New species for EU aquaculture

Deliverable Report

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Deliverable Title	Determining the effect of co-feeding ciliates and rotifers on digestive tract maturation and enzyme production		
WP No:		**WP Lead beneficiary:**	P4. IOLR
WP Title:	Larval Husbandry-grey mullet		
Task No:	19.2	**Task Lead beneficiary:**	P4. IOLR
Task Title:	Determine the effect of co-feeding ciliates and rotifers on digestive tract maturation and enzyme ontogeny		
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Objective: Determine the effect of co-feeding copepods and rotifers on growth, survival and digestive tract maturation and pancreatic and brush border enzyme ontogeny in larval grey mullet.

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1.0 Introduction

In the larviculture of grey mullet from first feeding to about 10 days post-hatching (DPH) mortality is very high, which has become a bottleneck to commercial larval rearing. After hatching, the pre-larvae depend completely on the endogenous nutrient reserves of the yolk sac for neural and organ development until its depletion. The end of endogenous feeding is synchronized with the pigmentation and function of the larval eyes as well as the opening of its mouth and anus. However, the larvae of species such as the grey mullet (*Mugil cephalus*), rabbitfish (*Siganus rivulatus*) and grouper (*Epinephelus aeneus*) have an exceedingly small mouth gape (*ca*. 100 µm) at first feeding. The zooplankton prey that is frequently provided in commercial hatcheries world-wide are small rotifers called *Brachionus rotundiformis* and range in size from 90-210 µm. This live food is readily consumed by the larger larvae of gilthead sea bream (*Sparus aurata*) and other commercial teleosts but can still test the physical limits of the larval mouth gape of grey mullet. As a general rule, larvae tend to seek out prey ranging from 25-50% of their mouth gape (Busch, 1996; Østergaard et al., 2005). It was generally believed that the prey organism at first larval feeding were the early instar stages of copepods, which would tend to be smaller than 100 µm and have superior nutrition to enriched rotifers. However, a number of authors have challenged this paradigm and argued that smaller protozoa such as soft bodied ciliates, which move slower than copepods, are actively selected for over copepods (Ikewake and Tanaka 1993) by first feeding larvae and form a major part of the diet during the first few days of this critical growth and survival period (Nagano et al. 2000; Rhodes and Phelps 2008). Successful first feeding is critical to normal neural development and growth where a delay of a day or even hours, depending on species and water temperature, can lead to abnormal behavior, atrophy of musculature, degeneration of the alimentary tract, reduced absorption efficiency and feeding activity leading to massive mortality (Gisbert et al. 2004).

Zo-Opt Ltd of Geva Ada, Israel had established know-how to sustainably mass produce, suitably sized (ca 60 µm), highly concentrated (50,000 individuals ml^{-1}) and clean cultures of ciliates (*Euplotes* spp.) as a first feed for the rearing of marine fish larvae. In the original DOW, this company was to be sub-contracted to supply ciliates to test on first feeding larval grey mullet. Apart from measuring larval performance in terms of growth and survival, this task was also planned to determine if digestive tract enzyme ontogeny, as a function of ingesting smaller prey immediately after the onset of exogenous feeding, was modulated.

Unfortunately, Zoopt closed a few years after the project "Diversify" began and it was impossible to carry out this task as originally planned. Consequently, **P4.IOLR** received permission from the EU to conduct a similar study but using copepods (*Tisbe* spp.) instead. At the time, P4.IOLR was developing protocols to produce sufficient quantities of these copepods in order to carry out small scale experiments that would co-feed these zooplankters with rotifers to early developing grey mullet larvae. The aim was to improve grey mullet larval survival, growth and advance the ontogeny of pancreatic and brush border enzymes.

