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Amyloodinum ocellatum in *Dicentrarchus labrax*: Study of infection in salt water and freshwater aquaponics

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ABSTRACT

This investigation is aimed to improve the knowledge on the physiological alterations occurring at morphological and molecular level in European sea bass naturally infected by *A. ocellatum* and reared at different salinities.

European sea bass juveniles (*Dicentrarchus labrax*) weighing 20 ± 0.5 g were divided in three aquaponics systems: CTRL, reared at 20 ppt salinity; AFI, reared in freshwater (0 ppt) and infected with the dinoflagellate *Amyloodinium ocellatum*; ASI, reared at 20 ppt salinity and infected with *A. ocellatum*. *Beta vulgaris* plants were introduced in each of the aquaponic systems. Temperature was increased 1 °C every second day from 18 to 25 °C during the experiment.

At the end of the trial, liver, brain, intestine and gills were sampled for molecular and histological analyses. *A. ocellatum* affected *D. labrax* growth (insulin-like growth factor I, IGF-I) and appetite (Neuropeptide Y, NPY) signals in ASI. Immune system was activated in ASI by the presence of parasites by producing higher levels of Interleukin-1 (IL-1) and Tumor Necrosis Factor α (TNFα). Peroxisome proliferator-activated receptor α (PPAR α), codifying for a protein involved in lipid metabolism, was upregulated in ASI because of the necessity to produce energy to maintain homeostasis. On the contrary, *A. ocellatum* did not cause signs of infection in AFI as confirmed by gene expression and histological analysis, that were similar to CTRL. However, in freshwater reared fish, a modification of lipid metabolism was observed through a reduction in PPARα gene expression and hepatic lipid content.

1. Introduction

Diseases control and management during fish productive cycle represents one of the main concerns in the aquaculture field [1–3]. Frequently, the infectious diseases onset led to serious economic losses [4–6]. The antibiotic use enhancement in aquaculture, albeit controlled, raises issues connected with the antibiotic resistance and with the risk associated to residues in environment and edible parts of the fish [7,8]. For these reasons, research mainly focuses on the optimization of diagnostic methodology and on the prevention of infectious disease in fish farming [9,10].

Aquaponics, a new aquaculture technique that integrates landless vegetables cultivation and fish rearing, is considered less susceptible to infection outbreaks compared to other culture methodologies. This advantage is mainly related to the high management control and reduced water exchange necessary for the system. Moreover, this kind of system is not suitable for the use of chemicals for disease treatment because both antibiotics and antiparasites may affect the biofilter activity and plant survivor [11]. However, the risk of pathogen introduction in aquaponics is very limited and mostly connected with the insertion of already infected animals or reservoirs deriving from other farming sites.

A. ocellatum represents a central concern in marine fish farming, either in water and land-based systems or in new technologies such aquaponics [12].

European sea bass is an important commercial fish species susceptible to *Amyloodinium ocellatum* [13,14], an obligate ectoparasitic dinoflagellate that causes sudden mortality in marine fish [15,16]. The life cycle of such dinoflagellate consists of three stages: the trophont stage or feeding stage (parasitic stage), present on the skin and on the gills of fish [17,18]; the tomont stage or reproductive stage; finally, the flagellate dinospores able to infect new hosts [19].

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A. ocellatum is not very susceptible to pharmacological treatments however, it is known that the trophont stage is inactivated by freshwater treatment.

The present study is aimed to increase the knowledge on the biological responses in European sea bass infected by *A. ocellatum* through a multidisciplinary approach. The study was conducted in aquaponics systems specifically designed and equipped in order to host both vegetable and animal species able to tolerate salinity and temperature changes. These characteristics are essential attributes for a safe and economic control of *A. ocellatum* infection in these systems.

2. Materials and methods

2.1. Experimental design

Three aquaponics systems, realized by Aquaguide s.a.s., were composed by a fish tank (500L) and a plants grow bed (2 m^2). The water from the fish tank, rich in nutrients, was pumped into in the plant grow bed filled with expanded clay. Expanded clay, both played as a mechanical and a biological filter. To maintain appropriate water parameters, *Beta vulgaris*, a salt tolerant plant, was cultivated in combination with fish, in each aquaponic system (50 seedling/m^2). pH was set at 8.3, ammonia, nitrate and nitrate were maintained within the threshold value to safeguard the fish health.

This systems acted as "ebb and flow technology", with a discontinuous flow activated for 15 min each hour at a rate of 600 L/h.

