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Stress effects of amyloodiniosis in gilthead sea bream *Sparus aurata*

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ABSTRACT: Amyloodiniosis is a parasitological disease caused by one of the most common and important parasitic dinoflagellates in fish, *Amyloodinium ocellatum* (Brown), that represents a major bottleneck for gilthead seabream semi-intensive aquaculture in Southern Europe. In this experiment, we analyzed some metabolic, osmoregulatory and stress indicators to elucidate some of the physiological responses of gilthead sea bream when exposed to an *A. ocellatum* outbreak. We observed significant differences between Control and Infection groups in the cortisol, lactate and gill Na^+/K^+-ATPase (NKA) activity levels but that glucose, osmolarity, pH and total protein did not present such differences. This could indicate that the presence of the parasite induced a stress response, possibly enhancing the metabolization of glucose and subsequently lactate to cope with the higher energy requirements of the organism. There was also a decrease in gill NKA activity possibly due to severe epithelial damage and increased mucus production caused by the parasite *A. ocellatum*, which could induce anoxia and osmoregulatory impairment in the organism. However, further works must be performed to fully understand the physiological reactions of fish for *A. ocellatum* outbreaks.

KEY WORDS: *Amyloodinium ocellatum* · Physiological responses · Osmoregulatory responses · Aquaculture · Gilthead seabream

INTRODUCTION

Gilthead sea bream *Sparus aurata* L. is an important commercial fish widely cultured in Southern Europe (Salati et al. 2016). Gilthead seabream aquaculture represents 11% of the European aquaculture production, corresponding to >86400 metric tons in 2015, with a market value of 434 million euros (EUMOFA 2016). Fish diseases are one of the major limitations to an increase in this marine fish production.

In Southern Europe, amyloodiniosis is an important ectoparasitic disease that represents a major bottleneck for gilthead seabream semi-intensive aquaculture (Soares et al. 2011). It is caused by one of the most common and important parasitic dinoflagellate in fish, *Amyloodinium ocellatum* (Brown). This parasite can affect almost all fish living within its ecological range (temperatures of 16 to 30°C and salinities of 10 to 60), causing serious morbidity and mortality in brackish and marine warm-water fish in different aquaculture facilities worldwide (Paperna et al. 1980). It is often considered the most consequential pathogen of marine fish, with extremely rapid outbreaks where, at the time of its detection, contaminated fish no longer respond to treatment, resulting in 100% mortality in a few days (Soares et al. 2011). The open design of many aquaculture systems also allows easy dissemination of this parasite to new places in their ecological range, where they find

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ideal conditions to cause disease outbreaks (Balcázar et al. 2006, Ivona 2006).

A. ocellatum has a direct life cycle which can be completed in <1 wk under optimal conditions. During the life cycle, the parasite develops in 3 phases: the actively feeding trophont (parasitic state); the reproductive encysted tomont (a cyst developed after the trophont leaves the fish); and the free-swimming dinospores (free-living state, released from the tomont) (Landsberg et al. 1994, Kuperman & Matey 1999, Woo 2007). Each tomont can produce up to 256 dinospores in 3 d at 25°C, each one capable of infecting a new host and producing a trophont (Brown & Hovasse 1946). Dinospores production and infectivity can occur over a broad range of temperatures (16 to 30°C) and salinities (10 to 60) (Paperna 1984a).

The clinical signs of this disease are changes in fish behaviour, with jerky movements, swimming at the water surface and decreased appetite (Soares et al. 2011). Behaviours of infected fish may also include increased respiratory rate and gathering at the surface or in areas with higher dissolved oxygen concentrations.

Fish mortality caused by this parasite is normally attributed to anoxia (associated with serious gill hyperplasia, inflammation, hemorrhage and necrosis) in heavy infestations (Lawler 1980). There are also lethal cases associated with apparently mild infestations that, according to Noga (2012), could be related to osmoregulatory impairment and secondary microbial infections due to severe epithelial damage. However, the information regarding host physiological responses to *A. ocellatum* infestation is scarce and poorly understood.