2.0 Materials and methods

Copepod culture

The protocol for the production of *Tisbe* spp. copepods was developed at **P4.IOLR**, where the culture system consisted of 40 plastic carboys where the top had been cut off to give a volume of *ca*. 17 l (**Fig. 1**). The carboys were filled with filtered (10 µm), UV treated, ambient seawater (40 ‰) at 27 ^{o}C and supplied with light aeration and illuminated with a light intensity of 500 lx before being seeded with a copepod culture. The copepods were given a mixture of *Isochrysis* and *Tetraselmis* spp. microalgae every 3 days while every 5 days the water was changed through filtering the copepods on a 40 µm mesh. This was a very labor intensive and time consuming process but resulted in a mixed culture of copepodites and adult copepods whose sizes are shown in **Fig. 2** and **Table 1**. However, before filtering, washing the copepods in filtered, UV treated seawater and feeding them to larval mullet, these zooplankters must be disinfected as they can be a vector for the red pigmented pathogenic bacterium *Pseudoaltermonas* spp., which can decimate or eradicate larval populations of grey mullet and other species (**Fig. 3**).

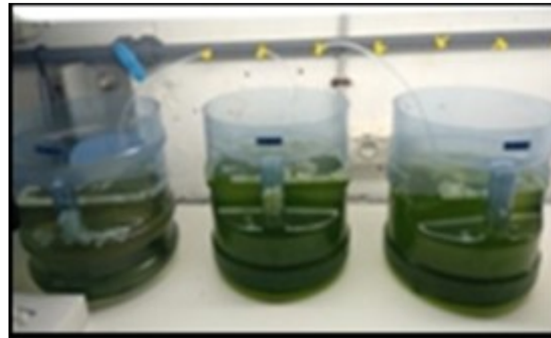
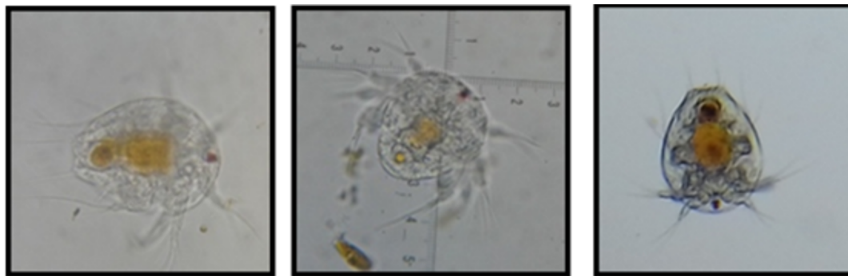


Figure 1 Copepod culture system consisted of 40 plastic carboys with tops cut off having a volume of 17 l.

(a)



(b)

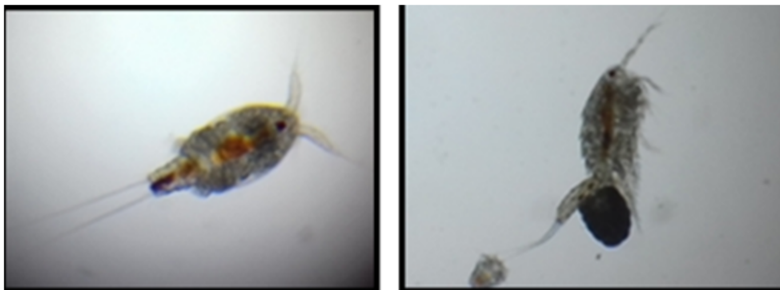


Figure 2 (a) 90 µm copepodites and (b) >400 µm adult copepods with egg sac.

Table 1 the weight, length and width of reared Tisbe copepods reared at P4.IOLR

	<100 µm	>300 µm	replicates
DW (µg)	3.21±0.05	10.87±0.06	50
Length (µm)	98.9±10.2	782.7±264	13
Width (µm)	30.7±2.7	244.3±141	13

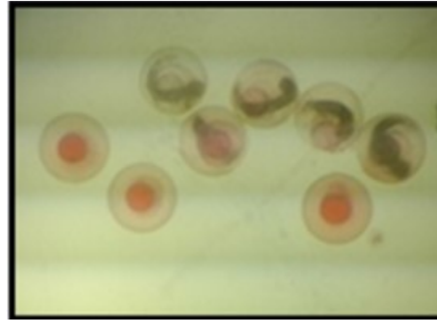


Figure 3 Pink pigmented *Pseudomonas* bacteria in eggs and killing mullet embryos.

