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Live prey enrichment, with particular emphasis on HUFAs, as limiting factor in false percula clownfish (*Amphiprion ocellaris,* Pomacentridae) larval development and metamorphosis: Molecular and biochemical implications

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ABSTRACT

In fast growing organisms, like fish larvae, fatty acids provided through live prey are essential to satisfy high energy demand and are required to promote growth. Therefore, in recent decades, a great amount of research has been directed towards the development of lipid enrichment in order to improve larval fish survival and growth. However, in fish, the biochemical and molecular processes related to highly unsaturated fatty acid (HUFA) administration are still poorly understood. In the current study, the false percula clownfish, a short larval phase marine species, was used as an experimental model and the effects of a standard and a HUFAs-enriched diet were tested through a molecular, biochemical, ultrastructural and morphometric approach. Our results support the hypothesis that HUFA administration may improve larval development through the presence of better structured mitochondria, a higher synthesis of energy compounds and coenzymes with a central position in the metabolism, with respect to controls. This higher energy status was confirmed by better growth performance and a shorter larval phase in larvae fed with an enriched diet with respect to the control. This strategy of rapid growth and early energy storage may be considered positively adaptive and beneficial to the survival of this species.

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1. Introduction

In fish larvae, fatty acids provided through live prey are essential to satisfy the high energy demand (Tocher et al., 2003) and are requested to promote their growth. This high energy status is usually characterized by the synthesis of a proper amount of energy compounds (ATP) and coenzymes with a central position in metabolism such as NAD/NADH, NADP/NADPH and acetyl-CoA (Sargent et al., 1999; Menghe et al., 2004). At this regard, a central role in ATP synthesis is played by mitochondria. Recently, it has been demonstrated that dietary lipids are able to influence the fatty acid composition of phospholipids from mitochondria as well as changing their respiratory capacities and thus influencing the animal's energy status (Guderley et al., 2008). For this reason understanding the effects of dietary lipids on fish energy status and mitochondria morphology may be of great interest.

Growth, and especially protein synthesis, is highly energy-demanding (Brafield and Llewellyn, 1982), and young fish larvae can exhibit very high growth rates relative to those of adult fish (Pérez-Domínguez and Holt, 2006). In fact, during the larval period, fish undergo complex morphological changes and remarkable size progression that takes them from larvae to competent juveniles in a very short time (Pérez-Domínguez and Holt, 2006). Since growth and ontogeny, and the addition of new/improved physiological competence follow a well-defined and genetically programmed sequence in which gene transcription and hormone regulation play a crucial role, clinical parameters and zootechnical indexes may be not sufficient to monitor fish growth and development. Therefore, besides the traditional markers (biochemical, histological, morphological and physiological), it may be important to look for alternative parameters such as molecular biomarkers. Consequently, gene expression can be used to generate useful insights linking biotic and abiotic conditions and individual's performance. The search for molecular markers can be approached looking for them among those genes whose expression could reasonably be modified by the different conditions, including

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nutrition (Olivotto et al., 2009). Among those genes, several studies showed that insulin like growth factors (IGFs) and myostatin (MSTN) are considered growth markers (Perrot et al., 1999; Patruno et al., 2006; Duguay et al., 1996; Avella et al., 2010; Olivotto et al., 2010); peroxisome proliferator activated receptors (PPARs) lipid sensing factors (Avella et al., 2007) as well as factors involved in ontogenesis, adipogenesis, lipid homeostasis, lipid metabolism regulation, lipid transport, lipid and glucose oxidation, peroxisomal biogenesis, immune functions, cell proliferation and epithelial cell differentiation (Burdick et al., 2006) and thyroid receptor (TR) metamorphosis indicators directly modulated by thyroid hormones concentrations (Kawakami et al., 2006).

It is well established that lipid nutrition is particularly important for marine fish (Bell et al., 2003; Copeman et al., 2002; Olivotto et al., 2006) since deficiencies in these molecules can cause a general decrease of larval health, poor growth, low feed efficiency, anemia and high mortality (Sargent et al., 1999; Bell et al., 2003; Olivotto et al., 2003, 2005, 2006; Faulk and Holt, 2005; Vagner and Santigosa, in press). The dietary requirements of HUFAs from the ω-3 family for marine fish larvae has long been known (Sargent et al., 1997, 1999; Olivotto et al., 2005) and in recent decades, a great amount of research has been directed towards the development of food enrichments (with particular emphasis on lipids) in order to improve larval fish survival and growth (Sargent et al., 1997, 1999). For these reasons conventional fresh water laboratory fish models such as *Danio rerio* and *Oryzias latipes* cannot be considered valid candidates. Among marine fish models, clownfishes may be considered good candidates since they are protandrous hermaphrodites (Kobayashi et al., 2010), they regularly spawn in laboratory conditions (Avella et al., 2007), larvae can be easily fed on conventional zooplankton (Olivotto et al., 2010), they are unable to synthesize HUFAs *de novo* and finally, their genome is partially available in Genbank (Olivotto et al., 2009, 2010; Avella et al., 2010; Kim et al., 2010; Kobayashi et al., 2010). The above-mentioned virtues warrant the possible utilization of clownfishes as a new laboratory model to better understand important biological processes during marine fish larval development.

The regulation of gene expression by dietary fat is extensively studied in mammals, as it has wide implications in human health (Cahu et al, 2003; Ibabe et al., 2004) but this regulation still needs to be deeply investigated in other experimental models including developing fish. HUFAs are able to act directly on the genome, via specific nuclear receptors, the PPARs (PPAR-α, -β, and γ) and for this reason they are considered lipid sensing factors primarily known for modulating energy metabolism, lipid storage and transport (Andersen et al., 2000; Ibabe et al., 2004; Batista-Pinto et al., 2009; Grimaldi, 2007; Nunn et al., 2007). Moreover, since IGFs are one of the PPAR target genes (Villeneuve et al., 2004) and fatty acids are able to modulate PPAR transcriptions, fatty acids provided through food intake are able to positively modulate fish growth (Avella et al., 2007; Kruger et al., 2010).

In fish, circulating IGF levels seem to play an important role in growth regulation and has also been shown to be nutritionally regulated (Duan and Hirano, 1992; Duan and Plisetskaya, 1993; Niu et al., 1993; Moriyama et al., 1994; Perez-Sanchez et al., 1995; Silverstein et al., 1998). For example, an increase in larval growth related to an up-regulation of IGF gene expression has recently been observed in clownfish larvae fed a diet rich in HUFAs (Olivotto et al., 2008). On the contrary, during negative energy balance status or when nutritionally inadequate live prey is provided, IGFI and /or IGF II decrease (Näntö-Salonen, 1993; Avella et al., 2007; Olivotto et al., 2008; du Dot et al., 2009).

Moreover, the release of hormones from the neuroendocrine system, which integrates environmental, physiological, and genetic information, plays a key role in fish growth and development. As a consequence, the release of these hormones is strictly related to an improvement/ reduction/cessation of growth (Reinecke et al., 2005; Canosa et al., 2007). The main (but not exclusive) hormones involved are the somatotropic and the thyroid ones. Hormones from the somatotropic axis include the Growth hormone (GH) which is a pluripotent hormone produced by the pituitary gland in teleost as in other vertebrates. Over the last two decades, many aspects of GH physiology have been the subject of intense research in fish such as the salmonids, cyprinids, and sparids (Björnsson, 1997). In fish, GH participates in almost all major physiological processes in the body including the regulation of ionic and osmotic balance, lipid, protein, and carbohydrate metabolism, skeletal and soft tissue growth, reproduction and immune function (Björnsson, 1997). Recent studies have indicated that GH affects several aspects of behavior, including appetite, foraging behavior, aggression, and predator avoidance, which in turn has ecological consequences (Björnsson, 1997; Pérez-Sánchez, 2000). Despite the vast body of knowledge which exists documenting GH action in teleost fish, the mode of GH action remains a major discussion topic. Generalized claims have been made that most/all GH effects are indirect, based on an outdated mammalian view where the pituitary/hepatic GH/IGF system was seen as an “axis” with IGFs mediating the physiological action of GH (Canosa et al., 2007).

