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NEW PERSPECTIVES IN THE CONTROL OF *AMYLOODINIUM OCELLATUM* INFECTION IN REARED SEA BASS (*DICENTRARCHUS LABRAX*)

Doctoral dissertation of

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Acta est fabula

(Augusto)

Ai miei genitori, a Luca,

alle mie famiglie, ai miei amici,

alle mie belve e a tutti coloro

che hanno reso possibile questa tesi

Summary

1. *Premise*

The increased scale of aquaculture production, characterised by intensive fish farming, has led to various disease problems for the main cultured species, including parasitic infections. Since there are a very few licensed medicines available and currently no effective vaccines against protozoan or metazoan parasites exist, the improvement of the understanding of fish-parasite relationship may contribute in developing innovative solutions and tools for the prevention, control and mitigation of the major parasites affecting the most valuable reared finfish species (i.e. Atlantic salmon, rainbow trout, common carp, European sea bass, gilthead sea bream and turbot).

The objective of the present PhD thesis was to collect novel information on the ectoparasite protozoan *Amyloodinium ocellatum* (AO), a globally distributed protist affecting various aquatic organisms in both brackish and full seawater environments. Amyloodiniosis (the infection provoked by AO) is a major threat for semi-intensive aquaculture (valliculture or inland brackish water farming) in Southern Europe and in different aquaculture facilities worldwide since, especially in the warmest months, mortality can reach 100%. In order to develop more targeted treatments against amyloodiniosis, several studies have been performed to deepen and clarify the interaction mechanisms underlying the host-parasite relationship. For the trials, European sea bass (ESB, *Dicentrarchus labrax*) was selected as target host as this species represents one of the most economically important reared finfish in the Mediterranean basin.

The investigations and challenges carried out during these three years explored: 1) environmental factors exploited by AO to survive/persist in the environment; 2) alternative protocols for the *in vitro* preservation of the parasite; 3) physiological data collected on the host immune response by using inedited technologies; 4) the effects of a panel of selected plant-derived compounds as potential immunomodulators or parasiticidal substances.

The results exposed in this PhD thesis are to be intended as preliminaries and further investigations will be necessary to confirm the data collected. Anyway, these findings contributed in a better comprehension on the ecology and biology of AO; furthermore, by these studies, it was possible to investigate the immune response mechanisms adopted by the ESB during the infection, describing and localising the recruited cell populations and some synthesised inflammatory mediators.

Experiment outcomes reported here may represent the starting point for the development of innovative solutions for the control of this parasitic disease.

This PhD thesis has been developed within the framework Horizon2020 project ParaFishControl (grant agreement 634429). Some of the experiments reported in this PhD thesis were funded by the Horizon2020 project Aquaexcel (grant agreement 652831).

2. *Premessa*

L'aumento della produzione nel settore acquacolturale, caratterizzato da sistemi d'allevamento intensivi, ha comportato anche un aumento della frequenza di varie malattie, tra cui le parassitosi, per le principali specie allevate. Attualmente pochi sono i medicinali disponibili e autorizzati in acquacoltura, e siccome non esistono ancora vaccini efficaci contro parassiti di specie ittiche quali salmone atlantico, trota iridea, carpa, branzino, orata e rombo, una migliore comprensione della relazione ospite-parassita potrebbe contribuire allo sviluppo di soluzioni e strumenti innovativi per la prevenzione, il controllo e la mitigazione delle principali malattie parassitarie in grado di limitare la produttività dell'industria acquacolturale.

L'obiettivo della presente tesi di dottorato era quello di raccogliere nuove informazioni sul protozoo ectoparassita *Amyloodinium ocellatum* (AO), un dinoflagellato distribuito globalmente che interessa vari organismi acquatici sia di acqua salmastra che marina. L'amyloodiniosi (l'infezione provocata da AO) è una delle principali minacce per l'acquacoltura semi-intensiva (vallicoltura o in bacini di terra) nell'Europa meridionale e in diverse realtà produttive in tutto il mondo; poiché, specialmente nei mesi più caldi, la mortalità può raggiungere il 100%. Al fine di sviluppare trattamenti più mirati contro l'amyloodiniosi, sono stati condotti diversi studi per approfondire e chiarire i meccanismi di interazione alla base della relazione ospite-parassita. Per le prove, il branzino (ESB, *Dicentrarchus labrax*) è stato selezionato come ospite di riferimento, poiché rappresenta una delle specie ittiche allevate economicamente più importanti nella produzione acquacolturale del bacino Mediterraneo. Le indagini e le prove svolte durante questi tre anni hanno esplorato: 1) i fattori ambientali sfruttati da AO per sopravvivere/persistere nell'ambiente; 2) protocolli alternativi per la conservazione *in vitro* del parassita; 3) dati fisiologici raccolti sulla risposta immunitaria dell'ospite utilizzando tecnologie inedite; 4) gli effetti di un pannello di composti selezionati derivati da piante quali potenziali immunomodulatori o parassiticidi.

I risultati esposti in questa tesi di dottorato sono da intendersi come preliminari e ulteriori indagini saranno necessarie per confermare/approfondire i dati raccolti. Ad ogni modo, questi studi hanno contribuito a una migliore comprensione dell'ecologia e della biologia di AO; inoltre, da queste investigazioni è stato possibile studiare i meccanismi di risposta immunitaria adottati dall'ESB durante l'infezione, descrivendo e localizzando le popolazioni di cellule reclutate e alcuni mediatori infiammatori sintetizzati. I risultati degli esperimenti qui riportati possono rappresentare il punto di partenza per lo sviluppo di soluzioni innovative per il controllo di questa parassitosi.

Questa tesi di dottorato è stata sviluppata nell'ambito del progetto Horizon2020 ParaFishControl (convenzione di sovvenzione 634429). Alcuni degli esperimenti qui discussi sono stati finanziati dal progetto Horizon2020 Aquaexcel (convenzione di sovvenzione 652831).

3. *Amyloodinium ocellatum*

3.1 PREFACE

Amyloodinium ocellatum (AO) is an ectoparasite protozoan belonging to the *Dinoflagellata* phylum. The phylum counts approximately 2000 living species, of which 140 are parasites of invertebrates (Debres, 1984). Dinoflagellates are unicellular organisms commonly found in both marine and freshwater aquatic ecosystems. The great majority are important primary producers and consumers in aquatic food webs, while a few are endosymbionts in invertebrates. Many dinoflagellates produce ichthyotoxins, which have caused mass mortalities in wild and cultured fish (Noga and Levy, 2006), and some are parasites, mainly of invertebrates. Anyway, only six or so genera are fish parasites (Noga, 2012); of these *Amyloodinium, Ichthyodinium* and *Piscinoodinium* are the genera on which attention is more focused, since these species can provoke significant productive losses and economic damages in fish facilities.

Amyloodinium ocellatum is the unique species belonging to *Amyloodinium* genus (class Dinophyceae, order Peridiniales and family Thoracosphaeraceae). The microorganism is worldwide distributed affecting brackish and seawater fish in tropical and temperate regions. Furthermore, AO is the only dinoflagellate capable to infect elasmobranchs as well as teleosts (Lawler, 1980). In fact, cumulative evidences show it has not a specific definitive host (Table 3.1), being isolated from four aquatic organism phyla: *Chordata*, *Arthropoda* (Aravindan *et al.*, 2007), *Mollusca* (de Souza, 2015) and *Platyhelminthes* (Colorni, 1994). The parasite represents a serious problem for both reared and aquarium fish (Landsberg *et al.*, 1994), since amyloodiniosis, the infection caused by the protozoan, can lead the host to death in less than 12 hours (Lawler, 1980) with acute morbidity and mortality around 100%. However, these two parameters considerably vary on the basis of farming typology, parasite burden, fish species and season considered (Overstreet, 1993; Kuperman *et al.*, 2001; Wang *et al*., 2001; Mladineo, 2006; Saraiva *et al.*, 2011; Bahri, 2012; Guerra-santos *et al.*, 2012; Bonucci-Moreira *et al.*, 2013).

3.1.1 Biological life cycle

AO biological life cycle is direct but triphasic (Fig. 3.2) and it can be completed in less than a week whether environmental characteristics are favourable (Noga, 1987).

The parasitizing stage is represented by the trophont (or trophozoite). In this phase, the protozoan is sessile and strictly anchored to host epithelia (gill or skin) by means of specific structures (rhizoids) (Fig. 3.1a,b). If the infection is severe, trophonts can be found also on eyes, fins and in the oral cavity (Lawler, 1980; Kuperman and Matey, 1999; Cruz-Lacierda *et al.*, 2004; Byadgi *et al.*, 2019).

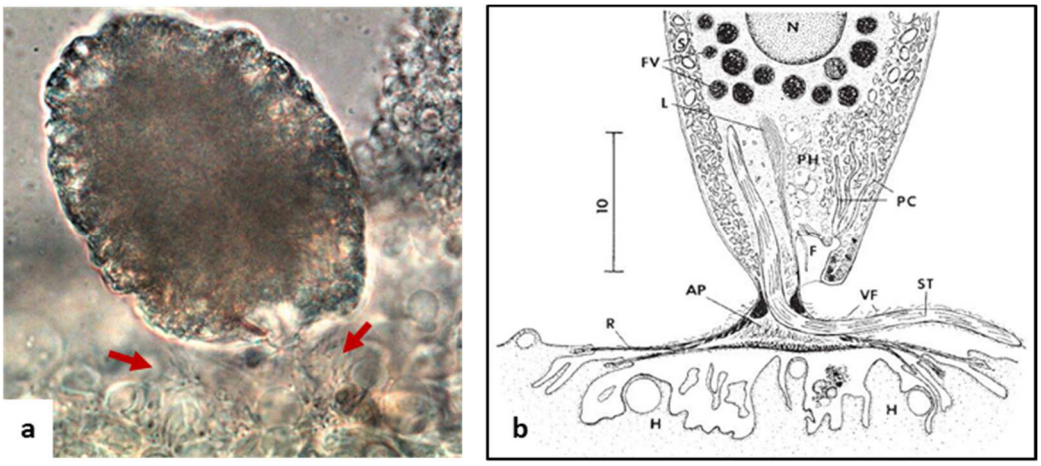

Figure 3.1. a) Trophont of Amyloodinium ocellatum anchored with rhizoids (arrows) to ESB gill epithelium. b) Schematic reconstruction of the basal portion of an attached trophont; scale is in µm; H, host cell; AP, attachment plate; R, rhizoid; ST, stomopode tube; VF, velum-like pellicular folds; F, flagellum; PC, pusular canal; L, fibrillar ledge; FV, food vacuole; N, nucleus; PH, phagoplasm. (From Lom and Lawler, 1973 and Noga and Levy, 2006).

An unusual and improbable location of trophont-like parasites was documented by Cheung *et al.* (1981). Authors sustained to have found the parasite in kidney and internal tissues of pork fish (*Anisotremus virginicus*). However, this report is incompatible with the biological characteristics of the ectoparasite and also Noga and Levy (2006) doubted the reliability of this finding. In fact, in Cheung *et al.*'s communication only tomont-like structures were seen and there was no strong morphological evidence that that organism was AO (Noga and Levy, 2006). Therefore, it was probably an artefact derived by the *post mortem* manipulation and processing of the samples.

The trophont feeds directly from the host, possibly through the prominent stalk, the stomopode (Fig. 3.1b) (Lom and Lawler, 1973; Noga, 1987; Noga and Levy, 2006); 2 to 6 days after feeding, the trophont detaches from the host and encysts transforming into the tomont, the reproductive stage. The protozoan reproduces asexually producing up to 256 new specimens (dinospores) in 2 to 4 days (Noga, 1987). The number of originated dinospores is directly correlated to the size reached by the trophont in the previous phase (Paperna, 1984a). The dinospore (8–13.5×10–12.5µm), which is the pre-infective stage, is capable to swim thanks to the presence of two flagella. After the adhesion to a new host, a dinospore transforms into a trophont within 5 to 20 minutes.

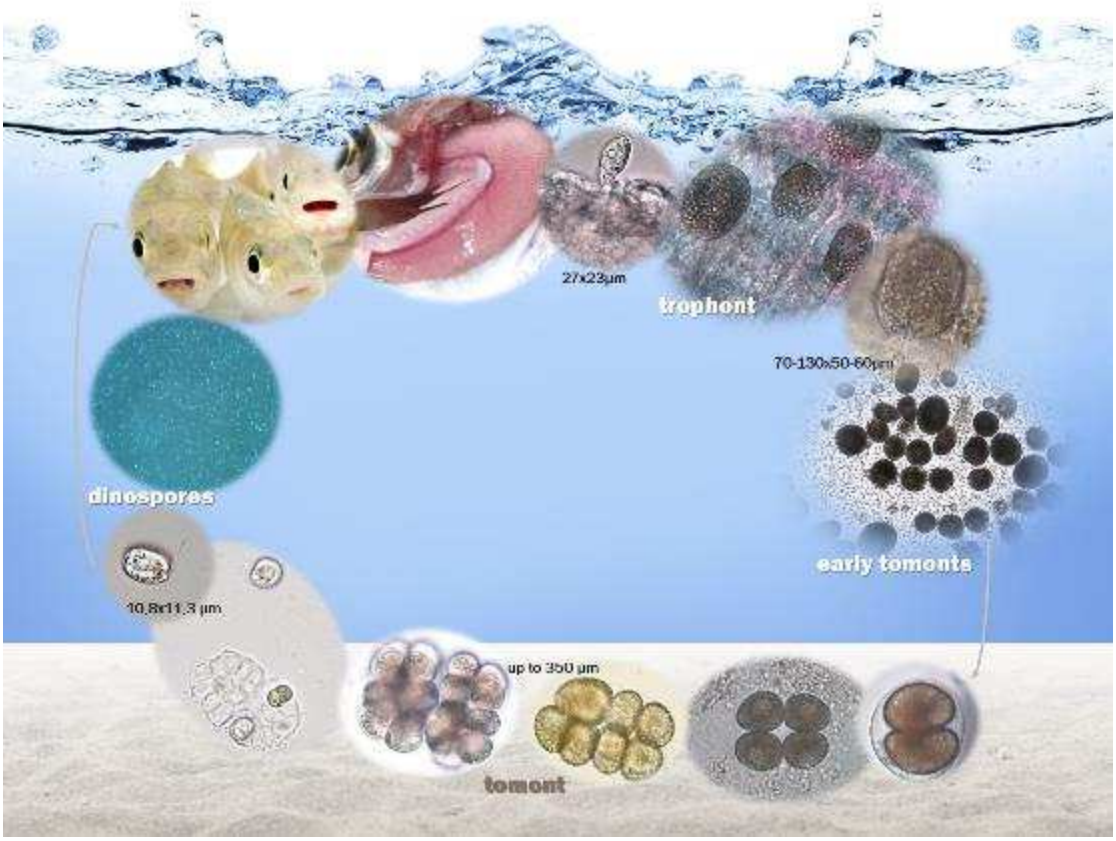

Figure 3.2. Diagrammatic representation of the lifecycle of Amyloodinium ocellatum.

3.1.2 Environmental conditions favouring amyloodiniosis

Since the 1930s, some investigations have been conducted to determine the most suitable climatic-environmental conditions for the parasite development (Brown, 1934; Nigrelli, 1936; Brown and Hovasse, 1946). Nevertheless, in the following decades AO has long been neglected, as emerged by the literature paucity, even if it can represent a limiting factor for several farmed fish species.

Paperna (1984a), studying the protozoan biology, demonstrated that the range temperature of 18-30°C guaranteed the optimal parasite development. Within this range, the dinoflagellate can complete its life cycle producing vital and active dinospores, though the minimum division rate was reported at 23-27°C. Deviating from these values, tomont division and tomites development decrease progressively to reversibly interrupt at 15°C. Lower temperatures (≤8°C) arrest completely the division and lead the parasite to death. On the other hand, over 30°C the tomont reproductive process is badly compromised with the insurgence of deformities, slowed-down or interrupted divisions and inactive dinospores release.

AO is tolerant to different salinities and this tolerance is proportionally dependent on the temperature (Paperna, 1984a; Kuperman and Matey, 1999; Kuperman *et al.*, 2001). Tomonts

division can happen within the 0.5-78 ppt salinity range, with the highest tolerance at 24-25°C. Nevertheless, infective and vital dinospores are originated only when salinity is comprised between 10-60 ppt, at the extreme values of this interval dinospores are less active (Paperna, 1984a; Bahri, 2012). An exception was documented during the summer 2017, in a facility located in the North-East of Italy where a lethal amyloodiniosis outbreak occurred even if water salinity was 7 ppt (Beraldo *et al.*, 2017). In that case, however, the infection was exacerbated also by the high water temperature and by the low dissolved oxygen concentration, which is another factor that can influence the dinoflagellate infectivity. The dissolved oxygen concentration effects on the different parasite stages have not been deepened yet; however, it is known that low oxygen levels can indirectly reinforce the negative impact of *A. ocellatum.* In fact, in a burden-dependent manner AO trophonts disrupt the host gill epithelium thus compromising the respiratory function (Sandifer *et al.,* 1993; Kuperman *et al.*, 2001).

Furthermore, some atmospheric events, such as storms and typhoons (Dequito *et al.*, 2015), have to be taken into account as possible promoters in the environmental spreading of the parasite (Beraldo *et al.*, 2017). In fact, dinospores can be transported in aerosol droplets (Roberts-Thomson *et al.*, 2006) thus contaminating other nearby facilities (Dequito *et al.*, 2015).

Finally, the parasitosis can be influenced also by the characteristics of the rearing site. Normally, sea cage reared fish are not affected by the infection (Diamant, 2001). However, an exception was documented by Rigos *et al.* (1998), a mass mortality of sharp-snout sea bream (*Puntazzo puntazzo*) reared in sea cages in Central Greece was caused by AO. The lethal outbreak was attributed by the authors to the inappropriate site selection as the distance between the lower part of the cage net and the sea bottom did not exceed 6-8 m; thus favouring the devastating effects of the parasitosis that had been exacerbated also by the high water temperature. In general, amyloodiniosis is tipically present in land- or lagoon-based rearing sites (valliculture or inland brackish farming) where shallow seabeds and poor water exchange/recirculation allow the parasite to reach its optimal proliferation values thus causing high mortality rates in Southern Europe especially in the warmest months.

3.1.3 Pathogenicity

AO is the etiological agent of the so called “marine velvet disease” or amyloodiniosis (in the past it was also named oodiniosis[1]). This infection is extremely dangerous and sometimes lethal for hosts,

[1] “Oodiniosis” derived from the previous genus name of the protozoan, which was known as *Oodinium ocellatum* (Brown, 1931). Anyway, it has not to be confused with the “freshwater *Oodinium* disease” caused by *Piscinoodium* (syn *Oodinium*) species in freshwater environments.

since it injuries the animals and promotes secondary bacterial infections. Usually juveniles are the most susceptible because they have not developed an acquired immunity against this pest yet.
The protozoan pathogenicity is associated with the trophont attachment to host tissues (Fig. 3.3a,b); trophonts constantly twist and turn slowly damaging and killing several host cells (Noga, 1987).

Figure 3.3. ESB infected gills by AO. a) Haemorrhages and a slight hyperplasia are visible; b) magnification of infected gills with numerous attached trophonts.

AO inflicts moderate-to-intense tissue reactions associated with serious gill hyperplasia, inflammation, haemorrhages and necrosis with subsequent death in less than 12 hours in heavy infected specimens (Lawler, 1980). However, some mortalities were documented also in subclinical or mild infections as a probable consequence of osmoregulatory impairment and secondary microbial infections due to the serious epithelial damage (Noga, 2012).
The symptomatology can be evaluated by some sudden host behavioural changes, which are more evident when the disease is severe. Amyloodiniosis is characterised by clinical signs such as apathy, dyspnoea, increased respiratory rate with laboured breathing and gathering at the water surface. Jerky movements and pruritis have also been documented as typical symptoms of the infection (Brown, 1934; Brown and Hovasse, 1946; Lawler, 1980; Noga, 1996), the latter is probably due to the effect of the digestive enzymes released by trophonts (Lom and Lawler, 1973). Anorexia and decreased appetite are frequently associated with prolonged infections as a possible consequence of the reduced production of the neuropeptide Y (Nozzi *et al.*, 2016). Another clinical sign of amyloodiniosis is the dusty appearance of the skin, even if fish death can occur before this clinical feature is evident.

3.1.4 Diagnosis

There are two major diagnostic approaches. Classic methodology (CM) is the most applied for amyloodiniosis diagnosis in field. Another approach, recently developed, utilises immunological and molecular techniques. Molecular identification can be applied as a second, confirmatory, diagnostic step in addition to clinical and microscopic identification by CM. It can be of further help when a differential diagnosis is required, e.g. for *Cryptocaryon irritans*, and as a routine screening for subclinical infections and new aquatic habitats before introducing susceptible fish species (Bessat and Fadel, 2018).

CM is easier to apply, it is immediate and reliable. CM consists of a direct diagnosis of the target tissues (fins, eyes, skin and gills) of the infection by means of microscopy or naked eye vision, in the latter case parasites can be detected by inspecting the fish against a dark background. Anyway, trophonts are best observed using indirect illumination, such as by shining a flashlight on the top of the fish in a darkened room (Noga, 2012). In fact, if the fish size is small, animals can be restrained in a dish of water and directly examined by means of a dissecting microscope. Generally, it is better to observe trophonts in their diagnostic attachment to epithelium (Noga, 2012), for this reason snips of gills are removed from living or recently dead fish. It is noteworthy to mention that fish are best examined while still living or immediately after death, as parasites often detach shortly after host death (Noga and Levy, 2006; Noga, 2012). However, trophonts can also be removed by gently brushing or scraping the skin or gills in order to observe the detached parasites by microscopy for trophont and tomont identification (definitive diagnosis) (Fig. 3.4).

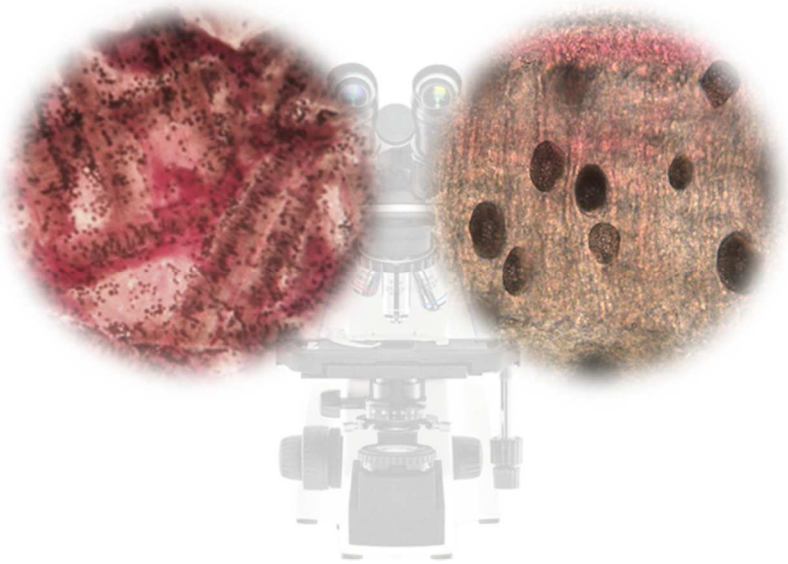

Figure 3.4. Representation of AO gills diagnostic features.

Trophonts removal can also be obtained through a fresh water bath of the infected fish or tissues, as freshwater promotes parasite dislodgement (Oestmann and Lewis, 1995; Noga, 2012). Anyway, since *Amyloodinium ocellatum* is sensitive to freshwater, salinity has to be rapidly restored.

Another approach that can be used for the detection of the parasite presence on skin and gills is the use of dilute Lugol's iodine. In fact, the iodine reacts with the starch-containing parasites (Noga, 2012; Fig. 3.5b) colouring AO in brown (Fig. 3.5a).

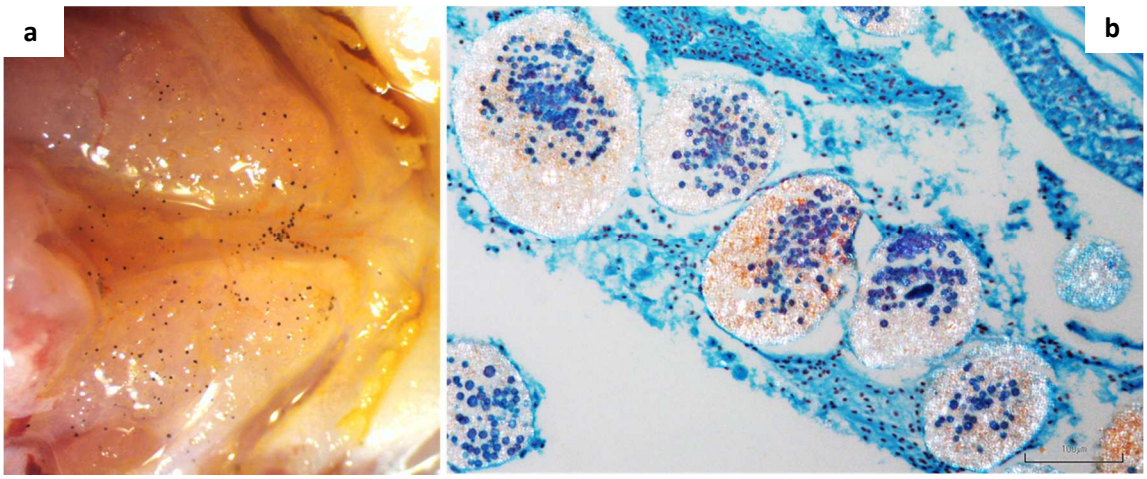

Figure 3.5. AO trophonts. a) Brownish staining of trophonts in ESB buccal cavity by Lugol's iodine solution; b) cytoplasmic starch granules highlighted with Twort Gram staining.

Molecular approaches (e.g. PCR, LAMP) of recent development has proven to be capable in specifically detecting the protozoan (Picón-Camacho *et al.*, 2013; Bessat and Fadel, 2018) also when

the parasite is present at lowest concentrations (e.g. 10 dinospores/ml of water) (Levy *et al.*, 2007). For this reason, these methods potentially allow for highly sensitive monitoring of pathogen load in susceptible fish populations (Noga, 2012). However, their application is still confined to laboratory contexts. In parallel, immunological approaches such as E.L.I.S.A. (enzyme linked immunosorbent assay) can detect the specific anti-AO antibody levels (Smith *et al.*, 1992, 1993, 1994; Cobb *et al.*, 1998a, b; Cecchini *et al.,* 2001) in fish recovering from amyloodiniosis outbreaks or that have been experimentally exposed to the parasite. The E.L.I.S.A. assay might be useful for monitoring levels of protection in susceptible populations, as elevated antibody titres have been associated with resistance (Cobb *et al.*, 1998 a,b). Finally, proteomic technology may contribute in determining the antigenic molecules of the parasite, which are still unknown. Their identification could help in the development of targeted therapies or more effective prophylaxis (e.g. vaccination).

3.1.5 Therapies and prophylaxis

Numerous efforts have been performed in order to control amyloodiniosis, but ineffectively. In fact, while dinospores are the most susceptible to treatments, trophonts and tomonts are relatively resistant (Lawler, 1977; Johnson, 1984; Paperna, 1984b; Noga, 2012), and for this reason, AO treatments' resolution is generally partial.

Actually, for food fish most of the cited practices remain suggestions or future perspectives, and usually their efficacy is contextualised to experimental investigations since some of them are expensive, with risks for the enviroment and not likely to be approved or not commercially available. For fish to be introduced in a new farm, a preventive strategy is the application of medicated baths by adding formalin or hydrogen peroxide (H_2O_2) to the water (Paperna, 1984a; Plumb, 1991; Ramos and Oliveira, 2001; Montgomery-Brock *et al.*, 2001; Fajer-Ávila *et al.*, 2003; Cruz-Lacierda *et al.*, 2004). Also freshwater baths showed to be effective against trophonts, because of the osmotic shock that determines their sudden detachment (Smith *et al.*, 1993; Cruz-Lacierda *et al.*, 2004; Abreu *et al.*, 2005; Roberts-Thomson *et al.*, 2006; Benetti *et al.*, 2008; Bonucci-Moreira *et al.*, 2013). Nevertheless, freshwater bath procedure remains impracticable for the majority of the facilities and some fish species cannot tolerate a similar treatment. Alternatively, some authors proposed the quarantine as a valid procedure to reduce the infection risk or possible cross-transmissions (Blaylock *et al.*, 2001; Ramos and Oliveira, 2001; Diamant, 2001; Calle, 2011). Another prophylactic approach was the molecular diagnostic technique proposed by Picón-Camacho *et al.* (2013). The AO loop-mediated isothermal amplification (LAMP) assay developed in their study showed to be a novel tool

for the sensitive detection of *A. ocellatum* in water and gill tissue samples, aimed at assisting in the early detection and control of amyloodiniosis episodes in aquaculture systems. LAMP may be useful for the evaluation of the parasite presence in batches under quarantine, but at the moment this approach is still confined to experimental contexts.

The infusion of salts, chemical compounds or chemotherapeutics in the water was recommended as a remedy in confirmed infection situations (Paperna, 1984b; Plumb, 1991; Aiello and D'Alba, 1986; Menezes, 1992; Oestmann and Lewis, 1996b; Cobb *et al.*, 1998b; Cecchini *et al.*, 2001; Montgomery-Brock *et al.*, 2001; Ramos and Oliveira, 2001; Ramesh Kumar *et al.*, 2015). The antimalarial chloroquine diphosphate (5-10 mg/L water) is effective against dinospores (Bower, 1987; Ramesh-Kumar *et al.*, 2015), and its oral administration in cultured red drum (*Sciaenops ocellatus*) suggested promising results in terms of oral medication (Lewis *et al.*, 1988). However, chloroquine is very expensive and is not likely to be approved for food fish.

To date, copper sulphate remains the most widely used treatment to control AO epidemics in aquaculture, due to the proven dinosporicide properties of free copper ion. The infusion of 0.75-1 g/m^3 of copper sulphate for a fortnight by dripping on ponds/tanks is lethal also to trophonts. However, it is necessary to constantly monitor copper ion levels in the water, adjusting them if needed in order to guarantee its therapeutic effects, since free copper ion is instable in seawater. Moreover, it is important not to use higher concentrations of free copper than those recommended for the control of AO epidemics, as it can be also toxic to fish as well as to most invertebrates and algae (Noga, 2012).

More eco-friendly was the treatment proposed by Oestmann *et al.* (1995). The authors promoted the usage of nauplii of brine shrimp *Artemia salina* as a bioremediation measure against the protozoan. Although the experiment had provided successful results, this remedy has not found a wide utilisation.

As preservative measures, Li *et al.* (2005) and Bahri (2012) suggested the application of immunostimulant compounds as additives to balanced or probiotic/prebiotic enriched diets. In fact, a diet supplemented with the live yeast *Debaryomyces hansenii* stimulated the immune system of juvenile leopard grouper (*Mycteroperca rosacea*) with a resulting enhanced resistance against AO (Reyes-Becerril *et al.*, 2008). Similar results were obtained by Buentello *et al.* (2010). They tested on red drum (*S. ocellatus*) juveniles the effect of several prebiotics added at 10 g/kg to their soybean-based diet noticing an improved protection against the disease.

At date, prospects for breeding resistant strains (Menezes, 1992; Bahri, 2012) are directed to the future as well as the formulation of a vaccine, which is still missing. Finally, within the Horizon2020 project ParaFishControl our research group is currently performing several investigations aimed at finding innovative and more targeted measures in the control of this dangerous pest (Massimo *et al.,* 2017a,b; Byadgi *et al.*, 2018).

3.1.6 Innate and adaptive immunity

As emerged from literature, certain wild fish species are naturally more resistant to amyloodiniosis, thanks to their capability in producing thick skin mucus or tolerating low oxygen levels (e.g. *Fundulus grandis*, *Anguilla rostrata* or *Poecilia latipinna*) (Noga and Levy, 2006; Noga, 2012).

Basically, there are no totally resistant specimens among farmed fish and juveniles are usually the most susceptible.

Trophonts feed exclusively on or within the epithelial tissues of the skin or gills. Hence, all host-parasite interactions (i.e. host recognition, defensive mechanisms responsible for protecting against these pathogens, etc.) are located in the mucus, or on/in epithelial cells and extracellular fluid of the epithelium (Noga, 2012). In an *in vitro* cell culture infectivity assay, Landsberg *et al.* (1992) demonstrated that the serum of blue tilapia (*Oreochromis aureus*), not previously exposed to the dinoflagellate, possessed parasiticidal activity at 1.25% concentration and was total inihibitory at 10% against AO.

Moreover, AO showed to be sensitive to some natural host-produced molecules with inhibitory properties, the histone like proteins (HLPs) (Noga *et al.*, 2001, 2002). These small proteins are secreted in skin and gills at high concentrations. Noga *et al.* (2001) evaluated the HLPs activity in hybrid striped bass determining their lethality on trophonts even if less effective on dinospores. On the other hand, in the same investigation, magainin 2, an antimicrobial peptide produced in the skin of the African clawed frog (*Xenopus laevis*) was equally toxic for trophonts and dinospores.

Antimicrobial peptides (AMPs) probably play a major role in protecting fish from several infections (Zasloff, 2002), having a broad-spectrum activity. One of the most common AMPs in fish are piscidins, a family of 26 amino acids long peptides with a highly conserved, histidine-rich, phenylalanine-rich N-terminus and a more variable C-terminus (Noga *et al.*, 2001). Piscidins have potent activity against viruses, bacteria, fungi and parasites. Colorni *et al.* (2008) isolated the piscidin2 from hybrid striped bass mast cells. Nonetheless, its efficacy against the dinoflagellate was variable since it is correlated with cations concentration in the water.

Another molecule class deputed in the innate defence of fish is represented by the toll-like receptors (TLRs). These receptors discriminate non-self signals by recognising the pathogen associated molecular patterns (PAMPs) of a variety of microbes and activate signalling cascades to induce innate immunity (Rauta *et al.*, 2014). TLR22 of yellowtail amberjack (*Seriola lalandi*) has been biomolecularly investigated by Reyes-Becerril *et al.* (2015). The authors defined its activity in preventing the fish species from a broad-spectrum of infections among which amyloodiniosis.

However, fish can also develop a specific immunity against the protozoan. This developed resistance was repeatedly confirmed through the detection of specific antibodies in fish sera by immunological assays as E.L.I.S.A., agglutination or western blot (Noga *et al.*, 1992; Smith *et al.*, 1992, 1993, 1994; Cobb *et al.*, 1998a; Cecchini *et al.*, 2001). Cobb *et al.* (1998a) demonstrated that some specimens of tomato clownfish (*Amphiprion frenatus*) developed a strong immunity to amyloodiniosis as a consequence of repeated nonlethal parasitic challenges. The same results were obtained by different fish species that were recovering from a spontaneous or experimental AO infection: blue tilapia (*Oreochromis aureus*) (Smith *et al.*, 1992, 1993), hybrid striped bass (*Morone saxatilis* × *M. chrysops*) (Smith *et al.*, 1994), European sea bass (Cecchini *et al.*, 2001). This is the possible explanation of the higher resistance of older fish to AO, since repeated sublethal exposures to the parasite have been associated with elevated antibody titres specific for AO (Cobb *et al.*, 1998a,b).

Table 3.1: List and classification of confirmed hosts of A. ocellatum derived from literature consultation. The table shows the site origin of the host, the nature of the infection and the relative published articles (author(s) and year).

Class	Order	Family	Species	Common name	Localisation (continent, state)	Nature of infection	Author, year
Actinopterygii	Anguilliformes	*Ophichthidae*	*Myrophis punctatus*	Speckled worm-eel	NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1980
			Ophichthus gomesii	Shrimp eel		Natural and experimental	
	Atheriniformes	*Atherinidae*	*Hypoatherina (syn. Atherina) harringtonensis*	Reef silverside	NORTH AMERICA (the UK), The Bermuda	Natural	Rand and Wiles, 1988
	Aulopiformes	*Synodontidae*	*Synodus foetens*	Inshore lizardfish	NORTH AMERICA, The U.S.A., Mississippi	Natural and experimental	Lawler, 1980
	Batrachoidiformes	*Batrachoididae*	*Opsanus beta*	Gulf toadfish	NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1980
			Porichthys porosissimus	Midshipman fish			
	Beloniformes	*Exocoetidae*	*Unknown* (flyingfish)		NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1980
	Beryciformes	*Holocentridae*	*Holocentrus ascenscionis*	Squirrelfish	EUROPE, The UK	Natural	Brown, 1931
							Brown, 1934
					NORTH AMERICA, The U.S.A., New York		Nigrelli, 1939
	Clupeiformes	*Clupeidae*	*Harengula jaguana*	Scaled sardine	NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1980
		Engraulidae	*Anchoa mitchilli*	Bay anchovy	NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1980
	Cyprinodontiformes	*Cyprinodontidae*	*Aphanius dispar*	Arabian pupfish	ASIA, Israel	Natural	Paperna, 1980
			Cyprinodon variegatus	Sheepshead minnow	NORTH AMERICA, The U.S.A., Virginia		Lom and Lawler, 1973
					NORTH AMERICA, The U.S.A., Mississippi		Lawler, 1980
		Fundulidae	*Fundulus heteroclitus*	Killifish	NORTH AMERICA, The U.S.A.	Experimental	Nigrelli, 1936
					NORTH AMERICA, The U.S.A., Virginia	Natural	Lom and Lawler, 1973
			Fundulus jenkinsi	Saltmarsh topminnow	NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1980
			Fundulus majalis	Striped killifish	NORTH AMERICA, The U.S.A., Virginia	Natural	Lom and Lawler, 1973
		Poeciliidae	*Gambusia affinis*	Gambusia	NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1980
			Poecilia reticulata	Guppy	NORTH AMERICA, The U.S.A.		Noga and Bower, 1987
	Gadiformes	*Gadidae*	*Merlangius (syn. Gadus) merlangus*	Merling	EUROPE, The UK	Natural	Brown, 1934
			Pollachius (syn. Gadus) virens	Saithe			
			Urophycis floridana	Southern Hake	NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1980
	Gobiesociformes	*Gobiesocidae*	*Gobiesox strumosus*	Skilletfish	NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1980
	Gonorynchiformes	*Chanidae*	*Chanos chanos*	Milkfish	ASIA, Philippines	Natural	Cruz-Lacierda *et al.*, 2004
							Dequito *et al.*, 2015
							Virgula *et al.*, 2017

Actinopterygii	Mugiliformes	*Mugilidae*	*Mugil cephalus*	Grey mullet	EUROPE, The UK	Natural	Brown, 1934
					ASIA, Israel		Paperna, 1980
					ASIA, Philippines		Baticados and Quinitio, 1984
					ASIA, Taiwan		Wang *et al.* , 2001
					ASIA, South Korea		Park *et al.* , 2006
					NORTH AMERICA, The U.S.A., Mississippi	Natural and experimental	Lawler, 1980
					NORTH AMERICA, The U.S.A., Hawaii	Natural	Montgomery-Brock *et al.* , 2000
					NORTH AMERICA, The U.S.A., Mississippi		Masson *et al.* , 2011
					NORTH AMERICA, The U.S.A.		Masson *et al.* , 2013
	Perciformes	*Acanthuridae*	*Acanthurus bahianus*	Ocean tang	EUROPE, The UK	Natural	Brown, 1934
			Acanthurus coeruleus	Atlantic blue tang	NORTH AMERICA, The U.S.A., New York		Nigrelli, 1939
			Paracanthurus hepatus	Surgeonfish			
			Zebrasoma flavescens	Hawaiian yellow tang	NORTH AMERICA, The U.S.A.		Cheung *et al.* , 1981b
		Apogonidae	*Apogon imberbis*	Cardinalfish	EUROPE, The UK	Natural	Brown, 1934
			Apogon maculatus	Spotted cardinal fish	NORTH AMERICA, The U.S.A., New York		Nigrelli, 1939
		Blenniidae	*Hypsoblennius hentz*	Feather blenny	NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1980
			Hypsoblennius ionthas	Freckled blenny		Natural and experimental	
			Chasmodes bosquianus	Striped blenny		Experimental	
		Callionymidae	*Pterosynchiropus (syn. Synchiropus) splendidus*	Mandarin dragonet	NORTH AMERICA, The U.S.A., Florida	Natural	Landsberg *et al.* , 1994
		Carangidae	*Caranx spp.*		EUROPE, The UK	Natural	Brown, 1934
			Caranx crysos	Blue runner	NORTH AMERICA, The U.S.A., New Jersey		Nigrelli, 1936
			Caranx hippos	Crevalle jack	NORTH AMERICA, The U.S.A., New Jersey	Natural	Nigrelli, 1936
			Caranx latus	Horse-eye jack	NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1980
			Chloroscombrus chrysurus	Atlantic bumper			
			Naucrates ductor	Pilot fish	NORTH AMERICA, The U.S.A., New Jersey	Natural	Nigrelli, 1936
			Oligoplites saurus	Leather jacket	NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1980
			Seriola dumerili	Greater amberjack	EUROPE, Italy	Natural	Aiello and D'Alba, 1986
							Rodgers and Furones, 1998
			Seriola lalandi	Yellowtail	NORTH AMERICA, Mexico	Natural and experimental (cross-transmission)	Reyes-Becerril *et al.* , 2015
			Trachinotus blochii	Silver pompano	ASIA, India	Natural	Ramesh Kumar *et al.* , 2015
			Trachinotus carolinus	Pompano	SOUTH AMERICA, Venezuela	Natural	Gomez and Scelzo, 1982
					NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1977b; 1980
			Trachinotus falcatus	Permit	NORTH AMERICA, The U.S.A., New Jersey	Natural	Nigrelli, 1936
		Centrarchidae	*Lepomis macrochirus*	Bluegill	NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1980
		Chaetodontidae	*Chaetodon capistratus*	Foureye butterflyfish	EUROPE, The UK	Natural	Brown, 1934
		Cichlidae	*Oreochromis aureus*	Blue tilapia	NORTH AMERICA, The U.S.A.	Experimental	Landsberg *et al.* , 1992
			Oreochromis mossambicus	Mozambique tilapia	ASIA, Israel	Natural	Paperna, 1980
					NORTH AMERICA, The U.S.A., California	Natural	Kuperman and Matey, 1999; Kuperman *et al.* , 2001

Actinopterygii	Perciformes	*Eleotridae*	*Eleotris pisonis*	Spinycheek sleeper	NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1980
		Ephippidae	*Chaetodipterus faber*	Atlantic spadefish	NORTH AMERICA, The U.S.A., Florida	Natural	Nigrelli, 1936
					NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1980
		Gerreidae	*Eucinostomus argenteus*	Spotfin mojarra	NORTH AMERICA, The U.S.A., Mississippi	Natural and experimental	Lawler, 1980
		Gobiidae	*Bathygobius soporator*	Frillfin goby	NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1980
			Gobioides broussonneti	Violet goby			
			Gobiosoma bosci	Naked goby		Natural and experimental	
			Gobiosoma robustum	Code goby		Experimental	
			Microgobius gulosus	Clown goby			
		Grammistidae	*Rypticus maculatus*	Whitespotted soapfish	NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1980
		Haemulidae	*Anisotremus virginicus*	Porkfish	NORTH AMERICA, The U.S.A., New York	Natural	Nigrelli, 1939
					NORTH AMERICA, The U.S.A.		Cheung *et al.* , 1981a
			Haemulon spp.		NORTH AMERICA, The U.S.A., New York		Nigrelli, 1939
			Haemulon album	White margate			
			Haemulon flavolineatum	French grunt			
			Haemulon macrostomum	Spanish grunt			
			Haemulon plumieri	White grunt			
			Haemulon sciurus	Blue striped grunt			
		Labridae	*Coris julis*	Rainbow wrasse	EUROPE	Natural (cross-transmission)	Menezes, 1992
			Labrus bergylta	Ballan wrasse	EUROPE, The UK	Natural	Brown, 1934
			Thalassoma (syn. Julis) pavo	Parrotfish			
		Latidae	*Lates calcarifer*	Barramundi	OCEANIA, Australia	Natural	Roberts-Thomson *et al.* , 2006
		Lobotidae	*Lobotes surinamensis*	Atlantic tripletail	NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1980
		Lutjanidae	*Lutjanus analis*	Mutton snapper	NORTH AMERICA, The U.S.A., New York	Natural	Nigrelli, 1939
					NORTH AMERICA, The U.S.A., Mississippi		Lawler, 1980
			Lutjanus apodus	Schoolmaster snapper	NORTH AMERICA, The U.S.A., New York		Nigrelli, 1939
			Lutjanus argentimaculatus	Mangrove red snapper	ASIA, Philippines		Cruz-Lacierda *et al.* , 2004
			Lutjanus campechanus	Red snapper	NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1980
					NORTH AMERICA, The U.S.A., Gulf of Mexico	Natural and experimental	Blaylock *et al.* , 2001
					NORTH AMERICA, The U.S.A.	Experimental	Masson *et al.* , 2011
							Masson *et al.* , 2013
					NORTH AMERICA, The U.S.A., Mississippi	Experimental	Picón-Camacho *et al.* , 2013
			Lutjanus griseus	Mangorve snapper	SOUTH AMERICA, Venezuela	Natural	Fuentes Zambrano *et al.* , 2003
					NORTH AMERICA, The U.S.A., New York		Nigrelli, 1939
					NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1980
			Lutjanus jocu	Dog snapper	NORTH AMERICA, The U.S.A., New York	Natural	Nigrelli, 1939
			Lutjanus suynagris	Lane snapper			
			Ocyurus chrysurus	Yellowtail snapper			
		Microdesmidae	*Microdesmus longipinnis*	Pink wormfish	NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1980
		Monodactylidae	*Monodactylus (syn. Psettus) argenteus*	Silver moonyfish	EUROPE, The UK	Natural	Brown, 1934