Copepod disinfection studies

Four experimental protocols to disinfect the copepods were tested. In experiment 1 the copepods in water medium were treated with the commercial product "Micro-control (Rich S.A., Faliro, Greece), which is a multipurpose aquaculture disinfectant for algae, rotifers and *Artemia*. A range of concentrations (ppm or µg l^{-1}) from 25 to 300 ppm were tested on copepod cultures with exposure periods of 5, 10, 15 and 20 minutes.

Experiment 2 tested 5 minute commercial polydine (10%) treatments using different concentrations of this product (0.5, 1.0, 3.0 and 5.0 ml l^{-1}) which resulted in total copepod mortality and therefore unsuitable as a disinfectant for this zooplankter. Experiment 3 tested the effect of the antibiotic nitrofurazone on the copepod culture. Various concentrations (1, 3, 5, 8 ppm) were added to the copepod medium where the zooplankters were exposed for 22 h.

Experiment 4 used ozone as a disinfectant and was the most successful of all these trials. Two concentrations (0.1 ppm and 0.47 ppm) were tested with a 3 min exposure period (CT values were 0.3 and 1.41, respectively). The ozone in the water medium was neutralized by using sodium thiosulphate (1 ppm O_3/2 ppm $Na_2S_2O_3 \cdot 5H_2O$). Copepod samples were taken and plated on agar plates, which were incubated at 25 ^oC for 22 h in order to monitor any bacterial growth.

Experimental system

The experimental system was comprised of fifteen 17 l aquaria, from a 60 aquaria assay system (**Fig. 4**), fed by filtered (10 µm), UV treated diluted seawater (25 ‰) at 25 ± 0.5 ^oC with an exchange rate of 3 aquaria volumes per day. This allowed the testing of the three rotifer: copepod ratio treatments; (1) control- 10 rotifers ml^{-1}: 0 copepods ml^{-1}, (2) 5 rotifers ml^{-1} : 30 copepods ml^{-1}, (3) 0 rotifers ml^{-1} : 60 copepods ml^{-1} in triplicate aquaria per treatment.

Figure 4 One side the sixty 17 l aquaria system with computer controlled salinity and temperature.

Larval stocking

On 4 separate occasions the 15 aquaria were stocked with hand-counted eggs or larvae; 1. eggs (100 eggs l^{-1}), 2. newly hatched larvae (1000 larvae $aquarium^{-1}$), 3. 14 dph larvae (1000 larvae $aquarium^{-1}$), 4. 14 dph larvae (500 larvae $aquarium^{-1}$). A fifth attempt was made with 25 dph larvae (50 larvae $aquarium^{-1}$), which did survive in the aquarium system, to test the effect of replacing *Artemia* nauplii with larger copepods (>300 µn). However, the copepod production could not produce sufficient numbers of these larger zooplankters to feed the fish in the aquaria according to the experimental design. On the other hand, grey mullet have already started weaning at 25 dph onto a dry food, which is completed, by 35 dph. As a result this study, had it succeeded, would be of limited value. Unfortunately, all eggs and larvae died within 24 h of the start of these aquarium trials and the objective of this study could not be completed.

3.0 Results and Discussion

The results of testing the "Micro-Control" product with exposure periods of 5, 10, 15 and 20 minutes with concentrations of 100, 190 and 300 ppm are shown in **Table 2**. All concentrations and exposure periods tested resulted in significant mortality and/or moribund copepods even at concentrations well below that recommended for disinfecting rotifers (50 ppm). In addition, the use of 10% polydine proved lethal to use and deemed unsuitable for disinfecting copepod cultures. In contrast, there was very little mortality in the nitrofurazone treatments but it was problematic to remove this antibiotic from the water, which is necessary as it is lethal for the larvae. The copepods could be removed from the medium through a filter but this was also a long process and the extended exposure to this antibiotic presented a potential health hazard to staff.

However, the ozone treatments were very promising at both CT values. The results are shown in **Fig. 5**, where **Fig. 5a** demonstrated that the filtered UV treated hatchery seawater, used for washing the copepods, had no bacterial growth, while the copepod medium that was not treated with disinfectant had a considerable number of colonies (**Fig. 2b**). On the other hand, the plates from the treatments having CT values of 0.3 and 1.41 demonstrated very few and no bacterial colonies, respectively (**Fig. 5c, d**). **Fig. 6** shows the copepod production after ozone treatment and washing with fresh, filtered, UV treated seawater.

