dietary and environmental inputs can modulate a number of physiological and biochemical events via gene-associated processes (Deng, Zhang, Lin & Cheng 2004; Pedroso, de Jesus-Ayson, Cortado & Ayson 2006). As in all vertebrates, development and growth in fish are governed by the neuroendocrine system through specific hormones, resulting in integration of environmental, physiological and genetic information (Moriyama, Ayson & Kawauchi 2000). In fish and mammals, the endocrine control of growth is affected by the interplay of positive signals, including the GH–IGF axis, and negative signals such as MSTN (McPherron, Lawler & Lee 1997; Wood, Duan & Bern 2005). In our larvae *igf1* was detected by real-time PCR already 1 dph; mRNA levels rose until 28 dph and became undetectable from 38 dph. These data agree with studies highlighting the important role of IGF-1 in postnatal growth modulation and as a GH mediator in fish (Moriyama *et al.* 2000; Wood *et al.* 2005; Patruno *et al.* 2008).

In contrast, *igf2* transcripts remained low and stable until 28 dph, they were much higher 38 dph, and declined again 48 dph. A similar trend was found for *mstn*, in line with several studies of teleost species (Funes, Asensio, Ponce,

Table 4 Lipid content in ballan wrasse larvae sampled 1, 10, 18, 28, 38 and 48 dph. EPA, DHA, AA content, DHA/EPA, AA/EPA ratios and total lipids/mg larvae. Values are mean ± SD

Fatty acid	1 dph	10 dph	18 dph	28 dph	38 dph	48 dph
EPA (C20:5n3)	13.18 ± 0.43	6.97 ± 0.32	5.09 ± 0.31	4.28 ± 0.02	7.47 ± 0.11	6.44 ± 0.22
DHA (C22:6n3)	31.88 ± 0.93	29.57 ± 0.41	27.49 ± 0.83	9.62 ± 0.59	17.23 ± 0.31	22.59 ± 0.41
AA (C20:4n6)	3.35 ± 0.11	3.85 ± 0.03	4.06 ± 0.23	2.99 ± 0.11	5.31 ± 0.04	6.4 ± 0.13
DHA/EPA	2.42 ± 0.02	4.24 ± 0.22	5.39 ± 0.11	2.24 ± 0.08	2.30 ± 0.15	3.51 ± 0.21
AA/EPA	0.25 ± 0.03	0.55 ± 0.01	0.79 ± 0.03	0.69 ± 0.03	0.71 ± 0.03	0.99 ± 0.12
Total lipids (mg mg^{-1} larvae)	82.01 ± 1.92	69.7 ± 7.21	67.6 ± 5.22	92 ± 12.03	119.2 ± 5.43	76.09 ± 4.89

EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

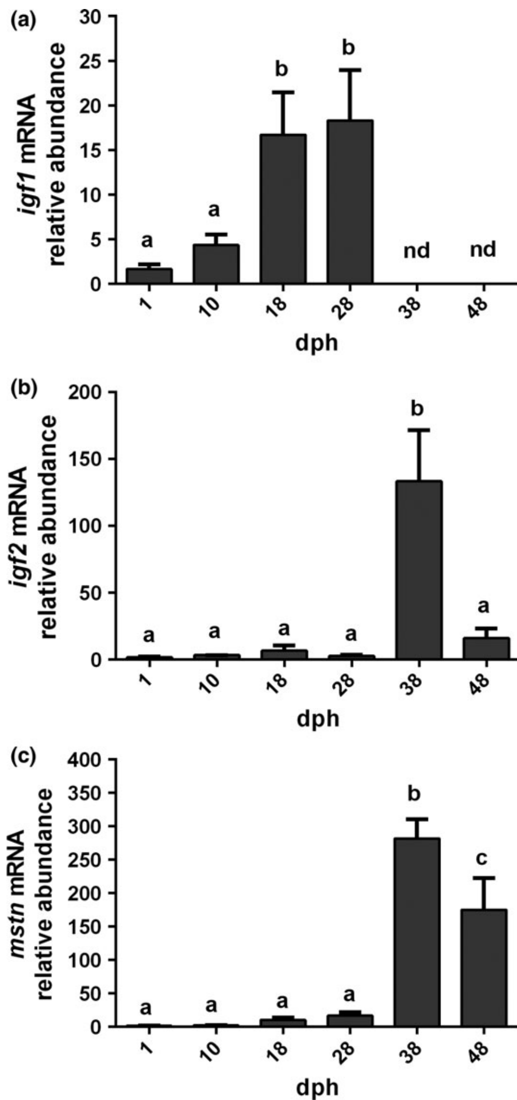

Figure 6 (a–c) Relative changes in mRNA transcript level of growth signals. *igf1*, *igf2* and *mstn* mRNA levels normalized against *ubq* and *rpl37* in ballan wrasse larvae sampled 1, 10, 18, 28, 38 and 48 dph. Error bars indicate mean ± SD; (a–c) indicate significant differences among sampling points analysed with one-way ANOVA followed by Tukey's multiple comparison test.

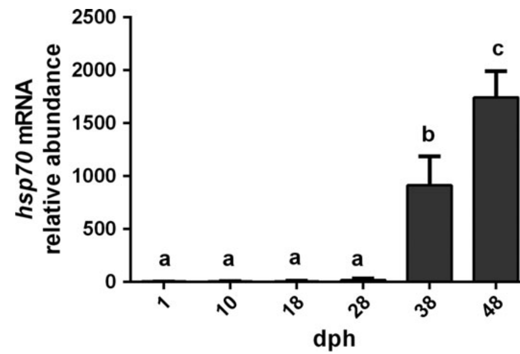

Fig. 7 Relative changes in mRNA transcript level of *hsp70* (stress marker). *hsp70* mRNA levels normalized against *ubq* and *rpl37* in ballan wrasse larvae sampled 1, 10, 18, 28, 38 and 48 dph. Error bars indicate mean ± SD; (a–c) indicate significant differences among sampling points as analysed by one-way ANOVA followed by Tukey's multiple comparison test.

Infante, Cañavate & Manchado 2006; Radaelli, Poltronieri, Bertotto, Funkenstein & Simontacchi 2008; Bertotto *et al.* 2011). The present findings indicate that in this species IGF-1 has a key role during early larval development and IGF-2 has a major role in late development, possibly as a local paracrine/autocrine tissue growth regulator. This result can also be related to a change in feeding: *igf1* was more abundant during the rotifer phase, while *igf2* during the *Artemia* one.

The molecular data match the biometric and histological findings, which document a steep increase in % DWI, especially from 28 to 38 dph. At this time, total lipids which are a key energy source (Olivotto *et al.* 2011), increased significantly in relation to the change from endogenous to exogenous feeding, the change in prey from rotifers to *A. salina* nauplii (which are richer in FA), and digestive tract development. In fact, starting on 28 dph histological analysis disclosed longer intestinal villi with an increased surface area, suggesting enhanced nutrient adsorption. At the same stage scattered goblet cells, which line the intestine with mucus and are involved in digestive enzyme neutralization, were detected in the intestinal epithelium. These data agree with the significant increase in most digestive enzymes reported in ballan wrasse larvae, particularly between 27 and 34 dph (Hansen *et al.* 2013).