Forty-five European sea bass juveniles (weighing 20 ± 0.5 g) were obtained from a local fishfarm which, at time of the experiment, experienced *A. ocellatum* disease in some tanks. In particular, 15 seabass were collected from tanks where no infection was observed, while 30 more fish were collected from tanks in which the farmer observed signs of *A. ocellatum* infection. The fish were acclimated for one week in two different tank systems (Panaque, Italy) equipped with mechanical and biological filtration and maintained at the following conditions: salinity 20 ppt; pH 8.0; temperature 18 °C; nitrate 2 mg/L; ammonia and nitrite were not detectable. Ten per cent of the water was changed weekly. This first week of acclimation was essential to ensure the uniformity of parasites load of infection in infected fish as well as to observe the absence of infection in the control group.

After the first week, the 15 healthy fish were stocked in an aquaponics system as CTRL and maintained in absence of the parasites at 20 ppt salinity, while 15 infected fish were moved in an aquaponic system at 20 ppt salinity (ASI groups), and 15 more infected fish were transferred in an aquaponic system and gradually acclimated to freshwater (AFI group, salinity 0 ppt). Salinity was decreased by 5 ppt every 48 h during the first week, 3 ppt every 48 h during the second week and 2 ppt every 48 h in the last acclimation week, by replacing salt water with freshwater.

The three groups of fish were fed with commercial pellets (Veronesi, Italy) (1.5% bw) once per day (corresponding to the amount of uneaten food in each experimental group was recorded as number of remaining pellets/tank after an hour from feed administration. The initial body weight was 20.2 ± 2.8; 20.3 ± 3.1 and 20.5 ± 1.8 in CTRL, AFI and ASI group respectivly.

Temperature was gradually increased (through the use of heaters, Prodac International, Italy) in all systems by 1 °C every second day up to 25 °C, starting from day 40 after the beginning of the experiment. This increase was necessary to in order to promote the parasite activity and multiplication.

Samplings (for biometric, histological and molecular analysis) were performed 60 days after the beginning of the experiment when fish exhibited an abnormal behaviour, resulting in rapid breath (perceptible by the fast operculum movement) and in the scratching on the tank walls. Abnormal behaviour was observed only in ASI group. The three fish groups were starved for one day and then anesthetized with MS222 (Sigma Aldrich) (1 g/L), following the guidelines of the European Union directive 2010/63/EU on the protection of animal for scientific purpose.

2.2. HIS and SGR determination

Total body and liver weight were recorded using a precision balance (Radwag®,±0,001 g) to determine the hepatosomatic index (HIS) and the Specific Growth Rate (SGR).

2.3. Histology and lipid content

Six samples of liver, intestine and gills from each experimental group were sampled and fixed in 4% PFA and stored at 4 °C for 24 h. Tissues were washed 3 times with PBS 0.1 M (pH 7.4) for 10 min and preserved in ethanol (75%). Samples were dehydrated by subsequent washing in ethanol (75, 95 and 100%), washed with clearing agent "Histo-Clear" (Bio-Clear, Bio Optica) and embedded in liquid paraffin (Bio-Optica, Milano, Italy) at 55–58 °C. Solidified paraffin blocks were cut with microtome (Leitz 1512) in order to obtain sections of 5 μm then stained with hematoxylin (Mayer) and eosin Y (Sigma-Aldrich). Sections were observed using Zeiss Axioskop optical microscope connected with a camera Canon EOS 6D. Resolution was 20× for liver and gills and 40× for intestine. Digital images were analysed with Image J ver. 1.49.

Hepatocytes vacuolization was measured to estimate lipid content in the liver, as described by Papadakis et al. [20] Microphotographs were used from different areas of the liver (n = 3) of each specimen (n = 6) from the three groups. During computer image processing, all tissue components that did not have any relationship with the hepatic tissue were manually excluded from the image. Finally, the area covered with lipid vacuoles (ACLV %) in each microphotograph was calculated automatically by the software.

2.4. Cortisol assay

Blood samples from 10 specimens randomly collected from each experimental group were added with heparin (0,1 ng/mL)

Table 1
List of Real Time primers designed on *D. labrax*.

Genes	Forward	Reverse
18s	5′-GTGAGGTTTCCCGTGTTGAG-3′	5′-GACCATAAACGGTGCCAACT-3′
IGF I	5′-TACAGGCTATGGCCCCAAT-3′	5′-TTGGCAGGTGCACAGTACAT-3′
NPY	5′-GGAGCTGGCCAAGTACTACTCA-3′	5′-GAGACCAGCGTGTCCAGAAT-3′
PPARα	5′-TGCAAGGGTTTCTTCAGGAG-3′	5′-GCAGTACTGCTTGTTGC-3′
IL-1	5′-AACTCCAACAGCGCAGTACA-3′	5′-AGACTGGCTTTGTCCACCAC-3′
TNFα	5′-GACTGGCGAACAACCAGATT-3	5′-GTCCGCTTCTGTAGCTGTCC-3′
GR	5′-GCCTTTTGGCATGTACTCAAACC-3′	5′-GGACGACTCTCCATACCTGTTC-3′