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Preserved copepods as a new technology for the marine ornamental fish aquaculture: A feeding study

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ABSTRACT

The aim of this study was to evaluate the potential use of preserved copepod as prey in *Amphiprion clarkii* larviculture. After hatching, *A. clarkii* larvae were divided in three experimental groups for feeding studies as follows: group A (control group) fed rotifers (*Brachionus plicatilis*) followed by *Artemia* nauplii; group B fed a mixed diet of rotifers-*Artemia salina* nauplii and preserved copepods and group C fed preserved copepods solely. In this study we observed a positive effect of feeding preserved copepods in *A. clarkii* larviculture as a supplement food to the traditional diet based on rotifers and *Artemia* nauplii. In group B larvae, fed a combination of rotifers/*Artemia* and copepods, a significant increase of insulin like growth factor I and II, peroxisome proliferator activated receptor α−β and thyroid receptor α and β gene expression together with a significant decrease of myostatin gene expression was evidenced by real time PCR compared to the other experimental groups. In this same group we also observed the best results in terms of growth (total length and weight) and survival. These preserved copepods may be considered a suitable food for marine fish larvae larviculture when used as a supplement to the traditional diet based on rotifers and *Artemia* nauplii.

1. Introduction

The main critical bottleneck that scientists have to face in rearing fish larvae is the transition from an endogenous to an exogenous feeding by the larvae. Most of the commercial species are reared using rotifers (*B. plicatilis*, *B. rotundifornis*) and *Artemia* nauplii since they can be cultured in large quantities at high densities. Unfortunately, using rotifers and *Artemia* during this early period in life history does not always promote optimal larval growth since these live prey may contain an inadequate fatty acid profile and, in some cases, be of an inappropriate size (Kahan, 1981; Sargent et al., 1999; Holt, 2003; Olivotto et al., 2003; Faulk and Holt, 2005). Because of this, there is a need for identification of alternative food sources that do not have these inadequacies and can promote satisfactory growth (Sun and Fleeger, 1995). It is well established that copepods, copepodites and nauplii are feeding items preferred by fish larvae and when used as live prey (solely or in combination with rotifers and *Artemia* nauplii), they usually dominate the gut content of the larvae (Holt, 2003). Copepods show a wide range of body size between nauplii and adults, typical movement that stimulate the predatory activity of the larvae, and they also show a high content of highly unsaturated fatty acids (HUFAs) (Delbare et al., 1996). 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). Deficiencies in these fatty acids can cause a general decrease of larval health, poor growth, low feed efficiency, anaemia and high mortality (Sargent et al., 1999; Bell et al., 2003; Olivotto et al., 2003, 2005, 2006b; Faulk and Holt, 2005;).Moreover, the retention time of copepods in the gut of fish is much higher than for the *Artemia* which passes through the digestive system very fast. This quality of copepods ensures that they are better digested and therefore offer more potential for nutrient uptake to the fish than *Artemia* (Pedersen et al., 1989).

For these reasons and with the increasing worldwide interest in aquaculture, copepods may be considered as a valid and alternative food source for the culture of many larval fish. The use of cultured copepods in intensive fish larviculture (Pedersen et al., 1989; Van der Meeren and Naas, 1997; Papandroulakis et al., 2005) has involved calanoids such as *Acartia* spp. (Schipp et al., 1999), *Eurytemora* spp. (Shields et al., 1999), *Parvocalanus* spp. (Olivotto et al., 2006a), *Centropages typicus*

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(Olivotto et al., 2008c, 2009) and harpacticoid copepods such as *Euterpina acutifrons* , *Tisbe* spp. (Kahan et al., 1982; Støttrup and Norsker, 1997; Olivotto et al., 2008a,b) and *Tigriopus japonicus* (Fukusho, 1980). The best results have been obtained using calanoid copepods which have a higher content of HUFAs, are entirely pelagic and thus more available as prey for marine fish larvae, and usually have very small naupliar stages which are more readily captured by fish larvae with small gapes at first feeding (Payne and Rippingale, 2001; Olivotto et al., 2008a,b; Olivotto et al., 2009). Unfortunately, there are several difficulties in culturing calanoid copepods on a continuous basis, since they are usually cultured at very low densities, in large tanks, and need to be fed different algal combinations (Holt, 2003).

Alternatives may be represented by harvesting live copepods from natural habitats: these productions are highly dependent on natural meteorological cycles which limit the annual production of fish in addition to be potentially affected by parasite that use copepods as an intermediate host. Prey selection in fish larvae is affected by a great number of factors related to the characteristics of both the larvae and the prey. These factors include visual acuity (Neave, 1984), visual threshold and spectral sensitivity (Drost, 1987), prey contrast and size (Dendrinos et al., 1984), shape, mobility (Holmes and Gibson, 1986), and concentration and by the presence of chemical stimulants (Knutsen, 1992).

