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Calanoid copepod administration improves yellow tail clownfish (*Amphiprion clarkii*) larviculture: biochemical and molecular implications

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Abstract. In this study we evaluated the effects of a standard rotifers/*Artemia* (control) diet and a copepod (*Centropages typicus*) based diet during *Amphiprion clarkii* larviculture. On day 11 post hatch, larvae fed *C. typicus* showed better survival and growth compared to those fed a standard rotifer/*Artemia* diet (87±2 vs 41±2%; 6.8± 0.2mm vs 6.1± 0.2mm; 5.9±0.3mg vs 4.5± 0.3 mg, respectively). Further molecular analysis have been carried out on 5 and 11 days post hatch larval samples to clarify the efficiency of a copepod based diet respect to control. In particular, the peroxisome proliferator activated receptors (PPAR-α, -β) gene expression, which are involved in several biological processes such as lipid metabolism, the retinoic acid receptor γ (RARγ), involved in vitamin A metabolism and the heat shock protein 70 (HSP70), a biomarkers of cellular stress, were analyzed by real-time PCR during the larval development. The results obtained are related to fatty acid composition of live preys used. The superiority of a *Centropages typicus* copepod diet respect to a standard rotifers - *Artemia* one has been demonstrated; these results are essential to improve captive production of *A. clarkii* through a closed system and in turn to preserve natural stocks.
Key words: copepods, fatty acids, clownfish, Artemia, rotifers.

Riassunto. In questo studio sono stati valutati gli effetti di una dieta standard a rotifer/*Artemia* e una a base di copepodi (*Centropages typicus*) durante la larvicoltura di *A. clarkii*. A undici giorni post schiusa le larve alimentate con i copepodi mostravano un tasso di crescita e sopravvivenza migliore rispetto a quelle alimentate con dieta standard (87±2 vs 41±2%; 6.8± 0.2mm vs 6.1± 0.2mm; 5.9±0.3mg vs 4.5± 0.3 mg, rispettivamente). Ulteriori analisi molecolari sono state condotte su campioni di larve a 5 e 11 giorni post schiusa, al fine di meglio comprendere l'efficienza dei copepodi come prede vive. In particolare l'espressione genica dei peroxisome proliferator activated receptors (PPAR-α, -β), coinvolti in diversi processi biologici come il metabolismo lipidico, del retinoic acid receptor γ (RARγ), coinvolto nel metabolismo della vitamenia A e dell'heat shock protein 70 (HSP70), un biomarker per la valutazione dello stress sono stati valutati mediante Real-Time PCR durante lo sviluppo larvale. I risultati ottenuti sono stati successivamente correlati alla composizione lipidica delle prede vive impiegate. E' quindi stata dimostrata la superiorità dei copepodi rispetto a una dieta standard e questi risultati sono indispensabili per migliorare la produzione in cattività di *A. clarkii* e preservarne le popolazioni naturali.
Parole chiave: copepodi, pesce pagliaccio, acidi grassi, Artemia, rotiferi.

Rezumat. În prezentul studiu am evaluat efectele dietei rotiferelor standard/*Artemia* (ca martor) și a unei diete bazate pe copepode (*Centropages typicus*) asupra creșterii larvelor speciei *Amphiprion clarkii* (peștele clown). În ziua a unsprezecea dupa ecloziune, larvele hrănite cu *C. typicus* au arătat o mai bună supraviețuire și creștere comparativ cu cele hrănite cu *Artemia* (87±2 vs 41±2%, 6.8± 0.2mm vs 6.1± 0.2mm, respectiv 5.9±0.3mg vs 4.5± 0.3 mg). Analizele moleculare ulterioare au fost effectuate între zilele 5 și 11 dupa ecloziune pentru a clarifica eficiența hrănirii cu copepode raportată la lotul martor. Expresia genelor PPAR-α și PPAR-β, care sunt implicate în câteva procese biologice precum metabolismul lipidic, a receptorului acidului retinoic (RARγ), implicat în metabolismul vitaminei A, și a proteinei de șoc termic 70 (HSP70), un biomarker de stres celular, au fost puse în evidență prin tehnica *real-time PCR* pe durata dezvoltării larvare. Rezultatele obținute sunt correlate cu compoziția acizilor grași a prăzilor vii utilizate. Valoarea superioară a copepodului *Centropages typicus* ca hrană față de rotiferele standard a fost demonstrată, iar rezultatele aduc îmbunătățiri substanțiale creșterii și reproducerii în captivitate a speciei de pește clown în sistem închis și deci la conservarea populațiilor naturale de *A. clarkii*.
Cuvinte cheie: copepode, acizi grași, peștele clown, Artemia, rotifere.

Introduction. One of the main factors that can negatively affect marine teleost growth and welfare is malnutrition, which has recently been included in the stressors list.

Heat shock proteins (HSPs) level has been found to be correlated to exposure to stressors within an ecologically relevant range, and thus it is suggested that stress response at the cellular level may play an important role in enhancing the survival of stressed fish (Iwama et al 1998). Therefore, HSPs have been proposed as biomarkers of cellular stress (Sanders 1993) or as nonspecific early indicators of toxic exposures (Goldstone et al 2006).