It is widely known that parasites can induce several physiological changes in the host, such as energetic reallocation associated with physiological processes involved in tissue repair, activation of immune system responses and homeostasis (MacKenzie et al. 1995, Minguez et al. 2009). To assess such changes, stress/metabolic or osmoregulatory indicators can be used because marine organisms are very sensitive to several stressors in nature and aquaculture conditions (Iwama 1998).

The stress response applies to a wide range of physiological mechanisms (e.g. gene and protein changes, metabolism, energetics, immunological, endocrinological, pathological, neurological) and even behavioural changes that try to overcome a stressful situation and then compensate for the imbalances produced by either the stressor or the consequences generated by the first array of responses (Tort 2011). It is broadly described in aquaculture that chronic and acute stress are associated with many aspects regarding biochemical or physical perturbations, with physiological effects on the organisms (Vijayan et al. 2009).

Numerous studies on stress and biological relationships in marine animals have been performed and several indicators along the stress reaction cascade in fish have been studied. This is the case of the activation of hypothalamus–pituitary–interrenal axis (Carragher et al. 1989, Pottinger & Mosuwe 1994, Alderman & Bernier 2009) that leads to the release of catecholamines (Einarsdóttir & Nilssen 1996), norepinephrine (Askarian & Kousha 2009) and glucocorticoid steroid hormones into the bloodstream (Weirich 1997, Martinez-Porchas et al. 2009, Vijayan et al. 2009). However, most studies use cortisol, glucose, lactate and total protein because these are the most prevalent stress indicators (Einarsdóttir & Nilssen 1996, Nolan et al. 1999a, Wells & Pankhurst 1999, Einarsdóttir et al. 2000, Pottinger et al. 2003, Fernandes-de-Castilho et al. 2008, Olsen et al. 2008). There have been several studies performed in parasite-infected fish using cortisol, glucose, lactate and total protein as stress/metabolic indicators. For instance, that is the case of the physiological response of Atlantic salmon *Salmo salar* L. to sea lice *Lepeophtheirus salmonis* Krøyer (Nolan et al. 1999b, Bowers et al. 2000) and *Caligus rogercresseyi* (Boxshall & Bravo) (González et al. 2016a,b) or the responses of rainbow trout *Oncorhynchus mykiss* (Walbaum) to experimental infection with the blood haemoflagellate *Cryptobia salmositica* (Katz) (Laidley et al. 1988).

There are also other mechanisms that can be studied in fish exposed to hypoxia. Fish possess mechanisms to cope with the demands for oxygen in order to adapt to the environment (Martemyanov 2013, 2015). During the initial stress stage, the levels of catecholamines in blood increase considerably (Mazeaud et al. 1977, Reid et al. 1998), increasing the demands for oxygen (Aardt & Booysen 2004, Fu et al. 2007). This can induce an enhancement of Na^+/H^+ countertransport, which increases the oxygen-carrying capacity of fish erythrocytes (Thomas & Perry 1994, Wendelaar Bonga 1997, Perry & Bernier 1999).

This regulation of ion channels/transporters during stressful situations can be studied using the Na^+/K^+-ATPase (NKA) activity (Cádiz et al. 2015). NKA is an ubiquitously expressed integral membrane protein that couples the exchange of 2 extracellular K^+ ions for 3 intracellular Na^+ ions (Armesto et al. 2014) and mediates the active secretory functions of chloride cells in fish by generating a chemical gradient for ion transport (Marshall & Bryson 1998, Lin et al. 2004,

Evans et al. 2005, Ostrowski et al. 2011). This is a very well-studied mechanism for salinity acclimation in gilthead sea bream and other teleosts (Ventrella et al. 1990, Borgatti et al. 1992, Lingwood et al. 2005, Bystriansky et al. 2006) and could be a good indicator of gill physiological impairment due to lesions caused by *A. ocellatum* infestation.

In this work, we analyzed the histopathological changes and the metabolic, osmoregulatory and stress indicators to elucidate some of the physiological responses of gilthead sea bream when exposed to an *A. ocellatum* infestation.