The aim of the present study was to test the efficiency of preserved copepods in *Amphiprion clarkii* larviculture as a possible alternative live prey to rotifers *Artemia* nauplii or alive copepods. Different feeding combinations and different sizes of preserved copepods have been tested in order to verify the efficiency of this innovative larval food on *A. clarkii* larviculture.

In this study, results obtained from morphometric analysis and live prey fatty acid composition are supported by a molecular approach. In particular, genes related to growth such as insulin like growth factor I and II and myostatin-(IGFI, II, MSTN), to lipid metabolism such as peroxisome activated proliferator receptors (PPAR-α, -β) and to metamorphosis (thyroid hormone receptor-α, -β (TR α, β) have been analyzed through real time PCR. Our results showed that preserved copepods maintained their nutritional characteristics and may be easily stored for a long time at −80 °C to be used upon request, and may improve larval fish survival and growth.

2. Material and methods

2.1. Animals and rearing system

Sexually mature fish (*A. clarkii*), measuring approximately 5–10 cm, were bought from a pet shop (Fauna Esotica, Civitanova Marche, Italy) in February 2005. The pairs were held in 200 L cubic breeding tanks in a closed recirculating system equipped with biological, UV and mechanical filtration (Panaque srl, Capranica, Italy).

The temperature in the breeding tanks was maintained at 28 ± 0.5 °C, salinity 30‰, pH 8–8.2 and NO_2 and NH_3 <0.03 mg/L. A photoperiod of 14 h light/10 h dark was provided by two 30 W incandescent lights suspended 20 cm above the water surface. Burned clay flowerpots (20 cm diameter) were placed in the tank as a substrate where the fish could spawn. The fish were fed twice a day using frozen adult *Artemia*, frozen plankton and chopped fish and shrimp.

Under these conditions, the fish started spawning 1 year after they were put in the broodstock tanks. Spawning occurred every 12 days and the egg clutches, each with about 350–400 embryos, were left to parental care for embryo development. Hatching took place at 28 °C, 144 h post fertilization. An hour before hatching, the flowerpot with the egg clutch was transferred to 20 L larval rearing tanks with the same chemical–physical characteristics of the breeding tanks. An air stone was gently put into the pot and the egg clutch was left in darkness for about 50 min. After this period synchronous hatching took place and the average percent hatch for the different clutches considered for the feeding studies was 96 ± 3%.

The water in the 20 L larval tank was gently replaced 10 times a day by a dripping system. The sides of the tank were covered with black panels to reduce light reflection, while the phytoplankton *Nannochloropsis oculata* was used (50,000 cells/mL) to condition the tanks from the first day after hatching.

2.2. Zooplankton culturing

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 necessary amount of rotifers was gently transferred to 50 L cone-shaped tanks for enrichment. Enrichment was performed with Algamac 3000 (Aquafauna Bio-Marine, Inc., Hawthorne, CA USA) using 0.5 g/million rotifers. Algamac 3000 was homogenized in 500 mL salt water for 1 min and then distributed to the rotifers for enrichment. As recommended by the company, enrichment lasted for 8 h.

AF 430 *Artemia salina* 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 24 h. After hatching, the nauplii were separated from the cysts by siphoning and enrichment was performed with Algamac 3000 (0.2 g/100,000 *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 10 times with clean seawater (salinity 30‰) in order to remove the remaining enrichment.

A mixture of Copepodites (CI–CIII) and nauplii (NIII–NVI) of the copepods *Temora longicornis* (76%), *Acartia clausi* (12%), *Centropages hamatus* (7%) and others (5%; *Pseudocalanus* spp., *Microcalanus* spp. and *Oithona similis*) were harvested outside Herøy in Northern Norway during spring 2009. The zooplankton were harvested using horizontally towed plankton net (10 m^2 mouth opening, 150 μm mesh size) at 1 knot speed for 6 h at 1 m depth. After landing of the catch, the zooplankton was screened on a 400 μm plankton net to remove large copepods. The size fraction 150–400 μm was thereafter further separated into size fractions 2–300 and 3–400 by sieving using a nylon filter mesh. 2–300 and 3–400 μm size fractions represents the width of the copepodites and nauplii, and their average length were approximately twice the width. The size fractions consisting of copepodites and nauplii were processed using a patent pending method, and thereafter packed in 20 ml plastic vials (polyethylene) and immediately frozen using liquid N_2. Number of copepodites/nauplii in the 2–300 and 3–400 μm size fractions were approximately 600 000 and 200 000 per gram, respectively. The preserved copepodites/nauplia were stored in a −80 °C freezer. Analysis showed a percentage dry weight (DW) of 10.5 ± 0.2 (SD) of the frozen copepodites/nauplii. Immediately after thawing of the copepodites/nauplii, the leaking of water soluble proteins were 1.5 ± 0.9 (mg/g wet weight). Leaking of water soluble proteins increased to 4.3 ± 0.1 (mg/g wet weight) when copepodites/nauplia were kept in sea water (12 °C) for 4 h.

The right amount of preserved copepodites/nauplii was defrozen before feeding the larvae in order to obtain a final concentration in the larval tank of 5 ind/mL.