Problems such as reduced growth and malformations are often encountered when fish are subjected to stress (Olivotto et al 2002). Malnutrition has recently been included in the stressors list and in particular the lack of essential fatty acids and micronutrients needed for optimal larval development and growth in the first food offered are of great relevance (Cahu et al 2003).

Usually, high survival and growth rates are characteristics of marine fish larvae reared on natural assemblages of marine zooplankton or on copepods (Støttrup & Norsker 1997; van der Meeren et al 2008; Olivotto et al 2008b). Copepods not only present a wide range of body size between nauplii and adults and typical movement, but they also have, respect to the most widely used rotifers and *Artemia* nauplii, a biochemical composition that matches the nutritional requirements of fish larvae (Evjemo et al 2003; Bell et al 2003; Delbare et al 1996; Olivotto et al 2008a; Olivotto et al 2008b).

At date, beneficial effects of copepod administration during early larval nutrition can be related to their lipid composition (Sargent et al 1997), in particular to the content and ratio of the polyunsaturated fatty acids (PUFAs). These fatty acids, in particular eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), are extremely important for larval fish survival and growth and several studies have demonstrated that they are essential in larval diets (Sargent et al 1999; Bell et al 2003; Olivotto et al 2003; Faulk & Holt 2005; Olivotto et al 2006a). Highly unsaturated fatty acids (HUFAs) are able to act directly on the genome, via specific nuclear receptors, the peroxisome proliferator activated receptors (PPAR-α, -β, and γ). PPARs are transcription factors belonging to the ligand-activated nuclear hormone receptor super-family. On binding ligands, PPARs, form heterodimers with RXR to facilitate the transcription of target genes. These nuclear receptors are involved in several biological processes such as skeletal development during ontogenesis (Burdick et al 2006; Grimaldi 2007), 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).

Since marine fish larvae are predominantly visual predators they require a functional retina at the onset of first feeding. In young developing mammals, dietary (n-3) PUFAs deficiency is known to impair visual acuity (Neuringer et al 1984; Hrboticky et al 1991). A similar loss in visual function has been observed in larval herring (*Clupea harengus* L.)(Bell & Dick 1993); DHA deprivation caused impaired visual performance, particularly at low light intensities when rod cells are active. Studies performed by Japanese researchers have suggested that, in addition to the provision of essential (n-3) HUFAs, supplementation with adequate vitamin A is vital for successful skin pigmentation and visual acuity (Kanazawa et al 1993; Miki et al 1990) in marine fish larvae. For this, the attention of scientists is now focused on the importance in the larviculture of some nutrients such as vitamins and pigments which are essential for a proper development and growth in marine fish larvae (Cahu et al 2003). A deficiency in dietary vitamin A, which is a precursor of rhodopsin, will disrupt transmission between the eye and the brain. Thus, vitamin A may be considered an important element in fish culture even if it has to be reminded that a surplus of vitamin A or its derivates (retinoids) may have detrimental effects on normal bone development (Cahu et al 2003). A valid and safe way to provide vitamin A to larval fish is the use of carotenoids which are important provitamin A compounds abundant in the plankton. Marine copepods are rich in carotenoid pigments (Shields et al 1999; van der Meeren et al 2008) including mono- and diesters of astaxanthin as well as unesterified astaxanthin, whereas *Artemia* contains lower quantities of the related carotenoid canthaxanthin, which is present only in the unesterified form

(Krinsky et al 1965; van der Meeren et al 2008). Although both canthaxanthin and astaxanthin can be converted to vitamin A in fish (Olson 1989), the higher quantity of total carotenoids usually present in copepods, may allow more efficient uptake and metabolism of these vitamin A precursors in fish fed copepods compared with those fed *Artemia.* Vitamin A and its bioactive metabolites have pleiotropic effects in all tissues of vertebrate organisms involving cellular development, proliferation, differentiation, metabolism and apoptosis (McGrane 2007). The understanding of the molecular mechanism of retinoid action was advanced when the nuclear retinoid receptors were cloned and characterized (Mengeldorf et al 1991). In the nucleus there are two distinct classes of retinoid receptors, the retinoic acid receptor (RAR) and retinoid X receptor (RXR). In addition to the multiplicity of RAR and RXR genes and their respective mRNA isoformes, their biological activity is also regulated by the interaction of the retinoid receptors with other nuclear receptors (NRs) (McGrane 2007). Thus, since RARs are ligand-dependent transcription factors, having the ability to repress transcription in the absence of ligand (McGrane 2007), a linear relationship between RAR expression and ligand amount can be stated.

The aim of this study was to compare the fatty acid composition of the different live preys used and to relate these results to *A. clarkii* survival, growth and modulation of some genes involved in lipid and retinoic acid metabolism and stress. This study is important to improve the survival and the production of captive born clownfish that in turn represent an environmental friendly alternative to wild caught specimens and may be useful for the conservation of wild populations.