In fact, in many fish species, blood levels of IGFs or tissue levels of its mRNA positively correlate with dietary ration, dietary protein content, and body growth rate (Duan and Plisetskaya, 1993; Perez-Sanchez et al., 1995; Beckman et al., 2004;). IGF-I increases in blood during the growing season in temperate fish showing seasonal growth (Mingarro et al., 2002), and is stimulated by increased temperature (Beckman et al., 1998) and day length (McCormick et al., 1992). Furthermore, treatment of fish with IGF-I implants stimulates growth (McCormick et al., 1992). IGFs in fish have been associated not only with growth, but also with metabolism (Castillo et al., 2004), development (Pozios et al., 2001) and reproduction (Weber and Sullivan, 2000).

On the contrary, myostatin (MSTN), a member of the transforming growth factor β (TGF- β) family, is considered as a negative factor that inhibits myoblast proliferation (McPherron et al, 1997; Rebhan and Funkenstein, 2008). MSTN expression in mammals is restricted predominantly to skeletal muscle (McPherron et al., 1997), lower level in adipose tissue (Gonzalez-Cadavid et al., 1998), mammary gland (Ji et al., 1998), and cardiac muscle. In contrast, a wider tissue expression has been described in different fish species. These tissues include muscle, intestine, brain, kidney, gills, heart, eyes, spleen, liver, ovaries, and testis (Roberts and Goetz, 2001; Kocabas et al., 2002; Ko et al., 2006; Helterline et al., 2007). This ample expression pattern in tissues other than muscle suggests that MSTN may have a wider function in fish. As a consequence, in fish, final growth is related to the interplay of these positive and negative signals.

In addition, metabolism regulation, growth and metamorphosis are deeply influenced by thyroid hormones [thyroxine or tetraiodothyronine (T4) and triiodothyronine (T3)] and this has been demonstrated in several studies (Eales and Sinclair, 1974; Peter and Marchant, 1995; Kelly, 2000; Pérez-Domínguez and Holt, 2006; Blanton and Specker, 2007). Their main molecular mechanism is the binding and activation of thyroid hormones to specific nuclear receptors, the TRs. The activation of TRs results in up- and down-regulation of several genes, e.g. the production of both thyroid hormones and its nuclear receptors is increased by the activation of the TRs. This autoinduction ensures a strong overall effect of the thyroid hormones (Shi et al., 1996; Gilbert, 2000). Early acting response genes induce the appearance of adult type cells, while late-acting responses induce the apoptosis or change of larval cells (Berry et al., 1998; Soffientino and Specker, 2003). The correct development of the hypothalamus and the pituitary is required for the axis' functionality and long-chain polyunsaturated fatty acids have been suggested to play an important role in growth related events in the central nervous system (CNS) by modulating the signal transduction

pathways. In fact, lack of polyunsaturated fatty acids during the sunrise dottyback (*Pseudochromis flavivertex*) larval phase results in an inadequate development of the CNS and hence, the hypothalamus–pituitary thyroid axis is not fully operational. Consequently, the developing larvae may not receive the correct signals through the increase of thyroid hormone production and increase time to metamorphosis (Olivotto et al., 2005).

Moreover, hypothyroidism, may have a deep impact on somatic growth since it is usually associated with low IGFs and IGF binding protein levels, with these changes being completely corrected by thyroid hormone replacement (Näntö-Salonen, 1993; Ramos et al., 2001) suggesting a direct effect of thyroid hormones on the hepatic secretion of IGFs. Thyroid hormones have been demonstrated to modulate the behavior of many metabolic pathways potentially relevant for the basal metabolic rate including uncoupling of cellular metabolism from ATP synthesis, or changes in the efficiency of metabolic processes downstream from the mitochondria.

False percula clownfish larvae, with their specific nutritional requirements, typical of marine fish larvae and with their short larval phase can thus be considered a valid model to study the effects of HUFA administration during marine fish larvae development and metamorphosis.

2. Materials and methods

2.1. Experimental model

The experimental model used in this study was the false percula clownfish (*Amphiprion ocellaris*, Pomacentridae). Clownfish pairs were kept in 200 L tanks and flower pots were supplied to the brood stock as a suitable substrate for egg laying. Photoperiod was maintained at 13 h light/11 h dark. Temperature was maintained at 28 °C, salinity at 30‰, pH at 8–8.5 and NH_3 and $NO_2^- < 0.02$ mg/L. Fish were fed twice a day with commercial frozen mysids and krill (ESCHEMATTEO, Italy). At these conditions, fishes spawned every 15 days and the embryo development was 168 h long. This study complies with the current laws about the use of animals for research purposes.

2.2. Hatching and larval rearing

On the hatching day, the flower pot with the egg clutch was transferred to a 20-L larval rearing tank presenting the same chemical–physical characteristics of the brood stock tank. A gentle air flow was provided to the egg clutch by an airstone to simulate the fanning activity of the male. The embryos were left in darkness for 50 ± 5 min and after this period hatching took place. During larval rearing, the water in the 20-L larval tank was gently replaced twice an hour by a dripping system (Olivotto et al., 2003, 2005) and the sides of the tank were covered with black panels to reduce light reflection, while the phytoplankton *Isochrysis galbana* was used (50,000 cells/mL) to condition the tank. Larvae were subjected to an extended photoperiod (24 L/0 D).

2.3. Experimental design

Larvae were divided in 2 experimental groups each of 300 ± 10 (5 tanks for each dietary group) fed on rotifers, *Brachionus plicatilis* (from day 1 to7 ph) and *Artemia salina* nauplii (AF 430, INVE Technologies, Belgium) (from day 7 to15 ph):

Group A: fed on not enriched live prey.

Group B: fed on HUFAs (Algamac 3000) enriched live prey.

Larval sampling was performed on day 6 ph (rotifer phase) and 11 ph (*A. salina* nauplii phase) at 8 a.m. before feeding the larvae. Live prey enrichment was performed with Algamac 3000 (Aquafauna Bio-Marine, Inc., Hawthorne, CA,USA) (0.5 g/million rotifers or 0.2 g/100000 *Artemia* nauplii). As recommended by the company enrichment lasted for 8–9 h.

2.4. Morphometric analysis

Ten larvae for each experimental group (five replicates each) on day 6 and 11 ph were sampled and anesthetized using MS222 (Sigma Aldrich) (1 mg/L) and total length (TL) and body mass (BW) were recorded using a Stemi 2000 micrometric microscope and microbalance OHAUS Explorer E11140 accurate to 0.1 mg. Every day, at 8 a.m. and 6 p.m., dead larvae were siphoned and counted to estimate survival rates. Between day 7–18 ph larvae were visually analyzed every day and when the orange banded pigmentation was evident the metamorphosis was considered completed.

2.5. Fatty acid analysis

Total lipids of enriched and not enriched rotifers and *A. salina* nauplii and larval fish samples (Group A and B) were extracted with chloroform/methanol (2:1 v/v) (Folch et al., 1957). Methanolic transesterification of lipids was carried out using 2 N methanolic KOH and hexane; the obtained FAME was analyzed by HRGC. A DANI 1000DPC gas-chromatograph (Norwalk, CT, USA), equipped with a split–splitless injector and with a flame ionization detector (FID), was used. The separation was obtained using a fused silica WCOT capillary column CP-Select CB for FAME (50 m × 0.25 mm i.d., 0.25 μm f.t.; Varian, Superchrom, Milan, Italy). The chromatograms were acquired and processed using Clarity integration software (DataApex Ltd., Prague, Czech Republic). The oven temperature was held at 180 °C for 6 min, then was programmed at 3 °C/min to 225 °C and held for 10 min, while the injector and the FID temperatures were set at 250 °C. Carrier gas (He) flow rate was 1 mL/min. FAs were identified by comparing the retention times of their methyl esters with standard FAME mixtures. Repeated injections of standard solutions were carried out to test the analytical precision. The relative standard deviations were less than 5% for all the FA, both considering the intradie precision, calculated on six repeated injections, and the interdie precision, evaluated over six days. All solvents and reagents were of analytical grade and were purchased from Carlo Erba Reagents (Milan, Italy). FAME standards were obtained from Supelco (Bellefonte, PA, USA). The use of an internal standard for the expression of fatty acid composition is recommended to reduce error propagation, in particular if one fatty acid is wrongly estimated, or omitted when unknown, the results for the other fatty acids are affected. Since we can exclude these occurrences, the fatty acid compositions have been expressed as weight percent (area normalization).