MATERIALS AND METHODS

Fish culture conditions

Gilthead seabream juveniles were reared at EPPO-IPMA (Aquaculture Research Centre, National Institute for the Sea and Atmosphere, Olhão, Portugal) originated from wild broodstock. After hatching, fish were reared following the protocol used in EPPO-IPMA for this species. When they reached a weight of 80 to 100 g, a set of 60 individuals with no history of ectoparasites were selected. The fish were kept in separate 1.5 m^3 tanks (30 individuals per tank) with a 12 light:12 h dark photoperiod (photophase from 07:00 h to 19:00 h). Water temperature was 20 ± 1°C, and water salinity was 37.5 psu. The fish were fed with the commercial feed Sorgal® (Aquasoja Balance, 5 mm) 3 times a day, until satiation.

Amyloodinium ocellatum tomont collection

A 600 l infection tank with closed recirculation seawater system, temperature of 22 ± 0.2°C, artificial aeration (100% dissolved oxygen) and 24 h light photoperiod was used to incubate the parasite *A. ocellatum* and obtain a maximal amount of tomonts. The initial inoculum of *A. ocellatum* tomonts was obtained from a gilthead seabream natural outbreak that occurred in an EPPO-IPMA earth pond. Several naive gilthead seabreams weighing 80 to 140 g were exposed to the parasite to increase the dinospore infective population of the tank and to obtain more *A. ocellatum* tomonts for future infestations. For tomont collection, we used an adaptation of the method described by Paperna (1984b). When the fish manifested jerky movements, we bathed the infested fish in freshwater for 1 to 2 min and passed the bath water sequentially through a 500 µm screen (to take the bigger debris), a 100 µm screen and finally a 60 µm screen. Filtered tomonts, with a diameter between 120 and 90 µm, were then placed into a Petri dish in clean seawater and counted. Finally, tomonts were preserved in 15 ml flasks with sterilized seawater at 4°C until further use.

Preparation of *A. ocellatum* infection tanks

Two 200 l plastic rectangular tanks were infested with 1000 to 1200 *A. ocellatum* tomonts, obtained from our tomont collection, and incubated until reaching an average of 5500 dinospores per ml, similar to the observed by Abreu et al. (2005) in a recurrent amyloodiniosis on Brazilian flounder *Paralichthys orbignyanus*. The water temperature was maintained at 22 ± 0.2°C, 37.5 psu of salinity and 100% dissolved oxygen in closed seawater systems, artificial aeration and 24 h light photoperiod. To avoid differences between tanks, the water from the 2 tanks were mixed before fish were introduced, to ensure the parasite load was equalised.

To test if the system was capable of infesting fish and to check the fish time of death and the progression of the infestation by *A. ocellatum,* some preliminary experiments were carried out. The results pointed out that fish died 18 to 24 h after exposure to *A. ocellatum*, with an estimated concentration of 5500 dinospores ml^{-1} in tank water (Moreira et al. 2017), with a load of 600 to 700 trophonts per gill arch. Based on these pilot data, we chose sampling timepoints of 12 and 18 h post-exposure, aiming for an expected trophont load of ~500 per gill arch, in order to maximize the possible physiological responses and avoid the risk of having dead fish at the time of sampling.

Experimental design

Fish were fasted for 24 h prior to the experiment. Nineteen fish (N = 19) were then transferred to each 200 l tank (Control group [C1 and C2] without *A. ocellatum* and Infection group [T1 and T2] with *A. ocellatum*).

Parasite burden was assessed 12 and 18 h after inoculation by microscopic observation of a wet mount of the first 2 branchial arches from 2 fish (N = 4) that were previously euthanized with 2-phenoxyethanol overdose (1000 ppm bath), according to the methodology described by Moreira et al. (2017). Fish were sampled at 18 h when the parasite load was expected to reach 500 trophonts per gill arch.

Sampling protocol

At 18 h (when *A. ocellatum* infestation reached 500 trophonts per gill arch), the remaining fifteen fish (N = 15) were anesthetized with 100 ppm of 2-phenoxyethanol. The time between capture and sampling of the fish was <5 min. Blood was collected from caudal arteria, into 1 ml heparinized syringes. Plasma was separated from cells by centrifugation (10 min, 5000 × *g*, room temperature), snap-frozen in liquid nitrogen and stored at −80°C until analysis of osmolarity, pH, hormonal (cortisol) and metabolic (glucose, lactate and protein concentrations) parameters. After sampling, infested fish were then euthanized with an overdose of 2-phenoxyethanol, according to EU Directive 2010/63/EU for animal experiments.