Actinopterygii	Perciformes	*Moronidae*	*Dicentrarchus labrax*	European sea bass	AFRICA, Egypt	Natural	Seoud *et al.* , 2017
					AFRICA, Egypt		Bessat and Fadel, 2018
					ASIA, Israel		Paperna, 1980
					EUROPE, The UK		Brown, 1934
					EUROPE, Italy	Experimental	Aiello and D'Alba, 1986
					EUROPE, Spain	Natural	Alvarez-Pellitero *et al.* , 1993
					EUROPE, Italy		Giavenni, 1988
					EUROPE, Portugal		Duarte *et al.* , 2000
					EUROPE, Italy		Cecchini *et al.* , 2001
					EUROPE, Turkey		Çağırgan, 2003
					EUROPE, Croatia		Mladineo, 2006
					EUROPE, Italy		Fioravanti *et al.* , 2006
							Levy *et al.* , 2007
			Morone saxatilis × M. chrysops	*Hybrids of striped bass*	NORTH AMERICA, The U.S.A.	Experimental	Bower *et al.* , 1987
					NORTH AMERICA, The U.S.A., North Carolina	Natural	Noga *et al.* , 1991
							Smith *et al.* , 1994
							Jenkins *et al.* , 1998
			Morone saxatilis	Striped bass	ASIA, Israel	Natural	Levy *et al.* , 2007
							Ullal and Noga, 2010
					NORTH AMERICA, The U.S.A., New Jersey	Natural	Nigrelli, 1936
					NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1980
		Mullidae	*Mullus barbatus*	Red mullet	EUROPE, The UK	Natural	Brown, 1934
		Polynemidae	*Polydactylus sexfilis*	Pacific threadfin	NORTH AMERICA, The U.S.A., Hawaii	Natural	Montgomery-Brock *et al.* , 2001
		Pomacanthidae	*Angelichthys isabelita*	Blue angelfish	EUROPE, The UK	Natural	Brown, 1934
					NORTH AMERICA, The U.S.A., New York		Nigrelli, 1939
			Centropyge loriculus	Flame angelfish	NORTH AMERICA, The U.S.A., Florida		Landsberg *et al.* , 1994
			Pomacanthus arcuatus	Gray angelfish	NORTH AMERICA, The U.S.A., New York		Nigrelli, 1939
			Pomacanthus imperator	Emperor angelfish	NORTH AMERICA, The U.S.A., Florida		Landsberg *et al.* , 1994
			Pomacanthus paru	French angelfish			Nigrelli, 1936
					NORTH AMERICA, The U.S.A., New York		Nigrelli, 1939
		Pomacentridae	*Abudefduf (syn. Glyphisodon) luridus*	Canary damsel	EUROPE, The UK	Natural	Brown, 1934
			Abudefduf (syn. Glyphisodon) saxatilis	Seargeant Major			
					NORTH AMERICA, The U.S.A., New York		Nigrelli, 1939
			Amphiprion clarkii	Yellowtail clownfish	NORTH AMERICA, The U.S.A., Florida		Landsberg *et al.* , 1994
			Amphiprion ephippium	Red saddleback anemonefish	EUROPE, The UK		Brown, 1934
			Amphiprion frenatus	Tomato clownfish	NORTH AMERICA, The U.S.A., Florida	Experimental	Cobb *et al.* , 1998a
			Amphiprion ocellaris	Clown anemonefish	NORTH AMERICA, The U.S.A.	Experimental	Bower *et al.* , 1987
					NORTH AMERICA, The U.S.A.	Natural	Levy *et al.* , 2007
			Amphiprion percula	Orange clownfish	EUROPE, The UK		Brown, 1931
			Chromis chromis	Damselfish			Brown, 1934
			Dascyllus aruanus	Whitetail dascyllus	NORTH AMERICA, The U.S.A., New York		Nigrelli, 1939

Actinopterygii	Perciformes	*Pomadasyidae*	*Orthopristis chrysoptera*	Pigfish	NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1980
		Pomatomidae	*Pomatomus saltatrix*	Bluefish	NORTH AMERICA, The U.S.A., New Jersey	Natural	Nigrelli, 1936
		Priachantidae	*Priacanthus hamrur (syn. P. macrophthalmus)*	Atlantic big-eye	EUROPE, The UK	Natural	Brown, 1934
		Rachycentridae	*Rachycentron canadum*	Cobia	SOUTH AMERICA, Brazil	Natural	Guerra-Santos *et al.* , 2012
							Bonucci-Moreira *et al.* , 2013
					NORTH AMERICA, The U.S.A., Miami		Benetti *et al.* , 2008
		Scaridae	*Scarus coeruleus*	Blue parrotfish	NORTH AMERICA, The U.S.A., New York	Natural	Nigrelli, 1939
			Sparisoma (syn. Scarus) cretense	Parrotfish	EUROPE, The UK		Brown, 1934
		Sciaenidae	*Argyrosomus japonicus*	Mulloway	OCEANIA, Australia	Natural	Fielder e Bardsley, 1999
			Argyrosomus regius	Meagre	EUROPE, Portugal	Natural (cross-transmission)	Soares *et al.* , 2012
			Bairdiella chrysura	American silver perch	NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1980
			Bairdiella icistia	Croaker	NORTH AMERICA, The U.S.A., California	Natural	Kuperman and Matey, 1999; Kuperman *et al.* , 2001
			Cynoscion arenarius	Sand sea trout	NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1980
			Cynoscion nebulosus	Spotted seatrout	NORTH AMERICA, The U.S.A., Mississippi	Natural and experimental	Lawler, 1980
					NORTH AMERICA, The U.S.A.	Experimental	Masson *et al.* , 2011
							Masson *et al.* , 2013
					NORTH AMERICA, The U.S.A., Mississippi		Picón-Camacho *et al.* , 2013
			Cynoscion regalis	Weakfish	NORTH AMERICA, The U.S.A., New Jersey	Natural	Nigrelli, 1936
			Larimus fasciatus	Banded drum	NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1980
			Leiostomus xanthurus	Spot fish	NORTH AMERICA, The U.S.A., New Jersey	Natural	Nigrelli, 1936
					NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1980
					NORTH AMERICA, The U.S.A.		Masson *et al.* , 2011
			Menticirrhus americanus	Southern kingfish	NORTH AMERICA, The U.S.A., Mississippi	Natural and experimental	Lawler, 1980
			Menticirrhus saxatilis	Northern kingfish	NORTH AMERICA, The U.S.A., New Jersey	Natural	Nigrelli, 1936
					NORTH AMERICA, The U.S.A., New York		Nigrelli, 1939
			Micropogonias undulatus	Atlantic croaker	NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1980
					NORTH AMERICA, The U.S.A.		Masson *et al.* , 2011
					NORTH AMERICA, The U.S.A., Mississippi	Natural	Picón-Camacho *et al.* , 2013
			Pareques (syn. Eques) acuminatus	Striped drum	NORTH AMERICA, The U.S.A., New York	Natural	Nigrelli, 1939
					NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1980
			Sciaenops ocellatus	Red drum	ASIA, Taiwan	Natural	Tu *et al.* , 2002
					NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1980
							Lewis *et al.* , 1988
					NORTH AMERICA, The U.S.A., South Carolina	Natural	Sandifer *et al.* , 1993
					NORTH AMERICA, The U.S.A., Florida		Landsberg *et al.* , 1994
					NORTH AMERICA, The U.S.A., Texas	Natural and experimental	Oestmann and Lewis, 1995; 1996a,b
							Oestmann *et al.* , 1995
							Li *et al.* , 2005
					NORTH AMERICA, The U.S.A., Florida	Natural	Levy *et al.* , 2007
					NORTH AMERICA, The U.S.A., Texas	Experimental	Buentello *et al.* , 2010

Actinopterygii	Perciformes	*Serranidae*	*Centropristis philadelphica*	Rock sea bass	NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1980
			Centropristis striata	Black sea bass	NORTH AMERICA, The U.S.A., New Jersey	Natural	Nigrelli, 1936
			Epinephelus adscensionis	Rock hind	NORTH AMERICA, The U.S.A., New York	Natural	Nigrelli, 1939
			Epinephelus morio	Red grouper			
			Epinephelus niveatus	Snowy grouper	NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1980
			Mycteroperca rosacea	Leopard grouper	NORTH AMERICA, Mexico		Reyes-Becerril *et al.* , 2008
			Prometopon cruentatum	Graysby	NORTH AMERICA, The U.S.A., New York	Natural	Nigrelli, 1939
			Rypticus saponaceus	Greater soapfish			
			Serraniculus pumilio	Pygmy sea bass	NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1980
			Serranus atricauda	Blacktail comber	EUROPE, The UK	Natural	Brown, 1934
			Serranus subligarius	Belted sandfish	NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1980
		Siganidae	*Siganus spp.*		OCEANIA, Fiji		Paperna, 1980
			Siganus rivulatus	Marbled spinefoot	ASIA, Israel	Natural	
		Sparidae	*Acanthopagrus schlegelii*	Japanese black porgy (black sea bream)			Colorni and Padros, 2011
			Archosargus probatocephalus	Sheepshead	NORTH AMERICA, The U.S.A., Mississippi	Natural and experimental	Lawler, 1980
			Dentex dentex	Common dentex	EUROPE, Turkey	Natural	Tokşen and Çilli, 2010
			Diplodus argenteus	Silver porgy	SOUTH AMERICA, Brasil	Natural	Soeth *et al.* , 2013
			Diplodus (syn. Puntazzo) puntazzo	Sharpsnout bream	EUROPE, Greece	Natural (cross-transmission)	Rigos *et al.* , 1998
					EUROPE, Croatia	Natural	Mladineo, 2006
					EUROPE, Spain	Natural (cross-transmission)	Sánchez-García *et al.* , 2014
			Diplodus sargus (syn. D. rondeletii)	Sargo	EUROPE, The UK	Natural	Brown, 1934
			Diplodus vulgaris	Common two-banded seabream			
			Lagodon rhomboides	Pinfish	NORTH AMERICA, The U.S.A., Mississippi	Natural and experimental	Lawler, 1980
			Pagellus bogaraveo	Blackspot sea bream	EUROPE, Croatia	Natural	Mladineo, 2006
			Pagrus auratus	Australian snapper	OCEANIA, Australia	Experimental	Roberts-Thomson *et al.* , 2006
			Pagrus major	Red sea bream			Colorni and Padros, 2011
			Sparus aurata	Gilthead sea bream	AFRICA, Tunisia	Natural	Bahri, 2012
					ASIA, Israel		Paperna, 1980
							Colorni, 1994
							Diamant, 2001
							Levy *et al.* , 2007
					EUROPE, France		Paperna e Laurencin, 1979
						Natural and experimental	Paperna, 1984a,b
					EUROPE, Italy	Experimental	Aiello and D'Alba, 1986
					EUROPE, Spain	Natural	Alvarez-Pellitero *et al.* , 1995
					EUROPE, Greece	Natural (cross-transmission)	Rigos *et al.* , 1998
					EUROPE, Turkey	Natural	Çağırgan, 2003
					EUROPE, Croatia		Mladineo, 2006

Actinopterygii	Perciformes	*Sparidae*	*Sparus aurata*	Gilthead sea bream	EUROPE, Italy	Natural	Fioravanti *et al.* , 2006
					EUROPE, Portugal		Pereira *et al.* , 2011
					EUROPE, Montenegro		Adžić, 2012
					EUROPE, Portugal	Natural (cross-transmission)	Soares *et al.* , 2012
					EUROPE, Spain	Natural	Sánchez-García *et al.* , 2014
					EUROPE, Portugal	Experimental	Moreira *et al.* , 2017
							Colorni and Padros, 2011
			Stenotomus chrysops	Scup	NORTH AMERICA, The U.S.A., New Jersey	Natural	Nigrelli, 1936
	Pleuronectiformes	*Achiridae*	*Trinectes maculatus*	Hogchoker	NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1980
		Bothidae	*Citharichthys spilopterus*	Bay whiff	NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1980
			Etropus crossotus	Fringed flounder			
		Cynoglossidae	*Symphurus plagiusa*	Blackcheek tonguefish	NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1980
		Paralichthyidae	*Paralichthys dentatus*	Flounder	NORTH AMERICA, The U.S.A.	Natural	Hughes and Smith, 2003
			Paralichthys lethostigma	Southern flounder	NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1980
					NORTH AMERICA, The U.S.A., South Carolina	Natural	Smith *et al.* , 1999
					NORTH AMERICA, The U.S.A.	Natural	Benetti *et al.* , 2001a,b
			Paralichthys orbignyanus	Brazilian flounder	SOUTH AMERICA, Brasil	Experimental	Abreu *et al.* , 2005
		Scophthalmidae	*Psetta maxima (syn. Scophthalmus maximus)*	Turbot	EUROPE, Portugal	Natural	Ramos and Oliveira, 2001
							Saraiva *et al.* , 2011
		Soleidae	*Achirus lineatus*	Lined sole	NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1980
			Solea aegyptica	Egyptian sole	AFRICA, Tunisia	Natural	Yemmen *et al.* , 2012
			Solea solea	Common sole			
			Solea senegalensis	Senegalese sole			
							Yemmen and Bahri, 2017
	Scorpaeniformes	*Scorpaenidae*	*Pterois miles*	Lionfish	ASIA, Israel	Natural	Diamant, 2001
			Pterois volans	Red lionfish	EUROPE, The UK	Natural	Brown and Hovasse, 1946
			Scorpaena brasiliensis	Barbfish	NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1980
		Triglidae	*Eutrigla (syn. Trigla) gurnardus*	Grey gurnard	EUROPE, The UK	Natural	Brown, 1934
			Prionotus spp.		NORTH AMERICA, The U.S.A., New York		Nigrelli, 1939
			Prionotus carolinus	Carolina sea robin	NORTH AMERICA, The U.S.A., New Jersey		Nigrelli, 1936
					NORTH AMERICA, The U.S.A., New York		Nigrelli, 1939
			Prionotus evolans	Striped sea robin	NORTH AMERICA, The U.S.A., New Jersey		Nigrelli, 1936
			Prionotus roseus	Bluespotted sea robin	NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1980
			Prionotus. rubio	Sea robin		Natural	
			Prionotus tribulus	Bighead sea robin		Experimental	

Actinopterygii	Siluriformes	*Ariidae*	*Ariopsis (syn. Arius) felis*	Hardhead catfish	NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1980
			Bagre marinus	Gafftopsail catfish			
		Clariidae	***Clarias batrachus****	Walking catfish	NORTH AMERICA, The U.S.A., Florida	Experimental	Noga and Hartmann, 1981
	Syngnathiformes	*Syngnathidae*	*Hippocampus erectus*	Lined seahorse	NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1980
			Syngnathus louisianae	Chain pipefish			
	Tetraodontiformes	*Balistidae*	*Balistes vetula*	Queen triggerfish	NORTH AMERICA, The U.S.A., New York	Natural	Nigrelli, 1939
			Stephanolepis (syn. Monacanthus) hispidus	Planehead filefish	NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1980
		Diodontidae	*Chilomycterus schoepfi*	Spiny boxfish	NORTH AMERICA, The U.S.A., New Jersey	Natural	Nigrelli, 1936
					NORTH AMERICA, The U.S.A., New York		Nigrelli, 1939
					EUROPE, The UK		Brown and Hovasse, 1946
					NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1980
			Didon hystrix	Spot-fin porcupinefish	NORTH AMERICA, The U.S.A., New York	Natural	Nigrelli, 1939
		Monacanthidae	*Aluterus schoepfi*	Orange filefish	NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1980
		Ostraciidae	*Lactophrys (syn. Acanthostracion) quadricornis*	Scrawled cowfish	NORTH AMERICA, The U.S.A., Mississippi	Natural and experimental	Lawler, 1980
		Tetraodontidae	*Canthigaster rostrata*	Caribbean sharpnose-puffer	EUROPE, The UK	Natural	Brown, 1934
			Sphoeroides annulatus	Bullseye pufferfish	NORTH AMERICA, Mexico	Experimental	Fajer-Avila *et al.* , 2003
			Sphoeroides maculatus	Whitespotted soapfish	NORTH AMERICA, The U.S.A., New Jersey	Natural	Nigrelli, 1936
					NORTH AMERICA, The U.S.A., New York	Natural	Nigrelli, 1939
			Sphoeroides parvus	Least puffer	NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1980
			Takifugu rubripes	Pufferfish	ASIA, Taiwan	Natural	Chang *et al.* , 2001
Chondrichthyes	Myliobatiformes	*Dasyatidae*	*Dasyatis sabina*	Atlantic stingray	NORTH AMERICA, The U.S.A., Mississippi	Experimental	Lawler, 1980
Malacostraca	Decapoda	*Penaeidae*	*Fenneropenaeus (syn. Penaeus) indicus*	Indian white shrimp	ASIA, India	Natural	Aravindan *et al.* , 2007
Monogenea	Monopisthocotylea	*Capsalidae*	*Neobenedenia melleni*		ASIA, Israel	Natural (hyperparasitism)	Colorni, 1994
Bivalvia	*Ostreoida*	*Ostreidae*	*Crassostrea gigas*	Pacific oyster	EUROPE, Portugal	Experimental	de Souza, 2015

**Clarias batrachus is mentioned as AO host as from its gill cells was developed the G1B cell line (ATCC® CRL-2536TM) used to the parasite in vitro cultivation.*

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4. *Findings on Amyloodinium ocellatum biology*

The research activities in this chapter sought to answer some key questions about some biological details of *A. ocellatum* (AO). During investigations, it emerged that despite removal of known adequate hosts, AO remains in the environment and is capable of successfully infecting hosts when they return.

Furthermore, it is known that AO can be continuously propagated *in vitro* on the G1B cell line (ATCC® CRL-2536TM). This method allows to continuously dispose of the infective stage (dinospore) in order to carry out studies on the host/parasite relation through experimental infections, and investigations on new alternative treatments. The AO *in vitro* propagation permits not to depend on natural or experimental infections for the parasite supply; on the other hand, it is expensive (purchase of the cell line and its maintenance), laborious and allows to produce just small quantities of dinospores. For this reason, the possibility of preserving tomonts over time has been explored.

4.1 ENVIRONMENTAL SURVIVAL OF *AMYLOODINIUM OCELLATUM*

4.1.1 Background

In Autumn 2015, at the Section of Animal and Veterinary Sciences facilities (Department of Agricultural, Food, Environmental and Animal Sciences - Di4A) of the University of Udine (UNIUD[2]), an experimental 300 L aquarium was set up to rear healthy European sea bass (*Dicentrarchus labrax*; ESB). In order to activate the biological filter quickly, filter materials were taken from an experimental recirculating system of the Di4A facilities in Pagnacco (UD, Italy), where gilthead sea breams (*Sparus aurata*) were farmed and previous amyloodiniosis outbreaks had occurred. Unfortunately, the filter material was contaminated by AO and in a few days all ESB died. *Post mortem* parasitological examination confirmed a massive branchial AO infection, then gill samples were collected and addressed to histological and immunohistochemical investigations that will be discussed in the following chapter of this thesis. Hence, for approximatively eight months the aquarium was maintained in operation without fish; but as soon as a new group of healthy ESB was introduced into the same aquarium (June 2016), an outbreak happened, characterised by the typical symptoms of amyloodiniosis (i.e. jerky movements, dyspnoea, increased respiratory rate with laboured breathing and gathering at the water surface). The infection was again confirmed by gill parasitological exams. However, the presence of the parasite was unexpected since in that aquarium, there had not been fish for eight months and water temperature fluctuated to values

[2] Note: the UNIUD acronym will be used in this thesis also to refer to the research group of the University of Udine in which I have been involved.

below AO optimal range (14-22±0.5°C). To comprehend how AO survived in the aquarium without fish, some investigations were carried out.

4.1.2 Materials and methods

After the second amyloodiniosis episode, 500 ml of water and sediment were periodically collected (5 times in one year) from the purge faucet of the investigated aquarium, and let to settle in a graduated cone for 1 hour. Then the supernatant was removed and the sediment was observed under a stereo microscope. Subsequently, the aquarium microfauna (small crustaceans, nematodes, foraminifera) was collected and analysed by light microscopy. Photos at different magnifications were taken using a light microscope (DMLB, Leica) supported by the digital camera LEICA ICC50 and LAS EZ V1 software.

4.1.3 Results

The collected microfauna was composed by ciliates, copepods, nematodes and foraminifera; moreover, it was possible to notice some swimming dinospores. Approximately 100 copepods were observed. However only two copepods collected from one sediment (late sample) out of the 5 examined, resulted positive to *A. ocellatum* as trophonts attached to the metasoma portion were identified (Fig. 4.1a,b).

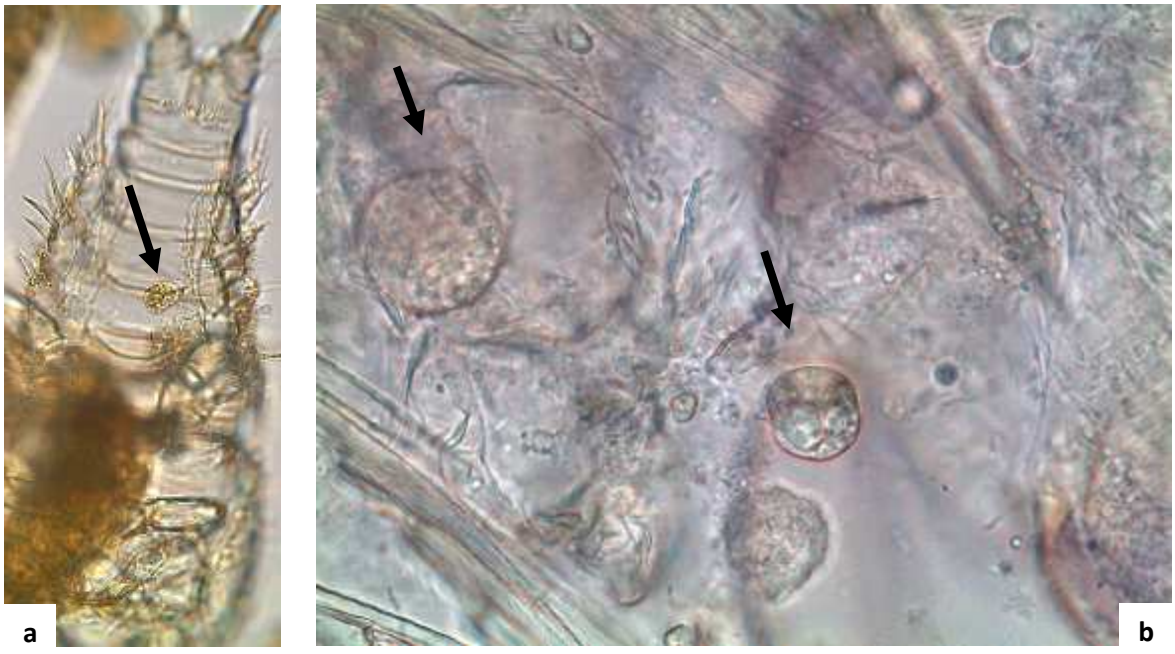

Figure 4.1. Crustacean isolated from the sediment of UNIUD aquaria. a) Trophont attached to the metasoma of the copepod (arrow); b) magnification of two attached trophonts (arrows).

4.1.4 Discussion

Amyloodinium ocellatum does not possess a specific definitive host and potentially can infect all the aquatic organisms living in its habitat as documented by numerous studies (Lawler, 1980; Colorni, 1994; Aravindan *et al.*, 2007; de Souza, 2015). The literature indicates that it has been tentatively identified from around 183 species of aquatic organisms belonging to 69 families (see Table 3.1 in the previous chapter). This low species specificity allows the parasite to complete the lifecycle even when these host-fish are absent in the environment.

The AO biological plasticity has also been confirmed by the present investigation on its capacity in persisting in the environment. Even if the prevalence of the infection was low, the presence of growing trophonts on copepods (*Crustacea*) (Fig. 4.1a,b) is a relevant observation. In fact, through this evidence, it was possible to understand how the parasite could have survived in our experimental aquarium for several months without fish, thus suggesting that AO may exploit this plasticity also to survive in the natural environment or when the environmental conditions are unfavourable.

4.2 TOMONTS PRESERVATION AND LIFESPAN

In order to deepen the knowledge about AO, some studies were carried out on the cystic phase of *Amyloodinium ocellatum*. In particular, based on issues related to ParaFishControl project (Horizon 2020) activities, tomonts deriving from natural outbreaks in fishfarms located in the North-East of Italy, were used to evaluate potential preservation methods (i.e. cryopreservation and refrigeration) or to elucidate how do they respond to adverse environmental conditions (i.e. dehydration). The focal aim of the trials was to find a technique to preserve viable AO in artificial conditions for several months, thus disposing of available aliquots of infective dinospores for different research activities: therapy (dinospore motility), ESB experimental infections, vaccination trials and coating of E.L.I.S.A. plates.

4.2.1 Materials and methods

4.2.1.1 Tomonts supply

Protozoa, used in the research activities here described, were obtained from gills of naturally or experimentally infected ESB (see Table 5.1 in the next chapter). Parasite collection was performed based on modified Oestmann and Lewis (1995). AO trophonts were detached by placing infected

fish (preferably of 20-50g of weight and a medium/high parasite burden) for 2-3 min in a clean 2-3 L plastic jar containing 0.5 L of freshwater. Then, 500 ml of 40‰ salt medium (or artificial seawater) was added to adjust the final salinity at about 20‰. Infected fish were let in the jar for 5-6 min for trophonts detachment. After this period, fish were removed from the jar and the content poured into a siliconized glass jar through a siliconized glass funnel lined with two layers of 100 µm nylon filter mesh, to remove large debris. The filtrate was set aside for 15–20 min to allow sedimentation and transformation of trophonts into early tomonts (before the first division); the saltwater overlay was removed and tomonts transferred in sterile 50 ml tubes. Tomonts were washed twice by centrifugation for 10 min at 150×g, saltwater overlay removed and pelleted tomonts re-suspended in sterile saltwater (20‰). Successively, 2 ml of the tomont suspension in sterile saltwater were gently layered onto 2 ml of Percoll® (P1644, Sigma-Aldrich) in 15 ml centrifuge tubes (Fig. 4.2), followed tomont purification by centrifugation for 10 min at 180×g. The supernatant was removed and the pelleted tomonts were washed three times with 15 ml of sterile saltwater, then they were immediately used or preserved for the research activity purposes.

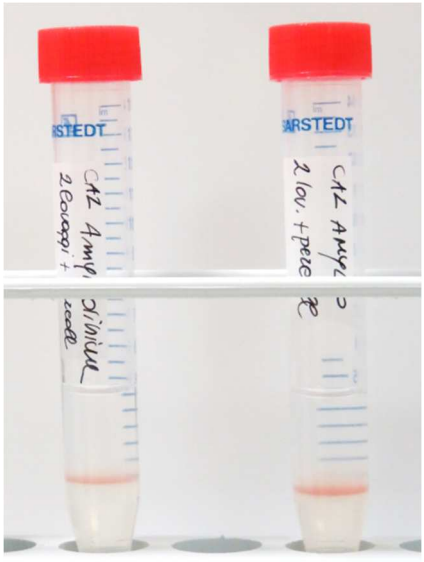

Figure 4.2. Early tomonts purification in Percoll® gradient.

4.2.1.2 Cryopreservation

Two samples of early tomonts were added with saltwater solutions containing glycerol at the final concentrations of 7.5 or 15% respectively in 15 ml tubes. The tomont equilibration was carried out at room temperature (RT) for three hours, then at 4°C overnight and finally stocked at -20°C for one month (Mutetwa and James, 1984; Miyake *et al.*, 2003). After this period, samples were re-equilibrated at 4°C overnight, the supernatant was removed and the tomonts transferred in Petri

dishes containing sterile saltwater (30‰), followed an incubation at 24°C for 2-3 days to allow the restoration of the cystic phase. During incubation, tomonts were constantly monitored through stereo and light microscopy observations.

4.2.1.3 Controlled hibernation

Three samples of early tomonts were incubated at different temperatures in Petri dishes containing sterile saltwater (30‰), respectively at 4±0.5°C, 14±0.5°C and 16±0.5°C. The incubation was initially set for 72 hours then extended to more days (7-10). During this period, tomonts were monitored under a stereo microscope and, after 72 hours or more, tomonts from each sample were incubated at 24°C for 2-3 days (or more days to elicit the reproductive process completion) in order to evaluate if they were still viable.

Successively, another sample of early tomonts was incubated at 16±0.5°C in Petri dishes with sterile saltwater (30‰) extending the incubation period to 2 months. At the deadline, a small quantity of tomonts was collected, transferred in other Petri dishes containing sterile saltwater (30‰) and incubated at 24°C for 2-3 days; in parallel, the original Petri dishes were maintained in incubator at 16±0.5°C for a further 2 months.

To avoid bacterial or mould contaminations, sterile saltwater was used adding to it a mixture of antibiotics: Amoxycillin (0.5 µg/ml), Flumequine (0.6 µg/ml), Penicillin/Streptomycin (2% v/v), Sulphamethoxazole/Trimethoprim (0.5 µg/ml) and Tetracycline (0.2 µg/ml).

4.2.1.4 Tomont drying

Early sedimented tomonts were transferred in 3 opened Petri dishes at RT (20-22°C) without saltwater (with the exception of the inevitable minimum amount of water transferred with the parasite) for 24, 48 and 72h respectively. At the different deadlines, sterile saltwater was added to each Petri dish and parasites were incubated at 24°C for 2-3 days to verify their viability.

4.2.2 Results

4.2.2.1 Cryopreservation

After the incubation time at 24°C only a very few tomonts (2%) were still vital showing a restoring of the asexual divisions. However, the reproductive process failed after the first divisions (1-2

maximum) in both glycerol concentrations (Fig. 4.3). The majority of tomonts (98%) did not survive after one month of cryopreservation at -20°C.

Figure 4.3. Tomonts appearance after cryopreservation. Only one tomont tried to restore the reproductive process (arrow).

4.2.2.2 Controlled hibernation

Hibernation at 4±0.5°C for 3 days adversely affected tomonts and no divisions occurred in parasites returned to 24°C and incubated for 2-3 days (or more days to elicit the reproductive process completion). Reproductive process was totally inhibited and tomonts maintained a normal appearance. On the other hand, they were not vital and capable of restoring the divisions to generate new dinospores.

During the hibernation period at 14±0.5°C, 95% of tomonts were vital but the reproductive process was partially inhibited as it started only at the end of the incubation. The detrimental impact of low temperature became evident after the incubation time and when tomonts remained at 14±0.5°C for more days (5-10). In fact, the division process was slowed down, but in general, after 3-4 asexual divisions it was completely interrupted. However, a very few tomonts (about 3%) were able to sporulate with a yield of limited dinospores, even if they were disvital and/or less motile.

Tomonts hibernated at 16±0.5°C showed a slowing down in the division process. In general, only a few tomonts originated dinospores, while the majority showed an asynchronous development with tomonts apparently interrupted at 4, 8 or 16 divisions. However, when transferred at 24°C for 2 days the reproductive process recovered and 100% of tomonts sporulated generating vital dinospores.

During long hibernation at 16±0.5°C (two months; fig. 4.4), the tomont divisions were considerably slowed down, even if some dinospores were generated throughout the incubation period. Anyway, the recovery of the reproductive process and sporulation occurred for the 95-98% of tomonts when

the incubation conditions returned to the optimal temperature of 24°C. Moreover, at that temperature dinospores were vital and infective, as confirmed by subsequent experimental ESB infections. This result was obtained also after 4 months of hibernation (at 16±0.5°C), but in that case there was a reduction of the 2-5% of the total number of tomonts capable to resume an effective reproductive process with a yield of infective dinospores.

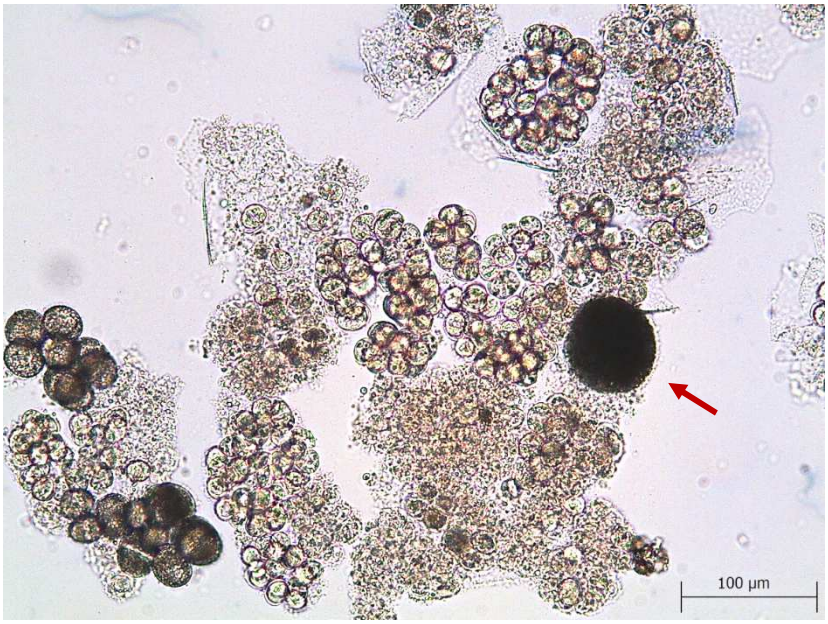

Figure 4.4. Extended controlled hibernation of early tomonts (16°C). In the picture are visible the asynchronous division stages and one dead early tomont (arrow).

4.2.2.3 The effect of drying on tomonts survival

All tomonts exposed to dehydratation at RT (20-22°C) for 24 to 72h, failed to divide when returned to adequate environmental conditions (24°C and 30‰).

4.2.3 Discussion

The main objectives of these surveys were to document the parasite ability to survive in an environment apparently in absence of the "perfect" host (fish), and to investigate techniques aimed at preserving vital tomonts, able to sporulate infective dinospores, over time. The latter study was very useful to perform the research activities that are going to be described in this thesis.

As already mentioned, *A. ocellatum* shows a wide range of potential hosts of which marine or brackish water fish are the most suitable. According to the literature (Lawler, 1980; Colorni, 1994; Aravindan *et al.*, 2007; de Souza, 2015), this dinoflagellate can infect aquatic organisms belonging to four different phyla (*Chordata*, *Arthropoda*, *Mollusca* and *Platyhelminthes*), and therefore not surprisingly the AO-parasitized copepods present in the experimental aquarium explained how the

parasite could have survived for several months without the fish host. Fish skin and gills represent the target organs for trophont growth, but the extreme biological plasticity, probably developed in adverse environments during AO evolution, enabled it to survive waiting for the "perfect" hosts. Anyway, for our knowledge, this is the first description of copepods infected by *A. ocellatum*.

To carry out the research activities on AO, it is necessary to dispose constantly of the parasite, especially of its infective phase, the dinospore. As already explained, the G1B cell line (ATCC® CRL-2536TM) permits to maintain continuously *in vitro* the AO biological cycle. However, with this system it is difficult to obtain a high number of dinospores as required for some studies (e.g. motility trial, vaccination, E.L.I.S.A. plates coating).

Cryopreservation is a process by which biological materials can be preserved at low temperatures, using specific additives (cryoprotectants). The storage in liquid nitrogen allows to obtain the best results since, at the temperature of -196°C, every biological activity is blocked. Cryopreservation is performed for many types of biological materials, such as blood cells or stem cells, cell tissues and cultures, embryos, sperm, bacteria and parasites (Rowe 1966; Hubàlek 2003). Despite some protozoa can be successfully cryopreserved, the method applied for tomont cryopreservation has to be considered as a pilot study and proved to be inadequate as all the tomonts died. As future perspective, this approach will be investigated again also by exploring the effects of liquid nitrogen storage on tomonts viability. Nonetheless, the successful results obtained by controlled hibernation make the cryopreservation improvement less urgent.

Furthermore, the results obtained from tomont hibernation experiments confirmed what already exposed by Paperna (1984). In general, temperatures far below 8°C caused irreversible damages to tomonts, which were unable to restore the reproductive process when incubated at 20-25°C. This condition happened also in our experiment after keeping AO at 4±0.5°C. In the same research, Paperna noticed that at 18°C tomonts appeared to exert their upmost division potential, for this reason in our investigations AO tomonts were hibernated at temperatures lower than this, at 14±0.5°C and 16±0.5°C. In the first condition, the reproductive process markedly delayed, but even if the condition was reversible, when transferred to 24°C the hatched dinospores were less vital and motile than those ones generated by tomonts hibernated at 16±0.5°C. From this result, it was decided to select this temperature (16±0.5°C) to successfully preserve AO tomonts for more extended periods. During the controlled hibernation, to avoid bacterial, protozoal and mould proliferations, which alter the vitality of dinospores and tomonts (Bower *et al.*, 1987; Noga and

Bower, 1987; Oestmann and Lewis, 1995), sterile saltwater (30‰) was always used adding to it a mixture of different antibiotics.

Hence, by tomonts' controlled hibernation at 16±0.5°C in sterile saltwater plus antibiotics, it is possible to obtain aliquots of infective dinospores also some months after their initial "tomontisation". Moreover, the infectivity of dinospores from these preserved tomonts was assessed during experimental infection trials.

As mentioned by Paperna (1984), the drying has a detrimental impact on the vitality of the early tomonts. This finding was confirmed by our experiments as parasites did not survive to the treatment, thus suggesting that drying can be a useful method for controlling the disease in confined rearing environments.

4.3 CONCLUSIONS

Research activities in this chapter have confirmed that *Amyloodinium ocellatum* can use copepods as hosts to survive in environments without fish.

These findings will drive further explorations on the associations that AO may have with organisms previously considered as non-target hosts, with a view to improve management of this parasite in farms. Moreover, these studies allowed to define a protocol for tomont controlled hibernation, letting to produce viable dinospores for different experiments over several months from parasite collection.

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5. *Host-parasite relationship*

5.1 PREFACE

The trophont is the parasitic stage of AO. In this phase, the protozoan is sessile and anchored to different host epithelia depending on the severity of the infection. The primary target sites of AO adhesion in the European sea bass (*Dicentrarchus labrax*, ESB) are the gills and oral cavity. Despite the fact that trophonts do not change location (Noga, 1987), they constantly twist and turn slowly, resulting in the destruction of epithelial cells. Consequently, the pathological effect is parasite density-dependent and is proportional to the extent of branchial epithelium alteration. Clinical signs of amyloodiniosis, mainly observed in advanced disease, include anorexia, dyspnoea, jerky and surface swimming. Mortality can be the consequence of osmoregulatory and respiratory impairment experienced and, in some cases, of secondary infections.

Previous authors have furnished detailed descriptions of gill epithelium alterations provoked by *A. ocellatum* on different host species, providing a uniform description of these lesions and alluding to mechanisms involved in the host response. Brown (1934) was the first author to publish a histological report on amyloodiniosis effects on the gill epithelium of some fish species kept in the Aquarium of the Zoological Society of London. The infection was characterised by a severe gill hyperplasia with subsequent lamellar fusion, inflammation, haemorrhages and necrosis of the distal extremities of the gill filaments. Same alterations were documented in juveniles and adults of gilthead sea bream (*Sparus aurata*), in turbot (*Scophthalmus maximus*), in cobia (*Rachycentron canadum*), in silver pompano (*Trachinotus blochii*) and in European sea bass (Paperna 1980; Saraiva *et al.*, 2011; Bahri 2012; Guerra-Santos *et al.,* 2012; Ramesh-Kumar *et al.*, 2015; Nozzi *et al.,* 2016). Ultrastructural studies better clarified the parasite relationship with host cells as reported by Lom and Lawler (1973), who investigated by electron microscopy the parasite behaviour and its feeding activity when attached to the gill epithelium of three fish species: *Fundulus heteroclitus*, *F. majalis* and *Cyprinodon variegatus*. Scanning electron microscopy was used by Kuperman and Matey (1999) and Kuperman *et al.* (2001) on naturally infected fish species living in the Salton Sea Lake. Through microphotographs, the authors could detail the local erosion and the gill epithelial distorsion in the sites of throphont attachment. In parallel, some researchers explored the host response against AO in terms of acquired immunity. Smith *et al.* (1992, 1993, 1994) developed an enzyme linked immunosorbent assay (E.L.I.S.A.) to evaluate the anti-AO antibody titres in sera of immunized blue tilapia (*Oreochromis aureus*) and hybrid striped bass (*Morone saxatilis × M. chrysops*) fish. The same assay was used by Cobb *et al.* (1998a) on sera of clownfish (*Amphiprion frenatus*) subjected to repeated non lethal AO infection challenges. Results confirmed the capability of the fish species in

developing a specific immunity to the dinoflagellate in agreement with the Western blot analyses performed by the authors in the same study.

Anyway, a very few information on ESB infected by AO are documented. Cecchini *et al.* (2001) investigated the anti-AO antibody response by means of E.L.I.S.A., while Nozzi *et al.* (2016) detected pro-inflammatory markers through biomolecular approaches. Furthermore, to our knowledge, there are no previous reports on immunohistochemistry (IHC) or *in situ* hybridization (ISH) approaches performed on ESB tissues and not even in other fish species infected by the dinoflagellate.

For this reason, in order to deepen our understanding on the mechanisms underlying the host-parasite interactions, several investigations have been performed by means of histology, immunoenzymatic surveys (IHC and E.L.I.S.A.), fluorescent *in situ* hybridization (FISH), fluorescence and confocal microscopy (CLSM) assessments. Infected tissues of ESB, from both spontaneous outbreaks and experimental infections set up at the University of Udine (UNIUD) facilities were employed for histology and immunohistochemistry (with the contribution of the University of Camerino and the University of Santiago de Compostela), in order to describe the lesions provoked by the parasite and the host tissue inflammatory response, in terms of cells recruitment and mediators synthesis. Dedicated paraffin-embedded blocks were addressed to FISH investigations aimed at the study of post-infection mucosal immune response, whereas some samples (paraffin-embedded blocks and/or slides stained following different staining protocols) have been analysed by confocal microscopy. Moreover, an indirect E.L.I.S.A. protocol was developed and applied to evaluate the specific anti-AO antibody titre in ESB sera collected from naturally and experimentally infected fish, as well as after the vaccination against AO.

5.2 MATERIALS AND METHODS

5.2.1 Parasite and fish samplings from natural and experimental infections

The AO trophonts and ESB biospecimens were collected during natural and UNIUD experimentally induced infections. Moreover, tissues of healthy ESB were also collected in order to have a comparison for some laboratory tests. The table 5.1 summarises the characteristics of the collected biospecimens, their origin and for which test they have been used. In all lethal samplings, fish were euthanized under Tricaine methanesulfonate (MS-222, 400 mg/L) (Sigma-Aldrich). All procedures were conducted in compliance with the Guideline of the European Union Council (Directive 2010/63/EU) and the Italian legislation (D.L. 26/2014) for the use of laboratory animals for experimental purposes.

5.2.1.1 AO natural infections

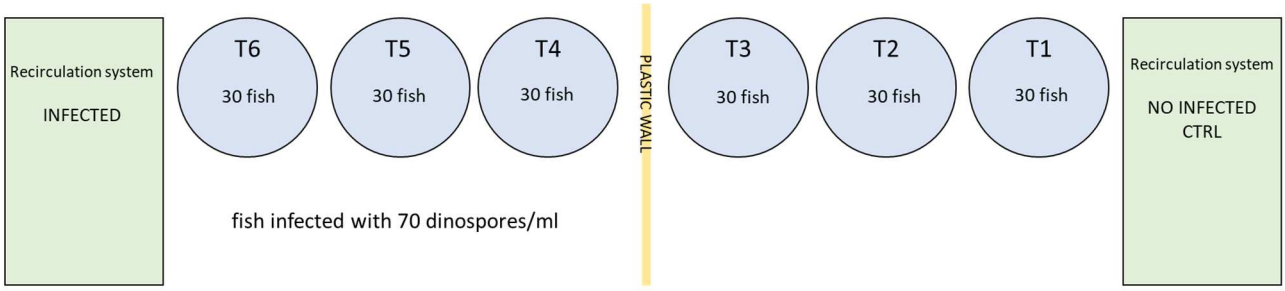
AO trophonts were collected from ESB (as described in paragraph 4.2.1.1) during summer 2016 and 2017 spontaneous outbreaks occurred in some fish farms located in the North-East and Centre of Italy. At the same time, during these AO episodes fish heads, gills and blood were collected from a total of 72 symptomatic, asymptomatic and healthy ESB (weight range 30-1500 g) (Table 5.1) to employ them for histopathological and immunohistochemical investigations.

5.2.1.2 Experimental infections

In accordance to ParaFishControl project purposes, experimental infections were carried out at UNIUD facilities in order to investigate the innate/specific immune response and the recovery mechanisms after amyloodiniosis, mainly with biomolecular approaches. However, biomolecular findings will not be covered by this PhD thesis. Experimental infections are here described as ESB tissue samples (gills and fish heads) were then employed for histology, IHC, FISH and CLSM studies.