2.6. RNA extraction and cDNA synthesis

Total RNA extraction from whole larval body was optimized with the best recovery being obtained with 30 mg of larvae (obtained from a pool of about 15 larvae, depending on the developmental stage) using the Minikit RNAeasy® (Qiagen) extraction kit following the manufacturer's protocol. Total RNA extracted was eluted in 15 μL of RNAse-free water. Final RNA concentrations were determined by spectrophotometer and the RNA integrity was verified by ethidium bromide staining of 28S and 18S ribosomal RNA bands on a 1% agarose gel. RNA was stored at −80 °C until use. Total RNA, was treated with DNAse (10 UI at 37 °C for 10 min, MBI Fermentas). A total amount of 5 μg of RNA was used for cDNA synthesis employing 0.5 μg oligo d(T) + adapter primer, 5′-GACTGCAGTCGACATCGATTTTTTTTTTTTTTTTTT-3′, in a buffer containing 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM of each dNTP, 40 units RNAse OUT (Invitrogen) and 200 units of Superscript II RT (Invitrogen, Life

Table 1
Endogenous NAD(P) levels, NMNAT activity at 28 °C and EC values in false percula clownfish larvae fed different diets at day 6 and 11 ph. NAD levels are about 2 times higher in Group B larvae with respect to Group A during both samplings, whereas a significant ($p<0.05$) increase of NADP level, NMNAT activity and EC values are detectable only in day 6 ph group B larvae with respect to control. Statistical significance is indicated with *.

	6 days ph		11 days ph	
	Group A	Group B	Group A	Group B
NAD (pmol/μg proteins)	1.28 ± 0.31*	2.58 ± 0.33*	2.37 ± 0.80*	4.67 ± 0.90*
NADP (pmol/μg proteins)	0.12 ± 0.05*	0.45 ± 0.03*	0.40 ± 0.09	0.36 ± 0.06
NMNAT activity (pmol NAD/min/mg proteins)	56.54 ± 6.00*	96.38 ± 7.00*	76.62 ± 11.00	87.60 ± 15.00
EC	0.77 ± 0.03*	0.87 ± 0.02*	0.80 ± 0.02*	0.82 ± 0.06

technologies, Milan, Italy). Cycling conditions were: 70 °C for 5 min, 42 °C for 52 min and 72 °C for 15 min.

2.7. Primer design

Sequenced regions containing the aforementioned markers for several species closely related to clownfish as well as outgroup species were aligned and the consensus sequences were used for make primers for real time PCR.

In particular the following primers were used at final concentration of 10 pmol/μL:

β-ACT: for: 5′-TTCCTCGATATGGAGTCCT-3′; Rev: 5′- TGGGGCAATGATCTTGATCTT-3′.

IGF I: for: 5′-AGTGCGATGTGCTGTATC-3′; Rev: 5′- CAGCTCACAGCTTTGGAAGCA-3′.

IGF II: for: 5′- CGGCAGAAACGCTATGTGGA-3′; Rew: 5′ TGCTGGTTGGCCTACTGAAA -3′.

MSTN: for: 5′-TTTTGAGCAAACTGCGAATG-3′; Rev: 5′- CACGTCGTACTGGTCGAGAA-3′

PPARα: for:5′-TTCAGCGACATGATGGAGCC-3′; Rew: 5′-CAGTTTCTGCAGCAGATTGG-3′.

PPARβ: for: 5′- AGGAGATAGGGGTACACGTG-3′; Rew: 5′- CAGGAACTCCCGGGTCACAA-3′.

TRα: for:5′-GGAAACAGAAGCGCAAGTTC-3′; Rew: 5′-TCTTCACAAGGCAGCTCTGA-3′.

TRβ: for: 5′-TGCATGGAAGATCATGTCG-3′; Rew: 5′-AGGCCTGGCTTCTTAAGCTG-3′.

2.8. Real time PCR

Triplicate PCRs were carried out for each sample analyzed. After real-time condition optimization (primers annealing temperatures and cDNA dilutions) the PCRs were performed with the SYBR green method in a Chromo 4 Real-time system (MJ Research). The reactions were set up in a 96-well plate by mixing, for each sample, 1 μL of diluted (1/20) cDNA, 5 μL of 2× concentrated SYBR Green PCR Master Mix (Finnzymes) containing SYBR Green as fluorescent intercalating agent, 0.3 μM forward primer and 0.3 μM of reverse primer. The thermal profile for all reactions was 15 min 95 °C followed by 45 cycles of 20 s at 95 °C, 20 s at 55 °C and 20 s at 72 °C except for PPARα 195 and PPARβ. The thermal profile for PPARα was 15 min 95 °C followed by 45 cycles of 20 s at 95 °C, 20 s at 51 °C and 196 20 s at 72 °C while for PPARβ the thermal profile was 15 min 95 °C followed by 45 cycles of 20 s at 95 °C, 20 s at 49 °C and 20 s at 72 °C. Fluorescence monitoring occurred at the end of each cycle. Additional dissociation curve analysis was performed and showed, in all cases, one single peak. A relative quantification of cDNA was made using β-ACT as a reference gene (internal standard). The data obtained were analyzed using the iQ5 optical system software version 2.0 (Bio-Rad). Modification of gene expression is represented with respect to the control and real-time PCR efficiencies were determined for each gene from the given slopes in Bio-Rad software.

2.9. Thyroid hormone fluctuations during the false percula clownfish development

Thyroid hormones were extracted from total body clownfish larvae (up to approx. 0.37 g depending on the developmental stage, the top mass used in determining extraction efficiency). Samples were placed in Teflon tubes on ice. Larvae were extracted in 2 mL of 95% ethanol containing 1 mM propylthiouracil (PTU) with homogenisation (Janke and Kunkel Ultra Turrax T25), sonication (Sonics and Materials Vibra-Cell at 50% output, 20 s), and vortexing. A further 2 mL ethanol containing PTU was added to larval samples before vortexing. All samples were centrifuged (10 min, 9980 × *g*, 4 °C) and supernatants were decanted into clean Teflon tubes. A further 2 mL of 95% ethanol containing PTU was added to larval sample pellets. Tubes were vortexed and re-centrifuged (10 min, 9980 g, 4 °C). The pooled supernatants were evaporated to dryness under nitrogen and stored at −80 °C. All extracts were re-suspended in 0.25 mL barbital buffer containing 2.5 mg mL^{-1} ANS, 0.25 mL ethanol, and 1 mL chloroform. Tubes were vortexed to solubilise all material, then centrifuged (10 min, 1500 × *g*, 4 °C) to separate the top aqueous layer containing the thyroid hormones (for a detailed procedure see Crane et al., 2004).

2.10. Nicotinamide mononucleotide adenylyltransferase (NMNAT, EC 2.7.7.1) activity

Ten fresh anesthetized larvae, in five replicates each, were homogenized in 500 μL 1% PBS buffer on ice for about 30 s. Protein concentration was determined by Bradford method (Bradford, 1976). The standard reaction mixture contained 28 mM Tris/HCl pH 7.5, 20 mM $MgCl_2$, 1 mM DTT, 1 mM of both NMN (Nicotinamide Mononucleotide) and ATP substrates, 10 mM NaF and a larvae lysate volume corresponding to 40 μg of total proteins. Reaction mixtures (final volume 100 μL) were incubated in a water bath at 28 °C for 2 h. A time-zero blank reaction was also assessed. To stop reactions, 50 μL

Table 2
Fatty acid % content in not enriched and Algamac 3000 enriched rotifers and *Artemia salina* nauplii based on GC-FID data and expressed as relative area percentage on total fatty acids.The best fatty acid profiles are showed by Algamac 3000 enriched rotifers and *Artemia*. Statistical significance is indicated with *.