Material and Methods

Zooplankton Culturing. *Centropages typicus* copepods were cultured at the Stazione Zoologica Anton Dohrn, in a 500 L re-circulating system (INNOVAQUA srl, Reggio Emilia, Italy) at a temperature of 19°C, 36 ‰ salinity and 12 h light:dark photoperiod from January to April 2008. Copepods were fed with a mixture of phytoplanktonic cells *Heterocapsa niei, Tetraselmis suecica and Isochrysis galbana* at concentrations of 5.5 x 10^3, 1.25 x 10^4 and 3.4 x 10^4 cells/mL, respectively, corresponding to about 1 mg cells/L of each algae. Nauplii and copepodites were collected at different developmental stages from naupliar stage I (NI) (110 µm length) to copepodids III (CIII) (560 µm length) and used for the feeding studies. Nauplii and copepodites were attracted into a 155 or 300 µm mesh net size filter cage using a light, automatically collected in a 200 L tank, and finally concentrated in 15 L of seawater. A further concentration was performed with a 50 µm mesh net, after this, nauplii and copepodites were re-suspended in 100 mL of 1 µm filtered seawater and counted in 5 sub-samples of 5 mL each, delivered in drops under a Leica stereomicroscope. With this method, we were able to count live nauplii and copepodites since they remained entrapped into small volumes of seawater. Before their administration to the larvae, nauplii and copepodites were diluted in a 10 L beaker in a 5 L seawater and gradually (8-12 h) acclimated to the chemical-physical conditions of the larval tanks. For this, the temperature was gradually (1.5°C/h) increased to 27°C and the salinity was gradually decreased to 32 ‰. After this, naupliar and copepodids survival was checked again.

Rotifers (*Brachionus plicatilis*) characterized by an average size of 239 µm were cultured on *N. oculata* (50.000 cells/mL) in 100 L tanks (salinity 30 ‰, pH 8.2, NO_2 and NH_3 < 0.03 mg/L) and subjected to constant light. Each day, the amount of rotifers was transferred to 50 L cone-shaped tanks for enrichment. Enrichment was performed with Algamac 2000 (Aquafauna Bio-Marine, Inc., Hawthorne, CA, USA) using 0.5 g/million rotifers. Algamac 2000 was homogenized in 200 mL salt water for 1 min and then distributed to the rotifers for enrichment. As recommended by the company enrichment lasted for 8-9 h.

AF 430 *Artemia* cysts (Inve Technologies, Belgium) were incubated and hatched following INVE instructions. The cysts were hatched in cone-shaped tanks, in artificial seawater (Prodac Int., Cittadella, Italy) (salinity 30 ‰, pH 8.2, NO_2 and NH_3 < 0.03 mg/L) at a density of 1-1.5 g cysts/L of water. The temperature in the hatching thanks

was maintained at 25°C and the photoperiod consisted of constant light at 2000 lux. The aeration was vigorous to keep all cysts in suspension throughout the hatching period and dissolved oxygen was maintained greater than 5 mg/L. Under these conditions, the cysts hatched in about 24 h. After hatching, the nauplii were separated from the cysts by siphoning and enrichment was performed with Algamac 2000 (0.2 g/100000 *Artemia* nauplii) in 10 L buckets filled with filtered seawater at a concentration of 200 nauplii mL^{-1} for 8 h at 25°C under continuous aeration and illumination. Before feeding to the larvae, rotifers and *Artemia* nauplii were concentrated on a 30 µm mesh and rinsed several times with clean seawater (salinity 30 ‰) in order to remove the remaining enrichment.

Experimental Design. Immediately after hatching, larvae were divided into two experimental groups (100 ± 5 larvae per group, in three replicates each) as follows:
-Group A (control): fed rotifers (*Brachionus plicatilis*) (10 ind/mL) from day 1 to day 7 post hatching (ph) followed by AF430 *Artemia* nauplii (6 ind/mL) introduced from day 7 ph till the end of the experiment.
-Group B: fed *C. typicus* nauplii (5 ind/mL) from day 1 to day 7 ph, followed by *C. typicus* copepodites (3 ind/mL) introduced from day 7 ph till the end of the experiment.

The feeding experiments were conducted in 20 L cubic larval tanks (Olivotto et al 2008c) connected to the broodstock tank system and the phytoplankton *N. oculata* (introduced at 0800, 1200, 1600, 2000 h) was used (50.000 cells/mL) to condition the water from day 1 to day 7 ph.

Sampling of Larvae. Samples of larvae (20 ± 1, in three replicates), for each experiment group, were collected 5 and 11 days ph. The experiment terminated on day 11 ph since *A. clarkii* undergoes metamorphosis around day 12-13 ph. All samplings were performed at 0900 h prior to feeding the larvae. Ten larvae were used for morphological measurements (total length TL and wet weight) using a Stemi 2000 micrometric microscope and a microbalance (OHAUS Explorer E11140 accurate to 0.1 mg), the remaining larvae were stored at -80°C for molecular analysis.

Every day at 0900 and 1600 dead larvae were siphoned from the tank bottom and counted in order to estimate survival rate.