Corticosteroids also play key functions during early vertebrate development. Several studies have documented the importance of cortisol in regulating teleost survival, growth and early development (Eriksen, Bakken, Espmark, Braastad & Salte 2006; Bertotto *et al.* 2011). The role of cortisol and glucocorticoid receptor in the stress response of adult fish has extensively been investigated (Wendelaar Bonga 1997; Rotllant, Ruane, Caballero, Montero & Tort 2003). However, knowledge of the HPI axis and its response

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to stressors during early ontogeny is limited. Studies conducted in other species have shown that *de novo* cortisol synthesis begins shortly after hatching, but a significant elevation in whole body cortisol in response to a stressor becomes obvious only days to weeks later, depending on the species (Barry *et al.* 1995; Deane & Woo 2003; Szisch *et al.* 2005). Various FA and phospholipids confer stress resistance in fish (Koven 2003; Lund & Steenfeldt 2011), although the mechanisms involved are still unclear. The modulation of cell membrane structure and/or function via diet-induced changes in phosphoacylglycerol levels probably exerts major effects on fish physiology including a stress-reducing action (Pavlidis *et al.* 2011; Piccinetti *et al.*, 2015). The consequences of dietary supplementation with certain HUFA on fish stress tolerance seem to be mediated by eicosanoids, which affect corticosteroid production (Ganga, Tort, Acerete, Montero & Izquierdo 2006; Ganga, Bell, Montero, Atalah, Vraskou, Tort, Fernandez & Izquierdo 2011). In fact, FA and eicosanoids serve as ligands for nuclear receptors that may affect the transcription of genes involved in lipid and energy homoeostasis, including cholesterol metabolism, which is central in steroidogenesis.

Three main HUFA, DHA (22:6n-3), EPA (20:5n-3) and AA (20:4n-6) (Sargent, McEvoy & Bell 1997), are required for normal growth and development of fish and all the vertebrates studied to date. They share broadly similar biochemical, cellular and physiological functions, including participation in cell membrane structural and functional integrity and a role as eicosanoid precursors. The major eicosanoid precursor in fish and mammals is AA, whereas the eicosanoids formed from EPA are less biologically active; moreover, EPA competitively inhibits eicosanoid formation from AA. As a consequence, the AA/EPA ratio is involved in the actions of eicosanoids in the organism (Sargent *et al.* 1997). High AA/EPA tissue ratios result in enhanced eicosanoid biological actions and cortisol synthesis (Koakoski, Oliveira, da Rosa, Fagundes, Kreutz & Barcellos 2012). In the present study the AA/EPA ratio increased from 0.25 ± 0.0 to 0.99 ± 0.1 in growing larvae; basal cortisol content matched the AA/EPA ratio and was similar to that reported in the gilthead sea bream, *S. aurata*, and other teleosts (Hwang, Wu, Lin & Wu 1992; Barry *et al.* 1995; Deane & Woo 2003). Cortisol content and AA/EPA ratio were both very low at hatching (1 dph) and increased slightly at first feeding (10 dph), indicating that in *L. bergylta* cortisol production begins near the shift from endogenous to exogenous feeding. Such poor cortisol synthesis in the early larval stages may protect larvae from the high metabolic demand involved by stress response at a time when the shift from endogenous to exogenous feeding is associated with limited energy stores. Whole body cortisol and the AA/EPA ratio increased after the initiation of exogenous feeding, and continued to rise at flexion and at all later stages, indicating that upon completion of notochord flexion *L. bergylta* larvae have a functional HPI.

As regards HSP, their ontogeny in post-hatching larval development remains to be established for several fish species, including ballan wrasse. Our real-time PCR data demonstrate that *hsp70* mRNA was unchanged from 1 to 28 dph and then rose as development advanced. These data are in line with previous studies of sea bream, sea bass and fruit mosquito (Deane & Woo 2003; Bertotto *et al.* 2011), which have hypothesized a physiological role for constant *hsp70* levels in promoting larval growth during early development.

Conclusion

This study found differences in gene expression of growth factors *igf1*, *igf2* and *mstn* at different stages of larval development. The low cortisol levels detected at the earliest larval stages reflect a poor stress-coping ability, a feature that may actually protect larvae from the elevated metabolic demands involved by stress responses, promoting faster growth and survival. These findings have concrete implications for commercial ballan wrasse farming and can be a starting point for future research evaluating the effects of different diets and environmental conditions during larval rearing.

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