Fatty acid	Rotifers	Rotifers + Algamac 3000	*Artemia* nauplii	*Artemia* nauplii + Algamac 3000
20:4 (n-6)	1.3 ± 0.2*	2.0 ± 0.2*	2.4 ± 0.2	2.9 ± 0.2
20:5 (n-3)	0.3 ± 0.1*	5.2 ± 0.6*	8.1 ± 0.7*	9.9 ± 1.1
20:5 (n-6)	0.9 ± 0.2*	2.9 ± 0.5*	2.8 ± 0.2	3.4 ± 0.4
22:5 (n-3)	0.3 ± 0.2*	0.8 ± 0.2*	1.8 ± 0.2	2.2 ± 0.2
22:5 (n-3)	3.4 ± 0.3*	14.8 ± 1.2*	9.5 ± 0.9	11.2 ± 1.2

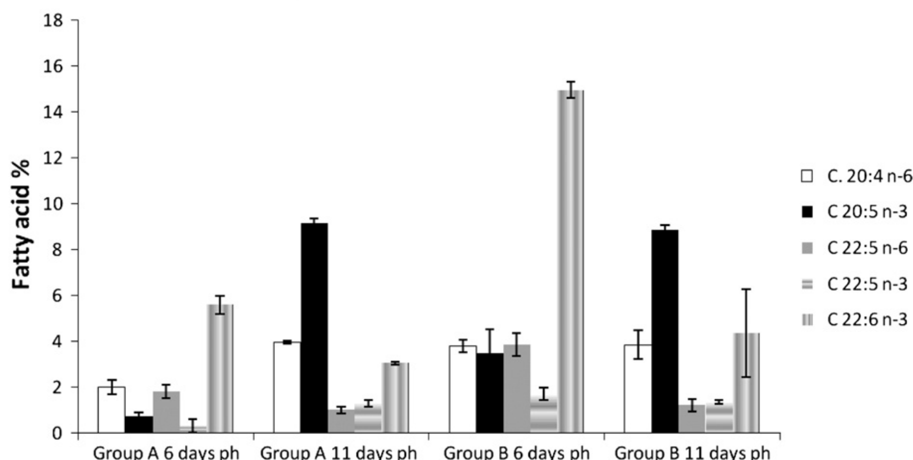

Fig. 1. Fatty acid % content in larvae fed different diets, based on GC-FID data and expressed as relative area percentage on total fatty acids. Analysis carried out on 6 days ph larval samples revealed that live prey enrichment with HUFAs can positively affect the lipid composition of the larvae. An increase of DHA, EPA, 20:4 n-6, 22:5 n-6 and 22:5 n-3% was evident in 6 days ph group B larvae respect to control. During the second sampling, performed 11 days ph (*Artemia salina* phase), no significant differences for all the analyzed fatty acids was observed in the two groups.

ice-cold 1.2 M $HClO_4$ were added to the single reaction mixtures. After 10 min on ice, the mixtures were centrifuged 2 min at 16000 × *g* and 140 μL aliquot of the perchloric acid supernatants were neutralized by the addition of 37.5 μL 0.8 M K_2CO_3. The neutralized reaction mixtures were frozen at −20 °C. The thawed samples were centrifuged 2 min at 16,000 × *g* and the supernatants were loaded on a 250 × 4.6 mm Supelcosil LC-18 DB 5 μm reversed-phase HPLC column (Supelco) using a gradient of 0.1 M KH_2PO_4 pH 6.0 (eluent A) and methanol (eluent B) (To improve the separation efficiency, the HPLC column was thermostatted at 18 °C) (Balducci et al., 2007). The NMNAT activity, expressed as picomoles of NAD produced per mg of total protein per minute, was determined from the differences between the areas of NAD peaks in the incubation mixtures and the zero-time blank. The enzymatic reaction was operating within its linear range as exhaustion of substrates was not observed and the NAD peak, after two hours incubation, was twice the one observed after a one hour incubation.

2.11. Endogenous nucleotide levels

Larvae lysates (100 μL) were acidified and neutralized by adding 50 μL ice-cold 1.2 M $HClO_4$ and 37.5 μL 0.8 M K_2CO_3, respectively, as indicated above. The HPLC conditions were the same as that utilized in the determination of the NMNAT activity. The ATP, ADP, AMP, NAD and NADP levels were analyzed. All the compounds were identified on the basis of their retention time, the spectrum profile at different wavelengths ranging from 200 to 400 nm and their coelution with standards. The EC was calculated as follows: EC = [ATP + ½ADP] / [ATP + ADP + AMP].

Table 3
Free T3 and T4 content in clownfish larvae fed not enriched (Group A) and enriched (Group B) diet. The T3 and T4 levels are low during the first sampling (6 days ph) but significant ($p<0.05$) differences in their abundance are evident between Group A and B at this stage with the highest content of both hormones in larvae fed enriched diet. At day 11 ph, T3 levels peak dramatically at metamorphic climax while, even if T4 peaks dramatically as well, there are no significant differences between the two groups. Statistical significance is indicated with *.

	6 days		11 days	
	Group A	Group B	Group A	Group B
T3 (pg/mg larvae)	9.2 ± 1.8*	13.8 ± 1.5*	41.9 ± 3.9*	59.8 ± 4.2*
T4 (pg/mg larvae)	18.2 ± 1.2*	23.6 ± 0.9*	42.6 ± 2.3	45.2 ± 1.9

2.12. Morphological investigations

Twenty larvae per sampling , in five replicates, were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 3 h at 4 °C, post-fixed in 1% osmium tetroxide in the same buffer, dehydrated in increasing ethanol concentrations and embedded in Araldite M (Fluka Chemie GmbH, Switzerland). Semi-thin sections were cut with a SorvallUltra Microtome MT 5000, stained with toluidine blue and observed with an Eclipse E600 microscope (Nikon). Ultra-thin sections, mounted on Cu/Rhogrids counterstained with aqueous uranyl acetate and lead citrate, were observed with a Philips CM10 TEM operating at 80 kV. Images were recorded by a TEM CCD camera (Veleta Soft Imaging System, Olympus Europa GmbH, Germany). In the present investigation striated muscle areas were selected. Specimens were cut along their longitudinal axes of muscle cells and transmission electron microscopy study was performed.

2.13. Statistical analysis

The real-time data were analyzed using the iQ5 optical system software version 2.0 (Bio-Rad). Modifications of gene expression are represented compared to a control sampled at the same time of the treatment, which is assumed to have the value of 1.

Results were expressed as the mean ± s.d. ($n = 5$). The significance of differences was determined using a one-way ANOVA, followed by Tukey's or Bonferroni's test for multigroup comparisons, with a statistical software package (SigmaStat 3.1; Systat software Inc.). A p value of <0.05 was regarded as statistically significant.

3. Results

3.1. NMNAT activity and endogenous nucleotide levels

NAD can be synthesized starting from NMN and ATP; this reaction is catalyzed by the enzyme NMNAT. The NMNAT activity levels at 28 °C showed a significant increase ($p<0.05$) in Group B with respect to control only at day 6 ph while on day 11 ph no significant differences ($p>0.05$) were observed (Table 1). However, endogenous NAD levels were about 2 times higher in Group B larvae fed HUFAs enriched live prey with respect to control (Group A) during both samplings (Table 1). Concerning NADP levels, a significant increase ($p<0.05$) was observed only in day 6 ph Group B larvae with respect to control, while 11 days ph no significant ($p>0.05$) differences were