5.2.1.2.1 Experimental design of AO infection-1 (AOinf-1)

Naïve ESB (N=30 randomly allocated to each tank, mean weight 14g) coming from the hatchery of Farm A (table 5.1) were acclimatised for one week in two separated recirculation systems, each consisting of three 120 L fiberglass tanks [temperature 22.5±2°C, salinity 30±2‰, pH 8.0, NH_4-N 0.02-0.03 mg/L, NO_2-N below the detection limit of the method (<0.015 mg/L), natural photoperiod]. The experimental infection design is illustrated in the scheme in the next page.



The 6 tanks were classified as follows: T1-T3 fish unexposed to *A. ocellatum* as controls (CTRL); T4-T6 fish to be infected with *A. ocellatum*.

Throughout the experiment, tanks were covered with a plastic sheet in order to avoid potential dinospore contamination between tanks by aerosol droplets (Roberts-Thomson *et al.*, 2006).

Viable dinospores, hatched from hibernated tomonts (paragraph 4.2.1.3), were counted by adapting Dehorty's (2003) protocol. Briefly, aliquots of 500 µl of the dinospore suspensions were stained with Lugol's iodine solution and counted using a counting cell chamber (S50 Sedgewick Rafter Cell, Pyser – SGI)[1][3].

Then, 3.5×10^6 dinospores were poured in each individual infection tank and left for 2h at a reduced volume of 50 L/tank, without water circulation (water temperature 26±2°C). The concentration of dinospores throughout the fish exposure was approximatively 70 dinospores/ml of water. After 2h, the level and the recirculation of water were restored. Control tanks were submitted to the same procedure, but in absence of the parasite.

The time-course of infection was determined by *in vivo* gill biopsy and, after the 2 hours of AO exposure, fish were already infected. The CTRL fish were also monitored *in vivo* to exclude the AO presence.

At 2, 3, 5, 7 and 23 days post infection (dpi) (23dpi was the last day of the AOinf-1) three fish/tank were sacrified by an overdose of anaesthetic (MS-222, 400 mg/L) to collect gills, kidney and spleen for biomolecular investigations (which, as previously mentioned, are not included in this thesis) and fish heads and gills were processed for histology.

[3] The dinospore concentration per ml was derived from the following formula: (number of dinospores in 50 grids×1000/12.5) × dilution factor; as reported in Dehorty (2003).

5.2.1.2.2 March 2018-AO infection (M18 AOinf)

In March 2018, a further experimental infection was carried out at UNIUD facilities. In this case, a small scale trial was performed to obtain ESB biospecimens to be addressed to FISH and confocal investigations. The infection was performed in a 300 L aquarium, half filled with seawater at 27±2°C. For the challenge, 10 healthy ESB (70±10g weight) were used. Dinospores were counted as mentioned above, then poured in the aquarium at a final concentration of 4 dinospores/ml. Fish were constantly monitored and at the onset of symptoms (5 dpi) euthanized (MS-222, 400 mg/L). Gill samples were collected and preserved in RNA later® (ThermoFisher Scientific) according to manufacturer's instructions for total RNA isolation and addressed to polymerase chain reaction (PCR) and electrophoresis for primers testing and FISH riboprobes production and to real time PCR for the evaluation of gene expression levels in the tissue. Gills were also fixed in 4% paraformaldehyde according to the University of Stirling's established protocol, to be subjected to histology, FISH, fluorescence and confocal microscopy insights.

5.2.1.2.3 Sampling from other AO episodes (AOeps)

In parallel to the AO spontaneous outbreaks (paragraph 5.2.1.1) and to the experimental infections (paragraph 5.2.1.2), some accidental amyloodiniosis episodes occurred.

The first episode, as previously mentioned in paragraph 4.1.1, was experienced in Autumn 2015 at UNIUD aquarium where healthy juveniles of ESB were introduced. In that case the infection was caused by the contaminated filter material used to rapidly activate the biological filter of the aquarium. Gill samples collected from those fish were addressed to histology and immunohistochemistry. However, the persistence of AO in that aquarium has been exploited to maintain the parasite *in vivo* during the subsequent three years by periodically introducing naïve ESB and dinospore aliquots. Fish, used as reservoirs of the dinoflagellate, were maintained in a premunition status, and gills and blood samples were collected for histological, immunohistochemical and E.L.I.S.A. surveys.

A second undesired amyloodiniosis episode occurred at UNIUD fish farm facilities in Pagnacco (Udine, Italy). In Summer 2017, during the quarantine (a separate sector of UNIUD fish farm facilities) of new purchased ESB juveniles, amyloodiniosis clinical symptoms appeared. The cause of the infection has not been completely clarified yet, anyway it was probably provoked by contaminated water coming from another infected recirculation system. Fish were promptly treated with copper sulphate. Two months after, blood was sampled to be examined by E.L.I.S.A. for specific IgM to AO.

Table 5.1. AO infection episodes in ESB. Collection of the parasite and of ESB biospecimens addressed to the analyses described in this chapter.

									Lab analysis					
		ORIGIN	REARING TYPE	PERIOD	SIZE	AO ts		samples	HIS	IHC	FISH	CLSM	LFL	ELISA
						Yes	No							
	healthy	Farm A1	lagoon	Summer 2017	adult		X	gills	X	X				
								head						
								blood						X
		Farm A2	sea cages	Summer 2016	adult		X	gills						
								head						
								blood						X
infection	natural	Farm A1	lagoon	Summer 2016	adult	X		gills	X	X				
								head						
								blood						X
		Farm B	land based	Summer 2016	adult	X		gills	X					
								head						
								blood						X
		Farm C	lagoon	Summer 2017	juvenile	X		gills	X	X				
								head	X	X		X	X	
								blood						
		Farm D	land based	Summer 2017	adult	X		gills						
								head						
								blood						
	experimental	AOinf-1	Di4A recirculating system	Autumn 2017	juvenile	X		gills	X	X				
								head	X	X				
								blood						
		M18-AOinf	Di4A aquarium	Spring 2018	juvenile	X		gills	X	X	X	X	X	
								head	X	X				
								blood						
	accidental	AOeps1	Di4A aquarium	2015-2018	juvenile/ adult	X		gills	X	X				
								head						
								blood						X
		AOeps2	Di4A tanks	Summer 2017	juvenile		X	gills						
								head						
								blood						X

Farm A1, A2, B, and C are located in the North-East of Italy; Farm D is situated in the Centre of Italy. AOts (A. ocellatum trophont sampling); HIS (histology); IHC (immunohistochemistry); FISH (fluorescent in situ hybridization); CLSM (confocal laser scanning electron microscope); LFL (lectin fluorescent labelling); ELISA (enzyme linked immunosorbent assay).

5.2.2 Vaccine efficacy trial

5.2.2.1 Antigen preparation and vaccination protocol

Thirty-nine naïve ESB juveniles (mean weight 62g) were reared and conditioned for two weeks in a three tanks recirculation system (13 fish/tank) at UNIUD [temperature 22.5±2°C, salinity 30±2‰, pH 8.0, NH_4-N 0.02-0.03 mg/L, NO_2-N below the detection limit of the method (<0.015 mg/L), natural photoperiod]. Post acclimatisation, fish were reared under controlled conditions (30‰ salinity and mean temperature 24°C) and fed daily with a commercial diet in two meals administered at 9:00 and 16:00 h, 6 days per week.

Dinospores were chosen for fish immunization based on previous experiments performed by other authors (Smith *et al.*, 1992, 1993, 1994), and also because it is supposed they share similar antigens with trophonts. In order to prepare the AO vaccine, vital dinospores hatched from hibernated tomonts (paragraph 4.2.1.3) were inactivated at -80°C overnight, then thawed and manually fragmented with a potter (Fig. 5.1) for 30 min in sterile conditions. Followed an overnight treatment with 0.6% formaldehyde to avoid bacterial contaminations. Then, fragmented dinospores were washed two times with sterile 1× phosphate buffered saline (PBS) and plated onto solid medium to assess their sterility.

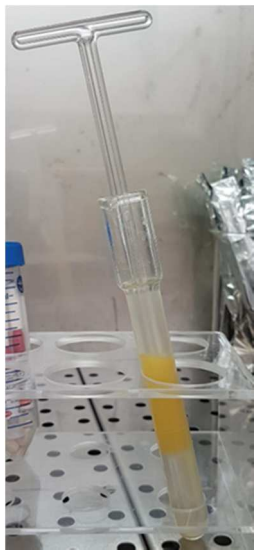
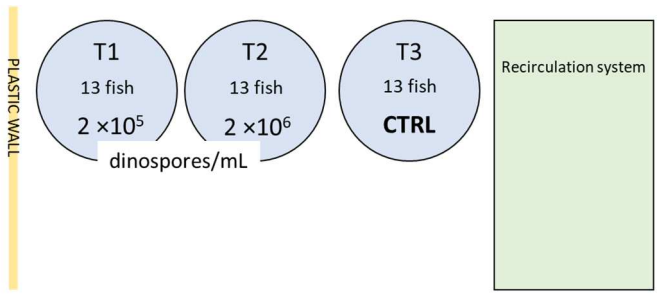


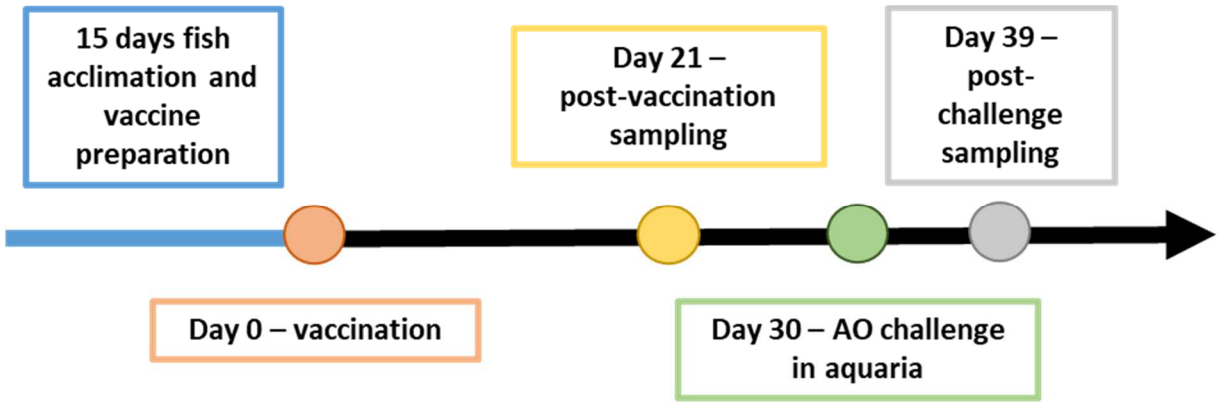
Figure 5.1. Manual potter used to fragment dinospores.

Two dinospores suspensions in 1× PBS were prepared, respectively at the concentrations of 2×10^5 and 2×10^6 dinospores/ml; these values derived from the initial dinospore concentrations as determined by adapted Dehorty (2003).



The vaccination protocol (illustrated on the left) included 3 groups (N=13), which were anaesthetised (MS-222, 65 mg/L) and submitted to intraperitoneal vaccination (250 µl/fish) as follows: T1 group, injected with low antigen dose (2×10^5 dinospores/ml); T2 group, injected with high antigen dose (2×10^6 dinospores/ml); T3 group (CTRL), injected with sterile 1× PBS used as placebo.

Three weeks after vaccination, blood was collected from the caudal vein of anaesthetised fish. Blood samples were centrifuged at 1,500×g for 15 min at 4°C. Carefully serum was collected and transferred in dedicated sterile microcentrifuge tubes (Sarstedt, Verona, Italy), then stored at -80°C. ESB sera were evaluated by E.L.I.S.A. to determine the specific antibody titres against AO.



Flowchart 5.1. Schematic representation of the vaccine efficacy trial protocol.

5.2.2.2 Potency test

As shown in the flowchart 5.1, on day 30 post vaccination an AO challenge was performed. Differently immunized and control fish were transferred in 3 marine water aquaria (200 L; 31‰ salinity), equipped with aerators, water pump and temperature control system (mean T 26°C). Vital dinospores, obtained as mentioned above, were added to each aquarium at the concentration of 3 dinospores/ml.

The infection time-course was monitored, recording clinical symptoms and specific mortalities within 10 days. Three sera were collected and pooled from moribund fish being part of group T2 (immunized with the highest dose of antigen).

Vaccination and potency test were carried out in compliance with the Guideline of the European Union Council (Directive 2010/63/EU) and the Italian legislation (D.L. 26/2014) for the use of laboratory animals for scientific purposes.

5.2.3 Histology

As reported in table 5.1, the ESB biospecimens collected from healthy or infected animals have been addressed to histological evaluation for the diagnostic confirmation of the infection and for a better characterization of the tissutal alterations. Tissue samples were alternatively fixed in Bouin's solution overnight at 4°C and in 4% buffered formaldehyde, embedded in paraffin by standard histological protocols, sectioned (4 µm in thickness) and stained with haematoxylin and eosin (H-E), Periodic acid Shiff (PAS)-Alcian blue, Masson and Azan trichrome, Cleveland trichrome, Giemsa and Twort Gram. The specimens were evaluated under light microscope (Leica DMLB) and relevant images were captured using a digital camera (LEICA ICC50) with imaging software (LAS EZ V1).

5.2.4 Immunohistochemistry (IHC)

In table 5.1 is reported the information about the origin of ESB samples used for the immunohistochemical investigations. Gill paraffin sections of healthy and infected ESB were collected on adhesive glass slides (TOMO® Matsunami, Germany), let to dry overnight and used for IHC tests.

For this purpose a panel of mono- or polyclonal antibodies specific for the following antigens was used: **CD3** (A0452, Dako, Agilent); **CD16** (H-80, sc-20627, Santa Cruz Biotechnologies); **CD35** (N-19, sc-7640, Santa Cruz Biotechnologies); **CD68** (M0814, Dako, Agilent); **Cyclooxygenase-2** (Cox-2) (H-62, sc-7951, Santa Cruz Biotechnologies); **Cyclooxygenase-2** (Cox-2) (M-19, sc-1747, Santa Cruz Biotechnologies); **Cytochrome P450** (CYP1A) (CO-226, Biosense Laboratories); **Cytokeratin** (ab9377, Abcam); **European sea bass IgM** (rabbit polyclonal, Univ. of Trieste, Italy); **Granulocyte Monocyte Colony Stimulating Factor Receptor alpha** (GM-CSFRα) (sc-690, Santa Cruz Biotechnologies); **Heat Shock Protein 70** (HSP70) (AS05 061A, Agrisera); **Histamine** (H7403, Sigma Aldrich); **Inducible Nitric Oxide Synthase** (iNOS) (RB-1605, Thermo Scientific); **Lysozyme** (A0099, Dako Agilent); **Proliferating Cell Nuclear Antigen** (PCNA) (SC-56, Santa Cruz Biotechnologies); **Toll-like Receptor 2** (TLR2) (ab1655, Abcam); **Toll-like Receptor 4** (TLR4) (76B357, Imgenex); **Tumor Necrosis Factor alpha** (TNF-α) (ab6671, Abcam).

IHC was conducted following a conventional protocol or using the EnVision™ FLEX system (K8009, K8021 and K8023, Dako, Agilent Technologies) to amplify the signal. Detection was performed using 3,3'-diaminobenzidine tetrahydrochloride (DAB) (D5905, Sigma-Aldrich) as chromogen and slides were counterstained with haematoxylin.

5.2.4.1 Conventional IHC

Sections were de-waxed in xylene baths (2x20 min) and gradually rehydrated through graded alcohols (100% ethanol 2 baths for 3 min each, 95% ethanol for 3 min, 80% ethanol for 3 min, 50% ethanol for 3 min) and a final bath in MilliQ water (ddH_2O) for 5 min. Endogenous peroxidase activity was blocked by the addition of 8% (v/v) hydrogen peroxide (H_2O_2) in ddH_2O for 30 min at room temperature (RT), then slides were washed in ddH_2O (3 min). Sections were subjected to antigen retrieval in TRIS buffer, 0.5% (w/v) trypsin and 0.5% (w/v) calcium chloride ($CaCl_2$) solution for 15 min at 37°C, washed with cold TRIS buffer (3 min) subsequently with RT TRIS buffer (3 min). The targeted sections were encircled by using a little piece of paraffin to keep reagents localised. All incubations were performed in a closed-lid humidified box. Nonspecific antibody binding was blocked by 1:20 normal goat serum (S26-100 EMD Millipore) in 1× PBS for 30 min at RT. After incubation with the primary antibodies (Table 5.2) appropriately diluted in 1% (w/v) bovine albumin serum (BSA) (A9647, Sigma Aldrich), 10% (v/v) normal goat serum in 1× PBS for 2 h at RT, sections were washed in TRIS buffer (3×5 min). Thereafter they were incubated for 30 min at RT with a biotinylated secondary antibody: goat-anti-mouse Ig (SAB3701051, Sigma Aldrich) diluted 1:400 in 1× PBS; or goat-anti-rabbit Ig (A9169, Sigma Aldrich) diluted 1:1,000 in 1× PBS; or rabbit-anti-goat Ig (B7014, Sigma Aldrich) diluted 1:800 in 1× PBS. Washes in TRIS buffer (3 × 5 min) followed the incubation. The reactions were developed using an ABComplex horseradish peroxidase (HRP) kit (PK-4000, Vectastain, Vector Laboratories) for 30 min at RT, followed by staining with DAB for 7 min and counterstaining with haematoxylin (1 min). Sections were washed with tap water and gradually dehydrated [50% ethanol for 3 min, 80% ethanol for 3 min, 95% ethanol for 3 min, 100% ethanol (2×3 min) and 2 successive xylene baths, 5 min each], coverslipped using a synthetic resin (Eukitt, O, KindlerGmbH). Slides were examined under a light microscope (Leica DMLB). Appropriate controls were included in all assays, and primary antibody was omitted in negative control sections.

5.2.4.2 EnVision™ FLEX

For some antibodies it was necessary to apply the EnVision™ FLEX protocol as the conventional IHC was not effective to recover the antigens. The protocol was the same described above for conventional IHC until the step of endogenous peroxidase inhibition. Then slides were washed with TRIS buffer (2×3 min). Antigen retrieval was performed using High pH or Low pH solutions (K8004 and K8005, Dako, Agilent) for 10 min at 90°C. Thereafter, slides were left for 15 min to cool to RT and washed with TRIS buffer (2×3 min). Targeted sections were encircled with paraffin to keep reagents localised. All incubations were performed in a closed-lid humidified box. Nonspecific antibody binding was blocked by 1:20 dilution of normal goat serum (S26-100 EMD Millipore) in 1× PBS for 30 min at RT. After incubation with the primary antibodies (Table 5.2) appropriately diluted in 1% (w/v) bovine albumin serum (BSA), 10% (v/v) normal goat serum in 1× PBS for 2 h at RT, sections were washed in TRIS buffer (3×5 min). The labelling was carried out using a horseradish peroxidase (HRP)-based anti-rabbit or anti-mouse kit (EnVision™ FLEX; Dako, Agilent) for 20 min at RT. Followed two washes with TRIS buffer (5 min each). The immunoreactive sites were visualized as reported above.

Table 5.2. Primary antibodies used in conventional and EnVision™ FLEX (Dako, Agilent) IHC tests, listed in alphabetical order.

Marker	Species	Protocol	Antigen retrivial	Optimal dilution
CD3	mouse	EnVision	low pH	-
CD16	rabbit	EnVision	low pH	1:100
CD35	goat	Conventional	-	-
CD68	mouse	EnVision	high pH	-
COX-2 (H-62)	rabbit	EnVision	low pH	-
COX-2 (M19)	goat	Conventional	trypsin	-
CYP1A	rabbit	EnVision	high pH	1:200
			low pH	
Cytokeratine	rabbit	Conventional	-	1:50
ESB IgM	rabbit	Conventional	trypsin	1:24.000
			-	
		EnVision	low pH	
GM-CSFRα	rabbit	EnVision	high pH	1:600
Histamine	rabbit	EnVision	high pH	-
			low pH	
HSP70	rabbit	EnVision	high pH	-
			low pH	
iNOS	rabbit	EnVision	low pH	1:200
Lysozyme	rabbit	EnVision	-	-
			high pH	
			low pH	
PCNA	mouse	Envision	low pH	1:50
TLR2*	goat	Conventional	trypsin	1:50
TLR4*	mouse	Conventional	trypsin	1:50
TNF-α**	rabbit	Envision	low pH	1:200

** IHC investigation performed at the University of Camerino by Dr. Magi. ** IHC investigation performed at the University of Santiago de Compostela by Dr. Ronza.*

5.2.5 Fluorescent mRNA *in situ* hybridization development

5.2.5.1 Fish samples and tissue processing

European sea bass (ESB) tissue samples (gills) were collected during an experimental infection carried out in the aquarium facilities of the University of Udine (UNIUD) in March-April 2018 (paragraph 5.2.1.2.2; Table 5.1). Samples were processed according to the Institute of Aquaculture (IoA) of the University of Stirling's (UoS) established protocols. Briefly, tissues were fixed in 4% paraformaldehyde in 1× PBS (pH 7.4) (16005 & P5368, Sigma-Aldrich) overnight at 4°C, then transferred to 70% ethanol and stored at -20°C. The paraffin embedding was performed at IoA and paraffin blocks stored at -20°C. Five µm sections were cut from the 4% paraformaldehyde-fixed, wax-embedded tissues, mounted onto Plus+ Frost positively charged microscope slides (MSS51012WH, Solmedia) and stored at -20°C for long-term usage. Samples for RNA expression analysis (gills) were stored in RNA later® Reagent (AM7021, Thermo Fisher Scientific) at -20°C until required.

5.2.5.2 Probes production

Total RNA was extracted from RNA later® preserved gill tissues (paragraph 5.2.1.2.2) using a TRI Reagent/Trizol (Ambion, Sigma, ABgene, Invitrogen) based protocol as per manufacturer's instructions. The RNA was quantified using the Nanodrop (ND2000c, ThermoFisher Scientific) and the quality was assessed by running 200 ng on a 1% agarose gel containing ethidium bromide (EthBr) (E1510, Sigma Aldrich) along with a 1Kb marker. The final concentration of EthBr was 0.05 µg/ml. A one-step reverse transcriptase polymerase chain reaction (RT-PCR) was performed with MyTaq One-Step RT-PCR kit (BIO-65408, BioLine) as per manufacturer's instructions, using 500 ng total RNA and 10 µM primers in a total volume of 50 µl. The primers Chemo2FW (5'-TCTCTGGAGAGGAACGGAGA-3'), Chemo2RV (5'-GGTGTTTTCATTGGCCGGAG-3'), Hepcid2FW (5'-AGTCAAAGGAGCTGACAAGAGTC-3') and Hepcid2RV (5'-TTTACAACCAGGAGTACAGTGGAA-3') were designed to amplify two selected immune-related transcripts (Chemokine CC1 and Hepcidin2). These molecules were selected on the basis of previous biomolecular results obtained by the UNIUD research group (Byadgi *et al.*, 2017, 2019), where both Chemokine CC1 and Hepcidin showed significantly higher levels of expression in ESB infected by AO than uninfected control ESB. This finding was also confirmed at IoA by repeating the real time PCR experiment on the RNA later® preserved fish gill tissues. Reactions were run using a thermal cycler under the following conditions:

1 cycle of 45°C for 20 min, 1 cycle of 95°C for 1 min, 40 cycles of 95°C for 10s, 60°C for 10s, 72°C for 30s, followed by 1 cycle of 72°C for 2 min and 1 cycle of 10°C for 30 s. 1.5 µl of each PCR product was visualised on 1% agarose gel containing EthBr (0.05 µg/ml) under UV light. DNA amplified by PCR was purified using the QIAquick PCR Purification Kit (28104, QIAgen), as per manufacturer's instructions with minor modifications. Purified PCR products were then sent to GATC Biotech (www.eurofinsgenomics.eu/en/custom-dna-sequencing/gatc-services/) for sequencing using their LightRun service. A second set of primers were produced to generate sense and anti-sense RNA probes by adding the T7 promotor sequence (5' TAATACGACTCACTATAG 3') to the nucleotide sequences (Table 5.3). The cycling protocol used was the same as that reported above. Digoxigenin-labelling was performed using the DIG-RNA Labeling Kit (11 175 041 910, Sigma-Aldrich) following manufacturer's instructions. DIG-labelled probes were aliquoted (1 µl) and stored at -70°C until required. To determine the yield of the DIG-labelled riboprobes a dot-blot analysis was carried out according to Sigma-Aldrich protocol (DIG Application Manual for Nonradioactive In Situ Hybridisation, p59-64).

Table 5.3. Primers plus T7 region used for designing the riboprobes for mRNA FISH.

	Oligo name	**Sequence (5' → 3')**
Chemokine CC1 sense probe	Chemo2_T7_FW	TAATACGACTCACTATAGGGTCTCTGGAGAGGAACGGAGA
	Chemo2RV	GGTGTTTTCATTGGCCGGAG
Chemokine CC1 antisense probe	Chemo2FW	TCTCTGGAGAGGAACGGAGA
	Chemo2_T7_RV	TAATACGACTCACTATAGGGGGTGTTTTCATTGGCCGGAG
Hepcidin 2 sense probe	Hepcid2_T7_FW	TAATACGACTCACTATAGGGAGTCAAAGGAGCTGACAAGAGTC
	Hepcid2RV	TTTACAACCAGGAGTACAGTGGAA
Hepcidin 2 antisense probe	Hepcid2FW	AGTCAAAGGAGCTGACAAGAGTC
	Hepcid2_T7_RV	TAATACGACTCACTATAGGGTTTACAACCAGGAGTACAGTGGAA

5.2.5.3 Fluorescent mRNA ISH (FISH)

Gills sections were dewaxed with xylene, rehydrated with a graded ethanol series, and then incubated in 2× Saline Sodium Citrate (SSC) (BP1325-1, ThermoFisher Scientifc) for 1 min. An Immedge hydrophobic barrier pen (H-4000, Vector Laboratories) was used to draw a circle around the sections and 10 µg/ml Proteinase K (P2308, Sigma-Aldirch) pipetted onto the tissue. The Proteinase K digestion was conducted in a humidified box at 37°C for 5 min, and then stopped by

immersing the slides in ice cold 4% paraformaldehyde (in 1× PBS) for 5 min. Two washes in 1× PBS for 2 min each at RT were then carried out and the slides were dried as much as possible before a GeneFrame (AB-0578, ThermoFisher Scientific) was placed over the sections to localise the reagents to the tissue. Slides were incubated with a Pre-hybridization solution (50% formamide, 20% 20× SSC and 30% nuclease-free ddH_2O) at 37°C for 10 min in a humidified box. The riboprobes (300-800 ng/ml final concentration) were resuspended in the following hybridisation buffer: 50% (deionised) formamide (F9037, Sigma-Aldrich), 5× SSC, 10% dextran sulphate (D8906, Sigma Aldrich), 5× Denhardt's solution (D2532, Sigma Aldrich), 250 µg/ml yeast tRNA (15401-011, Invitrogen), 500 µg/µl herring sperm DNA (D1811, Promega) and 1% blocking solution (11585762001, Sigma-Aldrich). The hybridisation mix + riboprobes were heated at 80°C for 5 min and then cooled on ice before approximately 150 µl was pipetted onto the appropriate slide and covered with a GeneFrame coverslip. Hybridisation was performed overnight at 60°C in a humidified box. The following day, the coverslips and Geneframes were removed by rinsing in 2× SSC, the slides were transferred into separate, individual 50ml centrifuge tubes and they were then washed twice in 2× SSC (30 min each) at RT on a rocking platform (Stuart Scientific). The sections were again delimited using an ImmEdge pen to contain the reagents. A high-stringency wash step was performed at 65°C for 30 min in 50% 2× SSC plus 50% deionised formamide, without agitation. This was followed by two washes in 2× SSC at 37°C for 10 min each on the rocking platform.

After post-hybridisation washes, transcripts were identified using the DIG nucleic acid detection kit (11175041910, Sigma-Aldrich) and DIG Wash and Block Buffer set (11585762001, Sigma-Aldrich). Details of solution preparation are supplied in the kit protocols. Sections were transferred to 1× Wash Buffer at RT for 5 min on rocking platform, followed by an incubation in 1× Blocking solution buffer at RT for 30 min with agitation. Sections were incubated for 2 h at room temperature with Anti-Digoxigenin-AP conjugate antibody (11175041910, Sigma-Aldrich) diluted 1:5,000 in 1× Blocking solution and then washed twice with 1× Wash buffer for 15 min at RT on rocking platform. Sections were dried as much as possible and equilibrated in 1× Detection buffer for 5 min at RT. Bound antibody was localised using Fast Red tablets (F4648, Sigma-Aldrich) dissolved in TRIS buffer (F4648, Sigma-Aldrich). 150 µl of Fast Red solution was added to each slide and incubated in the dark at RT in the humidified box (without agitation). Slides were monitored under a light microscope to prevent over-development and high background. As soon as a signal was detected or after a maximum of 30 min, the reaction was stopped by gently washing the slides with nuclease-free ddH_2O. Finally, the slides were dried and mounted with a coverslip using Vectamount AQ Aqueous

Mounting Medium (H-5501, Vector Laboratories). Coverslip edges were sealed with clear nail varnish and the sections were incubated overnight to dry. Light and fluorescence microscopy images were captured using ArcturusXT™ Laser Capture Microdissection System (Nikon) microscope with an attached digital camera. Negative control slides were also included to which hybridization mix only was added (i.e. no riboprobes); a positive control slide consisted of a salmon louse (*Lepeophtheirus salmonis*) intestine section labelled with a trypsin antisense riboprobe.

5.2.6 Confocal microscopy

Confocal investigations were performed at the Institute of Aquaculture (IoA) of the University of Stirling for the purposes of better detailing *A. ocellatum* (AO) anatomy and the host-parasite interaction by using ESB AO infected gills. For this study Bouin's fixed ESB AO infected gills, sectioned at 4µm, were prepared using standard histological protocols and stained with haematoxylin and eosin at UNIUD (paragraph 5.2.3, Table 5.1).

In parallel, some ESB AO infected gill samples were processed following the same protocol for FISH (paragraph 5.2.5.1, Table 5.1) with the exception of greater section thickness (10 µm) and were subjected to different staining and labelling protocols in order to obtain better resolution of the features of interest.

All slides were visualized using a Leica SP2 AOBS multi-spectral confocal laser scanning microscope (CLSM) and examined with a 405 nm diode laser and HeNe (543 nm) laser. By xyz mode scanning of samples at various focal planes along the Z-axis a three-dimensional data set was acquired for samples and the morphology of the parasite and interaction with host tissues were evaluated.

5.2.6.1 DAPI and TRITC-phalloidin staining

Ten µm 4% paraformaldehyde-fixed paraffin-embedded samples were dewaxed twice with xylene (3 min and 2 min) and rehydrated using 100% ethanol for 2 min, followed by immersion in methylated spirit for 1.5 min. Slides were then washed with tap water for 1 min and stained with eosin (5 min). Then, a proportion of the slides was mounted with 4,6-diamidino-2-phenylindole,dihydrochloride (DAPI) (H-1200, Vectashield, Vector Laboratories), while the remaining slides were mounted with TRITC (tetramethylrhodamine)-Phalloidin (H-1600, Vectashield, Vector laboratories) for nuclear and cytoskeleton counterstaining respectively.

5.2.6.2 Double staining with calcofluor white and propidium iodide

In this investigation, a double-staining method combining two fluorescent stains was also tested with the objective of better highlighting some morphological characteristics of AO trophonts. Ten µm 4% paraformaldehyde-fixed paraffin-embedded samples were dewaxed and rehydrated as described for the DAPI and TRITC-phalloidin staining. Slides were then washed with tap water for 1 min and stained with eosin for 1 min (since with 5 min immersion in eosin, the background was too strong). Then slides were rinsed for 1 min with distilled water (dH_2O) and incubated in the dark with 1:25 propidium iodide (PI) (BMS500PI, ThermoFisher Scientific) in dH_2O in a humidified box for 30 min at RT for nuclear counterstain. Then, in the last 5 min of incubation with PI, the specific chitin/cellulose binding fluorochrome calcofluor white (CFW) (F3543, Sigma Aldrich) was employed at a concentration of 1% CFW stock solution. CFW stock solution was prepared as described by Rasconi *et al.* (2009) and used with the intent of staining the cellulose wall of *A. ocellatum* trophonts. After incubation, slides were washed with distilled water for 1 min and mounted with an aqueous Antifade Mounting Medium (H-1000, Vectashield, Vector Laboratories). Finally, coverslip edges were sealed with clear nail varnish.

5.2.7 Fluorescent lectin labelling

This labelling approach was based on a protocol developed at IoA and tested on ESB AO infected gills samples. Wheat germ agglutinin (WGA) lectin obtained from fluorescein lectin kit I (FLK-2100, Vector Laboratories) and WGA plus rhodamine obtained from lectin kit I (RLK-2200, Vector Laboratories) were selected to label the carbohydrates of *Amyloodinium ocellatum* trophonts. Lectins were diluted to a final concentration of 5 µg/ml in the following lectin wash buffer (LWB): 50 mM Tris(hydroxymethyl)aminomethane, 150 mM sodium chloride (NaCl), 2 mM magnesium chloride ($MgCl_2$) and 1 mM calcium chloride ($CaCl_2$) pH 7.4 (all compounds were purchased from Sigma Aldrich). Then 5 µm sections fixed in 4% paraformaldehyde and paraffin-embedded were dewaxed into two changes of xylene for 3 min each, followed by rehydration using 100% ethanol (2 min) and 70% ethanol (2 min). After washing in dH_2O for 1 min, sections were encircled with an ImmEdge pen (H-4000, Vector Laboratories) to retain the lectin/buffer in place on the tissue preparation. Two-hundred µl of lectin solution was pipetted onto the dewaxed sections and incubated in a dark chamber for 2 h at RT. Thereafter, sections were washed in LWB three times for 5 min each. A negative control was used for each lectin labelling investigation and treated in the same way as test lectin sections, but with the use of LWB only. Slides were washed in LWB (3×5

min), then mounted in DAPI (H-1200, Vectashield, Vector Laboratories), coverslipped and sealed with clear nail varnish. Slides were then incubated in the dark for 30 min to let the mounting medium evenly distribute on the section. Slides were analysed using a fluorescence microscope (ArcturusXT™ Laser Capture Microdissection System, Nikon) and a Leica SP2 AOBS multi-spectral confocal laser scanning microscope (CLSM).

5.2.8 E.L.I.S.A. development

The enzyme linked immunosorbent assay (E.L.I.S.A.) used in this study was developed by adapting the method by Smith *et al.* (1992) as regards the coating antigen preparation and the method of Bakopoulos *et al* (1997) as regards the assay development. The test was applied on sera deriving from naturally infected ESB (Table 5.1) in the initial phase of set up; then sera from ESB experimentally infected and immunized ones (paragraph 5.2.2) were evaluated by following the definitive developed E.L.I.S.A. protocol. Sera from naturally infected fish were newly evaluated according to the developed E.L.I.S.A. protocol.

5.2.8.1 AO production for E.L.I.S.A. microplate coating

A. ocellatum tomonts purified and preserved as described in paragraphs 4.2.1.1 and 4.2.1.3, were transferred to RT to resume the reproductive process. Tomonts were constantly monitored under an inverted microscope to check dinospores development that generally required a couple of days. Free-swimming dinospores were collected with a Pasteur pipette and aliquoted in 50 ml centrifuge tubes, then inactivated at -20°C overnight. Inactivated dinospores were thawed and centrifuged at 1,210×g for 5 min at RT, supernatant (sterile sea water and antibiotics) was removed and the dinospore pellet suspended in sterile PBS (pH 7.2). Followed two washes (1,210×g for 5 min each at RT), dinospores were then resuspended in 5 ml of sterile PBS. Parasites were counted (adapted Dehorty, 2003) and adjusted to the final concentration of 1×10^6 dinospores/ml. Then the suspension was stored at -20°C. For coating antigen production, dinospores were subjected to sonication, homogenization or pottering.

5.2.8.2 AO sonication

As reported in literature (Smith *et al.,* 1992, 1993, 1994; Cobb *et al.,* 1998b; Cecchini *et al*., 2001), sonication was the most applied technology to disrupt armoured dinospores and obtain good amounts of protein. Inactivated parasites were treated with an ultrasonic bath sonication for 2 min

by the sonicator LBS2 (FALC Instruments) set as follows: 59 kHz of frequency and 95% power. Dinospores were then checked under an optical microscope. Because the wall was still intact, the treatment was repeated maintaining the same frequency but increasing both the power (100%) and the treatment duration (30 min). To avoid protein denaturation due to water temperature increment, the process was divided into three cycles of 10 min each with 15 min of pause between cycles. After light microscope visualization, the sample was centrifuged following the same parameters reported by Smith *et al.* (1992) (60×g for 5 min at RT). Then the protein content of the supernatant was measured with Bradford method (Bradford, 1976). A different sonication was conducted with a mini probe sonicator (UP450S, Hielscher) at 24 kHz frequency and 400W power for 10 min at 4°C. Dinospores integrity was checked under light microscopy, then parasites were centrifuged at 60×g for 5 min at RT and the protein content measured with Bradford method. The sample was finally aliquoted and stored at -80°C.

5.2.8.3 AO homogenization by Tissue Lyser II

Tissue Lyser II (85300, QIAgen) was used as an alternative treatment to sonication. For the trial, a dinospore sample was divided into two sterile screw cap micro tubes (72692, Sarstedt) filled with micro glass beads (G8772, Sigma Aldrich). Tubes were put on ice and subjected to two oscillation cycles (300 oscillation/s) of 150 s each, cycles were interspersed with pauses of 30 s during which tubes were put on ice to avoid protein denaturation. At the end of the treatment, the sample was visualized under a light microscope and tubes centrifuged at 18,000×g (5417R, Eppendorf Centrifuge) for 10 min at 4°C. The protein content was measured by Bradford assay.

5.2.8.4 AO pottering

Dinospores were processed as already mentioned in the vaccination trial paragraph (5.2.2) concerning the preparation of the vaccine. Therefore, in this case the antigen addressed to the microplate coating corresponded exactly to the antigen used for the *in vivo* immunization. Then parasites were adjusted to the concentration of 6.5×10^6 dinospores/ml, aliquoted and stored at -80°C

5.2.8.5 Reagents

3,3',5,5' Tetramethylbenidinedihydrochloride (TMB) substrate (42 mM); **Carbonate/bicarbonate buffer** [15 mM sodium carbonate (Na_2CO_3), and 35 mM sodium bicarbonate ($NaHCO_3$); pH 9.6]; **High Salt Wash Buffer (HSWB) 5X** [100 mM Tris aminomethane ($C_4H_{11}NO_3$), 2.5 mM sodium chloride (NaCl), and 5 mL Tween20; pH 7.7]; **Low Salt Wash Buffer (LSWB) 5X** [100 mM Tris aminomethane (C4H11NO3), 1.9 mM sodium chloride (NaCl), and 2.5 mL of Tween20; pH 7.3]; **phosphate buffered saline (PBS) 1X** [140 mM sodium chloride (NaCl), 7.9 mM disodium phosphate (Na_2HPO_4), 2.68 mM potassium chloride (KCl), and 1.47 mM potassium phosphate monobasic (KH_2PO_4); pH 7.4]; **Sodium acetate/citric acid** [109 mM citric acid ($C_6H_8O_7$), and 100 mM sodium acetate ($C_2H_3NaO_2$); pH 5.4];. All compounds were purchased from Sigma-Aldrich.

5.2.8.6 E.L.I.S.A. assay

One-hundred µl/well of 0.001% poly-L-lysine (P8920, Sigma Aldrich) in carbonate/bicarbonate buffer were placed into a 96-well flat bottom microtitre plate (NUNC Maxisorp) and then incubated for 1 h at RT. Followed three washes with 1× LSWB (200 µL/well). 200 µl of dinospore suspensions were added to each well as coating antigen. In the set up phase, dinospores sonicated with the miniprobe sonicator were used at the concentration of 5×10^3/ml; whereas in the definitive protocol, dinospores subjected to potter fragmentation were used at the concentration of 1.3×10^6/ml[4] (Fig. 5.2).

Figure 5.2. Microscopic observation of a microwell bottom after the coating with pottered dinospores.

[4] For the coating of E.L.I.S.A. plates were used only dinospores derived from the mini-probed sonication and from the pottering treatment. In fact, both bath sonication and homogenization were ineffective in fragmenting the parasites.

Hence, the plate was centrifuged at 210×g for 10 min at RT to allow the adhesion of the antigen to the wells bottom, and incubated for 1 h at RT. Plate was washed trice with 1× LSWB (200 µl/well) and post coated with 200 µl/well of 1% gelatine (G6650, Sigma Aldrich) in 1× LSWB for 3 h at RT. Followed three washes with 1× HSWB (200 µl/well), then 200 µL of 5% goat serum (S26-100, EMD Millipore) in 1× LSWB were placed in each well and incubated at 4°C overnight. Antigen-coated microtitre plate was washed trice with 1× HSWB (200 µl/well) and 100 µl/well of ESB test sera (in duplicate) (Table 5.4) diluted 1:10 in PBS and 0.1% Tween20 solution were added to the corresponding wells and incubated for 2 h at RT. Followed 4 washes with 1× HSWB (200 µl/well). Hence, primary monoclonal antibody anti ESB IgM (F01, Aquatic Diagnostic Ltd, UK) diluted 1:33 in 1% BSA and PBS solution was added (100 µl/well) and incubated for 1 h at RT. The plate was washed 4 times with 1× HSWB (200 µl/well), then 100 µl/well of a HRP conjugated antibody to mouse IgG (A4416, Sigma Aldrich) diluted 1:4000 in PBS and 1% BSA solution were placed and incubated for 1 h at RT. Followed 4 washes with 1× HSWB (200 µl/well), then 100 µl of freshly prepared colorimetric solution were placed into each well. The colorimetric solution composition was 150 µl of TMB (42 mM), 5 µl of hydrogen peroxide (H_2O_2) in 15 ml of pre-warmed (37° C) sodium acetate/citric acid buffer. The reaction was stopped after 6 min by adding 50 µl/well of 2M sulphuric acid (H_2SO_4) and the absorbance at 450 nm determined by spectrophotometer (TecanSunrise, Milan, Italy). Each plate included control wells (blanks) with omission of the serum, replaced by the dilution buffer.

For the vaccine efficacy trial, the antibody titre against AO was expressed as serum optical density (O.D.)/blank O.D.. The cut-off between negative and positive samples was set at the mean value of blank O.D.+ three standard deviations of blank O.D. (Engvall and Perlmann, 1971).

Table 5.4. List of ESB sera analysed by E.L.I.S.A. Fish origin data are reported in Table 5.1.

code	Health status	Origin	Size	Type infection
H1	Healthy	Farm A1	adult	-
H2	Healthy	Farm A2	adult	-
SPT	Symptomatic	Farm A1	adult	Natural
IS1	Infected but survived	Farm B	adult	Natural
IS2	Infected but survived	Di4A	juvenile	Experimental
IS3	Infected but survived	Di4A	juvenile	Accidental*
VLd	Vaccinated low dose	Di4A	juvenile	Experimental
VHd	Vaccinated high dose	Di4A	juvenile	Experimental
VCTRL	Vaccination CTRL	Di4A	juvenile	Experimental
VHS	Vaccination high dose survived**	Di4A	juvenile	Experimental
CI	Chronical infected	Di4A	juvenile	Experimental

**serum collection was performed two months after the infection outbreak. **VHS group are the survivors of the potency test and their sera have been pooled.*

5.2.8.7 Data analysis

The mean optical density (O.D.) was calculated for each sample tested in duplicate. From these values the O.D. average of blanks (serum omission) was subtracted. The data were then statistically analysed using SPSS Statistics v20 software (SPSS, Inc, Chicago, IL, USA). The normality of the data was verified by Kolmogorov-Smirnov test, then the data were compared by means of one-way analysis of variance (ANOVA) and Duncan *post hoc* test to identify any significant differences between the experimental groups ($P \leq 0.05$).

5.3 RESULTS

5.3.1 AO infection-1 outcomes

The AOinf-1 time-course was verified by *in vivo* or *post mortem* gill examinations, then confirmed also by histology. Fish were already infected after 2 hours of exposure (Fig. 5.3a), and 2 days post infection (dpi) the parasite burden was discrete with slightly evident clinical symptoms. During the infection, the maximum parasite burden was observed at 10-12 dpi (Fig. 5.3b), thereafter fish started to recover even if they were still infected. This experimental infection lasted 23 days and the cumulative mortality of infected fish was 18 % in all three tanks. Fish belonging to the control group were never infected (Fig. 5.3c) and no mortality was registered for them. Sampled gills were used for histological evaluations, while IHC investigations are in progress.