Lipid Analysis. Free fatty acids were extracted from lyophilized samples of rotifers, *Artemia* and copepods (nauplii and copepodites) in triplicates, by homogenizing the samples in 10 volumes of chloroform/methanol (2:1, v/v) using a glass/teflon homogenizer.

According to a modified Folch method (Hamilton et al 1992), the biological material was suspended in a chloroform/methanol mixture (2:1 v/v, 0.5mL) and sonicated for 1 min. After centrifugation for 1 min at 3000 rpm, the supernatant was transferred in a new tube and dried under nitrogen stream. The raw extract so obtained was dissolved with diethyl ether and methylated with diazomethane (CH_2N_2) for 60 min at room temperature. The reagent in excess was removed under nitrogen and the derivatized residue resuspended in *n*-hexane (100µL) for GCMS analysis (Focus GC-Polaris Q, Thermo) with a 30 m x 0.25mm ID x 0.25µm film thickness, capillary column (5% diphenyl/95% dimethyl polysiloxane). Elution of fatty acid methyl esters required a temperature programme starting with 50°C for 2.5 min followed by a 10°C min^{-1} ramp up to 100°C and then 5°C min^{-1} up to 290°C. Samples were directly injected (2µL) in a split (1:10) mode with a blink window of 3.5 min. The injection temperature was maintained at 270°C and the transfer line at 280°C. Full scan spectra were acquired from 60 to 600 *m/z*.

Individual methyl esters were identified by comparison with known standards (Supelco, PUFA-1 Marine source and Lipid Standards: Fatty Acid Methyl Ester mixtures C:4-C24:1) and by reference to published data.

RNA Extraction and cDNA Synthesis. Total RNA was extracted from the larvae using a Minikit RNAeasy® Qiagen extraction kit following the manufacturer's protocol. Final RNA concentrations were determined by optical density measurement at 260 nm, and the RNA integrity was verified by ethidium bromide staining of 28S and 18S ribosomal RNA bands on 1% agarose gel. Total RNA was treated with DNAse (10 UI at 37°C for 10 min, MBI Fermentas), a total amount of 1 mg of RNA was used for cDNA synthesis, employing iScript cDNA Synthesis Kit (Bio-Rad).

Primer Design. The following primers were used at final concentration of 10 pmol/μL: B-ACT: For: 5'-TTCCTCGATATGGAGTCCT-3'; Rev: 5'- TGGGGCAATGATCTTGATCTT-3'. PPARα: For: 5' -TTC AGC GAC ATG ATG GAG CC-3'; Rev: 5' - CAG TTT CTG CAG CAG ATT GG-3'. PPARβ: For: 5' - AGG AGA TAG GGG TAC ACG TG -3'; Rev: 5' - CAG GAA CTC CCG GGT CAC AA -3'. RARγ: For: 5' - ATG TGA AGG AGG AGG TGG TG -3'; Rev: 5' - CGT TTC TTG GTG AGC TTT GC -3'. HSP70: For: 5' - ACG GAG AGT CGA TTT CGA TG -3'; Rev: 5' - GAA GGA CAT CAG CGA CAA CA -3'.

Real-Time PCR. Triplicate PCR reactions were carried out for each sample analyzed. After real-time conditions optimization, the PCRs were performed with SYBR Green method in a iQ5 iCycler thermal cycler (Bio-Rad). The reactions were set on a 96-well plate by mixing, for each sample, 1 μL of diluted (1/20) cDNA, 5μL of 2X concentrated iQ TM SYBR Green Supermix (Bio-Rad), 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 and then 45 cycles of 20 s at 95°C, 20 s at 60°C and 20 s at 72°C. The fluorescence monitoring occurred at the end of each cycle. Additional dissociation curve analysis was performed and showed a single peak in each case. β-ACT was used as a house keeping gene in each sample in order to standardize the results by eliminating variation in mRNA and cDNA quantity and quality. No amplification product was observed in negative controls and no primer-dimer formation was observed in the control templates.

Data 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. The level for accepted statistical significance was $P<0.05$. Morphological and survival data are expressed as means ±sd and were analyzed by two-way ANOVA followed by Tukey's test. The level for accepted statistical significance was $P<0.05$.

Results and Discussion

Survival. On day 11 ph group A larvae (control) fed rotifers followed by *Artemia* nauplii had 41 ± 2% survival, while group B larvae fed a copepod nauplii followed by copepodites showed 87 ± 2% survival (Fig. 1). 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 6 and 8 when *Artemia* nauplii were firstly administered.

Morphometric Results. On day 5 ph no significant difference, either in TL or body weight, was observed between group A and B larvae (4.8 ± 0.3 vs 5.3 ± 0.2 mm and 1.7 ± 0.2 vs 2.0 ± 0.2 mg, respectively). In contrast, on day 11 ph, group B larvae fed copepods showed better growth both in terms of TL and body weight compared to group A larvae fed a standard rotifers/*Artemia* diet (6.8 ± 0.2 vs 6.1 ± 0.2 mm; 5.9 ± 0.3 vs 4.5 ± 0.3 mg, respectively).