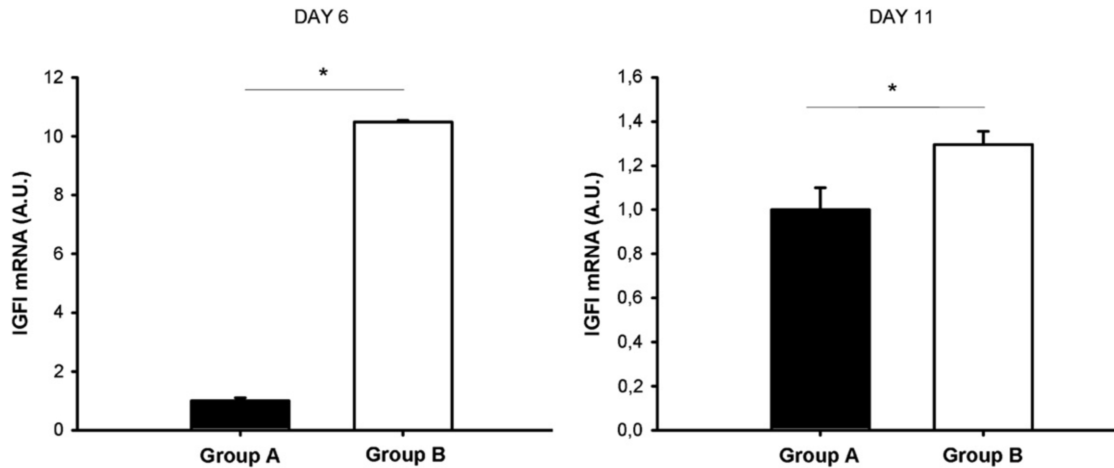

Fig. 2. A, B. IGF I gene expression in larvae fed different diets. On day 6 and 11 ph a significant ($p<0.05$) increase of IFG I transcript was observed in clownfish fed an enriched diet (group B) with respect to control. Statistical significance is indicated with *.

detected between the two groups (Table 1). Finally, concerning adenosine compounds, larval EC showed a significant increase only in 6 days ph Group B larvae with respect to control, while during the *A. salina* phase no significant differences ($p>0.05$) were observed among the two experimental groups (Table 1).

3.2. Lipid analysis

In Table 2 the fatty acid % contents of the different diets, based on GC-FID data and expressed as relative area percentage on total fatty acids, are reported. The best fatty acid profiles are showed by Algamac 3000 enriched rotifers and *Artemia*.

A similar result was observed in the fish larvae (Fig. 1). Analysis carried out on 6 day ph samples revealed that live prey enrichment with HUFAs can positively affect the lipid composition of the larvae. In fact, considering DHA and EPA % it is evident that, 6 day ph Group B larvae, showed a higher content of these two fatty acids with respect to control (14.95 ± 0.35 vs 5.6 ± 0.4 and 3.45 ± 1.06 vs 0.7 ± 0.2, respectively) (Fig. 1). In addition, at the same developmental stage, also 20:4 n-6, 22:5 n-6 and 22:5 n-3 always showed a higher content in Group B larvae fed enriched rotifers with respect to control group (Fig.1). During the second sampling, performed 11 days ph (*A. salina* phase), no significant differences for all the analyzed fatty acids was observed in the two groups (Fig. 1). However, a non significant increase ($p>0.05$) in DHA content was observed in Group B larvae with respect to control (4.35 ± 1.9 vs 3.05 ± 0.07, respectively) (Fig. 1).

3.3. Thyroid hormones analysis

T3 and T4 contents were determined in 6 and 11 days ph larvae and the results are shown in Table 3. The T3 and T4 levels are low during the first sampling (6 days ph) but significant ($p<0.05$) differences in their abundance are evident between Group A and B at this stage with the highest content of both hormones in larvae fed enriched diet. Subsequently, at day 11 ph, T3 levels peak dramatically at metamorphic climax, especially in Group B larvae while, even if T4 peaks dramatically as well, there are no significant differences between the two groups (Table 3).

3.4. Molecular analysis

Concerning molecular analysis, the expression of different genes was evaluated in this study and for each of them a partial nucleotide sequence was obtained and deposited in GeneBank (IGFI no. **EF620549**; IGFII no. **EF620550**; MSTN no. **EF620551**; PPARα no. **EF620552**; PPARß no. **EF620553,** TR α no. **EU676179;**TR ß **EU706337** and ß-ACT no. **EF620554**) (for details see Avella et al., 2010).Changes in transcript abundance for the various biomarkers supported the

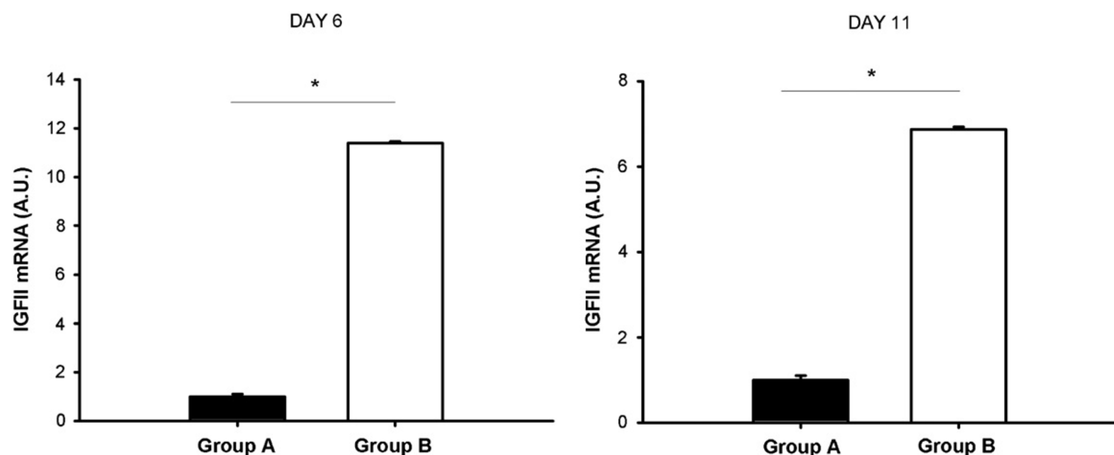

Fig. 3. A, B. IGF II gene expression. On day 6 and 11 ph a significant ($p<0.05$) increase of IGF II transcript was observed in clownfish fed an enriched diet (group B) with respect to control (group A). Statistical significance is indicated with *.

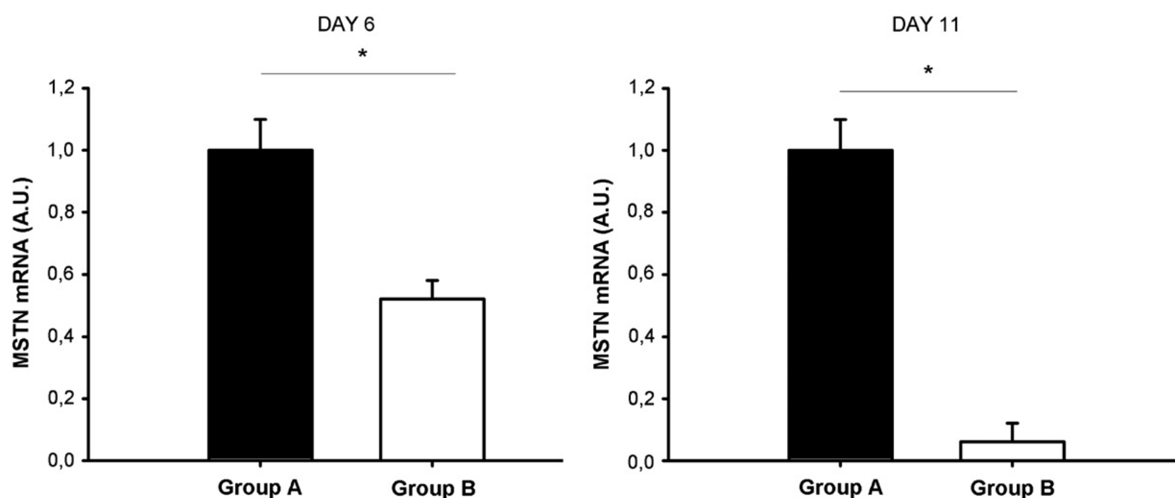

Fig. 4. A, B. MSTN gene expression. Myostatin gene expression was significantly ($p<0.05$) lower in group B larvae fed a HUFA enriched diet with respect to the control group (A) during both sampling times. Statistical significance is indicated with *.

changes in morphometric measurements. Concerning growth factors, on day 6 and 11 ph a significant increase of IGFI, IGFII gene expression was observed in clownfish fed an enriched diet (Group B) with respect to control (Fig. 2 A,B and Fig. 3 A,B). On the contrary, MSTN gene expression, was significantly lower in Group B with respect to the control group during both sampling times (Fig. 4 A, B). Concerning PPAR gene expression, on day 6 and 11 ph larvae fed enriched diet showed a significant increase for both subtypes α and β with respect to the control, as similarly observed for IGF gene expression (Fig. 5 A, B and Fig.6 A, B). Finally, also thyroid receptor ß (TR ß) was up-regulated in Group B with respect to control (Fig. 7 A, B). Surprisingly, on day 11 ph, the subtype α was not affected in Group B and no significant difference respect to control group was observed (Fig. 8 B) while on day 6 ph its gene expression was significantly higher in Group B larvae with respect to control (Fig. 8 A).