Then, from 5 fish, we removed the first 2 gill arches from the right dorsal side, to assess the final parasite burden according to the methodology described above.

From each fish, the first gill arch on the left dorsal side was also removed and dried with an absorbent paper, and 3 to 5 filaments were cut using fine-point scissors. Biopsy samples were placed into 100 µl of ice-cold sucrose–EDTA–imidazole (SEI) buffer and frozen at −80°C until NKA activity analysis.

Analytical procedures

Plasma glucose and lactate levels were measured using commercial kits from Spinreact (Glucose-HK Ref. 1001200; Lactate Ref. 1001330) adapted to 96 well microplates. Plasma proteins were determined in plasma samples diluted 1:5 (v/v) with the QCA Total Proteins kit (Química Clínica Aplicada, Barcelona). All assays were performed with a Tecan Sunrise microplate reader, using Magellan v.2.5 software for Windows (Tecan Austria, Salzburg). Plasma cortisol levels were quantified by an ELISA kit (EA65, Oxford Biomedical Research, Rochester Hills, MI) modified and adapted to fish, according to Herrera et al. (2015). Cortisol was extracted from 20 µl plasma in 200 µl diethyl ether. The lower limit of detection (81% binding) was 0.1 ng ml^{-1} plasma.

NKA activities were determined using the microassay method of McCormick (1993), with the modification for non-salmonid fish. Tissues were homogenized in 250 µl of SEI buffer with 0.1% deoxycholic acid and centrifuged at 2000 × *g* for 30 s. Duplicate 10 µl homogenate samples were added to 200 µl assay mixture in the presence or absence of 0.5 mM ouabain in 96 well microplates at 25°C and read at 340 nm for 10 min with intermittent mixing. Ouabain-sensitive ATPase activity was detected by enzymatic coupling of ATP dephosphorylation to NADH oxidation and expressed as µmol ADP mg $protein^{-1}$ h^{-1}.

The osmolarity of the plasma was analyzed with a cryo-osmometer (OSMOMAT 030, Gonotec), and pH was determined with a pH portable device (Oakton pH Spear®, Eutech Instruments, Vernon Hills, IL).

For histological analysis, we collected the first gill from 5 fishes from each tank (n = 10 per treatment). Following fixation in 10% formalin buffered (pH 7.2) in filtered seawater for 2 d, tissues were transferred to 70% ethanol. The tissues were processed in an automatic tissue processor Leica TP 1020 and embedded in paraffin wax blocks. Sections 5 µm thick were cut with a microtome slide Model Leica 5M 2000 R and stained with hematoxylin-eosin (H&E) according to the procedure described by Martoja & Martoja-Pierson (1967). Gill tissue was examined under a Nikon H550S microscope using bright-field illumination for the presence of *A. ocellatum* and possible pathological changes. Representative images were captured using Nikon® NIS Elements D imaging software.

Statistical analysis

For group comparisons, normality was assessed using the Shapiro-Wilks test, while the homogeneity of variance was assessed using the Bartlett tests. If the data were parametric, a Student *t*-test followed by a Bonferroni correction was done to detect differences between Control and Infested fish. Statistical significance was accepted at $p < 0.05$. These statistical tests were made using R studio (v.1.0.153). Values are expressed as mean ± SD.

RESULTS

Gill analysis

After 18 h, the observation of the gill arches indicated that fish from the Control group had no *Amyloodinium ocellatum* trophonts in gills. The Infection group had a total of 497 ± 15 *A. ocellatum* trophonts per gill arch. Fig. 1 shows some images of gilthead seabream gills from the Control and Infection tanks.

Stress, metabolic and osmotic indicators

The results of the different stress and osmoregulatory indicators analyzed are shown in Fig. 2.