Lipid Analysis. In Table 1 are reported the % of fatty acid in the different diets, based on GC-MS data and expressed as relative area percentage on total free fatty acids. In Rotifers and *Artemia* nauplii, both enriched with Algamac 2000®, the relative % of EPA and DHA ranged between 7 to 12.4 % respect to the other free fatty acids, with a ratio

DHA/EPA below to 2; the % of total w3 are less than 20% (Table 1). On the contrary, in *Centropages typicus* nauplii and copepodites the ratio DHA /EPA is always > 2, in particular, in the nauplii sample, % DHA was the highest recorded and the ratio is double respect to copepodites (4.58 vs 2.65). In this species % of w3 fatty acids is very high with the maximum value of 26.34 in *C. typicus* nauplii (Table 1).

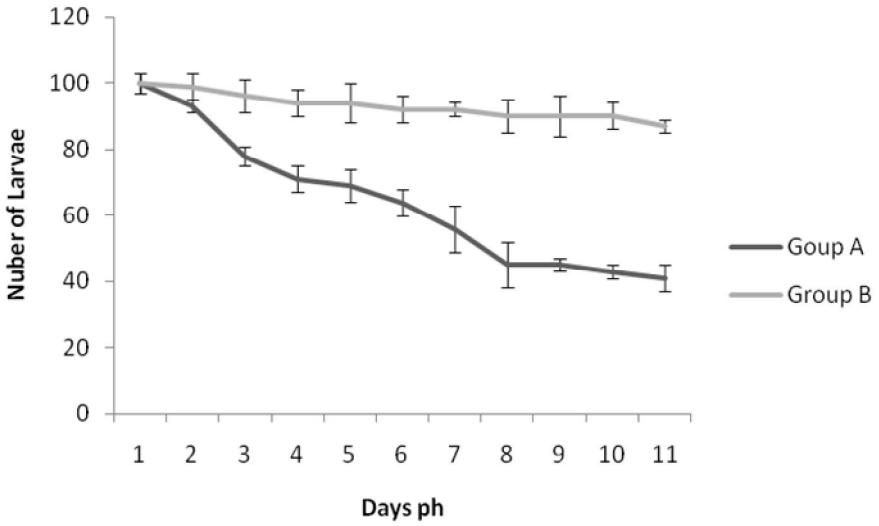

Figure 1. *A. clarkii* survival percentage in groups A and B larvae fed the different diets.

Table1

Percentage of relative fatty acid content in rotifers and *Artemia* nauplii enriched with Algamac 2000® and in *Centropages typicus* nauplii and copepodites (Fatty acid content was based on GC-MS data and expressed as relative area percentage on total free fatty acid area measured by peak integration. The best fatty acid profiles are showed by *C. typicus* nauplii and copepodites respect to rotifers and *Artemia*).

% relative total free fatty acid	*Rotifers+Algamac 2000*	*Artemia nauplii+Algamac2000*	*C. typicus nauplii*	*C. typicus copepodites*
EPA	7.00	7.8	4.71	5.09
DHA	12.4	9.8	21.62	13.52
DHA/EPA	1.77	1.25	4.58	2.65
%W3	19.4	19.1	26.34	18.61

Molecular Analysis. The expression of the different genes was analyzed by real-time PCR and a partial nucleotide sequences were obtained for each of them and after homology calculation they were deposited in the GenBank. In particular, RAR-γ (FJ393570) showed a 89% similarity with *Oryzias latipes* RAR-γ ; HSP70 (FJ393572), a 97% similarity with *Cichlasoma dimerus* HSP70; PPAR-α (FJ713638) a 90% similarity with *Dicentrarchus labrax* PPAR-α; PPAR-β (FJ713639) a 96% similarity with *Dicentrarchus labrax* PPAR-β; β-ACT (FJ713637) a 96% similarity with *Sparus aurata* β-ACT. On day 5 ph an increase ($P<0.05$) in PPARα, β, RAR γ gene expression was observed in group B larvae fed *C. typicus* nauplii (Fig. 2 A,B,C). On the contrary, on day 11 ph, a significant ($P<0.05$) increase in PPAR α and β gene expression was evident in group A larvae fed *Artemia* nauplii respect to group B larvae fed *C. typicus* copepodites

(Fig. 3 - A,B). Regarding RAR γ, its gene expression was significantly higher (P<0.05) in group B larvae in all the performed samplings (Fig. 2C, 3C).