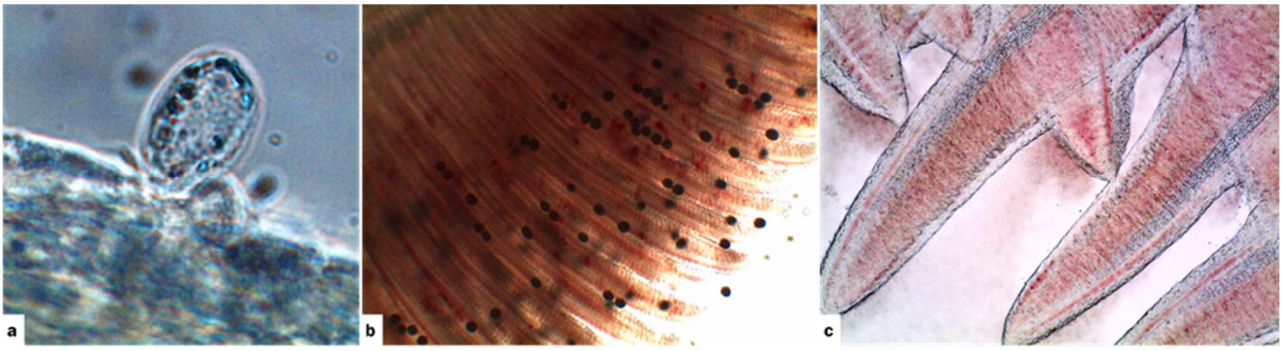


Figure 5.3. a) A very early trophont attached to gill epithelium after 2h post infectio; b) infected gills showing the maximum parasite burden detected at 10-12 dpi; c) CTRL gills without AO trophont.

5.3.2 Vaccine efficacy trial

The vaccination trial was performed by administering intraperitoneally two different doses of AO crude extract as antigen, then the effectiveness of the treatment was assessed by the E.L.I.S.A. based evaluation of specific humoral response against the pathogen after 3 weeks post vaccination (paragraph 5.3.8, fig. 5.21 and 5.22) and by recording the *in vivo* protection against a challenge with viable AO after 30 days post vaccination. Concerning the latter post challenge survey, both vaccinated and control individuals developed clinical sign ascribable to amyloodiniosis. Each mortality event was recorded and the level/specificity of infection were defined by observing the AO load on the gills of individual ESB under optical microscope. The mortality started on day 5 post infection in control unvaccinated fish, and in fish immunized with the low dose of antigen, whereas it started on day 7 post infection in the group vaccinated with the high antigen dose. The figure 5.4 illustrates the cumulative mortality data, and underlines a slight delay in the disease progression in

the case of fish vaccinated with the highest dose of AO crude extract. Still the post challenge mortality reached 100% in all the groups within the 9th day post infection.

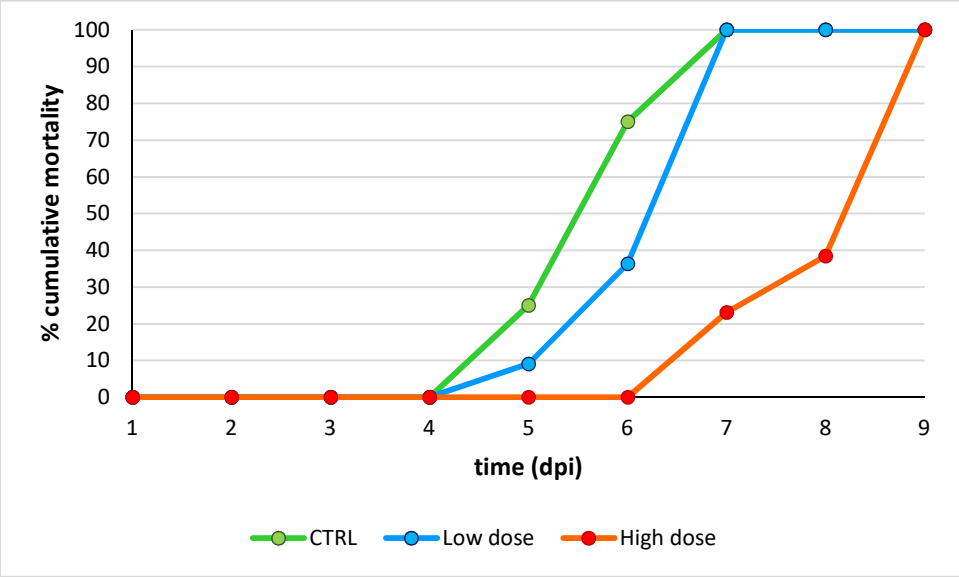


Figure 5.4. Cumulative mortality recorded in the three groups submitted to AO challenge.

5.3.3 Histology

All samples of ESB gills and heads, coming from naturally and experimentally infected fish (Table 5.1), contributed to the histological description reported below.

Gills and the entire oro-pharingeal cavity were the primary sites of the infection in ESB (Fig. 5.5b,c) and, albeit in some surveyed infections the skin was abundantly infected, it had not a dusty appearance (hence the name velvet disease), as observed in other fish species. AO infected ESB died without obvious gross skin lesions, also confirmed by histological observations.

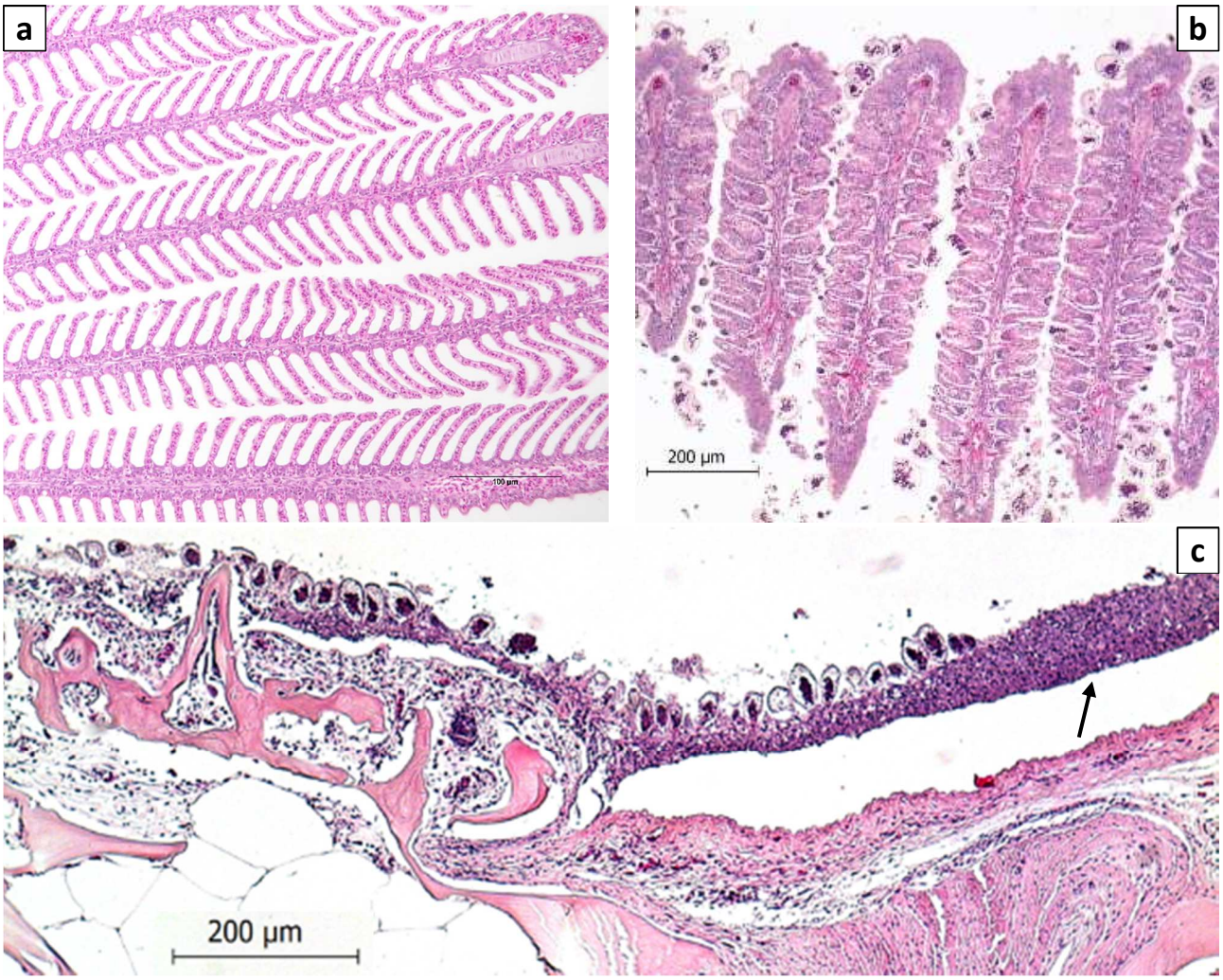


Figure 5.5. European sea bass. a) Some primary lamellae non infected by AO compared to (b) gills heavily infected; trophonts adhesion induced severe and diffuse epithelial hyperplasia and degeneration. c) Floor of buccal cavity showing anchored trophonts responsible of the epithelial hyperplasia (arrow) and necrosis. H-E.

In the early stages of infection or in slight infections (less than ten trophonts/primary lamella) no histological lesions were detectable, although it was observed a mild epithelial hyperplasia around the single trophont adhesion site associated to epithelial cells degeneration (Fig. 5.6).

Figure 5.6. Trophont anchored with rhizoids (arrows) to the gill epithelial cells, some of which are degenerated; scale bar = 10µm. H-E.

The parasite burden was considered very high when more than fifty trophonts were present on a singular primary lamella. In the first 36-48 h of heavy infection, severe and diffuse epithelium degeneration of oro-pharyngeal cavity was present, characterised by hydropic degeneration of gill epithelial cells and chloride cells, oedema and necrosis (Fig 5.7a,b); subsequently it was possible to observe hyperplasia of the gill epithelium especially in the distal third of the primary lamellae (Fig. 5.5b).

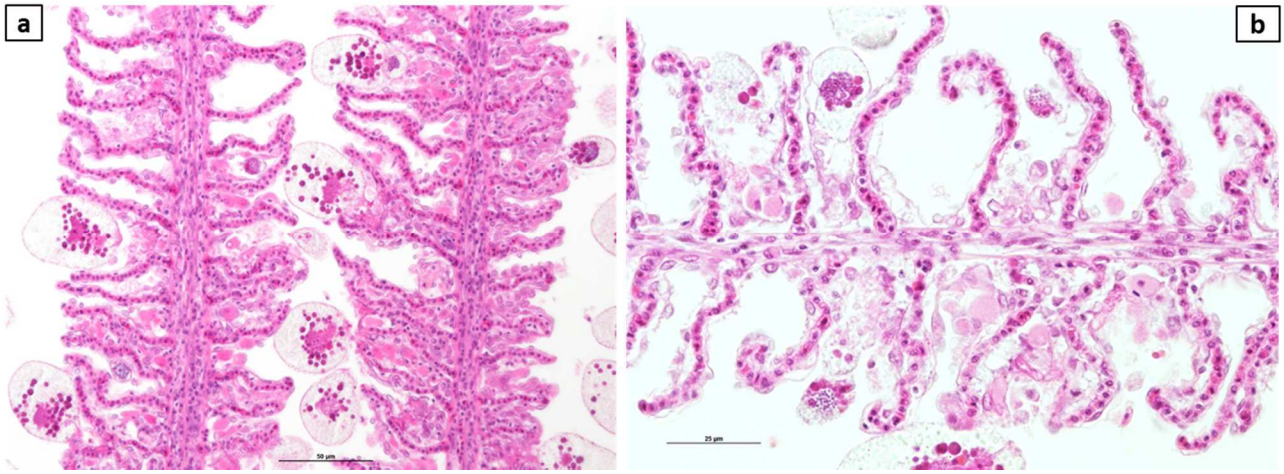


Figure 5.7. Gill epithelial cell degeneration characterised by hydropic cell degeneration (also of chloride cells) (a, scale bar = 50µm), oedema and necrosis (b, scale bar = 25µm). H-E.

The same histological pattern was observed in pseudobranchs (Fig. 5.8c), while marked epithelial hyperplasia was the primary alteration of buccal cavity (Fig. 5.5c, 5.8a), pharynx (Fig. 5.8b) and gill arches epithelium.

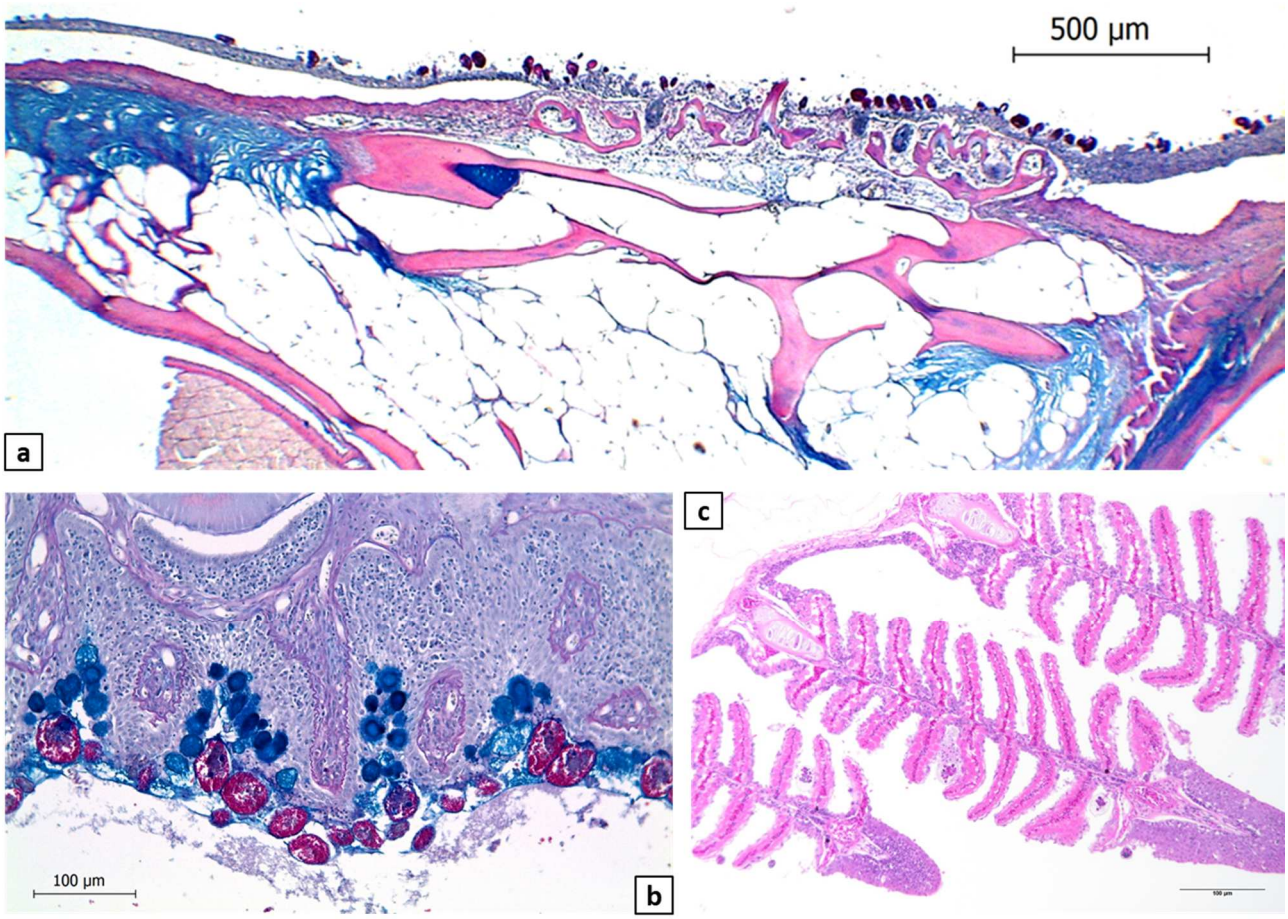


Figure 5.8. a) Buccal cavity floor showing attached trophonts and epithelial damage with hyperplasia. Pas-Alcian blue. b) Pharyngeal tract with attached AO trophonts in which starch granules are stained in purple-magenta and very abundant mucous cells (stained in blue) are visible in the epithelium. Pas-Alcian blue. c) Some trophonts attached to pseudobranchs where it is possible to observe moderate oedema and epithelial degenerative processes; scale bar = 100µm. H-E.

Trophonts were also well scattered in nasal cavities, where diffuse epithelial degeneration and cellular infiltration were observed. Marked gill epithelial hyperplasia induced a pattern in which synechiae were easily visible at lamellae tips (Fig. 5.9a). In the gill lamellae the inflammatory cells were difficult to differentiate, however along the filaments lymphocytes, macrophages, mast cells were discernible (Fig. 5.9b); the same cell infiltrate pattern was more evident in the buccal cavity and pharynx, where mucous cells were very abundant in the epithelium (Fig. 5.8b). Moreover, a discrete number of rodlet cells was often present along the lamellar epithelium, most of all at the base of secondary lamellae. *A. ocellatum* trophonts feed and anchor on multiple epithelial cells simultaneously, inducing extensive damage of the vascular system as well as provoking the rupture of pillar cells and formation of lamellar aneurysms or microhaemorrhages (Fig. 5.9c,d). These changes were observed at the necropsy in the form of anaemia in gills.

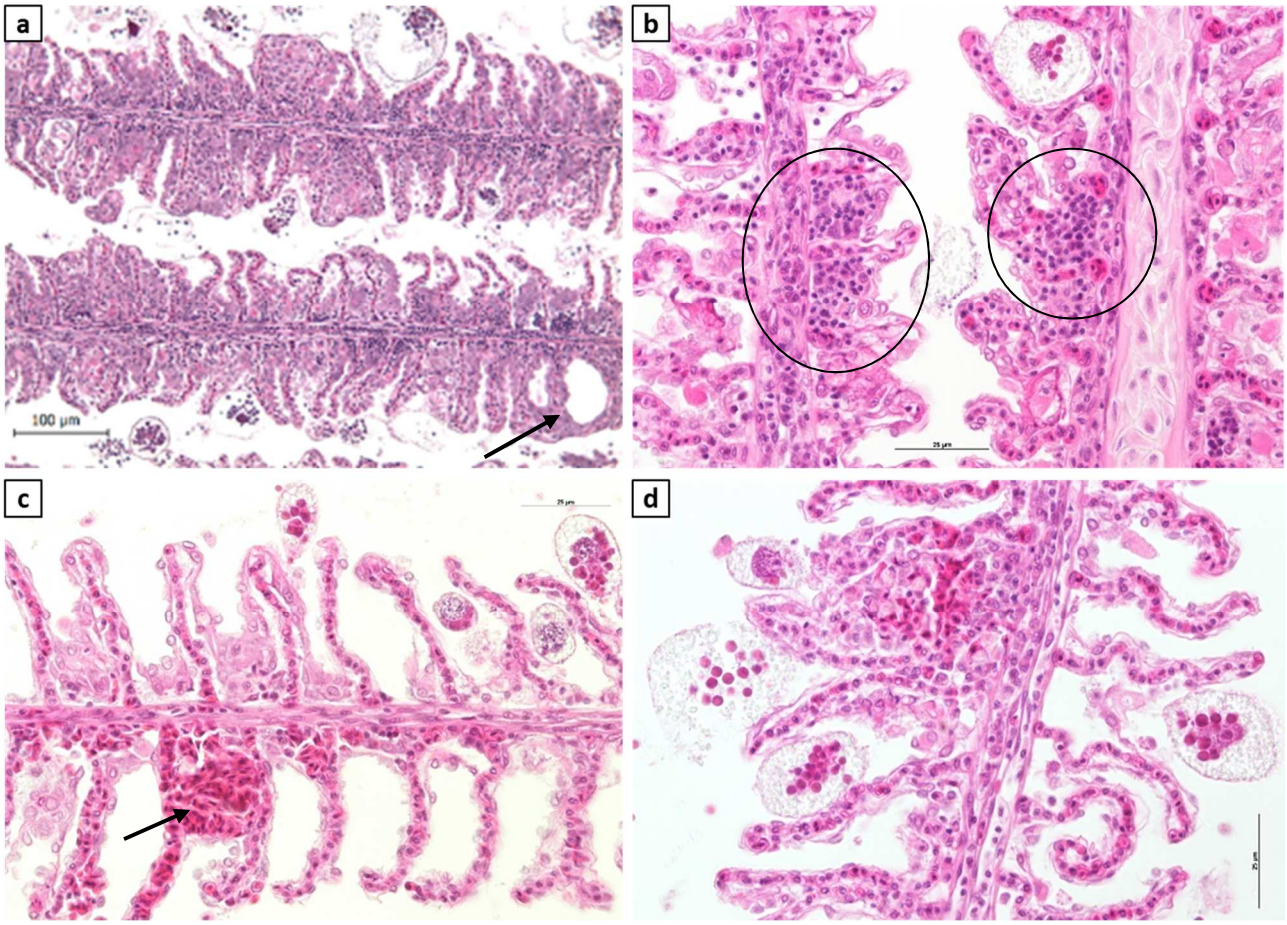


Figure 5.9. a) Marked gill epithelial hyperplasia causing the fusion of secondary lamellae in which synechiae are easily visible at lamellae tips (arrow). b) Focal lymphocytic infiltrate in gill lamellae; scale bar = 25µm. c and d) Vascular damages characterised by aneurysm formation (arrow) and microhaemorrhages; scale bars = 25µm. H-E.

5.3.4 Immunohistochemistry

The markers used in this study were selected based on their relevance as indicators of tissue inflammatory and proliferative response and on some previously assessed reactivities on ESB tissues in our laboratory.

The following description concerns the general IHC outcomes in specimens collected from both natural outbreaks and experimental infection trials (Table 5.1). Control sections of gills collected from diseased ESB, in which the primary antibody was replaced by dilution buffer, resulted always negative (Fig. 5.10).

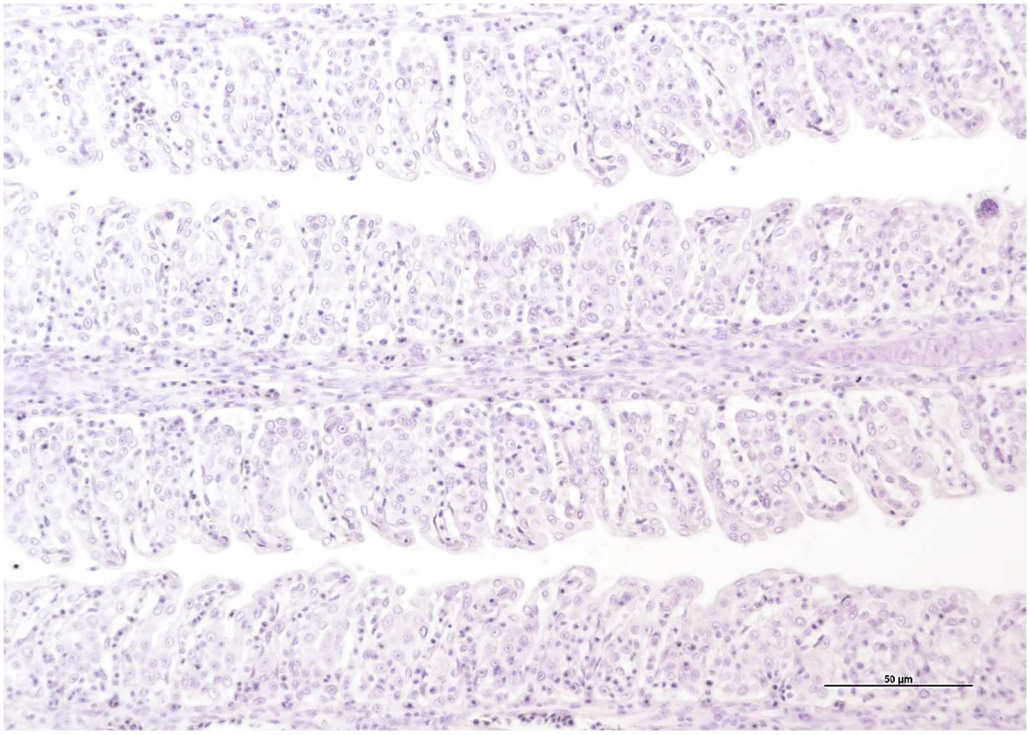


Figure 5.10. Absence of positivity in gills from diseased ESB submitted to IHC with the omission of primary antibody.

The hyperplastic gill lamellae revealed the presence of cell populations positive for the antibodies specific to CD16, CYP1A, cytokeratine, ESB IgM, GM-CSFRα, iNOS, PCNA, TLR2, TLR4 and TNF-α.

In detail, CD16, GM-CSFRα, iNOS, TLR2 and TLR4 labelled cellular populations morphologically ascribable to macrophages (Fig. 5.11a-f; Fig. 5.12a-f).

The CYP1A positive signal was evident in gill epithelial cells showing hyperplastic changes and localised necrosis (Fig. 5.13c). A relevant part of the hyperplastic epithelial cells resulted positive also for PCNA (Fig. 5.13e). The cytokeratin antibody marked very clearly the gill epithelial cells, underlying the condition of hyperplasia in the diseased individuals (Fig. 5.13f).

Macrophage-like cells positive for GM-CSFRα were not abundant, and their distribution was possibly associated with epithelial sites adjacent to the parasite adhesion (Fig. 5.11f).

The polyclonal antibody specific for ESB IgM marked cells whose morphology was ascribable to plasma cells and in some cases to macrophages diffusely distributed in the epithelium of branchial lamellae (Fig 5.13d).

TLR2 and TLR4 positivity was mainly observed in macrophage-like cells localised in the most inflamed and hyperplastic areas of the infected gills (Fig. 5.12a-d).

The macrophage-like cells positive for iNOS (Fig 5.11a-d) were mainly localized in the gill areas where lamellae were hyperplastic and fused. The positivity for TNF-α was observed in monocytes circulating inside the small gill vessels and the endothelial vessels walls (Fig. 5.13a,b).

Antibodies for TNF-α and iNOS reacted also with antigens expressed by the parasite. This positivity was especially evident for iNOS in trophonts at the early stage of adhesion (Fig. 5.11e).

The application of the reactive markers on gill tissues collected from healthy ESB (uninfected fish used as reference) allowed to detect a very few positive cells, whose number was negligible if compared to the one observed in the pathological samples (data not shown).

Some of the antibodies proposed as markers in this research (CD3, CD35, CD68, Cox-2 (H62), Cox-2 (M19), histamine, HSP-70 and lysozyme) did not react in the investigated gill samples.

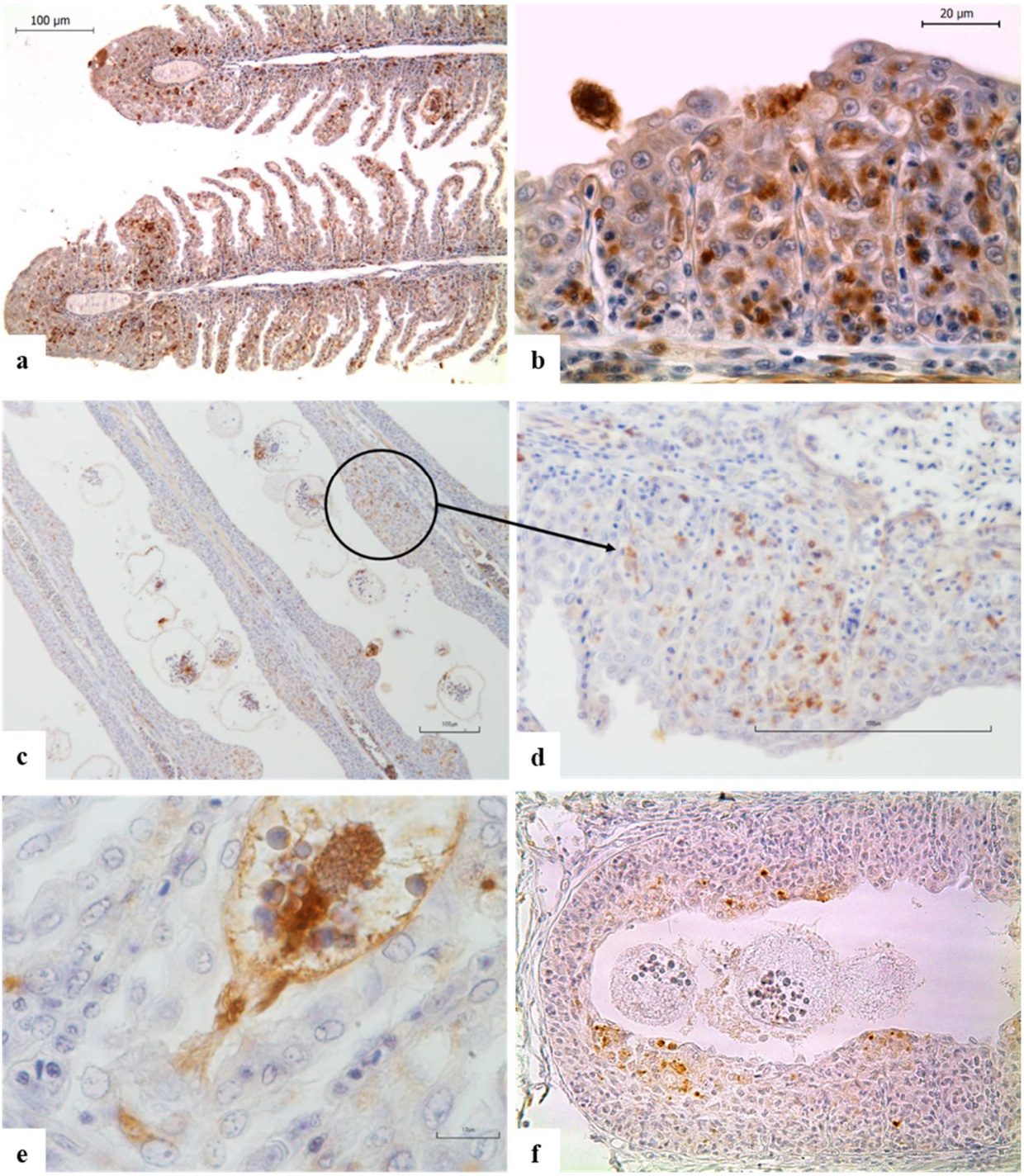


Figure 5.11. ESB gill tissue infected by A. ocellatum. a) to d) Macrophage-like cells positive for iNOS; e) trophont positive for iNOS; f) macrophage-like cells positive for GM-CSFRα, magnification 1000X.

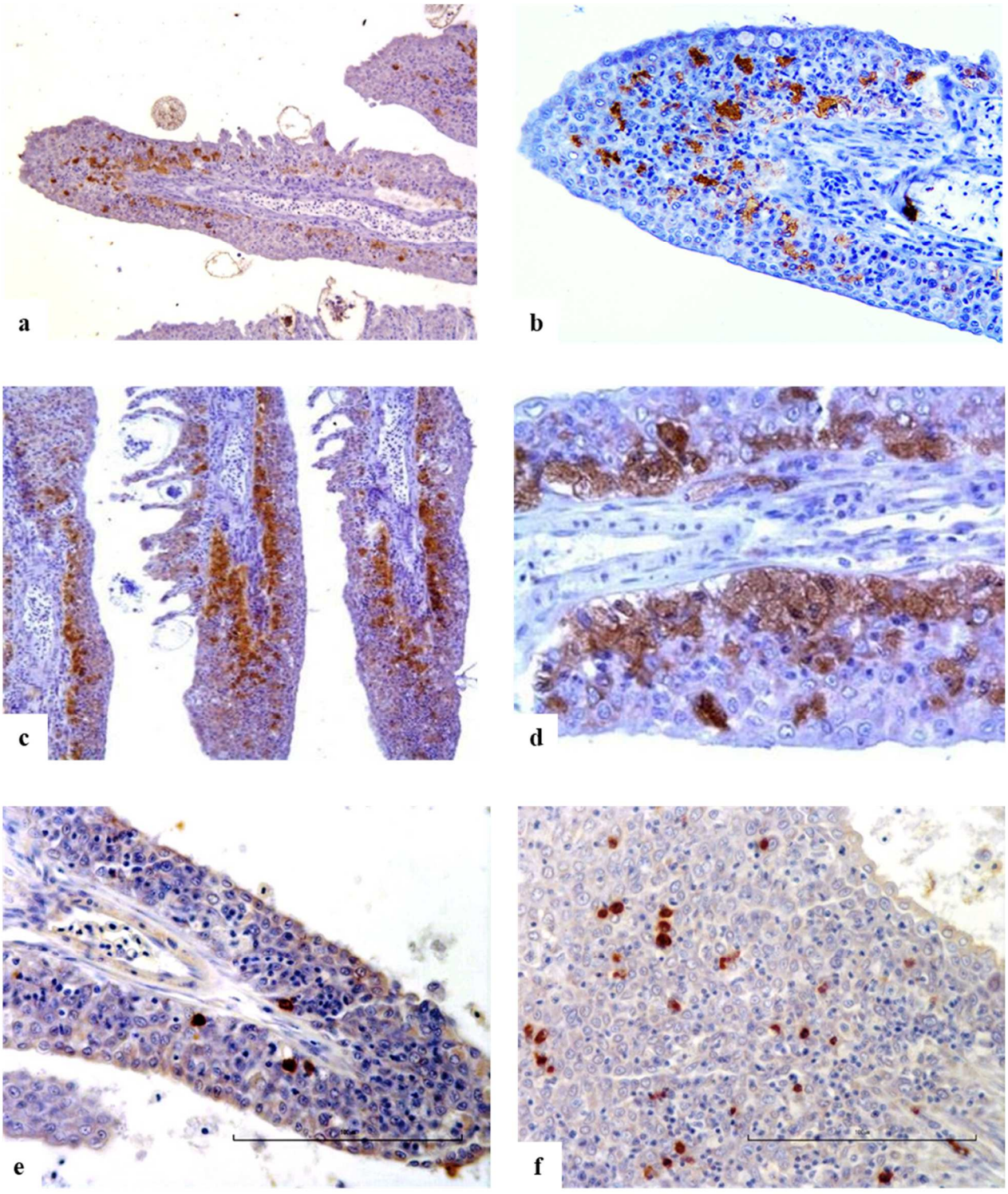


Figure 5.12. ESB gill tissue infected by A. ocellatum. a) and b) Macrophage-like cells positive for TLR2 (Courtesy of Dr. Magi); magnifications 20X and 100X respectively. c) and d) Macrophage-like cells positive for TLR4 (Courtesy of Dr. Magi); magnifications 20X and 1000X respectively. e) and f) Macrophage-like cells positive for CD16.

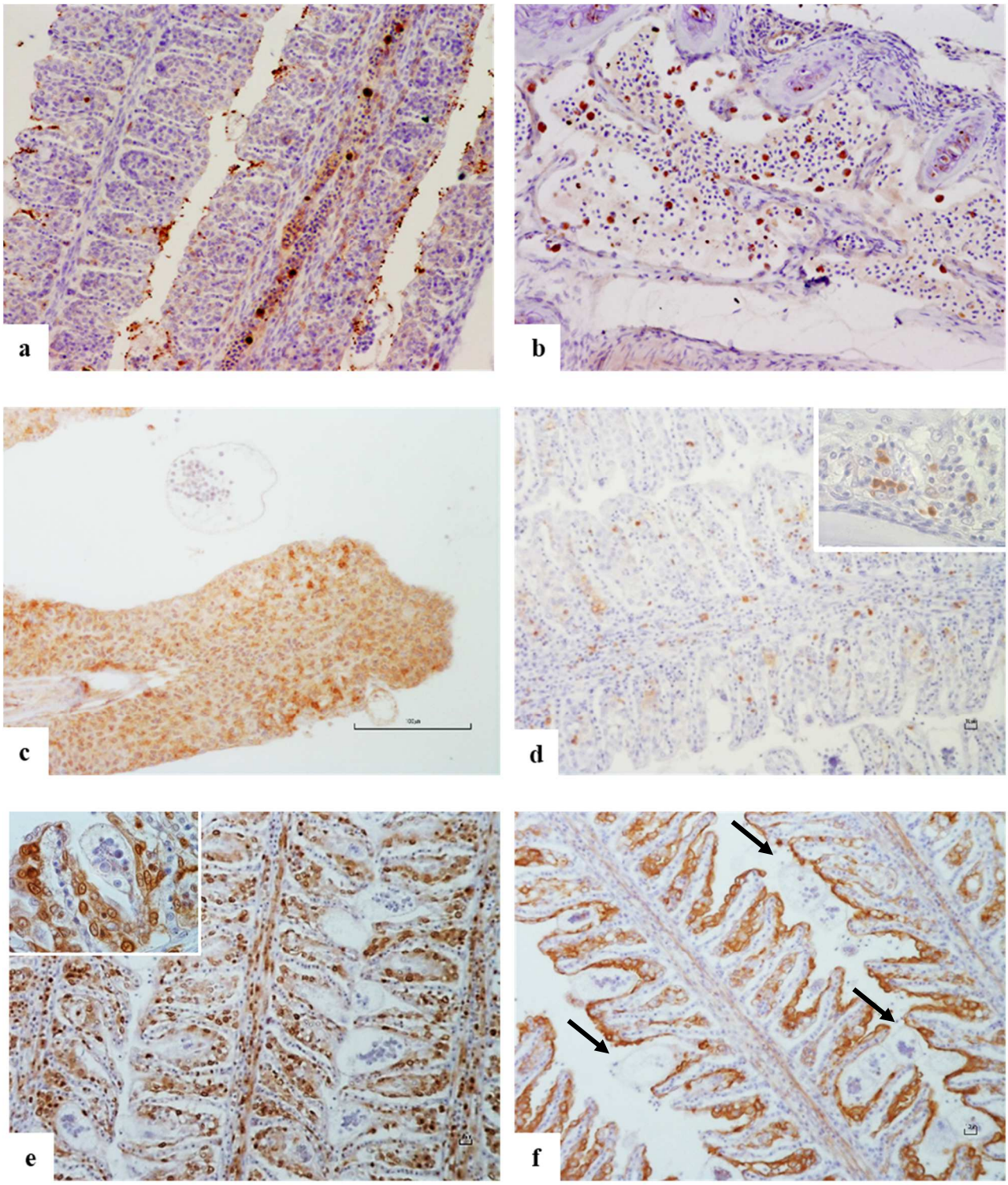


Figure 5.13. ESB gill tissue infected by A. ocellatum. a) and b) Vessel walls (endothelium) and monocytes circulating inside branchial vessel wall positive to TNF-α (Courtesy of Dr. Ronza); magnifications 40X and 1000X respectively. c) CYP1A immunolabelling of numerous epithelial cells in a hyperplastic area; d) plasma cells marked with the anti-ESB IgM antibody, in the upper corner on the right, cell positivity detail. e) Immunolabelling with anti PCNA antibody. The number of epithelial cells under turnover is relevant. The parasites are negative (detail in the upper left corner). f) Immunolabelling with anti cytokeratin antibody, pointing out very clearly the gill epithelial cells and underlying the condition of hyperplasia. The parasites between lamellae are negative (arrows).

5.3.5 FISH

The results obtained using mRNA *in situ* hybridization provided a better understanding of the pattern of localisation of leukocyte populations in the gills and supported the observed differential expression of Chemokine CC1 transcript between uninfected and infected sea bass. Briefly, in the uninfected control fish the signal was more evident in the gill associated lymphoid tissue (GIALT) (Fig. 5.14a,b), in the lumen of the central venous sinus and in the capillaries of the apical portion of the primary lamellae (data not shown).

In the infected fish a higher abundance of positive leukocytes was observed in the hyperplastic regions of the secondary lamellae (Fig. 5.15a-d) and in the vessel wall (diapedesis) of the central venous sinus of primary lamellae (Fig. 5.15a-d). Leukocytes positive for Chemokine CC1 were also detected in the cytoplasm of *A. ocellatum* trophonts (Fig. 5.15c,d).

In general, the signal intensity was lower in uninfected control fish, while in the infected subjects the signal was visibly higher.

In the gills of infected fish, no positive responses were detected in the close vicinity of trophont adhesion sites (Fig. 5.15c,d).

In the negative controls (no riboprobes) and in the samples hybridised with Chemokine CC1 sense probe, no positive signal was detected in the examined gill tissues (Fig. 5.14c-f; Fig. 5.16a-d).

No signal was observed on any of the gill slides incubated with the Hepcidin 2 probes.

Positive control slide consisted of a salmon louse (*Lepeophtheirus salmonis*) intestine section labelled with a Trypsin antisense riboprobe (Fig. 5.16e,f).

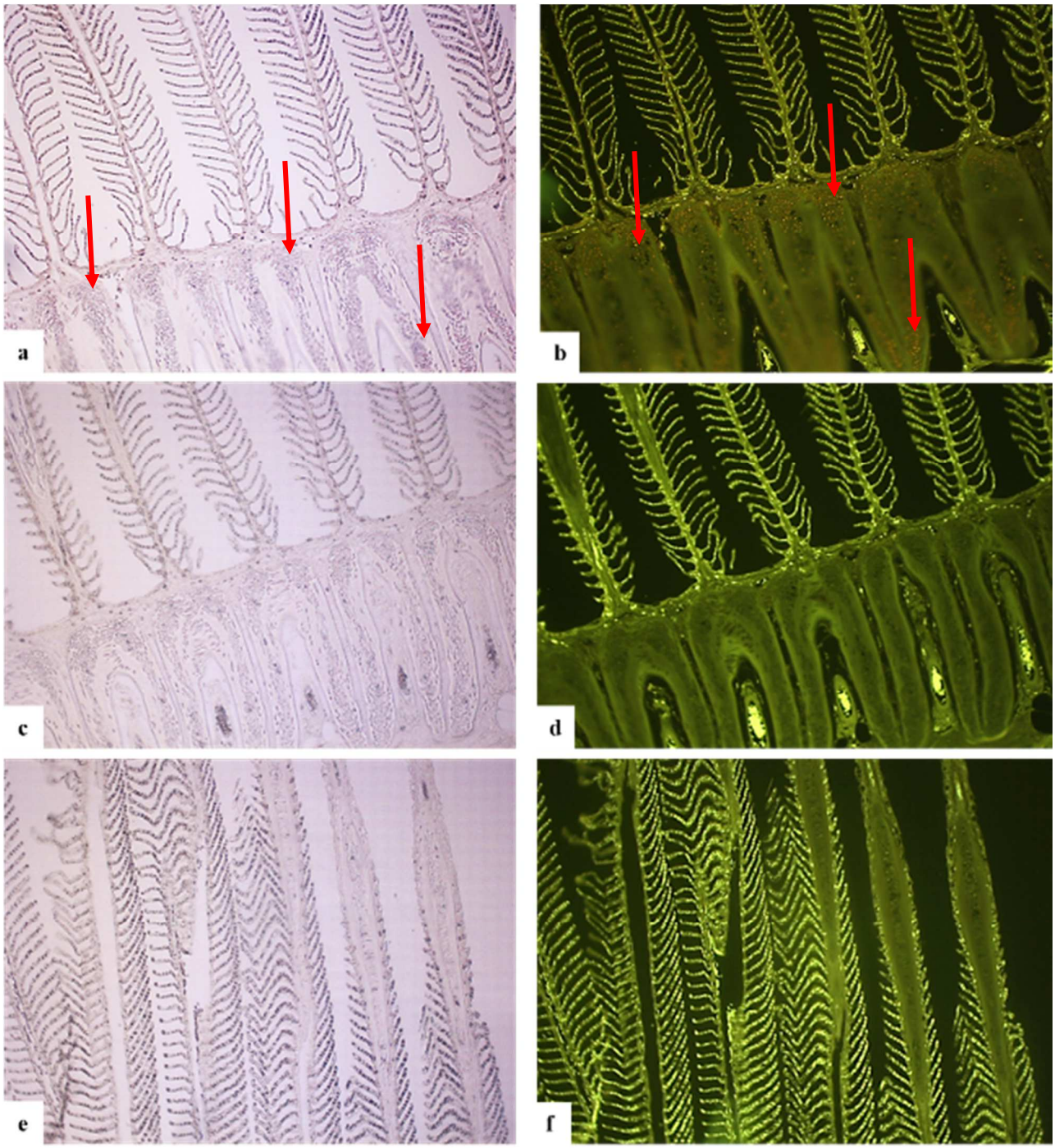


Figure 5.14. ESB gill tissue from uninfected control fish. a) and b) Leukocytes expressing Chemokine CC1 (arrows) in the GIALT (Gill Associated Lymphoid Tissue). c) and d) Negative control (no riboprobes). e) and f) sections incubated with Chemokine CC1 sense probe. Images captured in brightfield are on the left, images captured using fluorescence microscopy are on the right. All at 10X magnification.