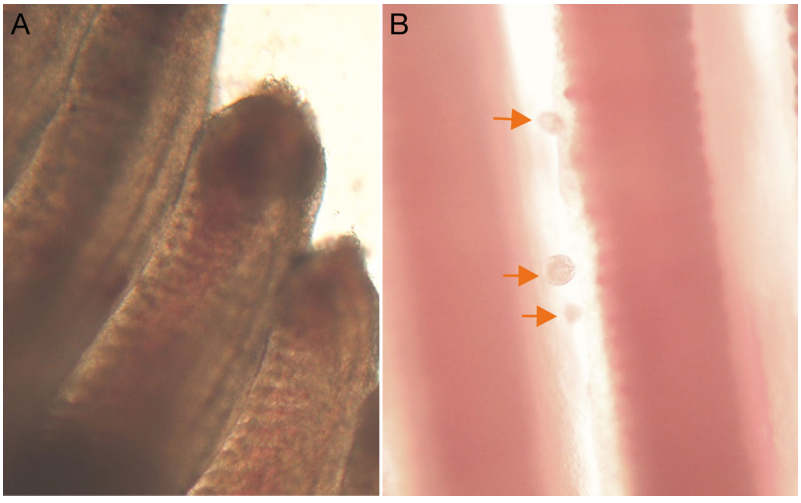

Fig. 1. Wet mount of gilthead sea bream *Sparus aurata* gills from 2 experimental groups: (A) Control (40×); (B) *Amyloodinium ocellatum* infested fish (40×). Presence of trophonts is indicated by red arrows

There were significant differences ($p < 0.05$) in the cortisol, lactate and gill NKA activity levels between fish from the Control and Infection groups. Fish in the Infection tanks had higher levels of cortisol and lactate and lower levels of gill NKA activity than those in the Control at 18 h post-infestation.

The rest of the stress/metabolic (glucose) and osmoregulatory (osmolarity, pH and total protein) parameters analyzed did not present any statistically significant differences ($p < 0.05$) between the Control and Infection groups at 18 h post-infestation.

Histopathological analysis of the gill

The gills of control fish showed a normal histology without any *A. ocellatum* trophonts detected. In contrast, infected fish had numerous histopathological alterations associated with the presence of *Amyloodinium* trophonts. The lesions observed were changes in primary and secondary lamellae, mainly caused by the hyperplasia of the lamellar epithelium with the consequent fusion of secondary lamellae. Other alterations were vacuolization and detachment of the lamellar epithelium and collapse of blood vessels (Fig. 3).

DISCUSSION

An outbreak of *Amyloodinium ocellatum* can cause mortality, mainly due to anoxia, osmoregulatory impairment and secondary microbial infections due to severe epithelial damage (Lawler 1980, Noga 2012). It is known that various fish pathogens cause a variety of general physiological responses in fish such as hyperglycaemia and decreases in haematocrit, plasma osmolarity, and plasma protein (Grimnes & Jakobsen 1996). We needed at least 500 trophonts per gill arc to achieve an experimental infection of *A. ocellatum* which maximized the physiological responses of the host. The results obtained in the gill analysis demonstrated that the Infection group had a similar population of trophonts and that the Control group did not have any trophonts of *A. ocellatum* or other parasites in the gills. The histopathological analysis of the gill also confirmed the presence of parasites in the gills of the Infection group and the absence of parasites in the Control group. Moreover, relevant histopathological alterations were observed only in the gills of the Infected group. Epithelial disruption and collapse of secondary lamellae should have observable effects on the respiratory functionality of the gills. These histopathological changes were similar to those reported by Guerra-Santos et al. (2012) in cobia *Rachycentron canadum* with amyloodiniosis and Kumar et al. (2015) in the broodstock of silver pompano *Trachinotus blochii*. In this experiment, we observed several significant differences ($p < 0.05$) in stress, metabolic and osmoregulatory indicators.

Parasites are natural stressors that have detrimental effects on their hosts (Combes 1996), so cortisol could be a useful physiological indicator to study parasitism (Triki et al. 2016). In this experiment, we observed a significantly ($p < 0.05$) higher concentration of cortisol in the fish exposed to *A. ocellatum*. This was expected because the presence of ectoparasites is normally associated with a higher concentration of cortisol in fish plasma (Triki et al. 2016), as reported in studies with sea lice infestations in Atlantic salmon (González et al. 2015, 2016a).