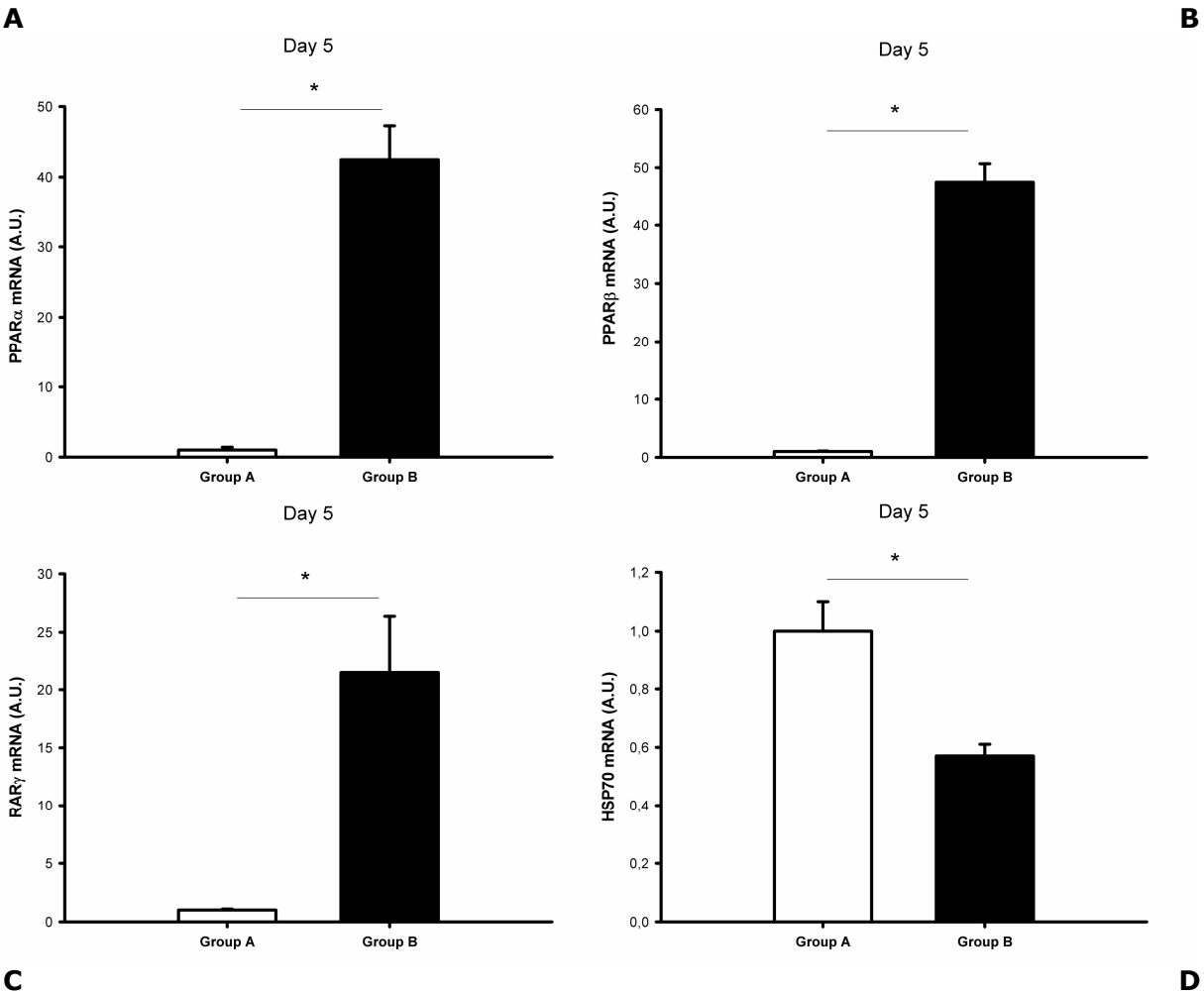

Figure 2. A,B,C,D - PPAR α and β, retinoic acid receptor γ and HSP70 gene expression in *A. clarkii* larvae fed different live prey at 5 days ph. Asterisk indicates statistical significance between group A and B (P<0.05).

Finally, HSP70 gene expression was always significantly (P<0.05) less expressed in larvae fed *C. typicus* during both samplings (Fig. 2D, 3D).

Marine fish larvae undergo major and morphological changes during this delicate moment of their life history. Several factors can interfere with the normal development of the larvae and affect their quality; among these of great interest is the quality of the first food offered.

It is well known that HUFAs, mainly EPA and DHA are essential for growth, development and survival of marine fish larvae (Sargent et al 1999; Olivotto et al 2006a; Olivotto et al 2006b). In particular, Sargent and coworkers recommend a DHA/EPA ratio in live feed of 2 or more in relation to the fatty acid composition of fish eggs (Bell et al 2003). This is supported by the fact that recently, it has been demonstrated that both live prey enrichment with HUFAs (Avella et al 2007) or copepods administration (which are known to be rich in HUFAs) (Olivotto et al 2008a; Olivotto et al 2008b) can promote larval fish growth.