3.5. Morphometric results

An enriched diet was able to accelerate development in false percula clownfish larvae, since in Group B, clownfish larvae started metamorphosis 3 days (day 9 vs day 12 in controls) earlier with respect to larvae from the control group. On day 6 ph significant difference ($p<0.05$), either in TL or BW, was observed between Group A and B larvae (4.51 ± 0.14 vs 5.92 ± 0.22 mm and 1.56 ± 0.11 vs 2.35 ± 0.30 mg, respectively). Also on day 11 ph, Group B larvae fed enriched diet showed better growth both in terms of TL and BW compared to Group A larvae (8.0 ± 0.87 vs 5.58 ± 0.67 mm; 7.40 ± 0.11 vs 4.98 ± 0.45 mg, respectively).

3.6. Survival

On day 15 ph Group A larvae (control) had $11\pm1\%$ survival, while Group B larvae fed enriched live prey showed a $58\pm4\%$ survival. Significant difference ($P<0.05$) in survival between these two groups was evident from day 3 ph. In particular, in Group A larvae, higher mortality was observed between day 4 and 8. The higher mortality observed in the control group is related to the fact that rotifers and *Artemia* are not the natural live prey of marine fish larvae and present an inappropriate fatty acid profile.

3.7. Mitochondria

In six days ph samples no differences in the degree of mitochondria structure were observed. In both samples obtained from 11 days ph larvae fed enriched and not enriched live prey, regular repeating regions of myofibrils organized in sarcomeres were clearly evident and mitochondria were longitudinally oriented along them. Fig. 9 A,B,C represents a section obtained from a 11 days ph larva fed enriched live prey, where well-structured mitochondria are detectable as elongated

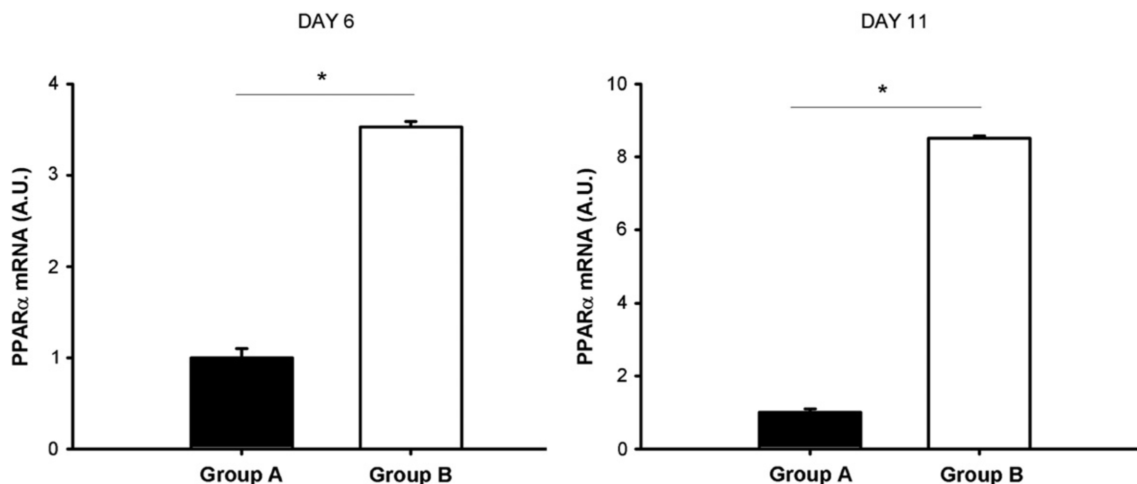

Fig. 5. A, B. PPAR gene expression in false percula clownfish larvae fed different diets. On day 6 and 11 ph for larvae fed enriched diet (group B) a significant ($p<0.05$) and relatively large increase for PPARs subtype α was detected with respect to the control (Group A). Statistical significance is indicated with *.

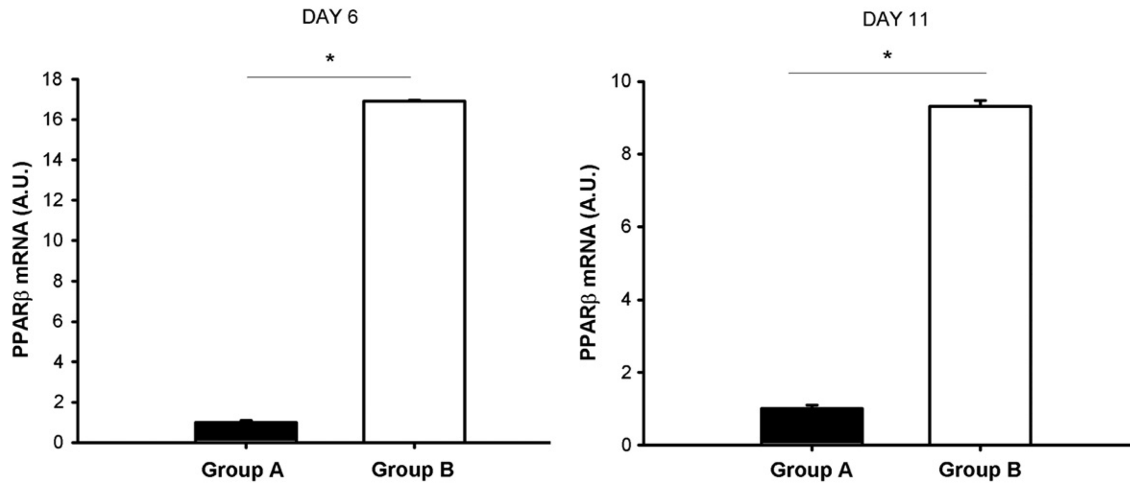

Fig. 6. A, B. PPAR gene expression in false percula clownfish larvae fed different diets. On day 6 and 11 ph for larvae fed enriched diet (group B) a significant ($p<0.05$) and relatively large increase for PPARs subtype β was detected with respect to the control (Group A). Statistical significance is indicated with *.

membrane-bound cylindrical organelles with a well evident outer and inner membrane forming numerous *cristae*. Glycogen deposits are detectable as electrondense bodies.

Sample sections obtained from 11 days ph larvae controls are shown in Fig. 10 A,B,C, where besides normal organelles, mitochondria with distorted and fused *cristae* were more frequently observed with respect to the fish fed HUFA enriched diet. Interdigitations of membranes and protrusions of part of one mitochondrion into another one were frequently detected in adjacent organelles (Fig. 10 A,B). Myelin-like figures ascribed to focal mitochondrial phospholipid membrane degeneration were also evident in theses samples. In perinuclear areas mitochondria of smaller dimensions and poorness of *cristae* appeared often evident at the ends of myofibrils close to large deposits of glycogen (Fig. 10 C).

4. Discussion

It is well known that the rate of biomass gain and the duration of the larval period of marine fish can greatly be affected by feeding conditions. The importance of larval experiences in determining growth rates, size and quality of individuals reaching metamorphosis has been demonstrated for terrestrial insects (Bradshaw and Johnson, 1995) and amphibians (Alvarez and Nicieza, 2002), in addition to aquatic invertebrates (Wacker and Von Elert, 2001). Recently, for a number of tropical and temperate fishes, researchers have shown that specific events during the larval phase (such as nutrition) can have profound impact on the number of successful individuals reaching the juvenile stage (Sargent et al., 1999; Campana et al., 1999; Jenkins and King, 2006; Avella et al., 2010). An important tradeoff occurs between growth and storage: with a fixed amount of energy, individuals can either allocate that energy toward growth or allocate it in important biological processes such as metamorphosis (Metcalfe and Monaghan, 2001).