As result of a stress challenge, cortisol induces higher concentrations of glucose caused by extended glycogenolysis and gluconeogenesis through the degradation of glycogen (Olsen et al. 2008), and suppression of immune responses, diminishing both the resistance to pathogens and fish survival (Portz et al. 2006). Nevertheless, in this experiment, we

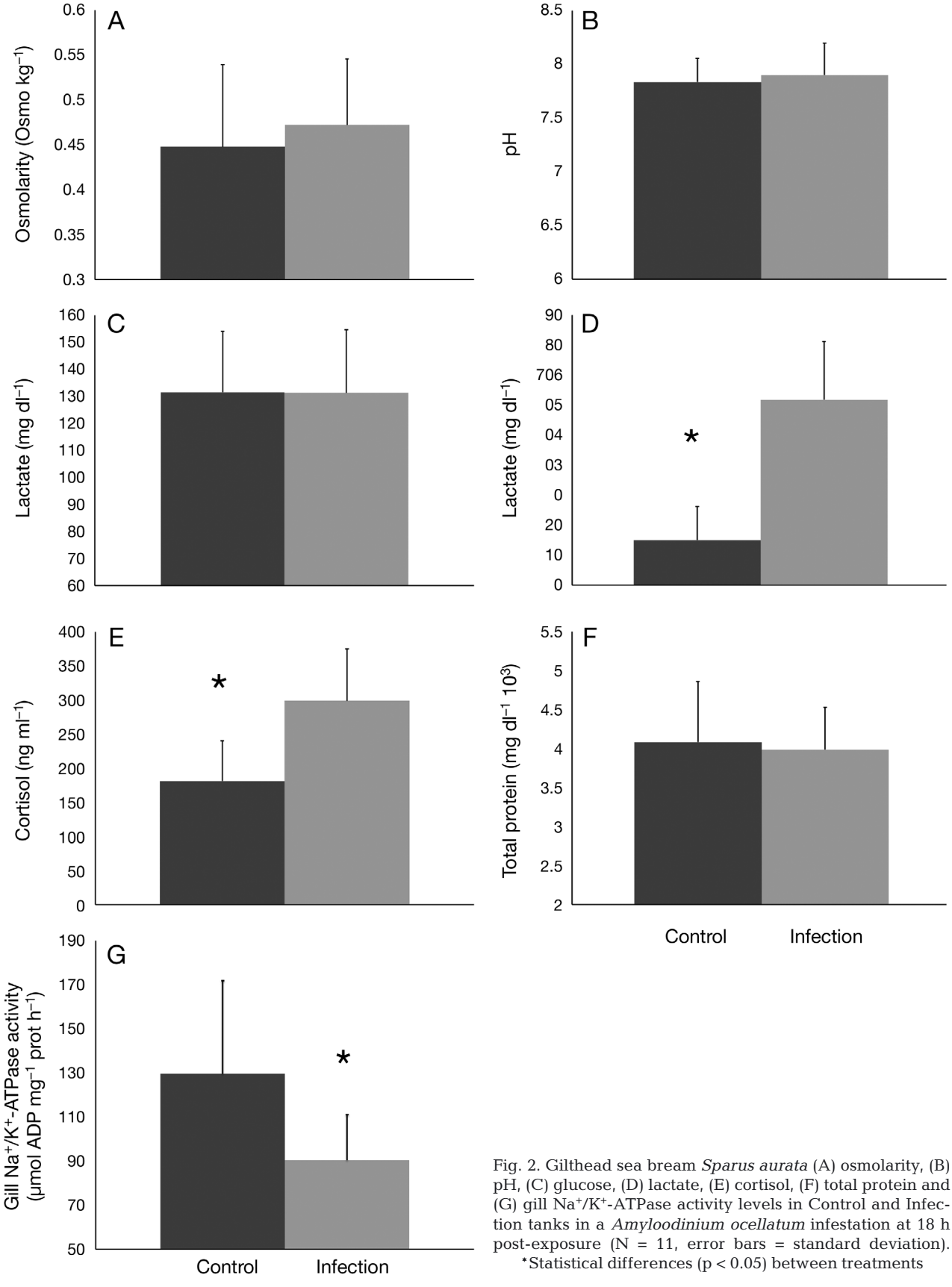

Fig. 2. Gilthead sea bream *Sparus aurata* (A) osmolarity, (B) pH, (C) glucose, (D) lactate, (E) cortisol, (F) total protein and (G) gill Na^+/K^+-ATPase activity levels in Control and Infection tanks in a *Amyloodinium ocellatum* infestation at 18 h post-exposure (N = 11, error bars = standard deviation). *Statistical differences ($p < 0.05$) between treatments