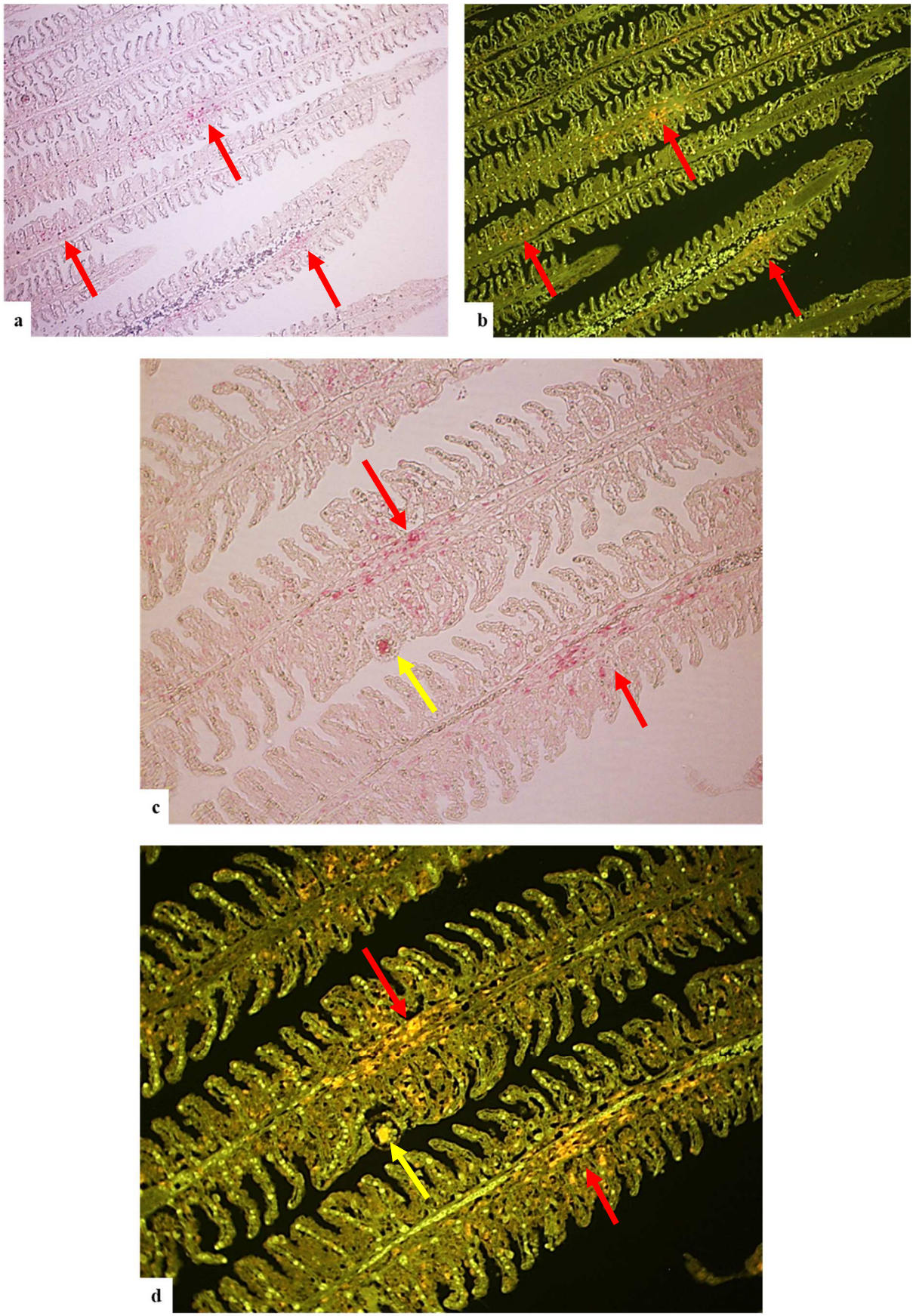


Figure 5.15. ESB gill tissue infected by A. ocellatum. a) to d) CC1 positive leukocytes (arrows) in the hyperplastic regions of the secondary lamellae and in the vessel wall (diapedesis) of the central venous sinus of the primary lamellae. c) and d) A positive signal for CC1 (red arrows) in the hyperplastic areas of secondary lamellae and within cytoplasm of A. ocellatum trophonts (yellow arrow). a) and b) 10X magnification; c) and d) 20X magnification. Images 5.5a and 5.5c were captured in brightfield, figures 5.5b and 5.5d using fluorescence.

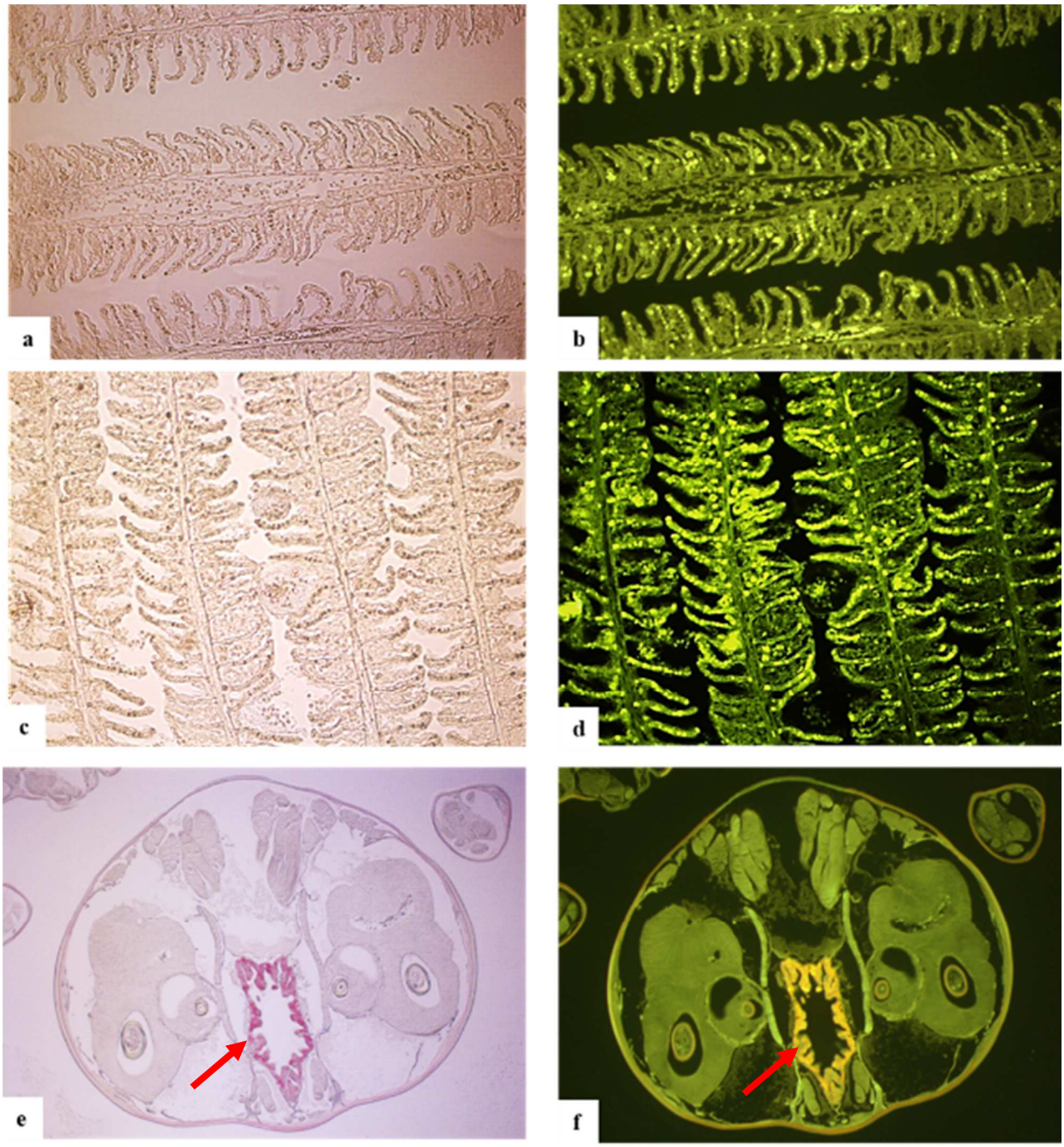


Figure 5.16. ESB gill tissue infected by A. ocellatum. a) and b) Chemokine CC1 sense probe, 10X magnification. c) and d) Negative control (no riboprobes), 20X magnification. e) and f) Reference positive control represented by sea louse (L. salmonis) intestine labelled with Trypsin antisense probe (arrow), 10X magnification. Images on the left captured with brightfield and on the right using fluorescence.

5.3.6 Confocal microscopy

Under confocal microscope eosin exhibits maximum absorption and emission at 527 and 550 nm respectively, with both peaks lying in the green light range. Four µm haematoxylin and eosin (H-E) slides from UNIUD archives provided good results even if the thickness of the sections (4µm) was not ideal for confocal reconstruction of the full trophont depth. The images captured from these samples gave the opportunity to better visualize the protein granules contained in the cytoplasm of trophonts, and in some parasites, also the stomopode conformation (Fig. 5.17a) and rhizoid disposition. On the other hand, the extent of the parasite wall was not clearly defined.

4,6-diamidino-2-phenylindole,dihydrochloride (DAPI) was used, with peak excitation wavelength at 360 nm, and blue fluorescence (460 nm). In the samples mounted with DAPI mounting medium it was possible to discriminate the nuclear details in the cells of the gill epithelium and the protein granules present in the cytoplasm of attached trophonts (Fig. 5.17b). In these preparations, the trophont wall was not clearly defined as well as the stomopode and rhizoid details. TRITC-phalloidin is excited at 544 nm and emits at 572 nm, producing an orange-red fluorescence. Slides mounted with TRITC-phalloidin did not provide a selective staining probably because of the overlapping of the fluorescence emission with eosin.

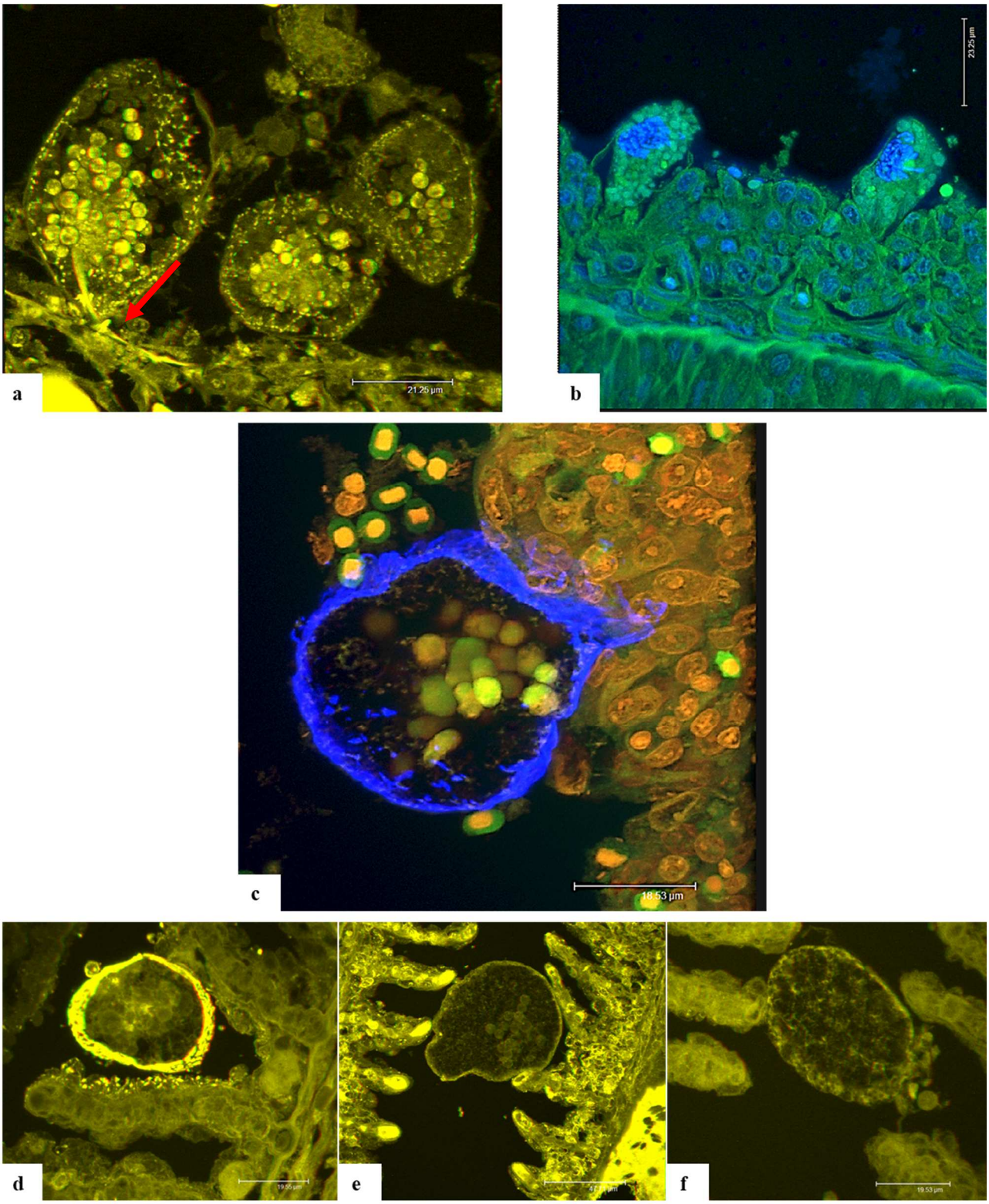
Calcofluor white (CFW) binds to β1-3 and β1-4 polysaccharides such as those found in cellulose. The absorption spectrum for aqueous CFW solution peaks at 347 nm and, when excited with UV radiation, fluoresces with an intense blue colour. Propidium iodide (PI), a nuclear stain, presents an excitation wavelength at 485 nm, and fluorescence with wavelengths greater than 595 nm, providing a red wavelength emission. In the samples processed by using the CFW and PI double staining, nuclear elements of the host gill cells appeared red stained in contrast with the green background. In the parasites a pale reddish colour was visible in the nuclear region even if covered by the eosin green emission of the protein granules. CFW specifically bound to the cellulose wall staining it in blue (Fig. 5.17c).

5.3.7 Lectins labelling

The lectins used for this investigation were conjugated to fluorophores that provided maximum fluorescence and optimal staining characteristics. In order to avoid fluorescence fading, slides labelled with fluorescein lectins were visualized on the same day by means of fluorescence and confocal microscopy. However, the best images were captured with the confocal microscope. Wheat germ agglutinin (WGA) contained in the Fluorescein Labeled Lectin Kit I shows an excitation

maximum at 495 nm and an emission maximum at 515 nm. Images captured using this lectin provided good results in terms of labelling specificity. WGA bound only to the cellulose wall of AO trophonts marked its thickness and delineated its shape (Fig. 5.17d). In this case, the trophont visualized was quite spherical suggesting it was in the process of transforming to a tomont stage, which is released from the gills. The other cellular components of both parasite and host gill epithelium presented no fluorescent labelling.

WGA plus rhodamine lectin showed slightly different fluorescent parameters compared to the previous lectin used, with a maximum excitation at 550 nm and an emission maximum at 575 nm. This protein labelled the cellulose wall of trophonts (Fig. 5.17e) even if the staining was less strong than that visualized with the fluorescein WGA. However, in this case it was also possible to observe the conformation of the armoured theca, which displayed the typical pear shape of trophonts. No labelling was detected in the negative controls (slides incubated with the dilution buffer only, fig. 5.17f).



Figures 5.17. A. ocellatum trophonts on the gill epithelium of ESB. Images captured with CLSM. a) H-E staining defined the AO cytoplasmic protein granules and the stomopode (arrow). Scale bar= 21.25 µm. b) Eosin and DAPI labelling, AO nucleus and gill cell nucleus light blue fluorescence due to DAPI. Scale bar = 23.25 µm. c) CFW and PI double staining. PI stained nuclei in red; the cellulose wall of the trophont is blue labelled by CFW; green fluorescence is due to eosin counterstaining. Scale bar = 18.53 µm. d) WGA labelling of AO cellulose wall in green fluorescence. Scale bar = 19.55 µm. e) WGA+rhodamine labelling of AO cellulose wall in green fluorescence. Scale bar =47.71 µm. f) Lectin labelling negative control, incubation with dilution buffer only. Scale bar = 19.53 µm.

5.3.8 E.L.I.S.A.

The E.L.I.S.A. set up was laborious especially regarding the coating antigen preparation. Various sonication attempts were performed according to Smith *et al.* (1992). The treatment with bath sonicator did not allow to disrupt the dinospores theca as confirmed by light microscopy observations (Fig. 5.18b). Similar results were obtained by homogenization with Tissue Lyser II (Fig. 5.18c) or with manual pottering. On the other hand, a partial fragmentation of the dinospores' wall was achieved after using the mini probe sonicator (Hielscher) (Fig. 5.18d) as observed by light microscopy. In all cases, Bradford's method resulted unsuitable to measure the purified antigen amount in terms of protein concentration; therefore, it was decided to express the antigen amount by referring it to the initial number of dinospores submitted to fragmentation.

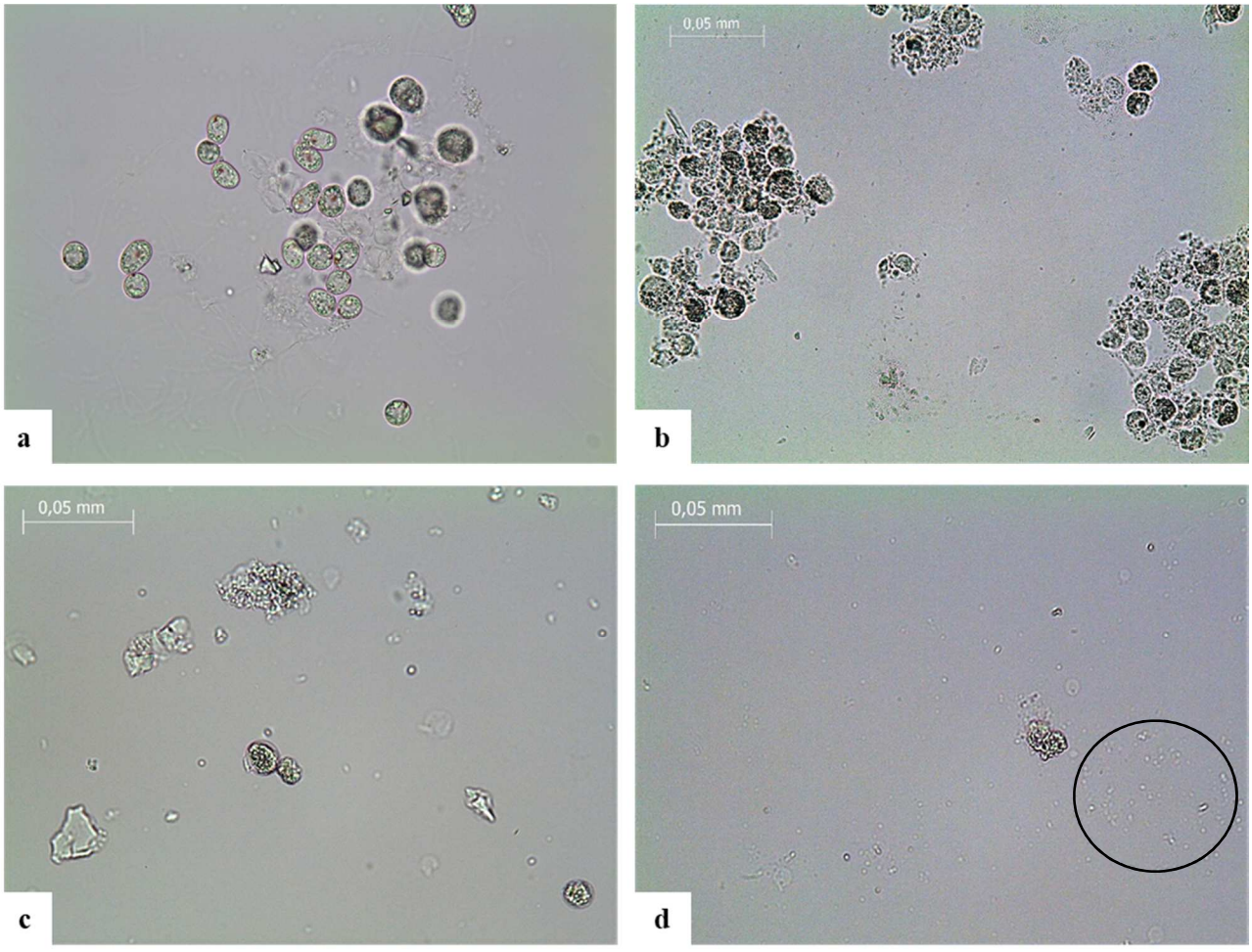


Figure 5.18. A. ocellatum dinospores subjected to the different fragmentation treatments. a) No treatments, dinospores showing intact thecal armour; image used as reference. b) Dinospores submitted to ultrasonic bath sonication. c) Dinospores submitted to TissueLyser II homogenization. d) Dinospores submitted to Hielscher mini probe sonication, in the image it is possible to notice the thecal debris.

The E.L.I.S.A. protocol was initially applied by coating the plate with an antigen concentration of 1×10^3 dinospores/well.

In this **first phase of investigation** the following sera were evaluated: groups **H1**, **H2**, **IS3** and **SPT** as codified in Table 5.4.

As shown in figure 5.19, the specific antibody titre expressed in terms of optical density (O.D.) (mean ± standard error, S. E.) of healthy fish reared in sea cages (**H2**) was 0.12±0.0206, whereas in healthy fish reared in earthen ponds (**H1**) was 0.13±0.0185. The symptomatic fish in the acute phase of infection (**SPT**) exhibited an antibody titre of 0.10±0.0089 O.D., while fish sampled 2 months after the infection outbreak (**IS3**) showed a significantly lower antibody titre (O.D. 0.04±0.0057) compared to that of the other groups examined (P≤0.05).

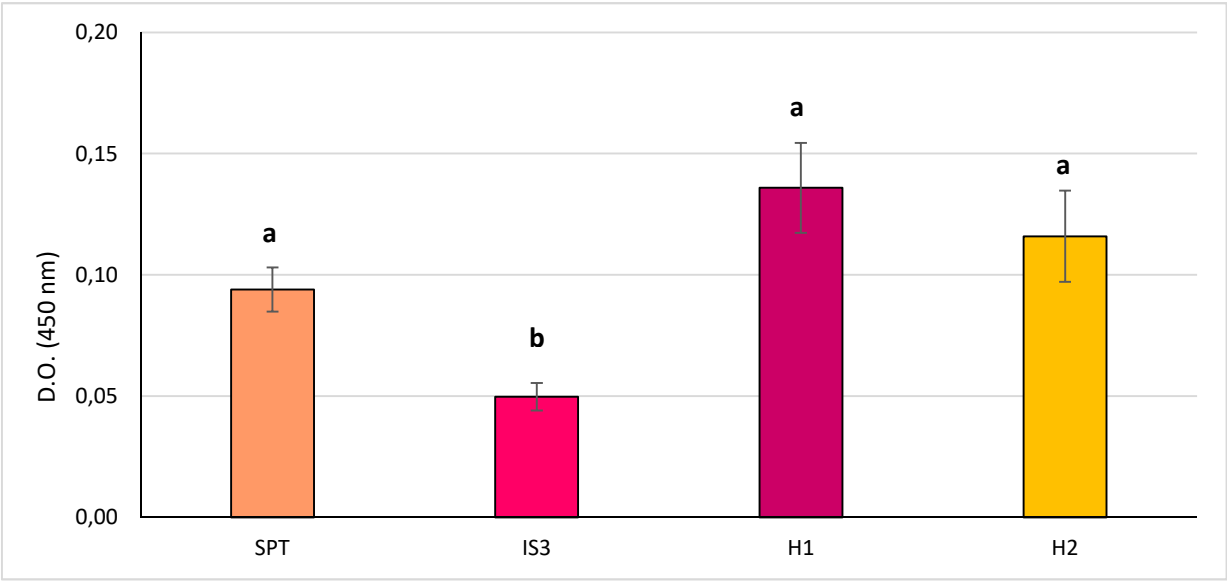


Figure 5.19. Anti-Amyloodinium ocellatum specific antibody titre in the surveyed fish groups (H1, H2, IS3 and SPT). Coating dinospores 1×10^3/well. Data are expressed as mean values (O.D.) ± SE (n=10). Data refer to diluted sera (1:10). Different letters indicate significant differences (P≤0.05).

Being the results of the first phase of E.L.I.S.A. based investigation (low dose of coating antigen) not always coherent with the foreseen immune status of fish groups under examination, the test was repeated in a **second phase of investigation** using a higher amount of AO antigen for the plate coating (2.6×10^5 dinospores/well). In this case, the samples addressed to titration were collected from groups **CI**, **IS1**, **IS2**, **SPT**, **VCTRL**, **VLd**, **VHd** and **VHS** as codified in Table 5.4.

As shown in figure 5.20, sera from fish recovering after AO infection (**IS2**) showed an O.D. equal to 0.11±0.0358. On the other hand, fish sampled in two farming sites and reared in earthen ponds (**SPT** and **IS1**) showed no statistical differences in O.D. values (0.34±0.0469 and 0.23±0.0873 respectively), while significant statistical difference (P≤0.05) was detected in specific IgM between **SPT** and **IS2** groups.

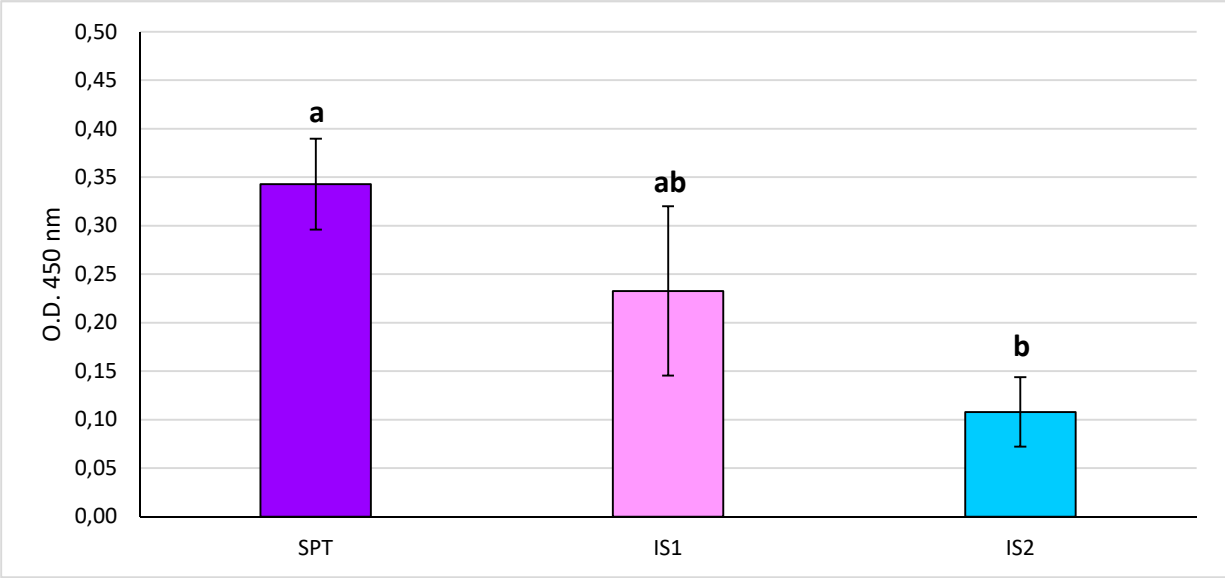


Figure 5.20. Anti-Amyloodinium ocellatum specific antibody titre in the surveyed fish groups (IS1, IS2 and SPT). Coating dinospores 2.6×10^5/well. Data are expressed as mean values (O.D.) ± SE (n=10). Data refer to diluted sera (1:10). Different letters indicate significant differences (P≤0.05).

Concerning the vaccine efficacy trial results are illustrated in figure 5.21 on the left side. The sera from control fish (**VCTRL**) showed an O.D. value of 0.06±0.0172. The sera from fish immunized with low (**VLd**) and high dose (**VHd**) of antigen exhibited O.D. values equal to 0.10±0.0163 and 0.16±0.0225 respectively. The response of fish vaccinated with the high dose of antigen in terms of specific IgM was significantly higher compared to that measured in control ESB and in ESB vaccinated with the low dose of antigen (P≤0.05).

On the right side of the figure 5.21 are reported the O.D. values of **VHS** and **CI** groups, which were not subjected to statistical analyses. The level of specific IgM measured in a pool of sera deriving from a few fish submitted to vaccination with the high dose and then challenged with AO (**VHS**) showed an O.D. value of 0.32. The E.L.I.S.A. was applied also to evaluate sera collected from ESB chronically infected by AO (**CI**) and kept at UNIUD aquaria as parasite reservoirs. This group showed an O.D. equal to 0.47±0.1105.

It is noteworthy to mention that a considerable variability among the ESB individuals was observed (Fig. 5.22).

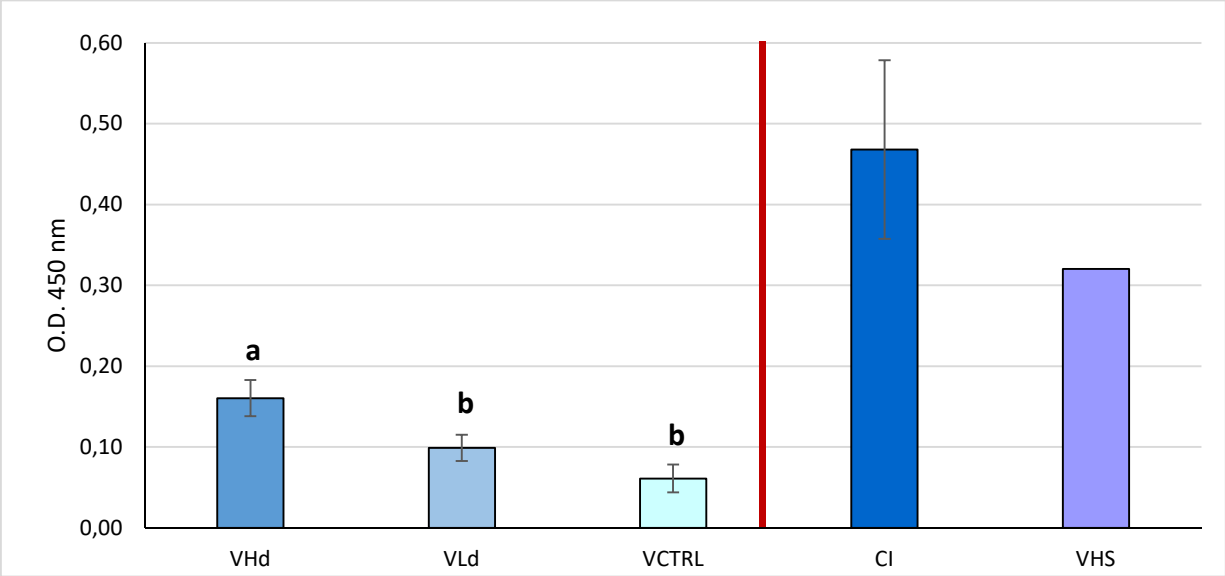


Figure 5.21. Anti-Amyloodinium ocellatum specific antibody titre in the surveyed fish groups (CI, VCTRL, VHd, VHS and VLd). Coating dinospores 2.6×10^5/well. Data are expressed as mean values (O.D.) ± SE (n=10). Data refer to diluted sera (1:10). Different letters indicate significant differences (P≤0.05) between the three groups on the left of the red line. Survived group VHS does not present the S.E. bar as sera had been pooled.

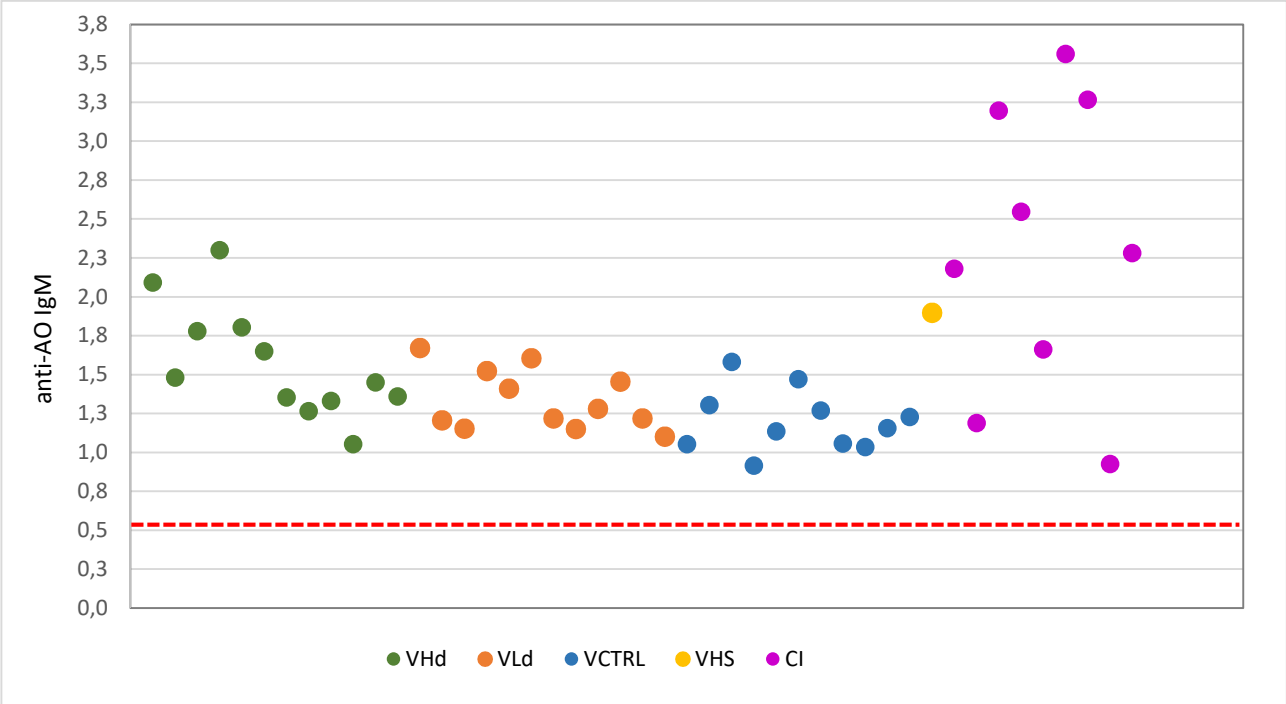


Figure 5.22. Scatterplot of the specific anti-Amyloodinium ocellatum antibody titre in the ESB subjects belonging to the surveyed groups (CI, VCTRL, VHd, VHS and VLd). Data are expressed as O.D. sample/O.D. blank ratio. The red line (O.D. = 0.554) represents the cut-off that is the blank mean O.D. + 3 standard deviations of the blank O.D..

5.4 DISCUSSION AND CONCLUSIONS

5.4.1 Vaccine efficacy trial

In spite of some preliminary experiments conducted in the '90 (Smith *et al.*, 1992, 1993; Cobb *et al.*, 1998a), aimed at investigating the immunological memory developed by fish submitted to immunization with AO antigens, currently there are no commercial or experimental vaccines against *Amyloodinium ocellatum*. Therefore, our research group conducted a first study to determine the efficacy of inactivated/fragmented AO dinospores as intraperitoneal vaccine in ESB juveniles. The vaccine effectiveness and potency were determined by challenging the fish with the pathogen; in parallel the specific IgM levels in the serum were evaluated (data discussion in paragraph 5.4.6). The challenge under controlled condition provoked a severe outbreak, in which both controls and immunized fish showed remarkable AO load and disease symptoms. The post infection mortality started on day 5 in control fish and in fish immunized with the low dose of antigen, whereas it started on day 7 in the group immunized with the high dose. It reached 100% in all the groups within the 9th day post infection, so the vaccination method was not effective. However, the survey underlined a slight delay in the disease onset and progression in the fish group treated with the high dose, suggesting that the antigen prepared as described in Materials and Methods and administered in consistent amount can be potentially protective against the infection, as probably it triggers the fish ability to limit the pathogenicity of AO at gill level. As a perspective for future studies it will be useful to further increase the antigen dose provided to each fish and to include an adjuvant in the vaccine formulation, in order to trigger a more effective and durable protection.

5.4.2 Histology

The histological results allowed to obtain an overall description of the pathological changes that characterize heavy infections in European sea bass, regardless of the fish size. The parasitic branchitis is the main inflammatory pattern occurred in heavy infections. Actually, gills and the entire oro-pharingeal cavity, included nostrils, were the primary site of the infection in ESB, while the skin, even if abundantly infected by trophonts, did not show relevant lesions nor the typical dusty appearance, as described in other species and from which derived the disease name (marine velvet disease).

Ultimately, the observed histological findings are coherent with those reported in previous studies conducted on other fish species naturally infected by *A. ocellatum* (Brown, 1934; Paperna, 1980; Kuperman and Matey, 1999; Kuperman *et al.,* 2001; Cruz-Lacierda *et al.*, 2004; Saraiva *et al.*, 2011;

Bahri, 2012; Guerra-Santos *et al.*, 2012; Ramesh Kumar *et al.*, 2015) and in *A. ocellatum* experimentally infected ESB (Nozzi *et al.*, 2016). In general, the inflammatory cells infiltrate was scarce in the gills, in agreement with the description provided by Paperna (1980) on gilthead sea bream (*Sparus aurata*).

In this thesis, histology was applied for the purpose of diagnostic confirmation of the infection.

5.4.3 Immunohistochemistry

Some markers resulted effective on ESB tissues when tested with the IHC classical method, while for others it was necessary the application of the EnVision™ Flex implemented method to obtain the labelling. On the other hand, CD3, CD35, CD68, Cox-2 (H62), Cox-2 (M19), histamine, HSP-70 and lysozyme did not cross-react with ESB tissues with both protocols. These results might depend on the ineffectiveness of the antigen retrieval processes in the fish tissue or to the absence of cross reactivity of antibodies. As far as we know, there are no documented data in which IHC was performed on ESB gills infected by *A. ocellatum* so the present data might represent a contribute to the morphological and functional description of gills reactivity to AO. Some of the markers selected for the present study have been already employed on ESB and other fish species for anatomo-pathological and immunological surveys. Immunoglobulin (IgM) bearing cells have been marked in ESB juveniles head kidney/spleen to evaluate the outcomes of a vaccination protocol against vibriosis (Galeotti *et al.*, 2013). The iNOS molecule was detected by immunohistochemistry in tissues of turbot (*Scophthalmus maximus*) in response to natural as well as experimental infection with *Aeromonas salmonicida* (Coscelli *et al.*, 2014, 2016) and upon vaccination against the same pathogen (Coscelli *et al.*, 2015). iNOS was also marked by IHC in wild bream (*Abramis brama*) during a natural infestation by *Ergasilus sieboldi* (Dezfuli et al., 2003), in wild *Hypostomus francisci* gills to investigate the impact of environmental pollutants (Ferreira Sales *et al.*, 2017) and in farmed turbot subjected to *Enteromyxum scophthalmi* infection (Losada *et al.,* 2012; Ronza *et al.*, 2015). Similarly, TNF-α positive cells have been labelled in tissues of turbot infected by *A. salmonicida* (Coscelli *et al.,* 2016; Ronza *et al.*, 2015) and in the gills of rainbow trout (*Oncorhynchus mykiss*) infected by *Loma salmonae* (Powell *et al.,* 2014). On the contrary, the antibody markers for TLR2 and TLR4 have never been used by other authors for immunohistochemical studies on fish, prior to this study. As reported in the Results section the iNOS antibody and the TNF-α antibody marked cells with a morphology ascribable to macrophages; whereas the ESB IgM antibody labelled cell populations referred as plasma cells or Ig bearing macrophages. These results suggest that the mucosal immune response adopted by ESB against AO includes the activation of GIALT (gill associated lymphoid tissue)

components such as phagocytic cells expressing also the pattern-recognition receptors (PRRs) TLR2 and TLR4, as well as the antibody producing cells. These populations are mainly located in the gill areas affected by the flogistic and hyperplastic processes triggered by the parasite. As other protozoa, AO can stimulate the host immune response promoting the release of pro-inflammatory cytokines and enzymes such as TNF-α and iNOS. Other than host tissue antigens, also anchored trophonts showed positivity for iNOS and TNF-α. This observation allows to speculate on the possible ability of the parasite itself to synthesize molecules that are ancestrally expressed also by very simple organisms. For example, the nitric oxide production pathway has been described in many organisms ranging from single cell moulds to humans, being a defence mechanism early developed in the evolution (Campos-Perez *et al.,* 2000). AO may possibly use these molecules to interact with the host gills to which it adheres. Moreover, it is also possible to hypotisize that the iNOS molecule labelled internally to the trophonts, as well as the positivity for the pro-inflammatory cytokine TNF-α, derives from material that the parasite incorporates from the gills through the stomopode. In fact, some dinoflagellates, among which *A. ocellatum*, are known as organisms able to perform "phagotrophy" in order to get nutrients from the host cell cytoplasms, as observed ultrastructurally by Lom and Lawler (1973). Several studies confirmed that there is cross-reactivity between mammalian and fish cytokeratins as well as mammalian and fish markers associated with cell proliferation (Bunton, 1993; Norte dos Santos *et al.*, 2014; Hermenean *et al.*, 2017; Sarasquete *et al.*, 2018). In this study, a better morphological description of the mucosal cell proliferation and consequent hyperplasia, which often characterises the amyloodiniosis infection pattern in the gills, was obtained by using the anti-cytokeratin and the anti-PCNA antibodies respectively. Anyway, further investigations will be necessary to determine the number of positive cells to the different markers and to correlate them with the lesion severity and parasite burden.

5.4.4 *In situ* hybridization

In this study, a fluorescent mRNA *in situ* hybridization (FISH) protocol was developed to investigate the host-parasite interactions through the presence or absence of Chemokine CC1 and Hepcidin2 mRNA sequences, as well as localising them to specific cells and allowing a semi-quantitative estimation of level of occurrence/expression. Based on our knowledge, there is no documented information about previous FISH approaches in ESB infected gills by *A. ocellatum* and so these data can be considered originals.

Chemokine CC1 is a cytokine belonging to the chemokine subfamily CC (Peatman and Liu, 2007) and is mainly contained in monocytes and macrophages. Chemokines or **chemo**tactic cyto**kines** (Laing

and Secombes, 2004) are a family of small molecular weight proteins that promote directional migration of leukocyte cell types, endothelial and epithelial cells to sites of infection or injury (Raman *et al*., 2011). Chemokines also play a significant role in wound healing, angiogenesis/angiostasis and in the development and metastasis of tumors (Raman *et al*., 2011). The expression of Chemokine CC1 was evaluated in Asian sea bass (*Lates calcarifer*) tissues infected by the protozoan ciliate *Cryptocaryon irritans* demonstrating that the gene was up-regulated under the inflammatory condition (Mod-Shaharuddin *et al.,* 2013). This finding is in agreement with the results reported by Bhatt *et al*. (2014) where the highest gene expression was noticed upon fungal (*Aphanomyces invadans*), bacterial (*Aeromonas hydrophila*) and viral (poly I:C) infections in snakehead murrel (*Channa striatus*). Furthermore, the authors found phylogenetic clustering with chemokine CC1 from the European sea bass, in which it acts in the same way in response to bacterial infections as reported by Yacoob *et al.* (2016).

In this study, the mRNA ISH results supported the observed differential expression of Chemokine CC1 transcript between uninfected and infected sea bass (Byadgi *et al.*, 2017, 2019) and provided a better understanding of the pattern of localisation of leukocyte populations in ESB gills.

In uninfected fish, a faint hybridization signal was detected in the GIALT, in the lumen of the central venous sinus and in the capillaries of the apical portion of the primary lamellae where normally leukocytes circulate. On the other hand, Chemokine CC1 signal was more evident in infected gill tissue, with positive cells detected in the hyperplastic areas of the secondary lamellae and in the vessel wall of the central venous sinus of the primary lamellae, typical localisations of leukocytes during inflammatory responses. Interestingly, a positive signal was also detected in the cytoplasm of some AO trophonts potentially confirming the "phagotrophic" nature of the dinoflagellate previously suggested by Lom and Lawler (1973), and also found in our IHC surveys (paragraph 5.4.3). On the other hand, no positive signal was detected in the areas close to anchored parasites. A possible explanation of the absence of positive cells in those areas may be due to evasive mechanisms adopted by AO to avoid detection by the host immune response, as documented for other fish parasites (Buchmann and Lindenstrøm, 2002; Sitjà-Bobadilla, 2008; Kumar *et al.*, 2013). However, more work is required to study this aspect in more detail.

The second riboprobe was designed for detecting Hepcidin 2 transcript. Hepcidin, a small cysteine-rich antimicrobial peptide, was originally isolated from human blood ultrafiltrate and named LEAP-1 (i.e. liver-expressed antimicrobial peptide, Krause *et al.,* 2000). In mammals, only one gene encodes hepcidin, which has a predominant role in iron homeostasis (Neves *et al.,* 2011, 2015).

Conversely, it is documented that teleosts could possess a large number of hepcidin genes (Hilton and Lambert, 2008; Masso-Silva and Diamond, 2014). Studying hepcidin in ESB, Neves *et al*. (2015) determined the presence of two isoforms. Hepcidin 1 (hamp1) is encoded by a single gene and is more highly expressed in the majority of the tissues, particularly in the liver and spleen-derived leukocytes; its function is to regulate the iron methabolism. Hepcidin 2 (hamp2) is encoded by several genes, has four isoforms and is predominant in the brain and gills where it exerts protective mechanisms against microbial infections.

Although previous research by Solstad *et al.* (2008) described a positive Hepcidin signal in gills and other tissues of Atlantic cod (*Gadus morhua*) experimentally infected by *V. anguillarum*, in the present study riboprobes, designed using the Hepcidin 2 gene sequence, did not hybridize to the ESB gill tissue from either infected or uninfected fish. However, the ISH protocol and primers sequences used in this study were different to those used by Solstad *et al.* (2008) and could have contributed to the different outcomes. Nevertheless, as previously mentioned, these findings are intended to be preliminaries and so further investigations are required to obtain more data and hopefully solve the problems encountered. However, the positive results obtained with this initial study provide a starting point for the development of novel probes for further FISH investigations, which will enhance our knowledge of the host-parasite interaction.