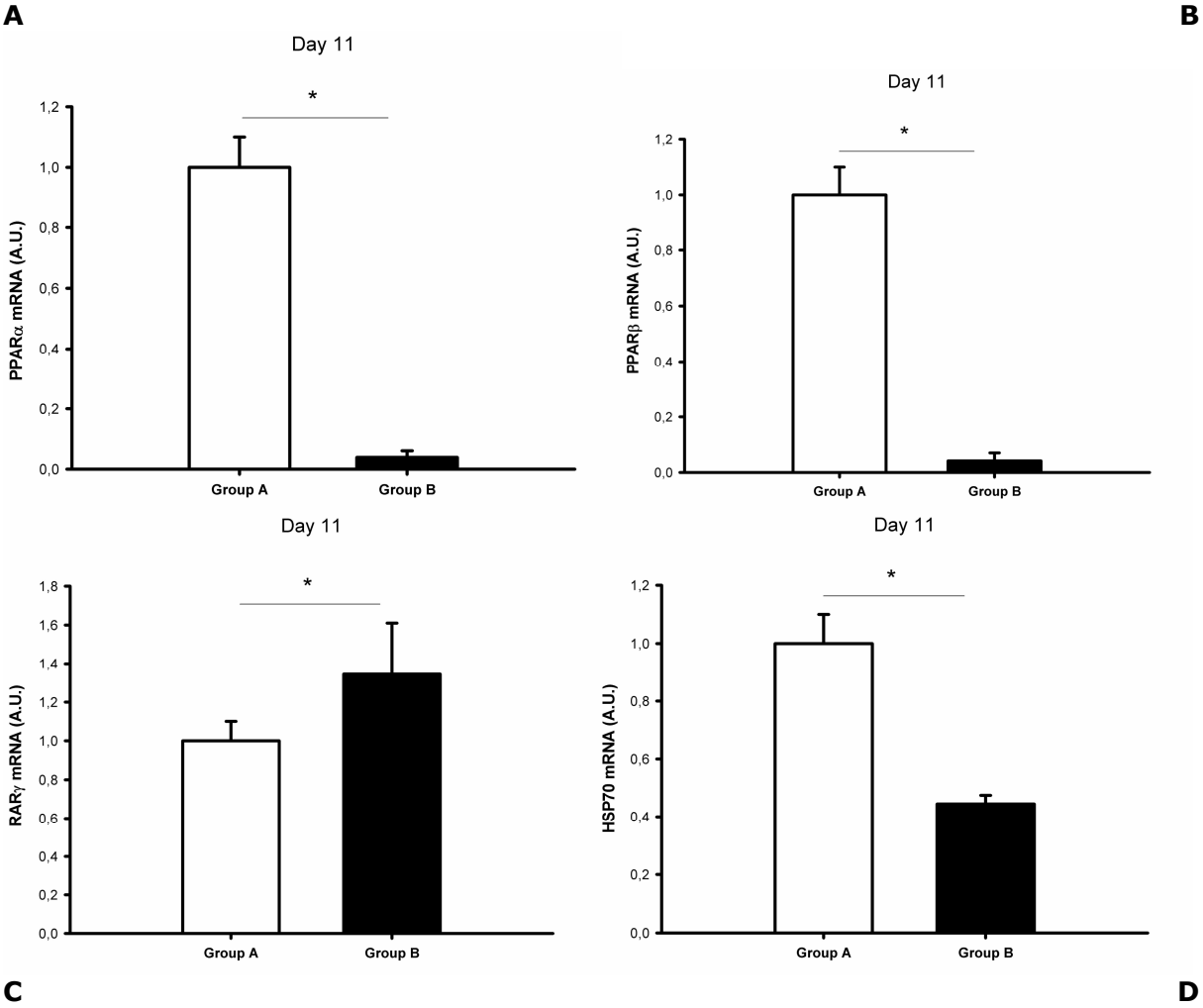

Figure 3. A,B,C,D - PPAR α and β, retinoic acid receptor γ and HSP70 gene expression in *A. clarkii* larvae fed different live prey at 11 days ph. Asterisk indicates statistical significance between group A and B ($P<0.05$).

Highly unsaturated fatty acids (HUFAs) act directly on the genome, via specific nuclear receptors, the peroxisome proliferator activated receptors (PPARs) which are thus considered good fatty acid sensors (Grimaldi 2007). The significant increase of PPARα and β gene expression observed in group B larvae, sampled 5 days ph and fed *C. typicus* nauplii respect to group A larvae fed rotifers, may thus be related to the fact that *C. typicus* nauplii present, at this developmental stage, a yolk sac. In fact, besides being the natural live prey for marine fish larvae, copepod nauplii are an important source of phospholipids and essential highly unsaturated fatty acids (Sargent et al 1997). Especially the two long chain polyunsaturated fatty acids, eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) are required in order to obtain normal growth and development, through maintaining the structural and functional integrity of cell membranes, and as precursors of eicosanoids (Sargent et al 1999). From the lipid analysis carried out on live preys, we observed that *C. typicus* nauplii are particularly rich in these two fatty acids and that the DHA/EPA ratio is particularly high in agreement with what suggested by Sargent et al (1999). These data are in accord with previous studies performed on different copepod species (Payne & Rippingale 2000; Evjemo et al 2003; Olivotto et al 2008a; Olivotto et al 2008b; van del Meeren at al 2008) in which it has been demonstrated that DHA was particularly abundant in the copepod naupliar stage indicating the importance of this fatty acid in the nutrition of newly hatched larvae. An higher and well balanced content of HUFAs in the copepod nauplii

respect to rotifers may thus result in a more efficient lipid oxidation and consequent larval growth; this in accord with the morphometric results obtained in this study (weight and length) and with the Insuline like growth factors/Myostatin gene expression analysis performed in a previous study (Olivotto et al 2008b).