The complete mortality following three attempts to transfer eggs, pre-larvae and end of rotifer feeding larvae to the aquaria was extremely frustrating as a protocol for the culturing of copepods of high quality with a low bacterial load was established and ready to be implemented. Although eggs and larvae transferred to the aquaria appeared of high quality and robust, we can only conclude that grey mullet larvae are extremely

sensitive and all handling should be avoided before 25 dph. Future studies should endeavor, when copepod production has been scaled up, to use the same tanks for stocking eggs and co-feeding the larvae with rotifers and copepods.

Table 2 The effect of different concentrations and exposure periods of copepods to the disinfectant product Micro-control (Rich S.A., Faliro, Greece)

Time	**100 ppm**	**190 ppm**	**300 ppm**
5	no mortality	no mortality	no mortality
10	no mortality	partial mortality	partial mortality
15	no mortality	partial mortality	complete mortality
20	complete mortality	complete mortality	complete mortality

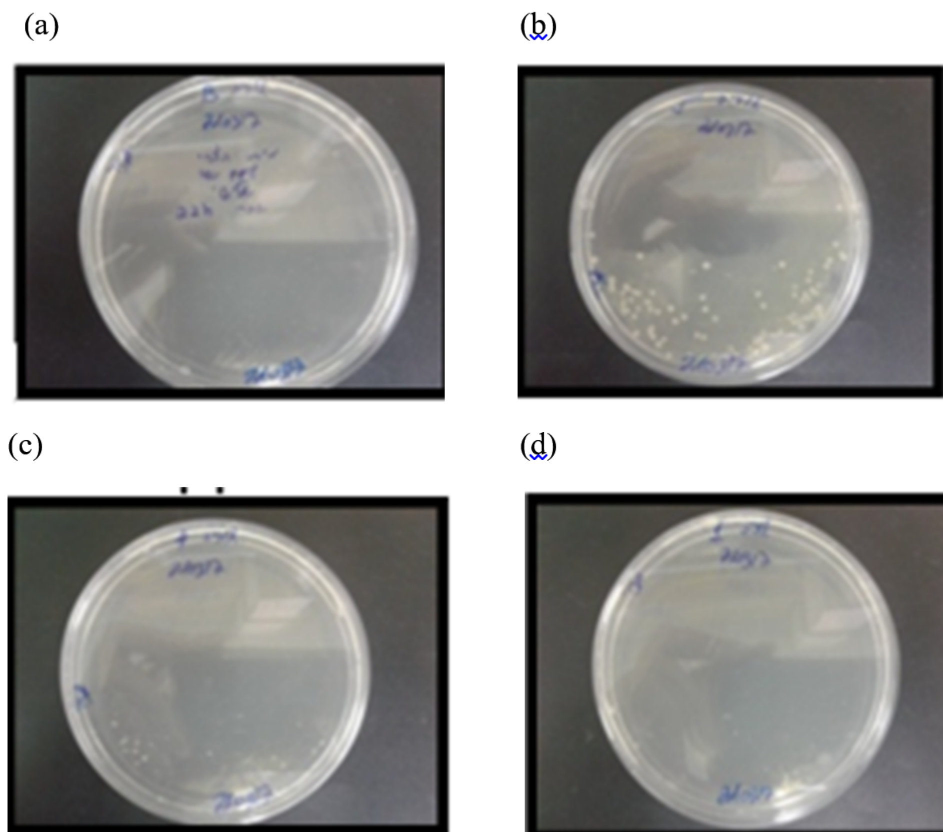


Fig. 5 Bacterial colonies on plates streaked with (a) filtered, UV treated water used for washing copepods, (b) copepods and medium without disinfectant treatment, (c) copepods and medium with CT 0.3 and (d) CT 1.41 ozone treatments

Figure 6 Copepods after ozone disinfectant and washing using filtered, UV seawater.

4.0 References

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