5.4.5 Confocal microscopy and fluorescent lectins labelling

Histological and fluorescent stains tested in this study have a long history of successful usage in fluorescence microscopy (von Provazek, 1914; Singer, 1932; Pick and Zuckerkandl, 1934 cited by Darken, 1961; Camplejhon *et al.*, 1989; Cano *et al.*, 1992; De Rossi *et al.*, 2007; Weli *et al.*, 2017); for this reason they have been applied.

Eosin is a known xanthene dye obtained by halogenization of fluorescein (Fleming *et al.,* 1977), usually it is not regarded as a fluorochrome, however, high-fluorescence emission has been described for this dye (Berlman, 1971).

Originally synthesized for trypanosomiasis treatment, 4',6'-Diamidino-2 Phenylindole (DAPI) was revealed to be a specific DNA fluorescent probe binding strongly to adenine-thymine rich regions (Kapuscinski, 1995), and producing a blue fluorescence when excited by UV light. Tetramethylrhodamine (TRITC)–phalloidin is widely used in fluorescence microscopy for cytoskeleton detailing. Thanks to the bicyclic heptapeptide Phalloidin, this stain specifically binds to F-actin filament ends (Cano *et al.*, 1992), while TRITC under UV light provides an orange-red fluorescence.

Calcofluor white M2R (CFW) or fluorescent brightner 28 is one of a group of compounds known as fluorescent brighteners or optical brighteners, or “whitening agents” as it is used to whiten and to prevent yellowing of papers and fabrics (Harrington and Hageage, 2003). CFW is a non-specific fluorochrome that binds to β1-3 and β1-4 polysaccharides normally found in chitin and cellulose, contained in the cell wall of fungi and other organisms including dinoflagellates such as *A. ocellatum*. In this study, CFW was used as a dual stain with propidium iodide (PI), an intercalating agent that binds to DNA or RNA, causing orange fluorescence (Mascotti *et al.,* 2000). PI is generally used in cell viability assays as it cannot permeate intact membranes but readily penetrates the membranes of nonviable cells (Sauch *et al.*, 1991; Mascotti *et al.,* 2000; Vasconcelos *et al.*, 2017).

The last fluorescent stains investigated were fluorescein labelled wheat germ agglutinin (WGA) and WGA+rhodamine; WGA is a plant lectin, which agglutinates various types of animal cells, including malignant cells and protease-treated cells (Monsigny *et al.,* 1979). WGA binds to N-acetyl-D-glucosamine and most strongly to its oligomers or polymers such as chitin (Goldstein *et al.*, 1975; Vierheilig *et al.,* 2005). The labelling results obtained for WGA and WGA+rhodamine are consistent with those obtained by using CFW.

Based on our knowledge, there are no previous documented reports about the use of confocal laser scanning microscopy (CLSM) on *A. ocellatum* trophonts. The present investigations were performed in order to develop staining protocols aimed at better detailing the anatomy of AO parasitic stage through an alternative approach. The purpose was to capture images of the stomopode and rhizoids to elucidate the way of interaction with host epithelium and to comprehend their composition. Through CLSM it was possible to capture anatomical particulars of the parasite and to obtain 3D images of AO. CFW, DAPI, PI and WGA provided specific labellings of the cellulose wall, cytoplasmic granules and nuclei. The anchoring organs and stomopode were not well stained and were visible only with eosin. Unfortunately, the thickness of H-E stained sections was not adequate for this technology, so it was not possible to appreciate the disposition of rhizoids in the gill cells nor the entire length of the stomopode. In any case, the results collected through this research suggest that both rhizoids and stomopode are composed by proteins. However, further studies will be necessary to clarify this finding by testing different stains specific for protein marking.

5.4.6 E.L.I.S.A.

As documented in literature fish submitted to immunization or after the natural exposure to AO can develop different levels of specific immunity against *A. ocellatum* (Smith *et al*., 1992, 1993, 1994; Cobb *et al.,* 1998a, b; Cecchini *et al*., 2001). This aspect appears interesting in the perspective of a

vaccine development. In fact, to date, no dedicated formulations have been yet proposed both experimentally and commercially, aiming at the prophylaxis against amyloodiniosis. However, vaccination will be very challenging since the response against parasites and the development of a specific immunity against them is complex due to the mechanisms by which parasites can evade or cope with fish immune response.

In this study, the set up of the E.L.I.S.A. was based on Smith *et al.* (1992) and Cecchini *et al.* (2001) protocols, especially regarding the antigen preparation. In both articles, *A. ocellatum* (dinospores and trophonts respectively) were subjected to sonication for the thecal disruption and protein content exposure. Based on this approach, our tests also availed of sonication technology. Nevertheless, it is known that the preparation of microplates for E.L.I.S.A. with antigens obtained from protozoa is more laborious compared to the use of bacterial or viral antigens. In fact, a problem encountered was the limited availability of the parasite, which also contains a few proteins that are expected to be immunogenic, and presents very strong cellulose armoured theca. The preliminary sonications performed in this study did not compromise thecal integrity as the homogenization with Tissue Lyser II or by manual pottering, even if this may have been due to the different technical characteristics of the instruments. In fact, using the mini-probe Hielscher sonicator, more similar to the sonicator model reported by the authors, better results were obtained. Furthermore, it was decided not to perform an enzymatic digestion of dinospores as enzymes could interfere with the assay and/or degrade antigens thus making them unavailable for serum antibodies binding.

The low quantity of proteins detected by the Bradford method on sonicated, homogenized or pottered dinospores could derive from the structural characteristics of the parasite, whose wall consists mainly of cellulose (Brown, 1934; Nigrelli, 1936; Noga and Levy, 2006). Furthermore, it cannot be excluded that the dinoflagellate possesses a few aromatic and basic aminoacid residues, being them the binding sites of Bradford's chromogen Coomassie Brilliant Blue. For this reason, it was not possible to correlate the dinospore concentration with a known amount of proteins as did by Smith *et al.* (1992). Hence, in our tests the antigen concentration was expressed in terms of number of dinospores/ml subjected to sonication or potter treatment.

In general, the application of E.L.I.S.A. in the set up phase (antigen coating with approximatively 1,000 dinospores/well) revealed that the results in terms of specific IgM were not always coherent with the expected fish group profile (reactivity in the post-infection phase or non reactivity in naïve/healthy individuals). A possible explanation of the low O.D. values recorded might be the scarce immunogenicity of *A. ocellatum*, which is able to escape the host defence mechanisms by

attenuating the specific immune response. Similar mechanisms have been already documented for other fish ectoparasites (Sitjà-Bobadilla *et al.*, 2008; Guardiola *et al.*, 2014; Henry *et al.*, 2015). Otherwise, we ascribed these findings to the low amount of antigen used for the microplate coating. Other authors used suspension coatings containing 0.15 µg (Smith *et al.*, 1992) or 0.5 µg (Cobb *et al.*, 1998b) per ml of protein deriving from sonicated dinospores, the former corresponding to 6×10^5 dinospores/ml.

Therefore, in the second phase of E.L.I.S.A. based evaluations the number of fragmented dinospores was increased and adjusted to a concentration of 2.6×10^5/well. In this phase sera from the amyloodiniosis episodes were re-analysed, whereas sera collected after the vaccination trial were firstly investigated.

Concerning the specific IgM response of ESB to AO infections, the **SPT** group exhibited the highest O.D. values. This finding is explainable by the fact that those fish were adults (up to 1,500 g) and sampled in the course of a severe amyloodiniosis field episode. Probably they also experienced during their lifespan, repeated exposures to AO at non-lethal doses, being the farm site located in an endemic area. In fact, a constant or repeated exposure to the parasite induces the development of a specific immunity as already reported by Cobb *et al.* (1998a) in the tomato clownfish (*Amphiprion frenatus*).

As regards to the post-vaccination response in ESB, the highest antibody titre at 3 weeks post treatment was observed in fish immunized with the high dose of AO antigen (**VHd**), resulting statistically different from that recorded in **VCTRL** and **VLd** groups. Even if this antibody titre was not capable in protecting fish from the AO challenge, still it determined a slight delay in the disease rising and in the mortality onset. For this reason, a further vaccination trial will be improved by adjusting the immunizing antigen dose, including an adjuvant to the formulation, and refining the infective dinospore dose for potency test.

In parallel the pooled sera from post-challenge fish (**VHS**), belonging to the group of ESB immunized with the high dose of antigen and individual sera from fish chronically infected (**CI**) were examined. In both cases, they displayed relevant specific antibody levels. Even if data could not have been statistically elaborated for a comparison with those deriving from the other three surveyed groups, these IgM titres could suggest the ability of ESB in synthesizing antibodies against AO as a consequence of repeated exposures to the parasite. This finding has already been documented in literature (Cobb *et al.*, 1998a,b) and confirmed by our analyses as derived by group **SPT**.

Finally, since gills and the oral cavity (and in a lesser extend the skin) are the main affected tissues, further studies aimed to explore the role of the mucosal immunity could be performed, thus complementing the E.L.I.S.A. results.

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6. *PLANT COMPOUNDS FOR THE CONTROL OF AMYLOODINIOSIS*

In this chapter are reported the results obtained by testing a panel of selected plant-derived compounds as potential immunomodulators or parasiticidal substances.

2^{I},4^{I}-dihydroxychalcone; 7-hydroxyflavone; artemisinin; camphor (1R); diallyl sulfide; esculetin; eucalyptol; garlicin 80%; harmalol hydrochloride dihydrate; palmatine chloride; piperine; resveratrol; rosmarinic acid; sclareolide; tomatine and umbelliferone were selected on the basis of their potential immunostimulant properties and their documented antibiotic, fungicidal or parasiticidal activity and provided to UNIUD by Zebra Fish Screens (Lieden, the Netherlands) one of the ParaFishControl project partners. The compounds have been tested *in vitro* to determine their effects on respiratory burst activity of head kidney (HK) leukocytes purified from European sea bass (ESB, *Dicentrarchus labrax*) juveniles and on the motility of *A. ocellatum* viable dinospores as immunomodulators or antiparasitic candidates respectively.

6.1 RESPIRATORY BURST

6.1.1 Preface

The increased scale of aquaculture production, characterised by intensive fish farming, has led to various disease problems for the main cultured species, including parasitic infections (Colorni, 2004; Buchmann, 2015). Since there are a very few licensed medicines available and currently no effective vaccines against fish protistan or metazoan parasites exist, the enhancement of the host immune responses could be a promising method for preventing parasite outbreaks in fish farms.

Recently, an interesting approach based on the inclusion of medicinal plant derived compounds in the diet has been widely proposed in several fish species in order to promote the innate defences of fish and increase their resistance to bacterial and parasitic infections (Alexander *et al.*, 2010; Sharma *et al.*, 2010; Talpur *et al.*, 2012; Zanuzzo *et al.*, 2015; Cerezuela *et al.*, 2016; Sukumaran *et al.*, 2016; Bulfon *et al.*, 2017; Liu *et al.,* 2017; Yao *et al.*, 2017; Zhou *et al.*, 2018).

Medicinal plants have been subjected to increasing consideration, due both to their antimicrobial and immunostimulant properties related to a high content of secondary metabolites with different biological activities (Reverter *et al.*, 2014) as well as to their proven benefits in human medicine for the prevention and cure of inflammatory and neoplastic diseases (Kohnen *et al.*, 2007; Costa *et al.*, 2008; Kim *et al.*, 2008). Moreover, plant derivatives are known to induce minimal side effects compared to conventional medicines, thus allowing to obtain more eco-friendly and acceptable treatments for the consumers (FAO/WHO/OIE 2006). Although in the last few years numerous *in vivo* studies have been carried out to investigate the potential use of medicinal plants in fish, the

strict recommendations of the European Union Commission (Directive 2010/63/EU) and the recent Italian guidelines (D.L. 26/2014) impose to limit the use of animals for experimental and other scientific purposes in order to minimize suffering and pain.

Therefore, in animal testing the cardinal principle of the 3Rs ("Replacement, Reduction and Refinement") should be applied, in accordance with the protocol on animal welfare: 1) replacement of animal models with *in vitro* models, when possible; 2) reduction of animal number used in the experiments; 3) use of less invasive techniques when the experiments involve the use of animals (Midtlyng *et al.*, 2011; Romberg *et al.*, 2012).

Consequently, *in vitro* or *ex vivo* approaches represent cost-effective preliminary investigations to subsequent *in vivo* experimentation. *Ex vivo* assays can be performed using primary cell cultures (e.g. leukocytes purified from lymphatic organs), whereas *in vitro* experiments exploit cell lines specifically developed for immunological research. In the case of *ex vivo* tests, particular attention should be given to the selection of healthy donors, with the cell reactivity being influenced by the physiological status of the fish (Bulfon *et al.*, 2018). Both these approaches can provide more consistent results as the experimental conditions can be highly controlled. Moreover, they permit the simultaneous screening of various products at different concentrations and allow to limit the number of animals to be sacrificed for experimental purposes (Fierro-Castro *et al.*, 2012; 2013). Thereby *in vitro* and *ex vivo* methods may constitute an effective instrument to elucidate the basic mechanisms of action of new immunostimulant candidates to be exploited in aquaculture (Galeotti, 1998).

The aim of the present research was to assess *in vitro* the effects of 16 active plant compounds (2^{I},4^{I}-dihydroxychalcone; 7-hydroxyflavone; artemisinin; camphor (1R); diallyl sulfide; esculetin; eucalyptol; garlicin 80%; harmalol hydrochloride dihydrate; palmatine chloride; piperine; resveratrol; rosmarinic acid; sclareolide; tomatine and umbelliferone) selected from the ZF-S library, on the respiratory burst activity of head kidney (HK) leukocytes purified from juveniles of ESB, which represent the most economically important fish species in the Mediterranean basin and a confirmed host of *Amyloodinium ocellatum*.

This research represents the starting point to determine if these compounds may be good candidates as food additives for ESB, for modulating the immune responses and its resistance to parasite infections such amyloodiniosis. Moreover, the results of this research may also prove informative for modulating responses in other cultured fish species.

6.1.2 Materials and methods

6.1.2.1 Reagents

5-amino-2,3-dihydro–1,4–pathalazinedione (luminol), dimethylsulphoxide (DMSO), Hank's Balanced Salt Solution without phenol red, Ca^{2+} and Mg^{2+} (HBSS), heparin (5 KU/ml), Histopaque® 1077, Histopaque® 1119, phorbol myristate acetate (PMA), sodium chloride, Tricaine methanesulfonate (MS-222), trypan blue, were purchased from Sigma-Aldrich (St. Louis, USA).

6.1.2.2 Plant compounds

Sixteen plant-derived compounds (2^{I},4^{I}-dihydroxychalcone; 7-hydroxyflavone; artemisinin; camphor (1R); diallyl sulfide; esculetin; eucalyptol; garlicin 80%; harmalol hydrochloride dihydrate; palmatine chloride; piperine; resveratrol; rosmarinic acid; sclareolide; tomatine and umbelliferone) were provided by a ParaFishControl project partner, Zebra Fish Screens (ZF-S). The substances under investigation belong to a range of classes of natural or synthesised phytochemical compounds, which were selected based on their potential immunostimulant properties, as resulted from reviewing the relevant literature (Table 6.1). Prior to use, compounds were dissolved in DMSO (10 mM) and stored in the dark at 4°C.

6.1.2.3 Fish

Healthy ESB juveniles were maintained in the aquarium facilities of the Department of Agricultural, Food, Environmental and Animal Sciences (Di4A) at the University of Udine (UNIUD). Fish were maintained in round fibreglass tanks, part of an indoor recirculating system supplied with filtered seawater, and fed daily with a commercial pelleted diet in two meals administered at 9:00 and 16:00 h 6 days per week. Water physico-chemical parameters were measured daily and maintained suitable for this fish species [temperature 22.5±2°C, salinity 30±2‰, pH 8.0, NH_4-N 0.02-0.03 mg/l, NO_2-N below the detection limit of the method (<0.015 mg/l), natural photoperiod]. Fish had not been previously exposed to immunization or immune-stimulation treatments. Animals were treated in compliance with the Guideline of the European Union Council (Directive 2010/63/EU) and the Italian legislation (D.L. 26/2014) for the use of laboratory animals for experimental purposes.

6.1.2.4 Head kidney leukocyte purification

Fish (average weight 80±10 g) were euthanized with an overdose of MS-222 (400 mg/L). Blood was collected from the caudal vein to reduce the blood supply to kidney tissue prior of the organ removal. Head kidney (HK) tissue was removed aseptically and placed in sterile plastic Petri dishes containing ESB isosmotic (372 mOsm) HBSS with 0.25% heparin. HK was selected because it provides a higher number of leukocytes compared to spleen and blood at all size stages of ESB, as demonstrated by our experience. The tissue was gently macerated with a sterile syringe piston. The resulting cell suspension was carefully layered over a gradient of isosmotic Histopaque-1119 and Histopaque-1077 and centrifuged (480xg for 25 min at 4°C). Subsequently, the leukocyte rich layer was collected and washed twice with isosmotic HBSS (300xg for 10 min at 4°C). Cell viability (usually higher than 95%) and concentration were estimated using the trypan blue exclusion method and a Thoma chamber (Volpatti *et al.*, 2014). Leukocyte concentrations were adjusted to $1x10^6$ or $5x10^6$ cells/ml (according to cell availability and experimental requirements) in isosmotic HBSS. Cells from different individual fish were not pooled but maintained as individual cell cultures throughout the experiments, in order to avoid possible mixed lymphocyte reactions (MLR) (Meloni *et al.*, 2006) and were used immediately following purification.

6.1.2.5 Leukocyte respiratory burst activity

Preliminary tests were performed in order to obtain an indication on the effect of each compound on ESB leukocyte respiratory burst and to define the optimal range of concentrations to be tested in the subsequent assays. The 16 plant compounds were tested in triplicate wells in sterile black 96-well plates (NUNC), at twofold serial dilutions (ranging from 50 µg/ml to 1.56 µg/ml, 100 µl/well depending on the compound). In particular, the compounds were diluted starting from a concentration at which their potential toxic effect or the toxicity due to DMSO solvent was negligible, on the basis of bibliographic data deriving from *in vitro* studies on both mammalian and fish cells (Castro *et al.*, 2008; Lou *et al.*, 2009) and our preliminary cytotoxicity tests (data not reported).

Leukocytes (100 µl/well) were then added to each well. Wells were supplemented with 50 µl/well of 2 mM luminol and 20 µl/well of 15 µg/ml PMA. Cells incubated without the extracts and without PMA were used as negative controls, while cells incubated only with PMA represented positive controls. The chemiluminescence (CL) emission was measured in terms of relative luminescence units (RLU) using a luminometer (Tecan S.r.l., MI, Italy). CL measurement was recorded every 3 min

for the first 15 min and every 5 min for the following 45 min at room temperature (RT) (Coteur *et al.*, 2002 with minor modifications).

Based on the results of these preliminary experiments, other tests were performed according to the same protocol to deepen the effects of 7 selected compounds (2^{I},4^{I}-dihydroxychalcone; esculetin; harmalol hydrochloride dihydrate; resveratrol; rosmarinic acid; 7-hydroxyflavone and camphor (1R)) on ESB respiratory burst by using cells purified from a statistically relevant number of fish (6 fish/substance), so as to limit the number of animals to be sacrificed for the experimental purposes. In this case, the tested concentrations were restricted to 1.56 µg/ml, 3.13 µg/ml and 6.25 µg/ml for the first five compounds, while the flavonoid 7-hydroxyflavone and the monoterpene camphor (1R) were tested at the concentrations of 12.5 µg/ml, 25 µg/ml and 50 µg/ml. The CL emission (relative luminescence units, RLU) was registered every 10 min for 120 min at RT.

6.1.3 Data evaluation and statistical analysis

Results are expressed as means ± standard error (SE). All statistical analyses were performed using the software SPSS Statistics v20 (SPSS, Inc, Chicago, IL, USA). Data were compared using one-way analysis of variance (ANOVA) after testing for normality using the Kolmogorov-Smirnov test. If results were significant, Duncan's *post hoc* test was applied for multiple comparisons. If the data were not normally distributed, the Kruskal-Wallis non-parametric test and the relative *post hoc* Mann-Whitney U test were used. Differences were considered statistically significant when P-value was ≤ 0.05.

Table 6.1. Classification of the active plant compounds used in this research.

ACTIVE PLANT COMPOUND	CLASSIFICATION
2^{I},4^{I} - Dihydroxychalcone	Flavonoid
7 - Hydroxyflavone	Flavonoid
Artemisinin	Sesquiterpene lactone (with a peroxide bridge)
Camphor (1R)	Monoterpene
Diallyl sulfide	Organosulfur compound
Esculetin	Lactone (from intramolecular cyclization of a cinnamic acid derivative)
Eucalyptol	Monoterpene cyclic ether
Garlicin 80%	Organosulfur compound
Harmalol hydrochloride dihydrate	Bio-active beta-carboline harmala alkaloid
Palmatine chloride	Protoberberine alkaloid
Piperine	Alkaloid
Resveratrol	Phytoalexin, stillbenoid (natural phenol)
Rosmarinic acid	Caffeic acid ester
Sclareolide	Sesquiterpene lactone
Tomatine	Glycoalkaloid
Umbelliferone	Phenylpropanoid

6.1.4 Results

As described in figures 6.1 a-d, the 16 plant compounds under investigation differently affected ESB leukocyte respiratory burst.

The preliminary experiments suggested that 2^{I},4^{I}-dihydroxychalcone; esculetin; harmalol hydrochloride dihydrate; resveratrol and rosmarinic acid possess evident antioxidant properties, as confirmed by the reduction in ROS production by ESB leukocytes exposed to these substances. On the other hand, 7-hydroxyflavone and camphor (1R) upregulated the ROS production, especially at the highest concentrations. The remaining compounds (artemisinin; diallyl sulfide; eucalyptol; garlicin 80%; palmatine chloride; piperine; sclareolide; tomatine and umbelliferone) did not show easily explainable effects on European sea bass leukocytes.

These preliminary results were confirmed by the subsequent *in vitro* studies, which were performed using cells deriving from a statistically relevant number of fish (6 fish/substance).

Regarding the respiratory burst kinetic response (Fig. 6.2a, 6.3a, 6.4a, 6.5a, 6.6a, 6.7a, 6.8a), the PMA alone induced a ROS production showing a maximum peak after ten minutes, then it gradually decreased during the remaining 110 min of the CL measurement. Cells treated with PMA plus the

five antioxidant compounds (2^{I},4^{I}-dihydroxychalcone; esculetin; harmalol hydrochloride dihydrate; resveratrol and rosmarinic acid) at different concentrations generally showed lower values of ROS release compared to positive controls (PMA stimulated leukocytes). Peak responses were generally reached after 30 minutes, with the exception of 2^{I},4^{I}-dihydroxychalcone, whose peak was registered at the third cycle of reading (10 min), to then mildly decrease during the last 20-30 min of the CL measurement. The response of leukocytes treated with PMA plus the flavonoid 7-hydroxyflavone resulted different. In fact, in this case the cells exhibited higher levels of ROS release compared to the positive control, with a maximum peak reached after 50 min of the CL measurement.

Figures 6.2b, 6.3b, 6.4b, 6.5b, 6.6b, 6.7b and 6.8b represent the cumulative response measured in leukocytes exposed to PMA or to PMA plus plant compounds. The respiratory burst activity was significantly reduced when leukocytes were incubated with PMA in the presence of 2^{I},4^{I}-dihydroxychalcone (Fig. 6.2b), esculetin (Fig. 6.3b), harmalol hydrochloride dihydrate (Fig. 6.4b), resveratrol (Fig. 6.5b) or rosmarinic acid (Fig. 6.6b), compared to the situation where cells were incubated only with PMA ($P \leq 0.05$). This effect was dose-dependent in all cases. In particular, significant differences in the respiratory burst activity among ESB leukocytes incubated with the 3 surveyed concentrations were detected when testing harmalol hydrochloride dihydrate and resveratrol ($P \leq 0.05$). Otherwise, the ROS release was significantly increased in the ESB leukocytes incubated with PMA plus the flavonoid 7-hydroxyflavone (Fig. 6.7b) in comparison to the cells stimulated only with PMA ($P \leq 0.05$) and this effect was dose dependent ($P \leq 0.05$).

About the monoterpene camphor (1R), the results of these studies did not confirm the immunostimulatory properties, which were supposed after the preliminary experiments (Fig. 6.8a and 6.8b), because no significant differences were observed in the ROS production between leukocytes incubated with PMA plus camphor (1R) and leukocytes stimulated only with PMA ($P > 0.05$).

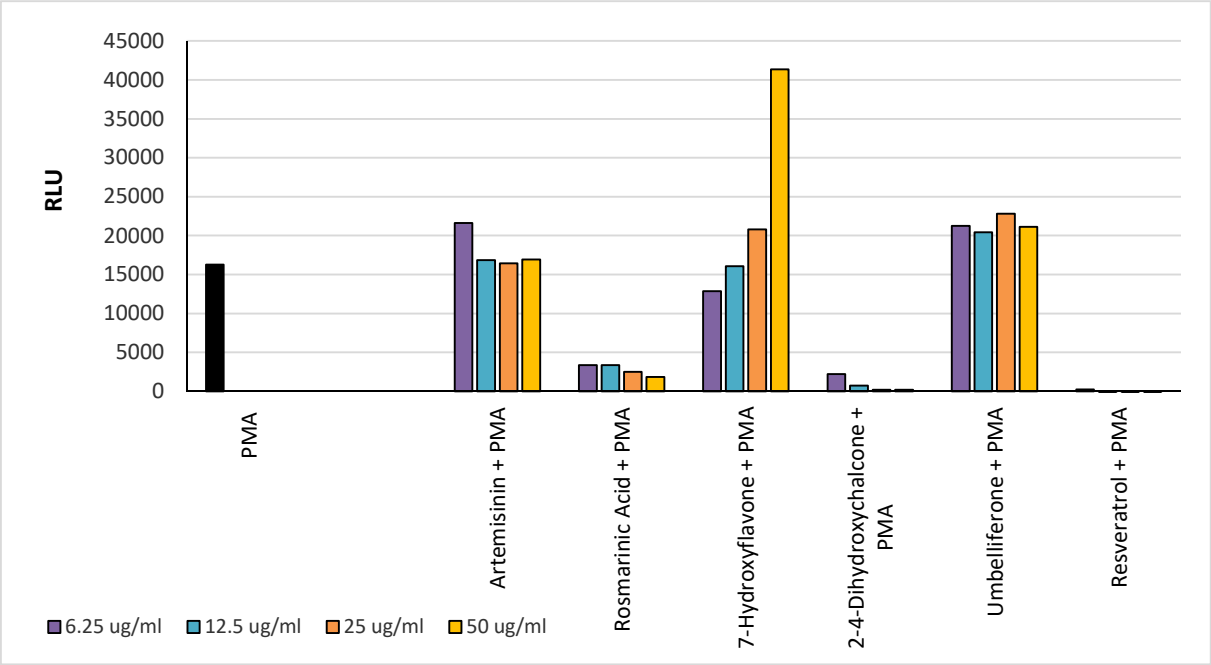


Figure 6.1a. Respiratory burst activity of HK leucocytes (1×10^6 cells/ml) exposed to PMA (1.25 µg/ml) and to PMA (1.25 µg/ml) in the presence of plant compounds at the concentration range of 6.25-50 µg/ml. Data refer to one individual fish and are expressed as cumulative response (RLU).

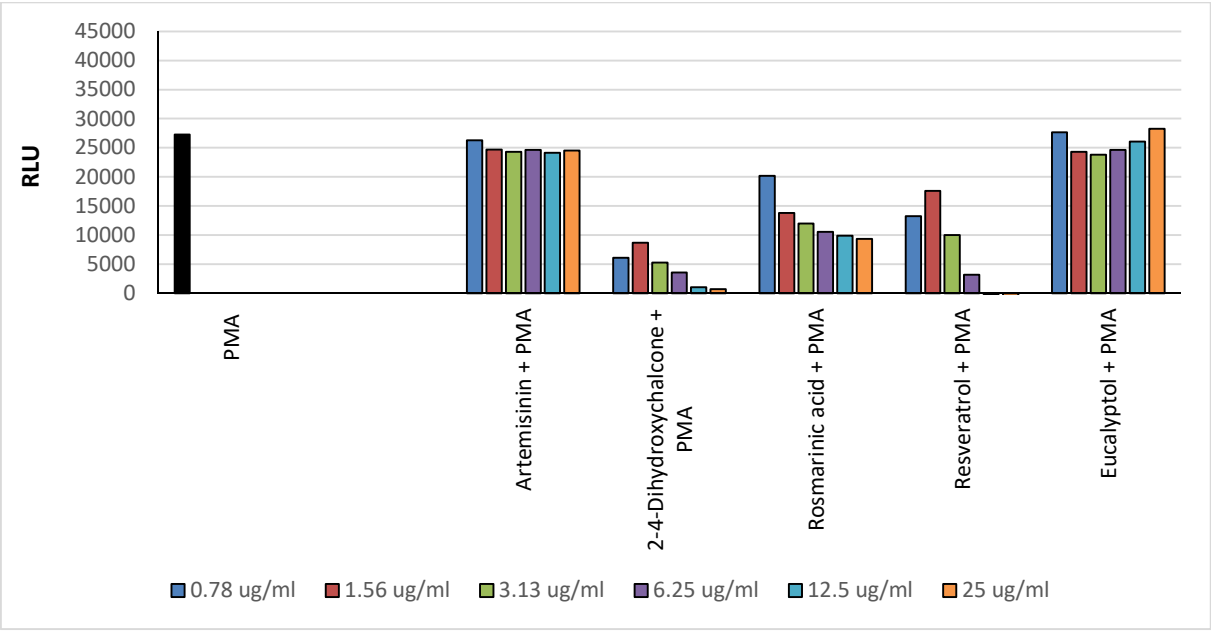


Figure 6.1b. Respiratory burst activity of HK leucocytes (5×10^6 cells/ml) exposed to PMA (1.25 µg/ml) and to PMA (1.25 µg/ml) in the presence of plant compounds at the concentration range of 0.78-25 µg/ml. Data refer to one individual fish and are expressed as cumulative response (RLU).

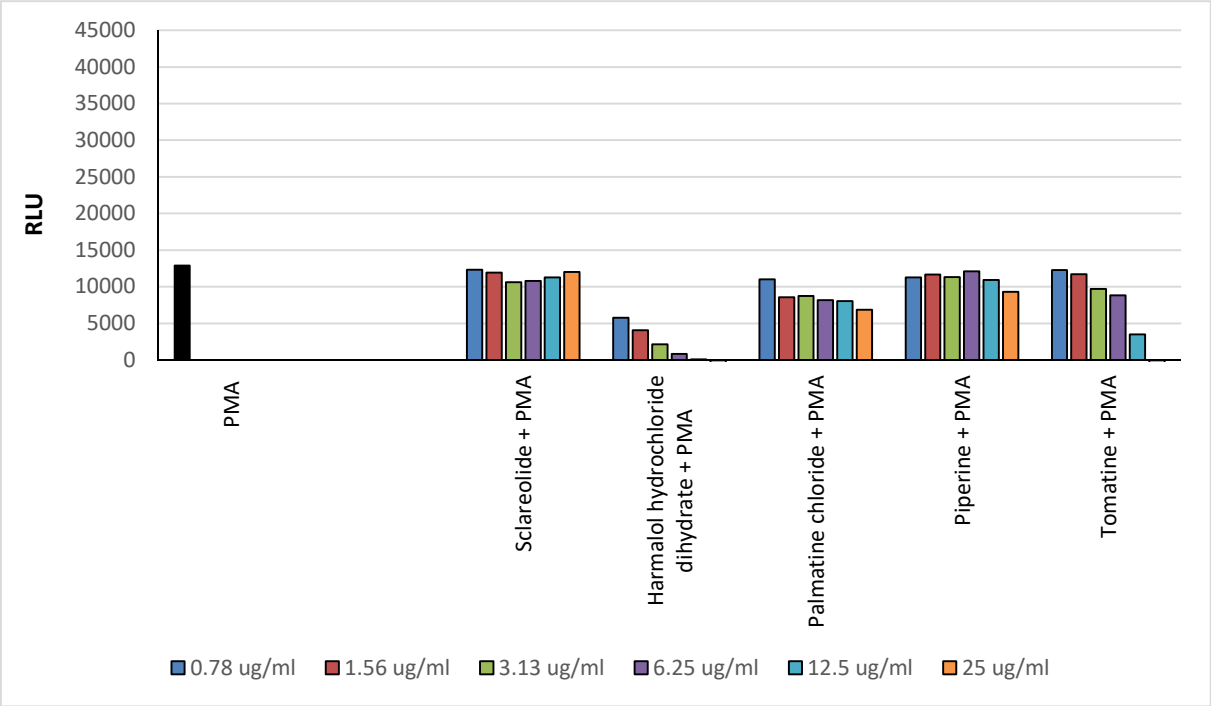


Figure 6.1c. Respiratory burst activity of HK leucocytes (1×10^6 cells/ml) exposed to PMA (1.25 µg/ml) and to PMA (1.25 µg/ml) in the presence of plant compounds at the concentration range of 0.78-25 µg/ml. Data refer to one individual fish and are expressed as cumulative response (RLU).

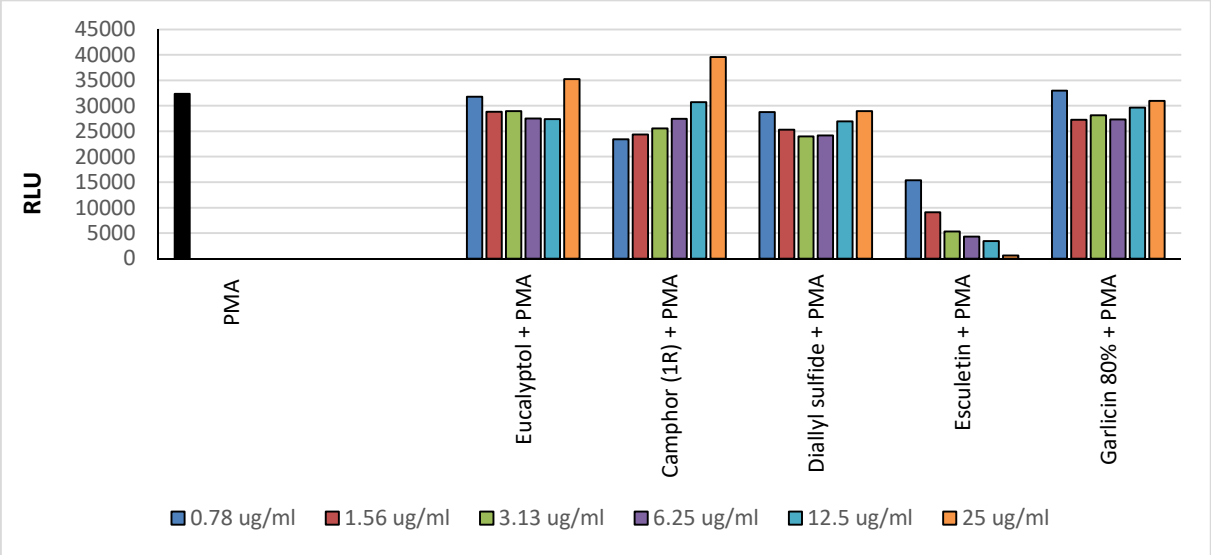
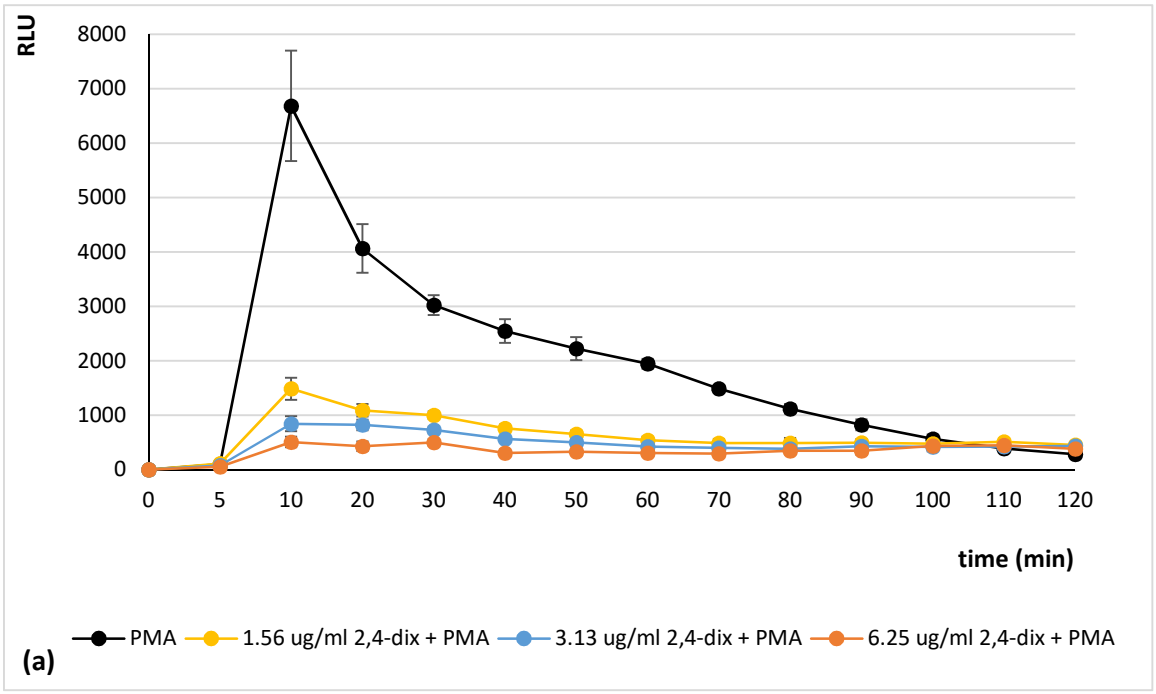
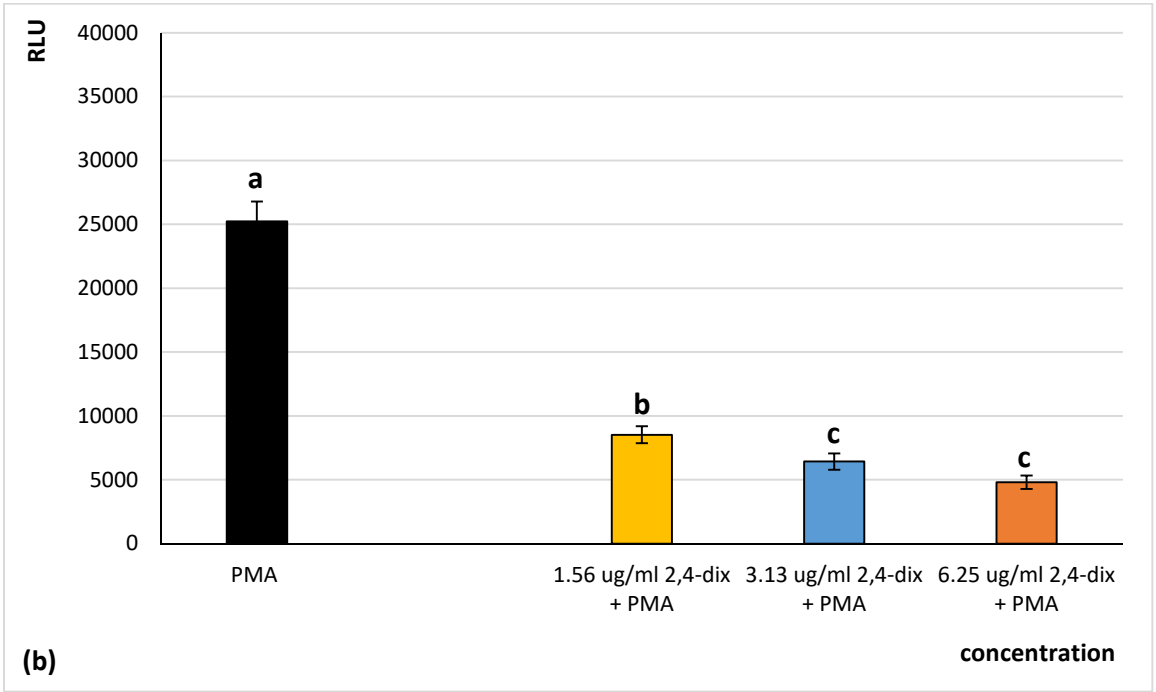
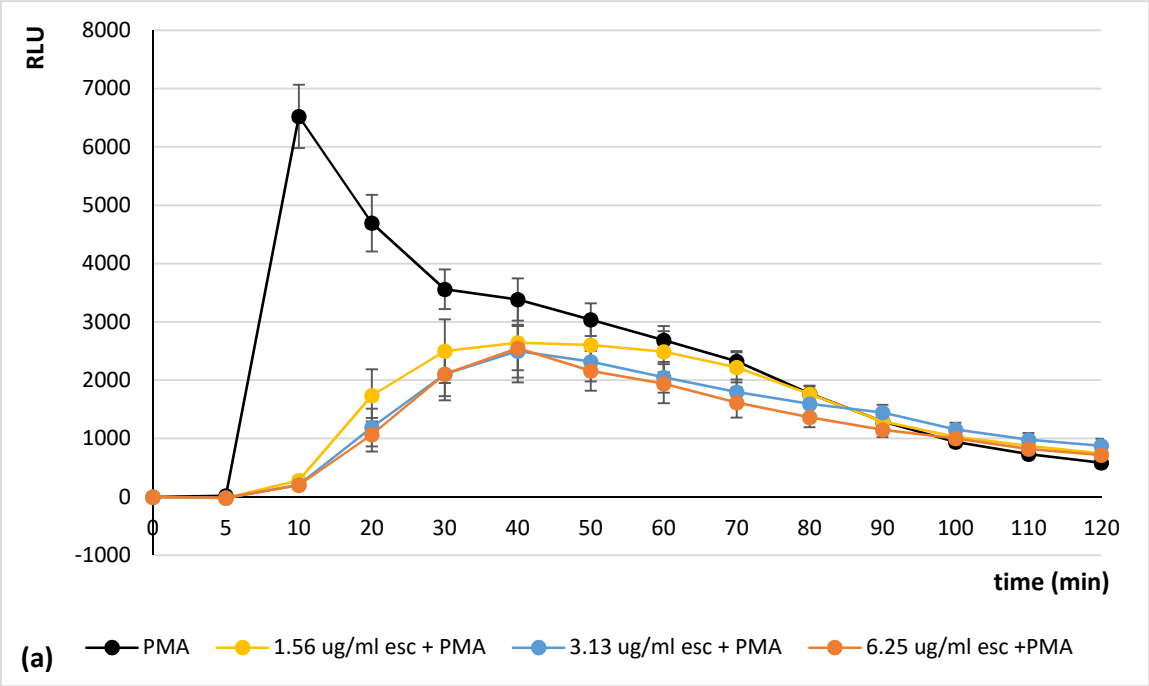


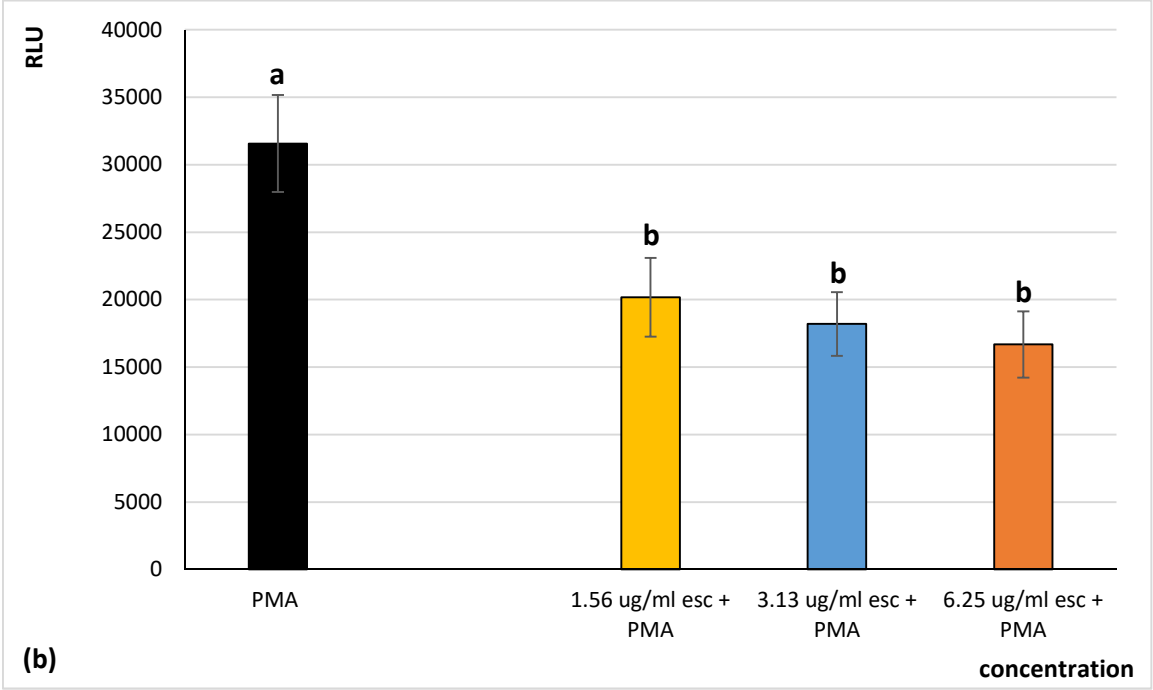
Figure 6.1d. Respiratory burst activity of HK leucocytes (5×10^6 cells/ml) exposed to PMA (1.25 µg/ml) and to PMA (1.25 µg/ml) in the presence of plant compounds at the concentration range of 0.78-25 µg/ml. Data refer to one individual fish and are expressed as cumulative response (RLU).