In marine fish larvae the great majority of this energy derives from fatty acid-oxidation and for this reason larvae are particularly sensitive to lipid deficiency (Izquierdo et al., 2008). Consequently, this study focused its attention on the live prey lipid profile used, and its effect on larval survival and development. In fast growing organisms, like fish larvae, a proper diet is essential to satisfy this high energy demand (Tocher et al., 2003) promote growth and finally undergo metamorphosis. Therefore, in the last decades, a large amount of research effort has been directed towards the development of food enrichments, with particular emphasis on lipids. Several studies have shown that providing fish larvae with enriched live prey may improve their survival, growth and reduce time to metamorphosis (Faulk and Holt, 2005; Avella et al., 2007; Olivotto et al., 2008, 2009). However, the biochemical and molecular processes related to HUFA administration are still poorly understood in fish. In the current study, we observed that HUFAs were able to improve the EC, growth performances (higher IGFs and lower MSTN gene expression) and shorten the larval phase in false percula clownfish larvae fed enriched

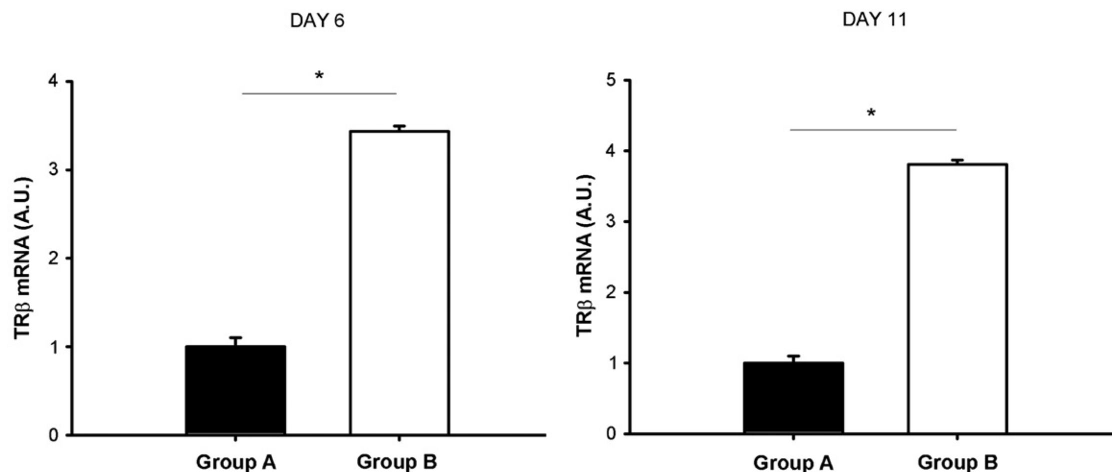

Fig. 7. A, B. TR ß gene expression. TR ß was significantly ($p<0.05$) up-regulated in group B with respect to control in both samplings. Statistical significance is indicated with *.

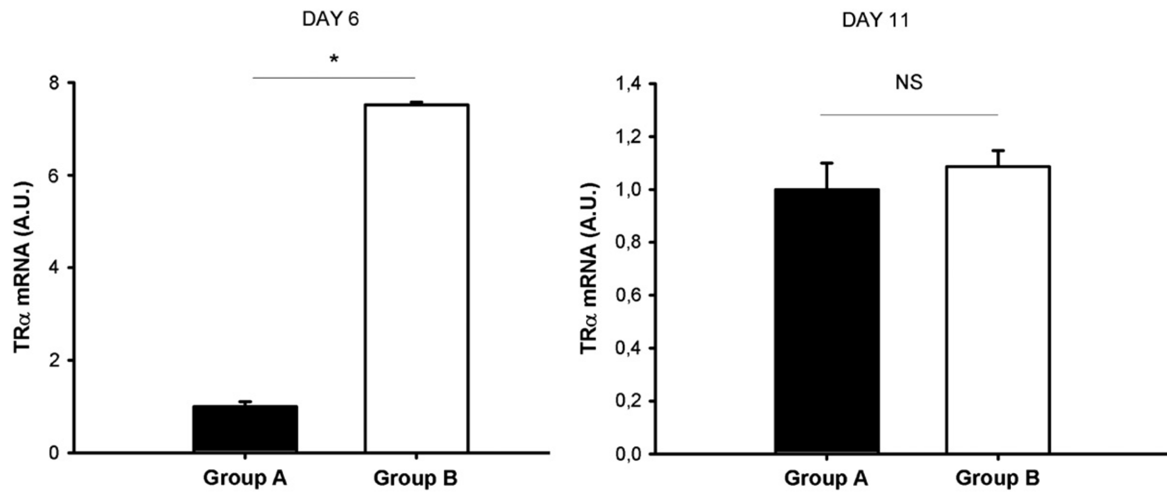

Fig. 8. A,B. TR α gene expression. Surprisingly, on day 11 ph, the subtype α was not affected in group B and no significant differences with respect to control group were observed ($p>0.05$) while on day 6 ph its gene expression was significantly higher in group B larvae with respect to control. Statistical significance is indicated with *.

live prey. Cells and organisms, generally maintain a homeostasis of EC and when EC is high, cells favor ATP-consuming pathways while when the EC declines below a set point, appropriate to any given cell, there follows a shift toward ATP-generating pathways (Smolensky et al., 1990; Dreanno et al., 1999; Zietara et al., 2004; Ishibashi et al., 2007). A central role in this process is played by mitochondria. They are involved in ATP synthesis and the presence of well structured mitochondria with well developed, numerous and elongated *cristae,* as observed in larvae fed HUFA enriched diet with respect to control, suggests the presence of fully active mitochondria.

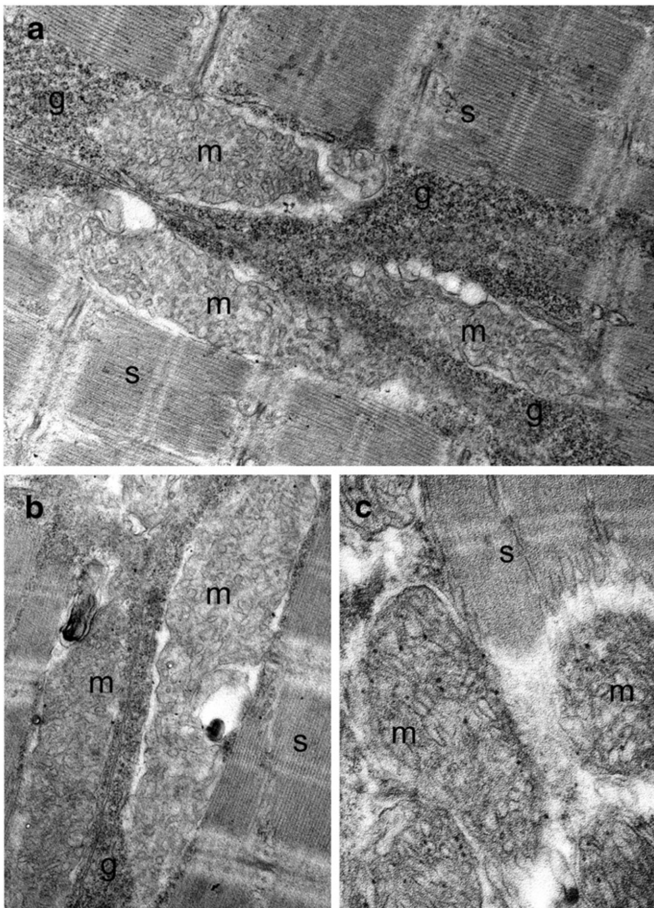

Fig. 9. A, B, C. TEM analysis of muscular tissue from an 11 day ph larva fed enriched live prey. A,B: longitudinal section muscle fibers. Normal structured mitochondria (m) with inner membrane forming numerous *cristae* were frequently detectable between myofibrils of sarcomeres (s). Glycogen (g) deposits are also evident as electrondense bodies. C: large and well structured mitochondria were the prevalent kind of organelles found in areas close to the end of myofibrils (31.000× original magnification).