On day 11 ph an inverse trend of PPAR α and β gene expression was observed. In fact, higher gene expression was observed in larvae fed *Artemia* nauplii rather than in larvae fed *C. typicus* copepodites. Total ω - 3 percentage is higher in enriched *Artemia* nauplii respect to *C. typicus* copepodites since at this developmental stage, *Artemia* nauplii still present a yolk sac rich in lipids while *C. typicus* copepodites have already exhausted it. Even if the total amount of HUFAs is higher in *Artemia* respect to copepodites, it is difficult to get a good fatty acid profile in this live prey (McEvoy et al 1998; Estevez et al 1999; Shields et al 1999; Evjemo et al 2001; Evjemo et al 2003; van der Meeren et al 2008) since in *Artemia,* EPA catabolism is lower than that of DHA. Also in this study we evidenced that the *Artemia* nauplii not only showed the lowest content of DHA but also (according to that suggested by Sargent and collaborators in 1999) the worst DHA/EPA ratio. Therefore, the superior performance of copepodites compared with *Artemia* nauplii reflects, at least in part, a relatively deficiency of DHA in the supplemented *Artemia*. This is supported by several studies performed on finfish (Reitan et al 1994; Shields et al 1999; Cahu et al 2003) and by our analysis.

Since marine fish larvae are visual predators they require a functional retina at the onset of first feeding. As already cited, it is well established that HUFAs deficiency may cause both in mammals and in fish a loss in visual function (Bell & Dick 1993; Rønnestad & Lie 1998; Shields et al 1999). The aldehyde form of vitamin A forms the photoreactive prosthetic group of opsins present in the retina. Animals cannot synthesize vitamin A de *novo* and a source must be available to them if normal retinal function has to be achieved. Natural prey of early stages of fish mainly includes copepods; however, several analyzed copepod species such as *Temora, Eurytemora, Acartia* and *Centropages* did not contain any form of vitamin A (Rønnestad & Lie 1998; Moren et al 2005; Hamre et al 2007). In fact, it is well known that in fish, vitamin A is mainly derived from precursors such as carotenoids present in planktonic preys (Olson 1989; Rønnestad & Lie 1998; Shields et al 1999; Hamre et al 2007; van der Meeren et al 2008). Copepods are usually rich in astaxanthin whereas *Artemia* contains lower quantities of the related carotenoid canthaxanthin (Shields et al 1999; Palace & Werner 2006; van der Meeren et al 2008) and both of them can be converted to vitamin A.

Since there is a linear relationship between RAR γ gene expression and ligand amount (McGrane 2007), we can state that the higher RAR γ gene expression observed in group B larvae fed *C. typicus* nauplii followed by copepodites may be related to a higher content of total carotenoids (especially astaxanthin) in the copepods respect to *Artemia.* The abundance of this pigment in copepods has mainly been related to its activity in copepod metabolism, e.g. antioxidant within food reserves, in photoadaptation and chemoreceptive processes Lotocka et al 2004) and is supported by several studies conducted on different copepod species (Shields et al 1999; Andersson et al 2003; Sommer et al 2006; van der Meeren et al 2008). Moreover, a study conducted on *Pseudocalanus acuspes* revealed a stage dependent content of astaxanthin in this copepod species indicating that both nauplii and copepodites are rich in this pigment: 321±98 μg/g of copepods in nauplii, 295 ±11 μg/g in copepodites I-III and 221 ±123 μg/g of copepods in copepodites IV-V (Lotocka et al 2004) .

Asthaxantin has also demonstrated profound antioxidant properties in fish larvae suppressing lipid peroxidation (Bell et al 2000). Impairment of survival and growth in fish fed oxidized lipids has been observed in several species including rainbow trout, yellow tail and African catfish (Murai et al 1988; Baker & Davies 1997). Antioxidants such as vitamin A are regarded to be very important for the prevention of lipid oxidation, since it is degraded to protect PUFAs against oxidation in larval fishes (Sargent et al 1997). Rapid growth and formation of cell membranes in fast growing larval fish count for high PUFAs requirement with the risk of high oxidative stress (Ahlemeyer et al 2001; Palace & Werner 2006). Oxidative stress is known to impair survival and growth in several species (Murai et al 1988; Baker & Davies 1997; Palace et al 1999). From HSP70 gene

expression analysis, we observed that fish fed copepods always show lower gene expression of this gene; this may be related to the higher content of vitamin A precursors (astaxanthin) and the better fatty acid profile of copepods rather than *Artemia* and rotifers.

Conclusions. In this study we evidenced that *C. typicus* nauplii administration positively affected the gene expression of PPAR-α, -β, and RAR γ in addition to significantly reduce the expression of HSP70. Moreover, both copepod nauplii and copepodites always showed a better content and ratio of DHA and EPA respect to the traditionally used rotifers and *Artemia* nauplii and a better content of carotenoids resulting in a better survival and growth of *A. clarkii* larvae. These results evidenced the superior quality of the Mediterranean calanoid copepod *C. typicus* as live prey for *A. clarkii* larviculture and are essential for a future development of the captive propagation of this species.

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