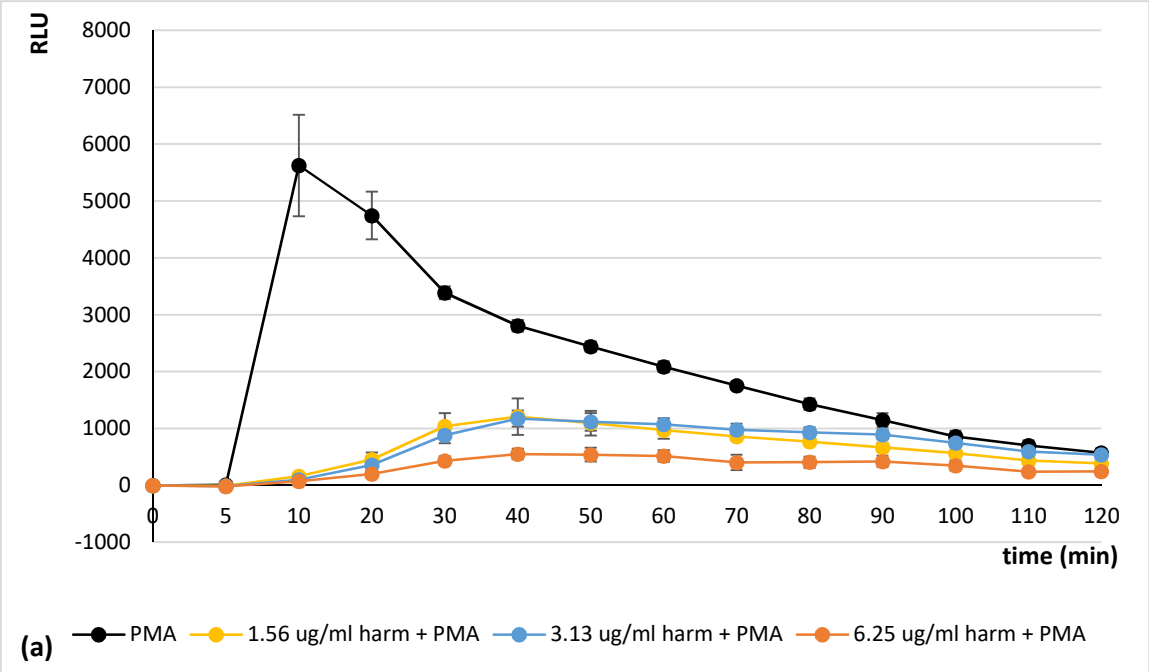


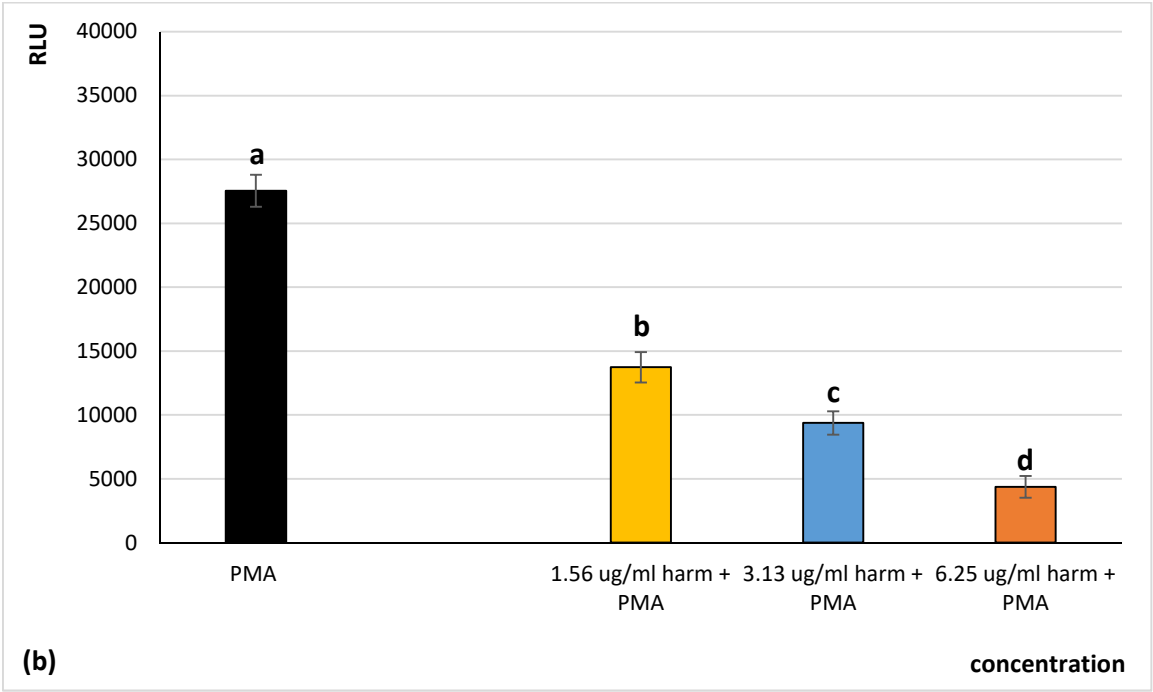
Figures 6.2a and 6.2b. Respiratory burst activity of HK leukocytes from European sea bass exposed to PMA (1.25 µg/ml) and to PMA (1.25 µg/ml) in the presence of 2',4'-dihydroxychalcone (2,4-dix) at 1.56 µg/ml, 3.13 µg/ml and 6.25 µg/ml, expressed as kinetic (a) and cumulative (b) response (RLU). Data are expressed as mean values ± SEM from n=6 independent fish (in triplicate). Different letters indicate significant differences (P≤0.05).



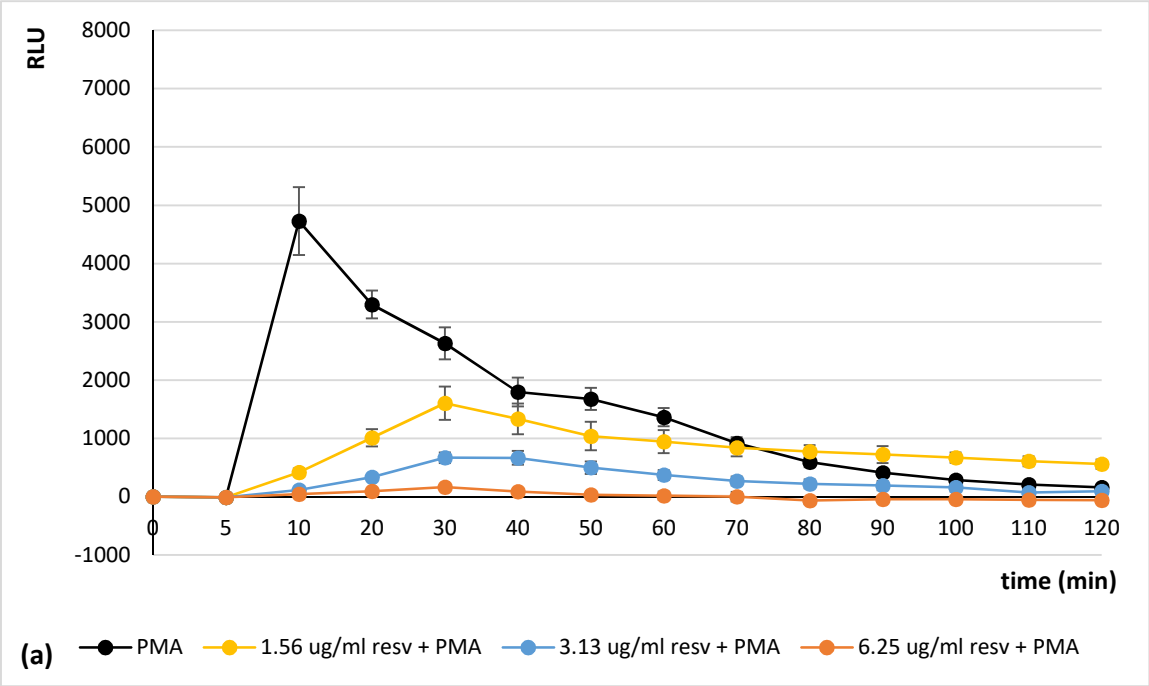


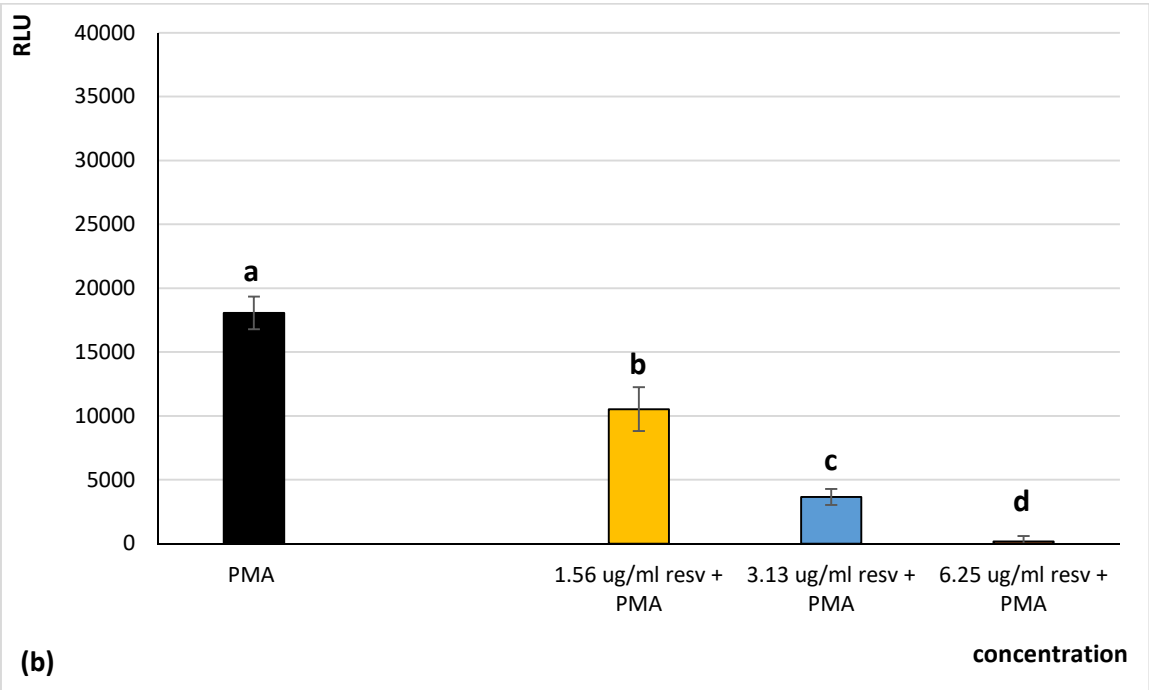
Figures 6.3a and 6.3b. Respiratory burst activity of HK leukocytes from European sea bass exposed to PMA (1.25 µg/ml) and to PMA (1.25 µg/ml) in the presence of esculetin (esc) at 1.56 µg/ml, 3.13 µg/ml and 6.25 µg/ml, expressed as kinetic (a) and cumulative (b) response (RLU). Data are expressed as mean values ± SEM from n=6 independent fish (in triplicate). Different letters indicate significant differences (P≤0.05).



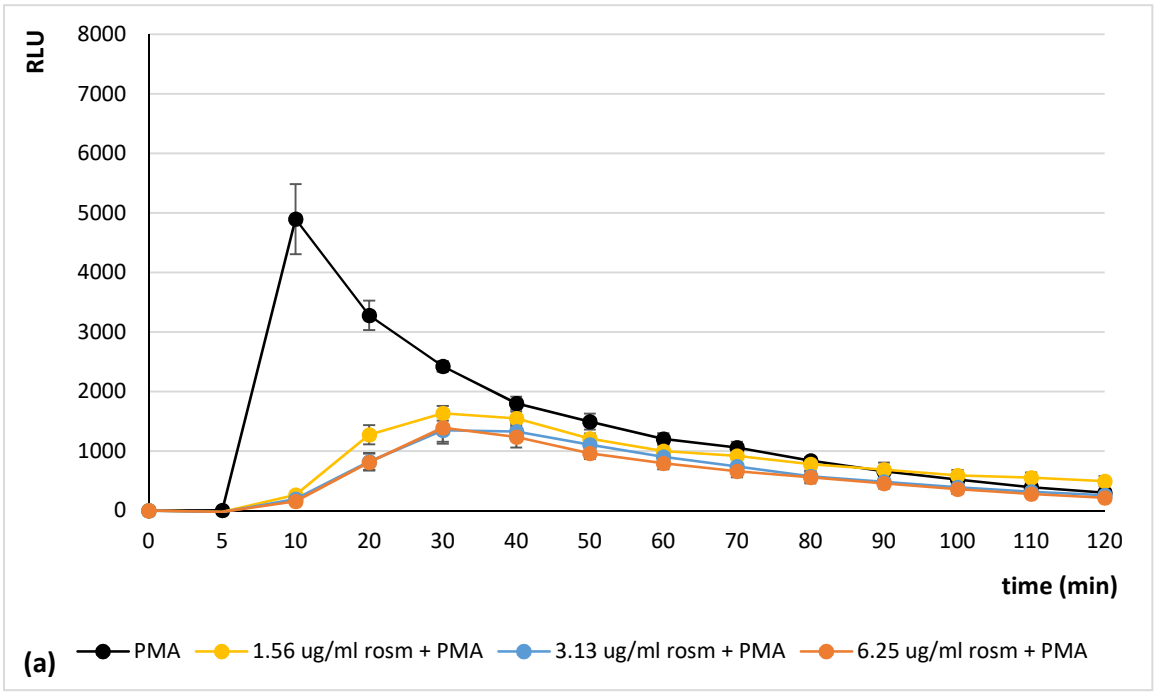


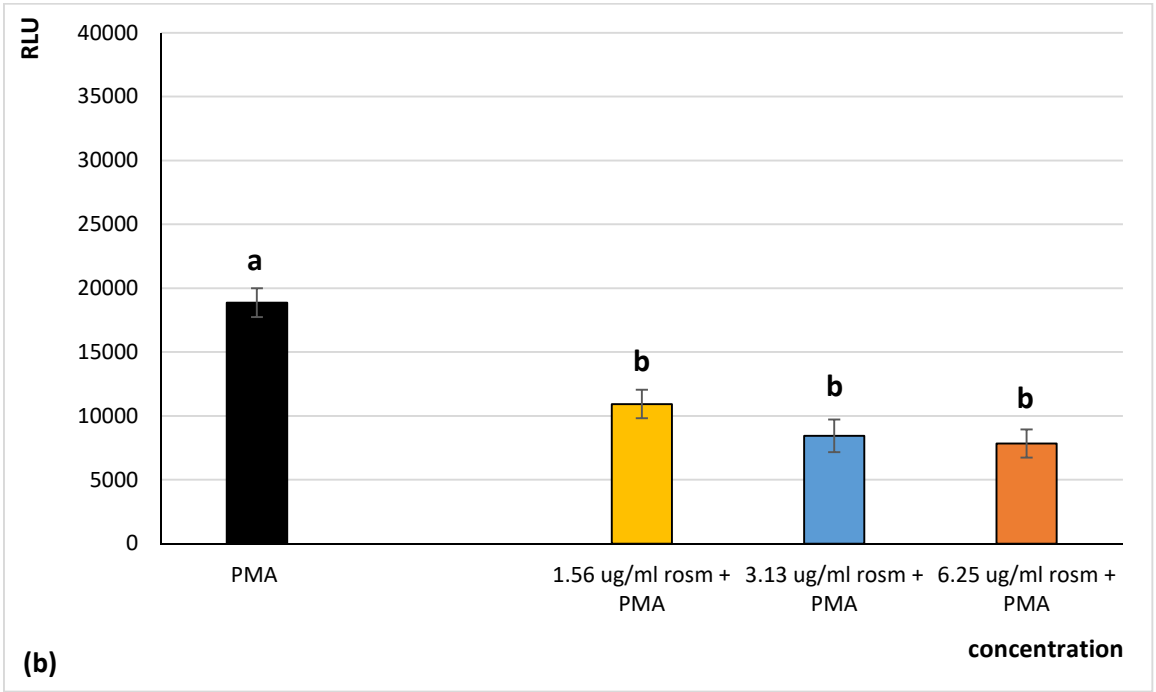
Figures 6.4a and 6.4b. Respiratory burst activity of HK leukocytes from European sea bass exposed to PMA (1.25 µg/ml) and to PMA (1.25 µg/ml) in the presence of harmalol hydrochloride dihydrate (harm) at 1.56 µg/ml, 3.13 µg/ml and 6.25 µg/ml, expressed as kinetic (a) and cumulative (b) response (RLU). Data are expressed as mean values ± SEM from n=6 independent fish (in triplicate). Different letters indicate significant differences (P≤0.05).



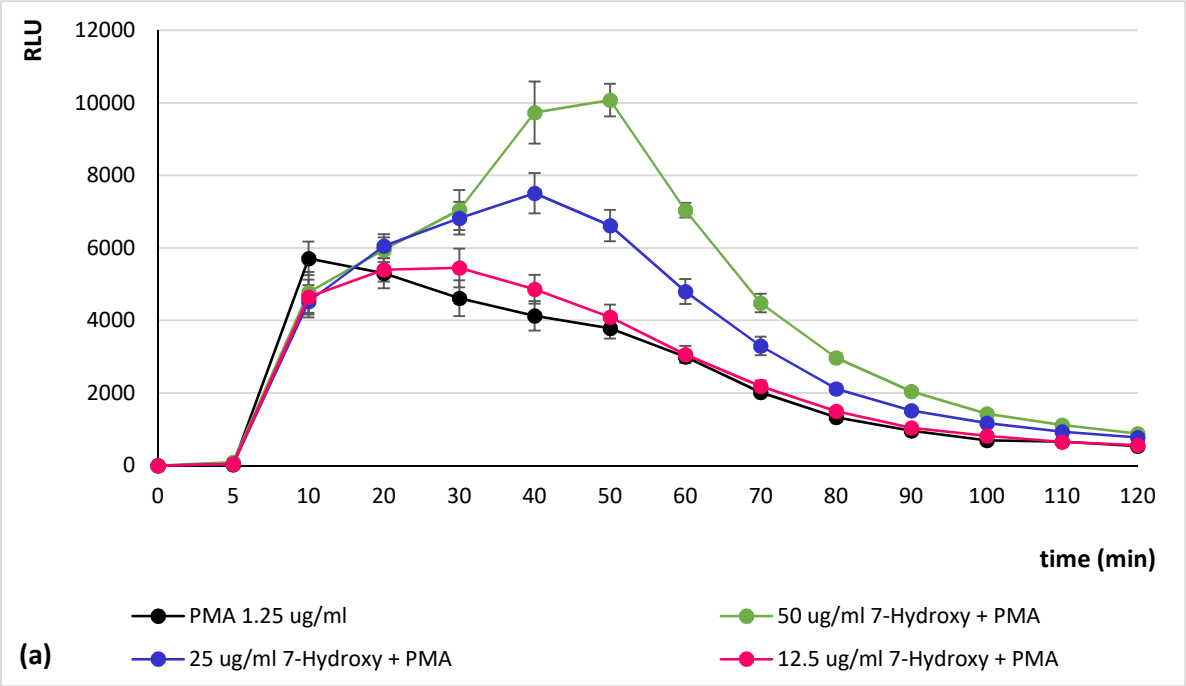


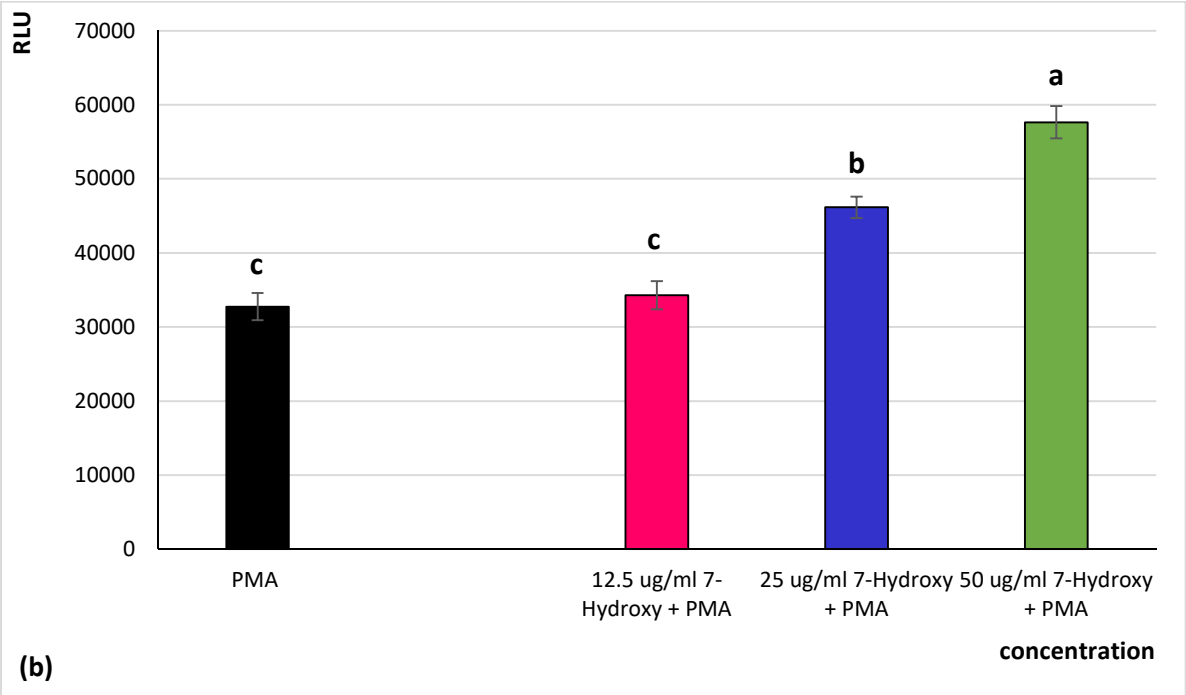
Figures 6.5a and 6.5b. Respiratory burst activity of HK leukocytes from European sea bass exposed to PMA (1.25 µg/ml) and to PMA (1.25 µg/ml) in the presence of resveratrol (resv) at 1.56 µg/ml, 3.13 µg/ml and 6.25 µg/ml, expressed as kinetic (a) and cumulative (b) response (RLU). Data are expressed as mean values ± SEM from n=6 independent fish (in triplicate). Different letters indicate significant differences (P≤0.05).



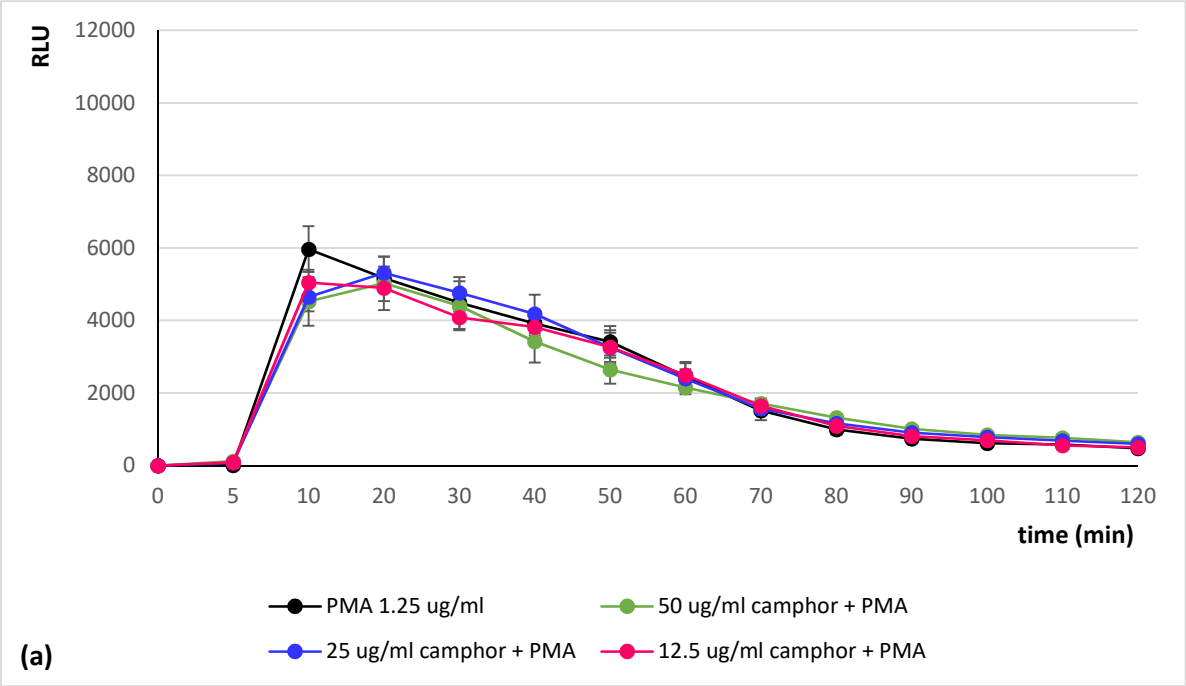


Figures 6.6a and 6.6b. Respiratory burst activity of HK leukocytes from European sea bass exposed to PMA (1.25 µg/ml) and to PMA (1.25 µg/ml) in the presence of rosmarinic acid (rosm) at 1.56 µg/ml, 3.13 µg/ml and 6.25 µg/ml, expressed as kinetic (a) and cumulative (b) response (RLU). Data are expressed as mean values ± SEM from n=6 independent fish (in triplicate). Different letters indicate significant differences (P≤0.05).





Figures 6.7a and 6.7b. Respiratory burst activity of HK leukocytes from European sea bass exposed to PMA (1.25 µg/ml) and to PMA (1.25 µg/ml) in the presence of 7-hydroxyflavone (7-Hydroxy) at 12.5 µg/ml, 25 µg/ml and 50 µg/ml, expressed as kinetic (a) and cumulative (b) response (RLU). Data are expressed as mean values ± SEM from n=6 independent fish (in triplicate). Different letters indicate significant differences among the concentrations (P≤0.05).



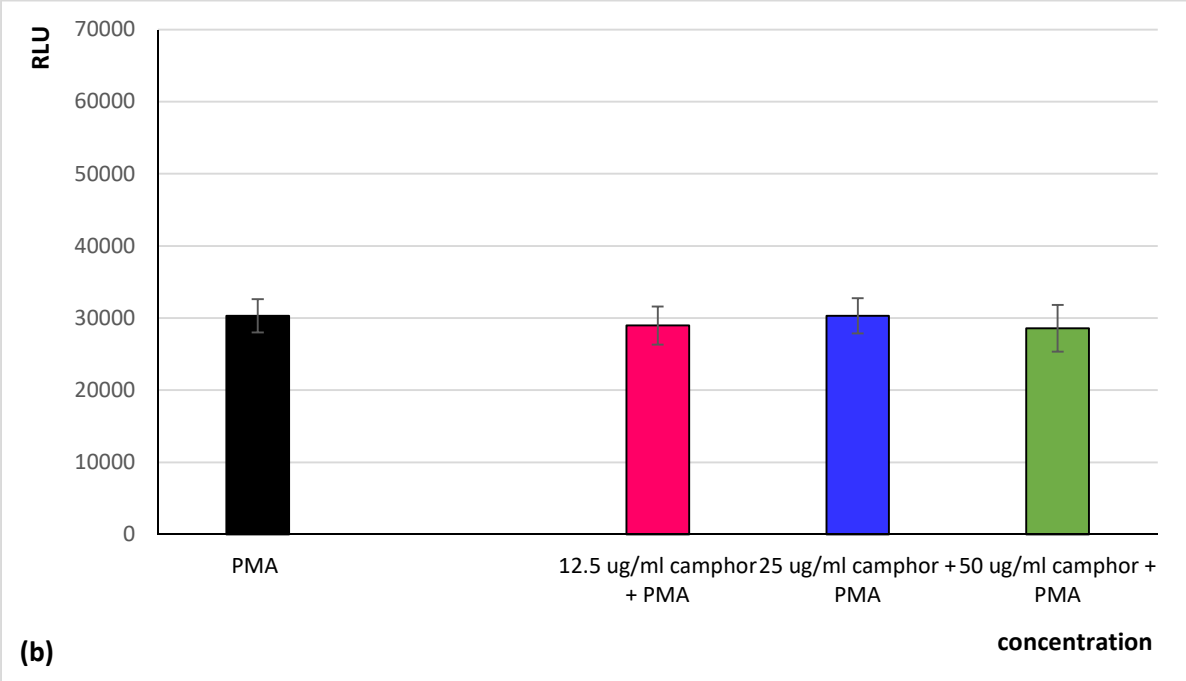


Figure 6.8a and 6.8b. Respiratory burst activity of HK leukocytes from European sea bass exposed to PMA (1.25 µg/ml) and to PMA (1.25 µg/ml) in the presence of camphor (1R) (camphor) at 12.5 µg/ml, 25 µg/ml and 50 µg/ml, expressed as kinetic (a) and cumulative (b) response (RLU). Data are expressed as mean values ± SEM from n=6 independent fish (in triplicate).

In order to compare the biological activity of these compounds, the reduction or the stimulation of ROS release by leukocytes incubated with PMA in the presence of plant substances relative to that of leukocytes incubated only with PMA was expressed as a percentage (Table 6.2 and 6.3). This approach normalised the data from different experimental runs to allow comparison.

In table 6.2 it is possible to observe that the five compounds had a dose-dependent antioxidant activity. At the concentration of 1.56 µg/ml 2^{I},4^{I}-dihydroxychalcone was the most effective by reducing the ROS release of 66.2%, while the other four compounds had statistically the same antioxidant effect. At 3.13 µg/ml escultin exhibited the weakest antioxidant activity (42.3%), instead resveratrol and 2^{I},4^{I}-dihydroxychalcone showed to be the strongest antioxidant compounds by reducing the ROS release up to 79.7%; harmalol hydrochloride dihydrate was as effective as 2^{I},4^{I}-dihydroxychalcone and rosmarinic acid. At the concentration of 6.25 µg/ml resveratrol exhibited the strongest antioxidant activity by reducing the ROS release of 99%, followed by 2^{I},4^{I}-dihydroxychalcone and harmalol hydrochloride dihydrate, while rosmarinc acid and esculetin displayed the weakest antioxidant activities.

In table 6.3 are reported the effects of 7-hydroxyflavone. The compound showed a dose-dependent stimulating effect, which was stronger at 50 µg/ml concentration by increasing the ROS release of 76%. Anyway, data were not statistically elaborated as this flavonoid was the unique surveyed compound displaying immunostimulating properties.

Table 6.2. Reduction in reactive oxygen species (ROS) production (%) induced by 2^{I},4^{I}-dihydroxychalcone; esculetin; harmalol hydrochloride dihydrate; resveratrol and rosmarinic acid. Data are expressed as mean ± S.E. from n=6 independent fish. Different letters indicate significant differences among active plant compounds for each concentration (P≤0.05).

ACTIVE PLANT COMPOUND	CONCENTRATION (µg/ml)		
	1.56	**3.13**	**6.25**
2^{I},4^{I}-Dihydroxychalcone	66.2±2.91 **a**	74.5±3.21 **ab**	80.9±2.13 **b**
Esculetin	36.1±5.00 **b**	42.3±3.99 **d**	47.2±4.69 **d**
Harmalol hydrochloride dihydrate	50.2±4.31 **b**	66.0±2.82 **bc**	84.2±3.03 **b**
Resveratrol	41.7±6.40 **b**	79.7±2.81 **a**	99.0±2.18 **a**
Rosmarinic acid	42.1±3.53 **b**	55.3±4.40 **c**	58.4±4.03 **c**

Table 6.3. Stimulation in reactive oxygen species (ROS) production (%) induced by 7-hydroxyflavone. Data are expressed as mean ± S.E. from n=6 independent fish.

ACTIVE PLANT COMPOUND	CONCENTRATION (µg/ml)		
	12.5	**25**	**50**
7-Hydroxyflavone	4.7±2.66	41.0±6.16	76.0±8.26

6.1.5 Discussion and conclusions

Fish defence against pathogens depends to a great extent on non-specific immune mechanisms (Tort *et al.*, 2003; Magnadottir, 2010; Rauta *et al.*, 2012). Among such non-specific responses, respiratory burst plays a central role. This process protects fish against invading pathogens through the production of reactive oxygen species (ROS), potent antimicrobial chemically reactive species produced by phagocytes (Secombes and Fletcher, 1992; Secombes, 1996). The production of ROS is also considered to play a key role in cell signalling to combat pathogen invasion (Forman and Torres, 2002). However, an excessive release of ROS may cause tissue damage if the process is not well controlled or it is modulated through pathogen activities. Evidences suggest that the respiratory burst can both be regulated by and can itself function to regulate inflammatory cytokines (Novoa *et al.,* 1996), suggesting a possible role for controlling the damage caused by excessive inflammatory responses to some parasite species in the host (Abowei *et al.,* 2001; Iwanowicz 2011).

In the current research, the respiratory burst activity of European sea bass HK leukocytes was selected as a key indicator of fish aspecific immune response and it was evaluated to investigate the potential *in vitro* immunomodulatory properties of a panel of plant-derived compounds that were selected for their known capacity to promote reactivity of mammalian cells (Lojek *et al.*, 2014). For this purpose, the respiratory burst activity of ESB HK leukocytes was measured using a luminol-enhanced chemiluminescence assay, after cell incubation with the single substances in the presence of PMA. PMA is a fast activator of protein kinase C (PKC), so it activates directly the enzymes responsible for ROS production bypassing all previous events involved in the signal transduction cascade (Tsumbu *et al*., 2012). Luminol can cross biological membranes due to its chemical structure, allowing the detection of extracellular and intracellular production of ROS (Vera-Jimenez *et al.,* 2013). Screening tests were performed to evaluate the properties of the 16 plant compounds provided by ZF-S (partner of ParaFishControl project). Currently the available literature provides little data concerning *in vitro* immunomodulatory effects of plant products on primary fish cell cultures (Castro *et al.*, 2008; Leiro *et al.*, 2010; Zanuzzo *et al.*, 2012; Domínguez *et al.*, 2013; Baba *et al.,* 2014; Soltani *et al.*, 2017) so that the observations provided by this research are both novel and informative. Moreover, analogous studies seem not to have been carried out on ESB purified cells.

Results indicated that five of the selected compounds (2^{I},4^{I}-dihydroxychalcone; esculetin; harmalol hydrochloride dihydrate; resveratrol and rosmarinic acid) displayed significant antioxidant effects in terms of reduced leukocyte PMA dependent ROS production. On the other hand, 7-hydroxyflavone upregulated the ROS production of cells incubated with PMA.

Resveratrol is a polyphenolic phytoalexin found in numerous plants especially in the skin of grapes and has been reported to have antioxidative, nephron-protective, anticarcinogenic, antiangiogenic, vascular-targeting, anti-obesogenic, and anti-ageing properties in several fish models (Zheng *et al.*, 2017). In the present research, it exhibited the strongest antioxidant activity (up to 99% reduction of ROS release), confirming previous data already documented in literature for HK leukocytes purified from turbot (*Scophthalmus maximus*) (Castro *et al.*, 2008). *In vitro* studies on mammalian leukocytes stimulated with PMA demonstrated that polyphenols are capable of entering cells and reducing ROS production, either through a direct stoichiometric relationship or by modifying leukocytes' reactivity to PMA (Leiro *et al.*, 2002; Kohnen *et al.*, 2007; Franck *et al.*, 2008; Tsumbu *et al.*, 2012). Furthermore, several mammalian *in vitro* studies have determined that pre-treatment with different plant derivative compounds, such as resveratrol (Qureshi *et al.,* 2012; Zong *et al.*, 2012), can modify the macrophage inflammatory and oxidative response to lipopolysaccharide (LPS), a pathogen associated molecular pattern (PAMP) that stimulates an antibacterial immune response. Similarly, Smith *et al.* (2018) demonstrated that resveratrol decreased the expression of relevant genes codifing for anti-bacterial and inflammatory molecules, the number of phagocytic cells and the ROS production in Atlantic salmon (*Salmo salar*), following leukocyte stimulation with LPS. In the same way, earlier studies in turbot and in Nile tilapia (*Oreochromis niloticus*) demonstrated the capacity of this polyphenol to induce a modulation of expression of several genes involved in the immune responses and inflammation (Domínguez *et al.*, 2013; Zheng *et al.*, 2017).

Harmalol hydrochloride dihydrate reduced the ROS release by up to 84%, demonstrating an activity slightly lower than that of resveratrol. This compound is an alkaloid extracted from the widely-distributed plant *Peganum harmala*. *P. harmala* roots and seeds, which are rich in alkaloids, have been used for the treatment of several human diseases (Bukhari *et al.*, 2008) and as an antiseptic and disinfectant agents (Fathiazada *et al.*, 2006; Arshad *et al.*, 2008). Moreover, it has been reported that this plant shows antibacterial, antifungal and antiviral effects. However, information concerning its effect on fish immune responses is very scarce. Poor *et al.* (2014) studied its immuno-modulatory effects in rainbow trout (*Oncorhynchus mykiss*) fed with the seed extracts of *P. harmala* at the concentration of 100 mg/kg feed for two weeks, observing enhanced phagocytotic index and lysozyme activity.

The flavonoid 2^{I},4^{I}-dihydroxychalcone is an active compound contained in the Tibetan plant *Oxytropis falcata,* which has been used for thousands of years in Chinese and Tibetan medicine. In the present study, this flavonoid reduced the release of ROS by ESB leukocytes up to 75%. From the

available literature, there is no previous information relating to its *in vitro* activity on fish cells, however some studies reported well-known anti-inflammatory properties of *O. falcata* (Mao *et al.*, 1986, cited by Lou *et al.*, 2009; Jiang *et al.*, 2006).

Rosmarinic acid, is a phenol that possesses numerous biological activities, including astringent, anti-oxidative, anti-inflammatory, anti-mutagenic, antibacterial and antiviral properties (Petersen and Simmonds, 2003). Rosmarinic acid is commonly found in plant species belonging to the Lamiaceae such as *Rosmarinus officinalis*, its properties had not been previously documented through *in vitro* trials with fish cells. In the current study, the compound exhibited antioxidant activities up to 58%. In previous studies, Gültepe *et al.* (2014) investigated the effects of *R. officinalis* extract inclusion in the feed of Mozambique tilapia (*Oreochromis mossambicus*) and observed a considerable increase of phagocytotic activity but no significant changes in the respiratory burst activity were noticed. Due to these different observations, further investigations on both *R. officinalis* extracts and rosmarinic acid should be performed in order to better characterise their activities in fish.

Esculetin is a derivative of coumarin and in the present study demonstrated the less potent antioxidant activity, halving the production of ROS species. The anti-oxidant activity of this lactone has been previously documented in *in vitro* experiments with mammalian cells, resulting in the protection of cells from lipid peroxidation, protein carbonyl and DNA damage induced by H_2O_2 (Kim *et al.*, 2008). Till now, no information concerning its potential immunomodulatory effects in fish is available in literature.

7-hydroxyflavone is a synthetic chromophore and prototype of all naturally occurring flavonoids (Sengupta *et al.*, 2017). This compound is a hydroxyflavonoid in which the flavone nucleus is substituted at position 7 by a hydroxy group. Among different substances, flavonoids are known to protect several cell types from oxidative stress by scavenging activities (against ROS and reactive nitrogen species) or through transcriptional induction of genes with antioxidant properties such as heme oxygenase-1 (HO-1) (Akhlaghi *et al.*, 2009) or the mitochondrial manganese superoxide dismutase (MnSOD) (Korkmaz *et al.*, 2010). Recently, Sengupta *et al.* (2017) determined, *in vitro*, the antioxidant mechanism of 7-hydroxyflavone on the rat renal proximal tubule cell line NRK52E exposed to nicotine oxidative stress. Authors confirmed the protective effects of the flavonoid both by limiting ROS release and by inducing the transcription of HO-1 gene. The results of our research appear to be in disagreement with these findings, because the incubation of ESB HK leukocytes with 7-hydroxyflavone resulted in a significant stimulation of ROS production. However, some flavonoids have been found to possess anti-protozoal, insecticidal, antifungal and antibacterial activities other

than anti-inflammatory and antioxidant activities (Ingham, 1983 cited by Choudhary *et al.*, 2008). These different properties may provide a possible explanation of the diverse activities exerted by our two investigated flavonoids: 2^{I},4^{I}-dihydroxychalcone and 7-hydroxyflavone.

Finally, we also investigated the possible modulation of ESB HK leukocytes by camphor, a monoterpene constituting of essential oils of many aromatic and medicinal plants, including *Cinnamomum*, *Eucalyptus*, *Artemisia*, *Salvia* and *Thuja* species. Camphor is known to act as an insecticidal, anti-microbial, anti-viral, anti-coccidial, analgesic, anticancer and anti-tussive agent (Chen *et al.*, 2013). However, the literature on camphor's effects on animals is limited and shows inconsistent results (Sedaghat and Torshizi, 2017). For example, Nikolić *et al.* (2015) demonstrated that at low doses, camphor could exert anti-mutagenic effects on bacterial and mammalian cells, but when applied in higher concentrations it was genotoxic. In our investigation, the preliminary experiments seemed to indicate that it was immunostimulant, but when the test was repeated with a statistically representative number of fish, the compound was neither immunostimulant nor antioxidant, suggesting that it was ineffective in modulating the ROS release by HK leukocytes at the used concentrations.

This is the first study examining the effects of these functional plant compounds on European sea bass head kidney leukocyte function. In conclusion, the present investigation suggests that 2^{I},4^{I}-dihydroxychalcone, esculetin, harmalol hydrochloride dihydrate, resveratrol and rosmarinic acid, display relevant anti-oxidant activity *in vitro* as determined by the significant reductions in leukocyte ROS production. Their role in the down-regulation of this aspecific immune parameter may suggest that these plant compounds can be important in restraining the excessive inflammatory responses that lead to pathological condition in ESB during *A. ocellatum* infections. On the other hand, the dose-dependent *in vitro* immunostimulant activity of 7-hydroxyflavone makes this flavonoid a good candidate to be used in aquaculture as immunostimulant for the control of infectious diseases caused by current and emerging pathogens. Additional appropriate *in vivo* studies will be necessary to test these hypotheses and determine if these substances are capable of modulating other key immune parameters (i.e. cell proliferation, MPO release and cytokine expression) involved in the effective ESB response against parasite pathogens, and consequently if they could be used as feed additives in aquaculture.

6.2 EFFECTS OF PLANT COMPOUNDS ON AO DINOPORE MOTILITY

6.2.1 Preface

Amyloodinium ocellatum (AO) dinospores are the pre infective stage of this dangerous parasite. Dinospores are originated by tomonts (AO cystic and reproductive stage) after a certain number of cell divisions. When formed, antero-posteriorly compressed dinospores break gradually the tomont envelope and come out quite gently (Brown, 1934). At this stage, the protozoan has two flagella, one transverse and one longitudinal that allow the dinospore to swim in the water in order to find a new host. The dinospore swimming is characterised by whirling movements alternated to sudden sprints directed to a casual direction. Viable dinospores can also show motionless periods, even if to a more scrupulous observation it is possible to notice the undulating beats of the transverse flagellum in the girdle sulcus (Landsberg *et al.,* 1994), thus discriminating them from dead dinospores whose flagella are completely still. *In vitro*, dinospores remain viable for approximatively 6 days, even if some specimens naturally die before this period (UNIUD personal communication).
When a dinospore finds a new host, in a couple of minutes transforms into a trophont (Noga, 1987), the AO parasitic stage responsible of severe damages to host epithelia.
Till now, numerous efforts have been performed in order to control amyloodiniosis with the use of chemical drugs, but ineffectively. In particular, most of the studies have been focused on trying to reduce the dinospore vitality, so as to contribute in limiting AO impacts on aquaculture. In fact, dinospores seem to be the most susceptible stage to chemical treatments (Lawler, 1980; Paperna, 1984a), whereas trophonts and tomonts are relatively resistant to several experimented therapeutants (Lawler, 1977; Johnson, 1984; Paperna, 1984b). For example, Bower (1983) and, more recently, Ramesh-Kumar *et al.* (2015) proposed the antimalarial chloroquine diphosphate as an effective dinosporicide. However, this compound showed evident ecotoxic effects being harmful to aquatic organisms (Zurita *et al.*, 2005). In 1995, Oestmann *et al.* reported a bioremediation measure based on the use of *Artemia salina* nauplii against AO dinospores. However, even if this treatment was more eco-friendly than chemical baths, its usage remained confined to an experimental context.
In general, the majority of the compounds mentioned in literature for the treatment of amyloodiniosis are very expensive, polluting, not commercially available or they are not likely to be approved for the use in fish that are intended for the consumer. To date, copper sulphate remains

the most applied therapy as dinospores are particularly sensitive to this compound (Noga, 2010; Bessat and Fadel, 2018).

In order to try limiting the AO impacts on aquaculture and to reduce the accumulation of chemical or synthetic residues in the environment and in fish tissues, which can be harmful to public health, in the context of the Horizon2020 ParaFishControl project, some investigations have been planned for the research of new eco-sustainable therapies against *A. ocellatum* with a particular attention on animal welfare.