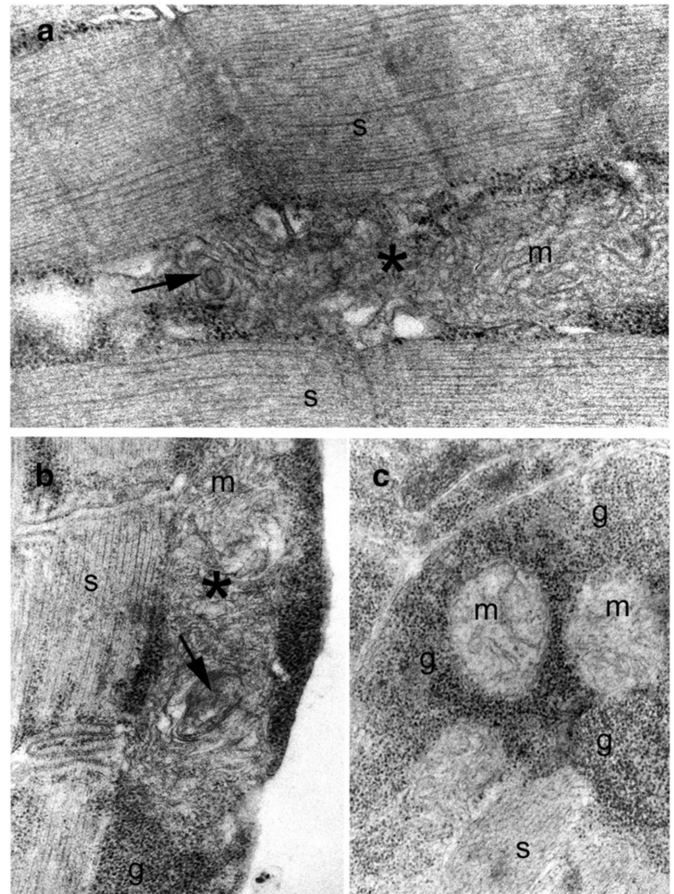

Fig. 10. A, B, C. TEM analysis of larval tissue obtained from 11 days ph larvae fed not enriched live prey. A, B: mitochondria (m) dispersed among sarcomeres (s) showing aspects of membrane interdigitation (*) and myelin-like figures (↑). Larger deposits of glycogen (g) are also evident in this sample with respect to that obtained from larvae fed enriched live prey. C: mitochondria showed smaller dimensions and fewer, poorly organized *cristae* with respect to the previous samples. (56,000× original magnification).

On the other hand, several glycogen deposits occupying cytoplasmic zones close to poorly developed mitochondria can be observed in the control larvae, sustaining the hypothesis of a lower activity grade of mitochondrial metabolism. Moreover, these same mitochondria show aspects of membrane interdigitation and myelin-like figures. The impact of dietary lipid composition on mitochondrial structure and function has mainly been examined in mammals and birds (Comte et al., 1976; Ballantyne and George, 1977; Renner et al., 1979; Angulo-Monroy et al., 1991). Cardiac mitochondria from chicks fed diets containing sunflower oil had higher membrane levels of 18:2n-6, higher rates of ATP synthesis and were better coupled than those from chicks fed diets containing rapeseed oil (Renner et al., 1979). In addition, feeding rats with sardine oil, changes the fatty acid composition of cardiolipin (22:6n-3 rises and 18:2n-6 falls), decreases the oxidative capacity and cytochrome c oxidase activity of heart mitochondria, and increases the F1F0-ATPase activity (Yamaoka et al., 1988). The present study represents the first report about the effects of HUFA administration on mitochondrial ultrastructure in a marine species showing a possible involvement of fatty acids in a regular mitochondrial morphology.

Beside the higher EC value, on day 6 ph, fish larvae fed enriched diet always showed higher content in both NAD and NADP with respect to control. NAD(H) is an essential and ubiquitous coenzyme that plays a fundamental role in cellular redox reactions. Beyond this pivotal role as a redox cofactor in energy transduction, numerous recent studies have demonstrated the importance of the non-redox functions for NAD, including its role as a substrate for covalent protein posttranslational modifications such as protein deacetilation and mono- and poly-(ADP-ribosyl)ation (Magni et al., 2004). Owing to the dual role played by NAD, as energy and signal transducer, it is clear that its synthesis and its endocellular levels have significant impact on the cellular metabolism.

NAD biosynthetic pathway is dependent on the NMNAT enzyme activity, catalyzing the transfer of the adenylyl moiety of ATP to NMN (Magni et al., 2004; Zhai et al., 2009). Its activity, in 6 days ph larvae, was 2 times higher in fish larvae fed HUFA enriched live prey, with respect to control, supporting the higher concentration of NAD detected in these larvae. Differently from NAD, NADP(H) is involved in most reductive biosynthetic reactions, in cellular defense against oxidative stress and through its deaminated form, NADP, in calcium signaling. The higher levels of NADP in group B larvae can be correlated to the activation of cellular anabolic pathways, leading to the synthesis of new biological macromolecules.

Since clownfish larvae fed enriched live prey metamorphosed 3 days (day 9 vs day 12 in controls) earlier than the control group, we can hypothesize that HUFAs may represent a valid energy source to undergo this process. The requirement of nutritional reserves to undergo metamorphosis has been confirmed in a variety of marine organisms (Youson, 1988; Pechenik and Cerulli, 1991; Pfeileret, 1999) with thyroid activity considered crucial in this regard. In our study we observed that a better fatty acid profile was associated to higher EC, better mitochondria development and higher PPAR α and ß gene expression, conditions that seem to be essential to accumulate enough energy, activate the hypothalamus–pituitary–thyroid axis and undergo metamorphosis. Moreover, in this species, the energy to undergo the metamorphosis process is stored in the early part of the larval cycle (already at 6 days ph) since differences between biochemical and molecular parameters in the two experimental groups are much more severe at this sampling point.

It is well established that hormonal secretion and action of the thyroid hormones and those of the GH/IGF axis are interdependent (Akin et al., 2009). Thyroid hormones influence growth in part by increasing the GH/IGF secretion that, in turn, affects growth and function of the thyroid as well as thyroid hormone metabolism (Akin et al., 2009). Several studies have reported alterations of the GH/IGF axis in animals showing hypothyroidism, with the main alterations reported in hypothyroid being related to low IGF levels and slower growth (Akin et al., 2009). Similar results were obtained in this study: highest levels of IGFs were always associated with highest free T3 and T4 content and TR α and β gene expression. TRs mRNA levels are directly modulated by thyroid hormone concentrations (Kawakami et al., 2006) as already demonstrated in several fish species where significantly higher levels of TRs have been evaluated during the metamorphosis event, with the highest levels simultaneously occurring with metamorphosis climax (Yamano and Miwa, 1998; Galay-Burgos et al., 2008).

In conclusion, a strategy of rapid growth and early energy storage may be adaptive and beneficial to survival for this species. From the moment of conception, individuals vary in development and growth rates that will pre-dispose some individuals to a lower probability of surviving later developmental stages. These initial differences in life-history characteristics are parentally driven, being either genetically programmed, or maternal non-genetic contributions to nutrition (McCormick, 1993), immunology (Lung et al., 1996) or metabolism (Agrawal, 2001). These initial effects interact with environmental conditions to result in high variability in growth and performance characteristics of the organism (Relyea and Hoverman, 2003). High larval growth may lead to size advantages in gaining access to limited resources and/or evading and escaping predators (Leggett and Deblois, 1994). Recent studies on tropical marine fishes have shown that the advantages associated with a high larval growth rate lead to enhanced survival through to metamorphosis and settlement to the juvenile population (Wilson and Meekan, 2002). Organisms vary widely in phenotypic traits that may influence survival (Moran and Emlet, 2001). These characteristics may include size (Schmitt and Holbrook, 1999), growth rate (Vigliola and Meekan, 2002), overall body condition, fat content (Booth and Hixon, 1999), sensory development (McCormick, 1993) and burst speed (McCormick and Molony, 1993). This suggests that during the high mortality that occurs during the larval phase, survival may be strongly influenced by the energy status of the larvae.

If we can consider survival as a balance between utilizing energy to grow rapidly out of vulnerable sizes and allocating sufficient energy for other demands (such as metamorphosis), in the species here selected a supplement of HUFAs is particularly important during the first week of life. A supplement of HUFAs induced a better mitochondria development, higher EC and ATP synthesis and thus positively influenced growth and metabolism inducing as final result a faster development a shorter larval phase and better survival.

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