Specifically, the aim of this study was to assess if 16 substances derived from medicinal plants (2^{I},4^{I}-dihydroxychalcone; 7-hydroxyflavone; artemisinin; camphor (1R); diallyl sulfide; esculetin; eucalyptol; garlicin 80%; harmalol hydrochloride dihydrate; palmatine chloride; piperine; resveratrol; rosmarinic acid; sclareolide; tomatine and umbelliferone) could exert *in vitro* antiparasitic effects on viable dinospores of AO.

6.2.2 Materials and methods

6.2.2.1 Reagents

Citric acid (100814N, BDH Laboratory Supplies), copper sulphate (451657, Sigma Aldrich), formalin, Hank's Balanced Salt Solution without phenol red, Ca^{2+} and Mg^{2+} (HBSS)(H8264, Sigma Aldrich), Instant Ocean (IO2)(Noga, 1989), Lugol's iodine (L6146, Sigma Aldrich).

6.2.2.2 Plant compounds

2^{I},4^{I}-dihydroxychalcone; 7-hydroxyflavone; artemisinin; camphor (1R); diallyl sulfide; esculetin; eucalyptol; garlicin 80%; harmalol hydrochloride dihydrate; palmatine chloride; piperine; resveratrol; rosmarinic acid; sclareolide; tomatine and umbelliferone. Prior to use, compounds were dissolved in DMSO (10 mM) and stored in the dark at 4°C.

6.2.2.3 Dinospores

For the motility tests, dinospores originated by hibernated tomonts and fresh ones were used. Tomonts were collected as previously mentioned (paragraph 4.2.1.1) from the gills of naturally infected ESB. Hibernated tomonts have been kept in controlled conditions (paragraph 4.2.1.3) for three months, then incubated at 24°C in order to resume their reproductive process. Instead, fresh tomonts were maintained at 24°C from their collection to dinospores' hatching and never

hibernated. In both cases, for the whole duration of the reproductive process tomonts have been constantly monitored under an inverted microscope to check dinospores hatching that generally required a couple of days. Then, dinospores were counted as previously mentioned in paragraph 5.2.1.2.1. Parasites were adjusted to a concentration of 5,200 dinospores/ml in 1:1 HBSS/IO2 medium (Noga, 1989).

6.2.2.4 Incubation of dinospores with plant compounds

The 16 plant compounds were tested twice in duplicate wells in sterile 96-well plates (Sarstedt), at twofold serial dilutions (ranging from 50 µg/ml to 0.39 µg/ml; 100 µl/well) (Fig. 6.9). The compounds were diluted in HBSS/IO2 medium starting from a concentration at which the toxicity due to DMSO solvent was negligible, on the basis of bibliographic data deriving from *in vitro* studies (Hutchinson *et al.*, 2006; Qi *et al.,* 2008). Dinospores (100 µl) were then added to each well, those incubated without the extracts, but in the culture medium only, were used as negative controls. Copper sulphate chelated with citric acid (100 µl/well) at a final concentration of 1 µg/ml and 100 µl/well of formalin (4µg/ml) were included in the plate as inhibitory positive controls. Then plates, covered with lids, were maintained at room temperature for the whole duration of the experiment (24 h).

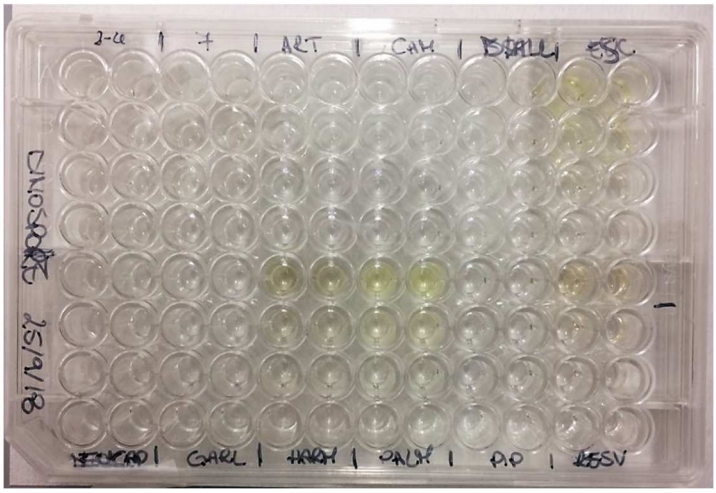


Figure 6.9. Arrangement of the microplate used for AO dinospore incubation with the diluted plant compunds.

6.2.2.5 Motility test

The behaviour of the parasites was observed under an inverted microscope (Fig. 6.10). At 1, 6 and 24 hours after the beginning of the incubation with the compounds, aliquots of 50 µl were taken from each well and transferred into urinary deposit chambers (Vacutest Kima precision cell) in order

to facilitate the visualization and counting of the dinospores. Aliquots were inspected under light microscopy.

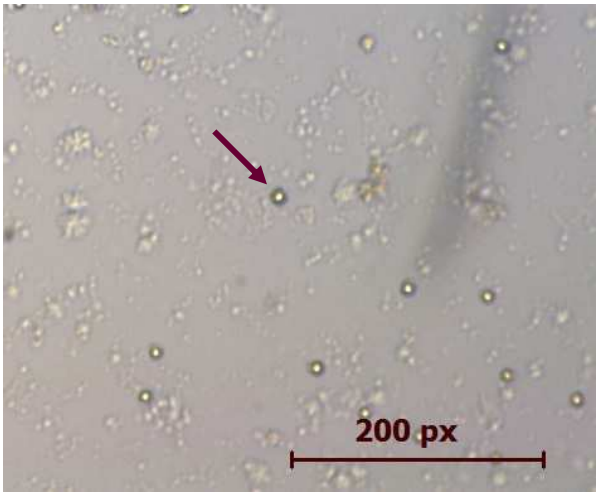


Figure 6.10. Dinospores incubated with tomatine (50µg/ml); in the picture, dinospores appear as iridescent dots (arrow).

6.2.2.6 Data elaboration

Only non motile dinospores and whose flagella did not vibrate were counted. Based on non-motile counted dinospores it was derived the percentage of motile dinospores, calculated for every compound concentration and at the different times of evaluation. Motility percentages were then compared to the motility of dinospores detected in the negative control to whom it was attributed the motility value of 100%[5]. Results are expressed as mean values ± standard error (S.E.).

[5] Immotile dinospores counted in the negative control wells are to be ascribed to the spontaneous death of the parasite.

6.2.3 Results

The substances under investigation belong to a range of classes of natural or synthesised phytochemical compounds, as reported in Table 6.1 (information deriving from literature). In the present investigation, all the 16 plant compounds were initially tested twice on dinospore motility at concentrations ranging from 50 µg/ml to 6.25 µg/ml. The preliminary results demonstrated that 14 out of 16 compounds (7-hydroxyflavone; artemisinin; camphor (1R); diallyl sulfide; esculetin; eucalyptol; garlicin 80%; harmalol hydrochloride dihydrate; palmatine chloride; piperine; resveratrol; rosmarinic acid; sclareolide and umbelliferone) showed no considerable effects on dinospore motility as described in figures 6.11-6.13. In fact, the percentage of dinospore movements in the wells with these substances were comparable to those recorded for the negative controls.

Instead, 2^{I},4^{I}-dihydroxychalcone and tomatine had an evident inhibitory action on dinospores, for this reason they have been subsequently tested extending also the range of concentrations from 50 µg/ml to 0.39 µg/ml as reported in figures 6.14-6.16. These results showed that 2^{I},4^{I}-dihydroxychalcone inhibited the dinospore motility at the highest tested concentrations (50 and 25 µg/ml) after 1 h of incubation. After 6 h of incubation, only the three highest surveyed doses (50, 25 and 12.5 µg/ml) stopped the activity of dinospores, whereas motility inhibition was halved at 6.25 and 3.13 µg/ml, while the remaining concentrations did not demonstrate a relevant inhibitory activity on dinospore motility. After 24 h of incubation, 2^{I},4^{I}-dihydroxychalcone at a concentration range of 50 to 6.25 µg/ml inhibited the motility of all the dinospores in the well, while at 3.13 µg/ml the 4% of dinospores still moved. Conversely, 0.39, 0.78 and 1.56 µg/ml concentrations of this chalcone did not inhibit dinospore motility.

Tomatine displayed more evident inhibitory effects on dinospore motility than 2^{I},4^{I}-dihydroxychalcone for the whole duration of the experiment (24 h) in the range doses 6.25-50 µg/ml. In fact, after 1 h of incubation, the highest concentrations of this substance ranging from 50 to 6.25 µg/ml completely inhibited the dinospore motility. On the other hand, in the concentrations ranging from 3.13 to 0.39 µg/ml the percentage of swimming dinospores was 89-95%. Similarly, after 6 h and 24 h of incubation no activity of dinospores was observed in the wells with the highest tomatine concentrations (50 to 6.25 µg/ml) as noticed after 1 h, whereas the lowest concentrations resulted less or no effective, and capable of inhibiting dinospore motility up to 9%.

Chelated copper sulphate (1 µg/ml) was effective for the whole duration (24 h) of the experiment; similarly also formalin (4µg/ml) showed an inhibiting activity on the dinoflagellate but at a long term

exposure. In fact, after 1 h of incubation only the 2% of dinospores was immotile. However, at 6 and 24 hours no swimming dinospores were observed (Fig. 6.17).

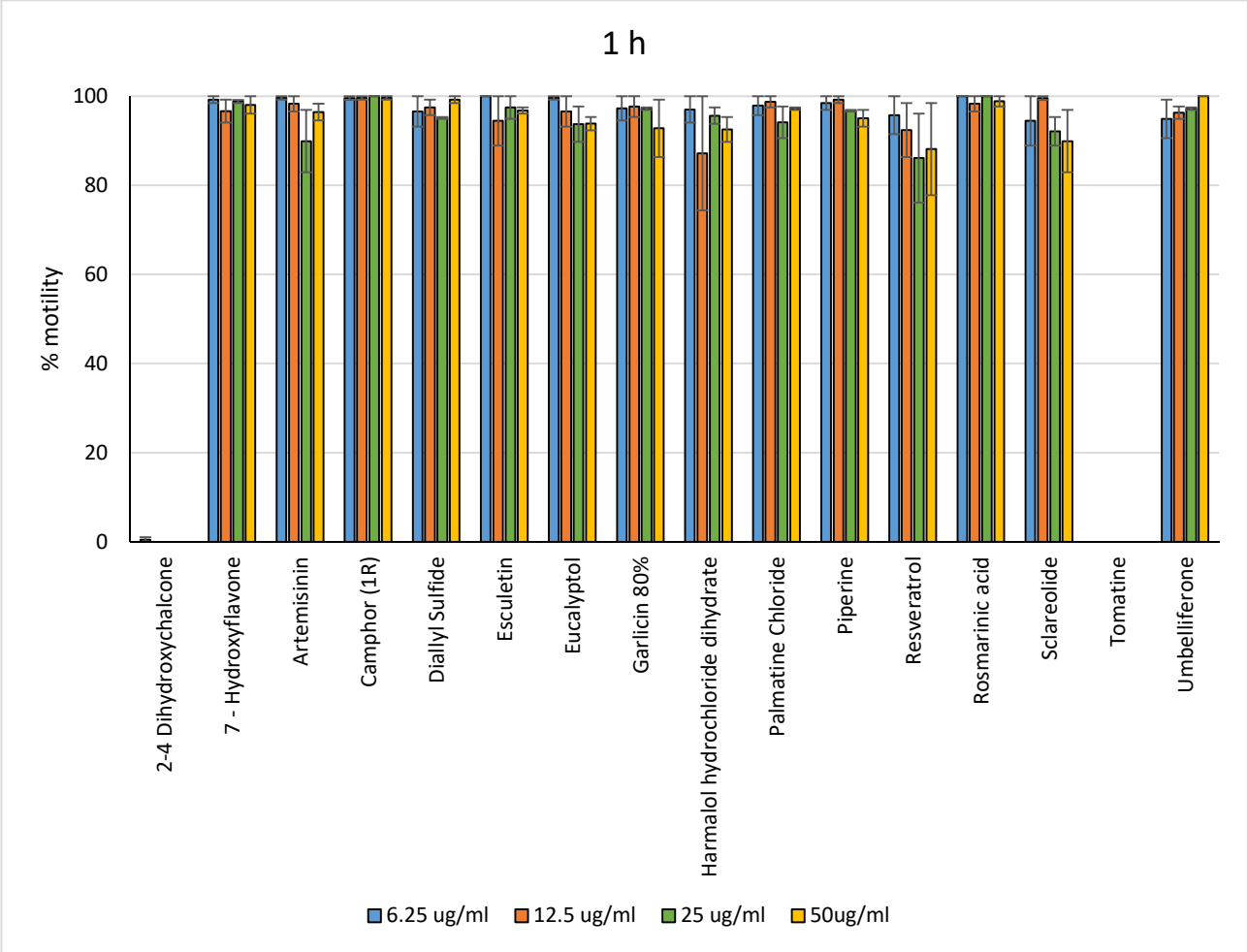


Figure 6.11. Motility of dinospores of A. ocellatum incubated at room temperature with different concentrations (6.25 – 50 µg/ml) of 16 plant derived compounds. The motility is expressed as percentage of motile dinospores on the total number of dinospores present per well after 1 hour of incubation. Then, values have been related to the motility observed in the wells assigned to negative control and corresponding to 100%.

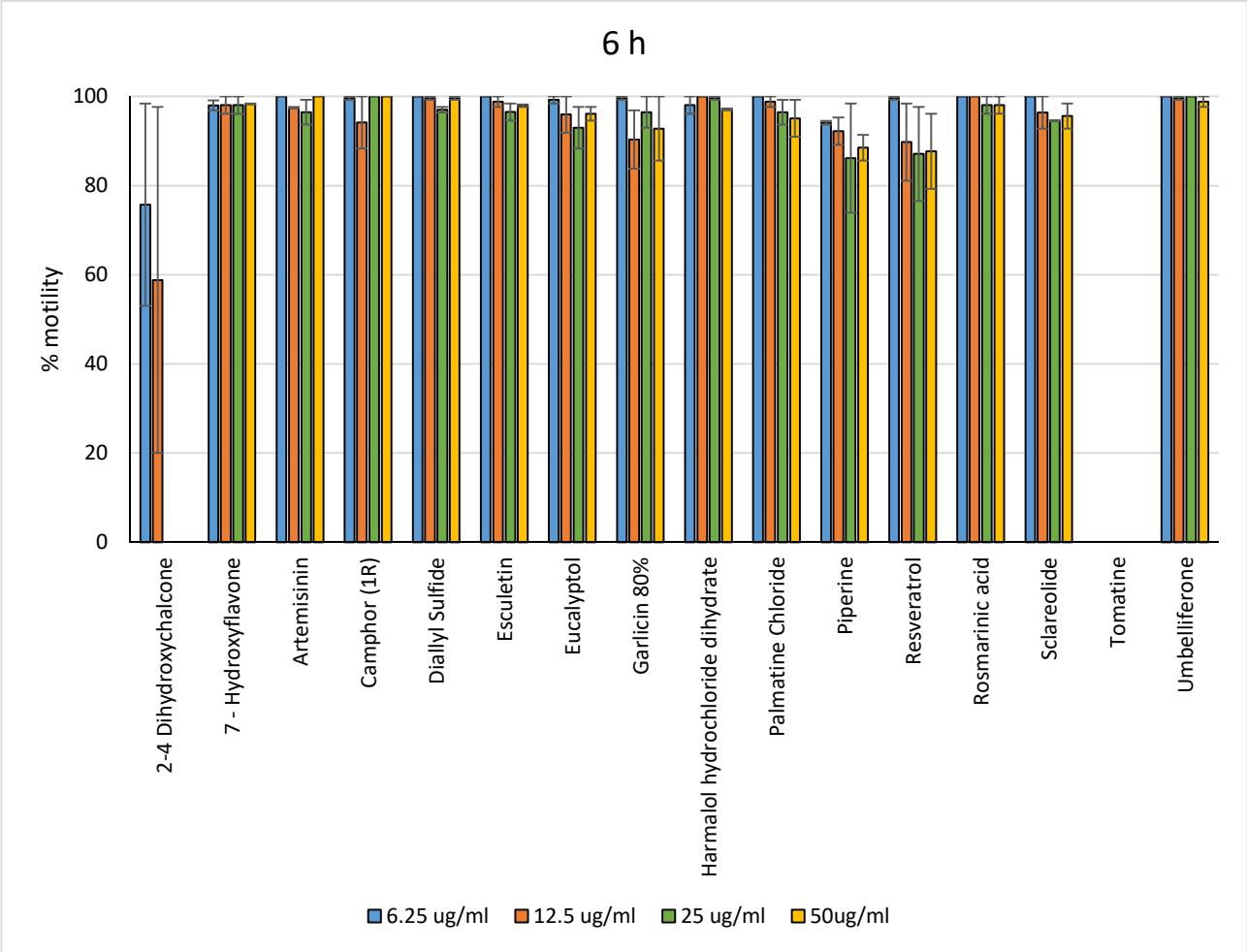


Figure 6.12. Motility of dinospores of A. ocellatum incubated at room temperature with different concentrations (6.25 – 50 µg/ml) of 16 plant derived compounds. The motility is expressed as percentage of motile dinospores on the total number of dinospores present per well after 6 hours of incubation. Then, values have been related to the motility observed in the wells assigned to negative control and corresponding to 100%.

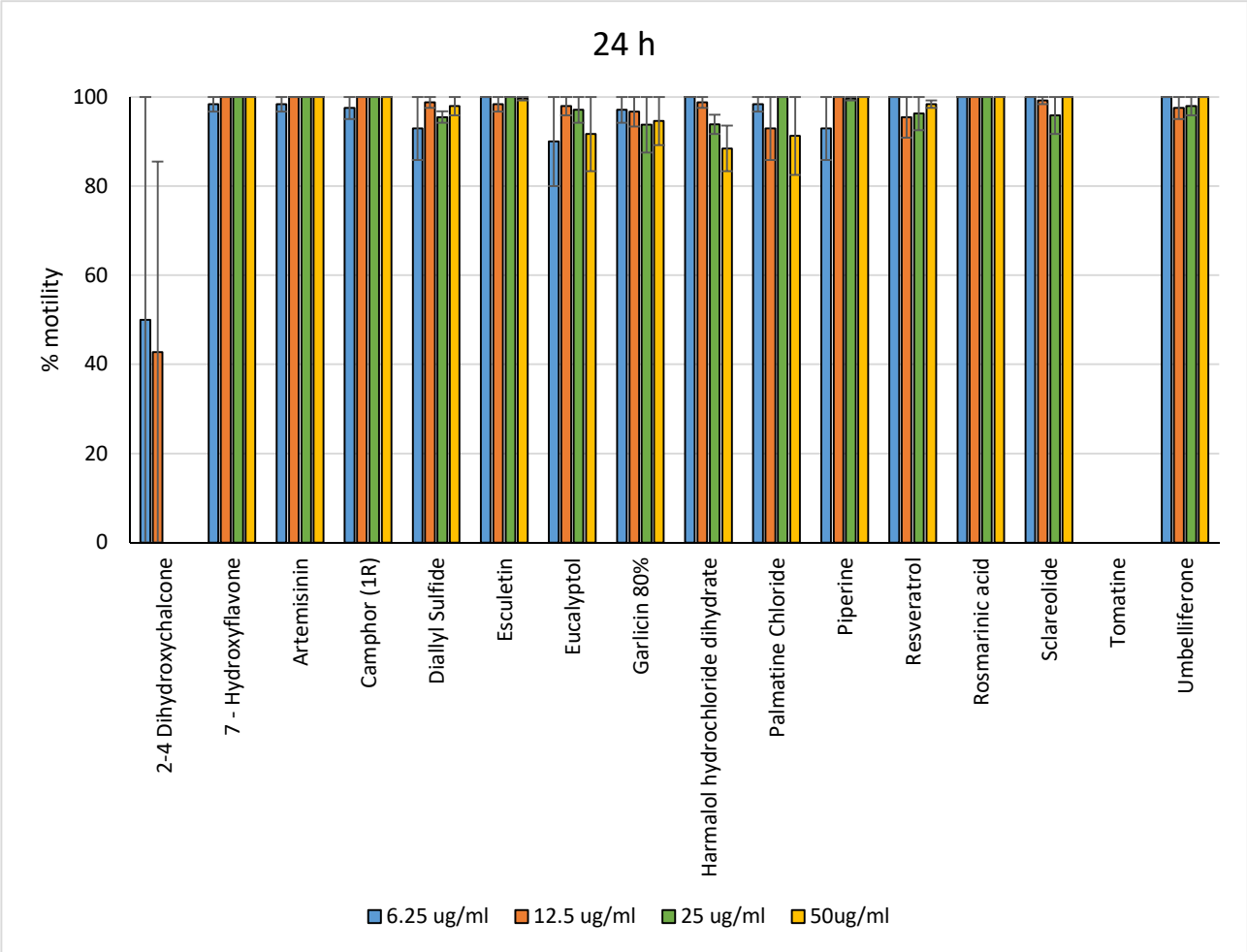


Figure 6.13. Motility of dinospores of A. ocellatum incubated at room temperature with different concentrations (6.25 – 50 µg/ml) of 16 plant derived compounds. The motility is expressed as percentage of motile dinospores on the total number of dinospores present per well after 24 hours of incubation. Then, values have been related to the motility observed in the wells assigned to negative control and corresponding to 100%.

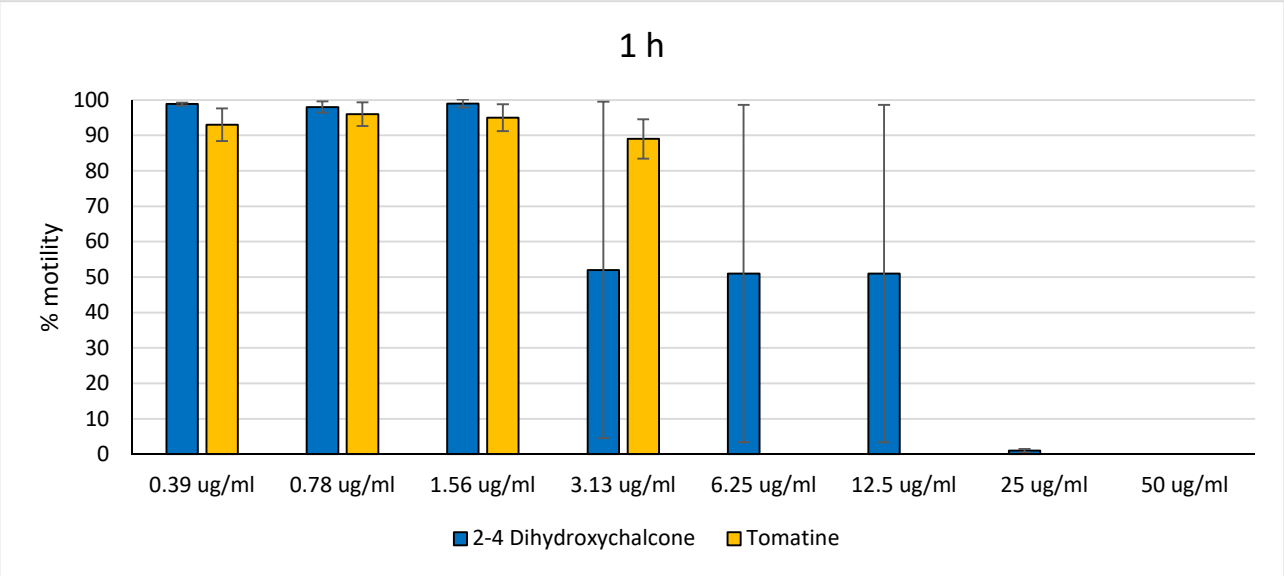


Figure 6.14. Motility of dinospores of A. ocellatum incubated at room temperature with different concentrations (0.39-50 µg/ml) of 2'-4'-dihydroxychalcone and tomatine. The motility is expressed as percentage of motile dinospores on the total number of dinospores present per well after 1 hour of incubation. Then, values have been related to the motility observed in the wells assigned to negative control and corresponding to 100%.

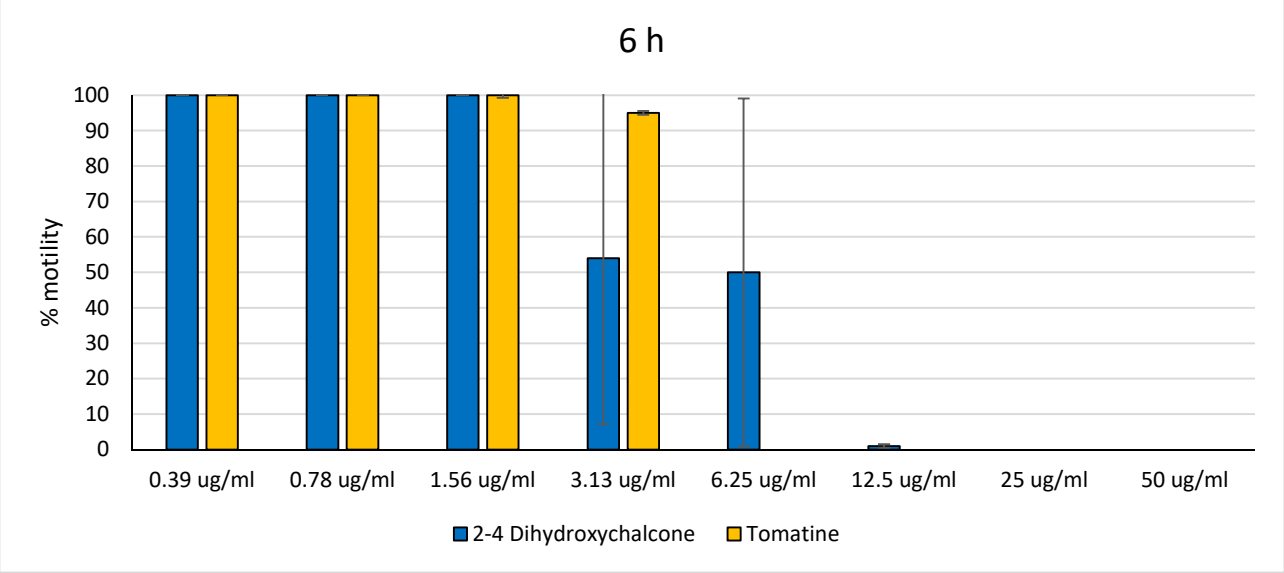


Figure 6.15. Motility of dinospores of A. ocellatum incubated at room temperature with different concentrations (0.39-50 µg/ml) of 2'-4'-dihydroxychalcone and tomatine. The motility is expressed as percentage of motile dinospores on the total number of dinospores present per well after 6 hours of incubation. Then, values have been related to the motility observed in the wells assigned to negative control and corresponding to 100%.

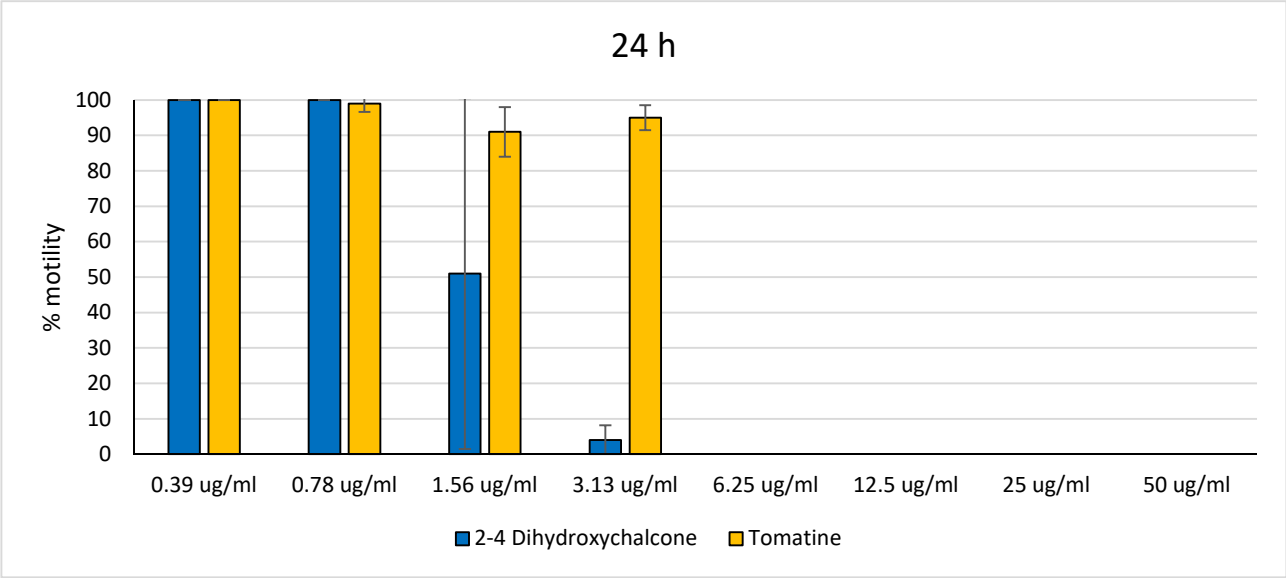


Figure 6.16. Motility of dinospores of A. ocellatum incubated at room temperature with different concentrations (0.39-50 µg/ml) of 2'-4'-dihydroxychalcone and tomatine. The motility is expressed as percentage of motile dinospores on the total number of dinospores present per well after 24 hours of incubation. Then, values have been related to the motility observed in the wells assigned to negative control and corresponding to 100%.

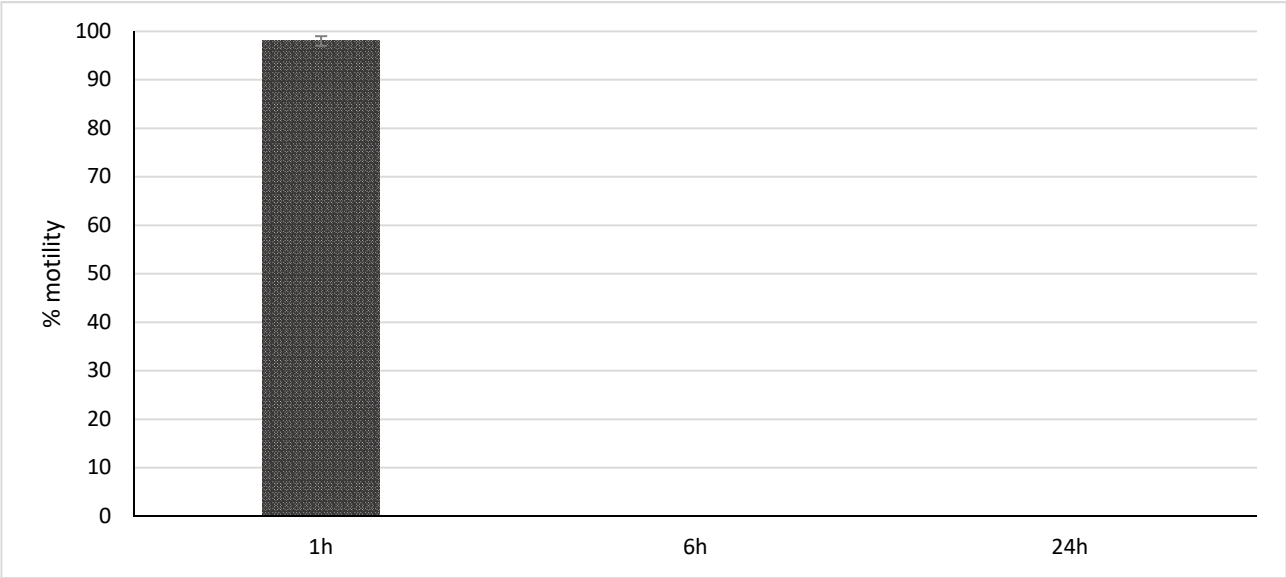


Figure 6.17. Motility of dinospores of A. ocellatum incubated at room temperature with formalin (4 µg/ml). The motility is expressed as percentage of motile dinospores on the total number of dinospores present per well at 1, 6 and 24 hours of incubation. Then, values have been related to the motility observed in the wells assigned to negative control and corresponding to 100%.

6.2.4 Discussion and conclusions

Amyloodinium ocellatum (Brown, 1931) (AO) is a cosmopolitan ectoparasitic dinoflagellate (Dinophyceae, Peridiniales, Thoracosphaeraceae) of numerous aquatic organisms living in brackish and seawater environments, that is endemic in temperate and tropical areas. This microorganism is capable of successfully adapting to a variety of different environments and to a great number of hosts, as detailed in the Introduction of this thesis. Amyloodiniosis (the infection caused by AO) is a major threat for semi-intensive aquaculture (valliculture or inland brackish farming) in Southern Europe and in different aquaculture facilities worldwide since, especially in the warmest months, it can cause high mortality rates (Cruz-Lacierda *et al.,* 2004; UNIUD personal data).

Several treatments alternative to copper sulphate (the most widely used for epidemics control) have been tested in experimental trials in order to manage amyloodiniosis, but the effects were not resolutive and in some cases neither fully clarified. Paperna (1984b) tested both *in vitro* and *in vivo* formalin (25-200 ppm), Nitrofurazone [nitrofural] (10-50 ppm), Furanace [nifurpirinol] (1-5 ppm), malachite green (0.1-100 ppm) and acriflavine (0.1-10 ppm). The author reported that these compounds induced, *in vitro*, a gradual inhibitory effect on the rate of tomont divisions, impairing or preventing its sporulation. However, *in vivo* treatments up to 9 hours with these substances did not prevent reinfection of gilthead sea bream (*Sparus aurata*). Montgomery-Brock *et al.* (2001) tested the effect of hydrogen peroxide (H_2O_2) as an amyloodiniosis treatment in a field trial with pacific threadfin (*Polydactylus sexfilis*). At 75-150 mg/L the compound showed to be effective determining trophonts' detachment without fish losses. On the other hand, doubling the concentration the fish mortality reached the 100%, suggesting that this drug had a relatively low therapeutic index. Furthermore, the authors confirmed the unknown effects of hydrogen peroxide on tomonts, so the resolution of this treatment has still to be demonstrated.

Among different developmental stages of *A. ocellatum*, only dinospores are susceptible to fish-safe chemical bath treatments (Lawler, 1980; Paperna, 1984b; Bessat and Fadel, 2018), while trophonts and tomonts are more resistant. In this context, there is a strong need to search for new strategies to limit the spread of the disease in fish farms, with a particular attention on animal welfare and environmental impact.

To try to reduce the severity of the infection, repeated or long-term treatments are required in commercial-scale aquaculture (Blaylock and Whelan, 2004), with consequent eco-toxic effects by the applied compounds on aquatic biomass (Caldwell *et al.,* 2011; Zurita *et al.,* 2005; Silva *et al.,* 2018). Another potential risk of long-term exposure treatments could be the development of AO

drug resistance as happened for *Plasmodium falciparum* against most of the drugs currently used to treat malaria (Birru *et al.,* 2017). The antimalarial chloroquine diphosphate, for example, has proven dinosporicide activity (Bower, 1983; Ramesh-Kumar *et al.,* 2015).

For this reason, in order to reduce the impacts of chemical and synthesised compounds on the environment and with the perspective to avoid possible AO drug resistance development, this study investigated the potential antiparasitic effects of 16 plant derived compounds on viable AO dinospores compared to the activity of copper sulphate and formalin (elective treatments). For this purpose, some *in vitro* experiments were performed to study the reduction in the motility of dinospores after their exposure to the various substances under investigation.

Motility test results showed that 14 compounds (7-hydroxyflavone; artemisinin; camphor (1R); diallyl sulfide; esculetin; eucalyptol; garlicin 80%; harmalol hydrochloride dihydrate; palmatine chloride; piperine; resveratrol; rosmarinic acid; sclareolide and umbelliferone) had a limited inhibitory activity against AO (up to 14%). On the other hand, the flavonoid 2^{I},4^{I}-dihydroxychalcone and the glycoalkaloid tomatine considerably reduced dinospores' motility.

2^{I},4^{I}-dihydroxychalcone completely inhibited dinospore motility at the highest concentrations (50 and 25 µg/ml) after 1 h of incubation, to then being inhibitory till 3.13 µg/ml in long term exposure (24h). Specifically, at 50 and 25 µg/ml this compound completely stopped the dinospores swimming until the end of the experiment (24 h of incubation), whereas at 12.5 µg/ml it reduced more than 99% the dinospore movements after 6 h of incubation and no motile parasites were observed at 24 h. Lowering the concentration, the inhibitory effects of the compound decreased in a dose-dependent way, while the range concentrations 6.25-1.56 µg/ml stopped more than the 50% of viable dinospores after 24 h of incubation, in 0.78 and 0.39 µg/ml wells the motility was 100% for the whole duration of the experiment. Chalcones, also known as a-b-unsaturated ketones, are the central core for a variety of important biological compounds other than important precursors for synthetic manipulations. The variety of biological properties displayed by these aromatic ketones is well documented in literature. In fact, chalcones possess anti-inflammatory, antioxidant, antitumoral, antimicrobial, antifungal, anti-leishmanial, anti-malarial and antiviral activities (Dimmocks *et al.,* 1999; Go *et al.,* 2005; Nowakowska, 2007). For this reason, 2^{I},4^{I}-dihydroxychalcone, derived from medicinal plants such as *Oxytropis falcata* (Yang *et al.,*2008) or *Zuccagnia punctata* (Isla *et al.*, 2016) but also contained in propolis (Ordóñez *et al.,* 2011), has a long history of use in traditional medicine. Moreover, from *in vitro* studies this molecule has proven to possess *in vitro* anti proliferative action on human gastric cancer MGC-803 cells (Lou *et al.,* 2009,

2010), whereas in combination with oxycillin it demonstrated antibacterial activity against *Staphylococcus aureus* strains (Talia *et al.*, 2009). In particular, these authors determined that a dose of 10 µg/ml added to 2-8 µg/ml of oxycillin, 2^{I},4^{I}-dihydroxychalcone was capable in totally inhibiting the bacterial growth, furthermore the chalcone and the semi-synthetic penicillin showed an effective synergic action.

Recently, 2^{I},4^{I}-dihydroxychalcone has been tested *in vitro* on isolates of *Saprolegnia parasitica* and *S. australis* (Flores *et al.,* 2016). Results demonstrated an anti-oomycete activity of the compound with MIC and MOC values of 6.25 µg/ml and 12.5 µg/ml respectively. These findings are in agreement with our results, but other experiments are necessary to clarify if *A. ocellatum* dinospores may be killed by this compound at higher concentrations or if their motility is only temporarily inhibited in the presence of this substance. However, the results of Flores *et al.* (2016) suggest that the chalcone 2^{I},4^{I}-dihydroxychalcone may display dinosporicide activity at concentrations higher than 25 µg/ml, whereas at lower concentrations it could inhibit the dinospore movements only for a certain period of time (as demonstrated in the present investigation for at least 6 h). Nevertheless, since there are no published reports of 2^{I},4^{I}-dihydroxychalcone usage on ESB, further researches will be necessary to determine the effects of this compound on fish in the perspective of its application in aquaculture as remedy against fish parasites.

The second effective compound investigated in this study was the glycoalkaloid tomatine, which displayed inhibitory effects on dinospore motility for the whole duration of the experiment (24 h) in the range doses 6.25-50 µg/ml. Conversely, at concentrations lower than 6.25 µg/ml this glycoalkaloid was no or less effective. Glycoalkaloids, which are commonly referred to as saponins, are known to possess antimicrobial and antifungal activities that act as plant defences against pests, pathogens and invasion by neighbouring plants (Medina *et al.,* 2015). Tomato plants (*Solanum lycopersicum*) produce tomatine, a tetrasaccharide linked to the 3-OH group of the aglycone tomatidine. Immature green tomatoes contain up to 500 mg of tomatine/kg of fresh fruit weight, nevertheless the compound is largely degraded as the tomato ripens until it reaches levels in mature red tomatoes of 5 mg/kg of fresh fruit weight (Friedman, 2002). Tomatine administrated *in vivo* at a dose of 2000 ppm showed anticarcinogenic effects against dibenzo[a,l]pyrene (DBP)-induced liver and stomach tumours in rainbow trout (*Oncorhynchus mykiss*) without adverse effects on animals (Friedman *et al.,* 2007).

Ito *et al.* (2007) determined *in vitro* the antifungal properties of this glicoalkaloid on *Fusarium oxysporum* by inducing apoptotic mechanisms in the phytopathogen. Tomatine has also

documented inhibitory effects on human and animal pathogenic protozoa (Liu *et al.*, 2016). In particular, the reported doses of tomatine which inhibited the growth of the three surveyed trichomonads are in line with the concentrations we have tested in the present trial. And as mentioned for 2^{I},4^{I}-dihydroxychalcone also for tomatine it may be possible to affirm that it can possesses dinosporicide properties. However, also in this case further studies will be necessary to understand if tomatine displays a dinosporicide activity against *A. ocellatum* at the surveyed effective concentrations.

Copper sulphate is the most widely used treatment for amyloodiniosis, even if its therapeutic doses are also toxic to most invertebrates and algae (Noga, 2012). Copper sulphate is usually administered at 0.12–0.15 mg/L for 10–14 days to control epidemics in aquaculture. However, free copper ion that is the active component is instable in seawater; therefore it is necessary to constantly monitor copper ion levels in the water, adjusting them if needed in order to guarantee its therapeutic effects. An alternative is the usage of chelated copper compounds, which increase the ion stability (Montgomery-Brock *et al.*, 2000; Noga, 2012). For this reason, in the present investigation, copper sulphate was used chelated with citric acid and considered as positive inhibitory control due to its well documented dinosporicide properties (Paperna, 1984b; Bessat and Fadel, 2018). At the concentration of 1 µg/ml the compound was capable in completely interrupting dinospore motility for the whole duration of the experiment (24 h).

Formalin was tested as second positive control at a concentration of 4 µg/ml. This concentration was selected on the basis of Fajer-Ávila *et al.* (2003) *in vivo* investigations. Authors, surveying the antiparasitic properties of the compound against ectoparasites (among which *A. ocellatum*) of bullseye puffer fish (*Sphoeroides annulatus*), reported that 4 mg/L was the dose at which the compound had less adverse effects on fish in long-term exposure, and contemporary reduced the parasite burden on the skin and gills after 7 h of treatment.

For our knowledge, the plant derived compounds investigated in this study have never been used before on viable dinospores of *A. ocellatum*. Hence, the observations provided by this research are to be considered preliminaries and further *in vitro* investigations will be necessary to better explore the mechanisms of the two effective compounds: 2^{I},4^{I}-dihydroxychalcone and tomatine. Furthermore, *in vivo* small scale trials should be arranged in order to select doses of these compounds, which are effective against *A. ocellatum* infections but at the same time non-toxic to marine fish.

Anyway, even if these preliminary results may be considered promising, the very expensive costs of 2^{I},4^{I}-dihydroxychalcone and tomatine make their usage prohibitive in farming realities.

Indeed, within the Horizon 2020 ParaFishControl project, based on UNIUD acquired experience in the last three years, this research line has been interrupted as potential results are not transferable to aquaculture, most of all in the rearing sites where amyloodiniosis is very prevalent. Since prevention is better than cure (multiple benefits when applied, minimising the impacts of disease upon host populations, reducing economic and welfare impacts post-infection and mediating against the development of drug resistance in parasite populations), which is the philosophy adopted by the project, UNIUD is now focusing on the research of a vaccine development against *Amyloodinium ocellatum*, as demonstrated by the pilot study reported in this thesis.

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7. *Conclusions*

The experimental contribution of the present thesis allowed to:

- deepen our understanding on the biology and ecology of *A. ocellatum* (AO) through *in vitro* and *in vivo* experiments that contributed to aquire new information on the dinoflagellate (e.g. capability of AO to adapt to alternative hosts and to persist in the environment; possibility to hibernate tomonts in order to conserve them for at least 6 months);
- investigate a discrete number of commercially available antibodies by classical immunohistochemistry or EnVision™ Flex implemented methodology. This approach was applied for the first time in this thesis in order to study the host-parasite interactions and the involvement and role of cell populations recruited and chemical mediators released in the branchial inflammatory response of European sea bass to *A. ocellatum* infection;
- develop an inedited florescent mRNA *in situ* hybridization protocol aimed at complement the UNIUD IHC and *real time* PCR results, by localising specific transcripts expressed by cell populations recruited during the ESB inflammatory response to AO and evaluating the difference in the expression level between uninfected and infected fish;
- develop fluorescent staining protocols to observe some anatomical details of the parasite by means of approaches alternative to the conventional histology;
- study the specific immune response to AO in infected and vaccinated ESB by E.L.I.S.A.;
- investigate the effects of selected plant-derived compounds, determing the most suitable candidates as immunomodulators or antiparasitics.

Further investigations will be necessary to:

- increase the number of markers to be used in IHC investigations, implementing antigen retrieval and colorimetric detection methods;
- correlate the severity of the histological lesions and the number of IHC positive cells with parasite burden;
- develop new riboprobes to increment FISH results in order to investigate other cell populations recruited during the host inflammatory response to amyloodiniosis;
- use new markers and fluorescent stain protocols to detail the anchoring organs of trophonts in order to deepen the mechanism of interaction of the parasite with its host and to determine their composition;
- improve the vaccination protocol in order to trigger a more effective and durable protection.

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