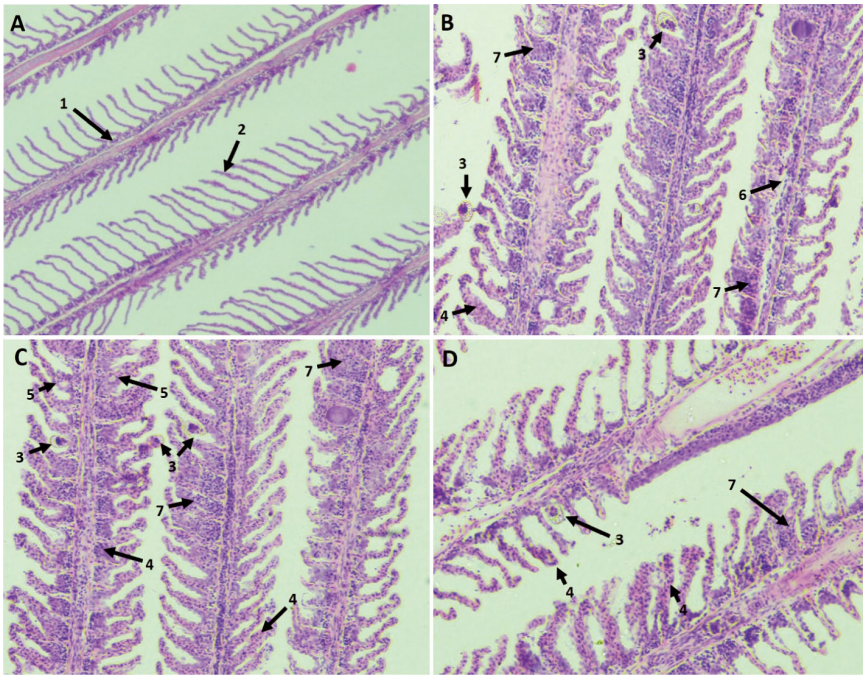

Fig. 3. Histological section of H&E stained gills from gilthead sea bream *Sparus aurata* from 2 experimental groups: (A) Control fish, with normal gills (1: primary lamella; 2: secondary lamella, 100×); (B,C,D) *Amyloodinium ocellatum* infected fish gills, with parasite trophonts (3) and histopathological alterations: hyperplasia of the lamellar epithelium (4) with vacuolization (5) and detachment of the lamellar epithelium (6) and fusion of secondary lamellae (7)

did not find statistical differences in glucose levels between treatments. This is in agreement with the data obtained by Grutter & Pankhurst (2000) for the tropical labrid *Hemigymnus melapterus* (Bloch) infected with gnathiid parasites and (González et al. 2016b) for *Salmo salar* after infection with *Caligus rogercresseyi.*

Lactate is a well-established indicator of stress (Mesa et al. 1998, Philp et al. 2005), being an end product of glycolysis formed under anaerobic conditions, and acts as a metabolite, playing an important role in fish cellular and organelle redox balance (Philp et al. 2005). Plasma lactate levels also increase in response to stressors resulting from increased activity or a decrease in oxygen (Thomas et al. 1999), with a possible role in hypoxia signaling and being responsible for collagen deposition and gluconeogenesis in non-hypoxic tissues (Philp et al. 2005). In this experiment, lactate levels were significantly higher ($p < 0.05$) in the fish plasma exposed to *A. ocellatum*. This agrees with the lactate levels obtained by Mesa et al. (1998) for stress responses in juvenile chinook salmon *Oncorhynchus tshawytscha* (Walbaum) experimentally infected with *Renibacterium salmoninarum* and in other studies with fish parasites (González Gómez et al. 2016). Moreover, this fact could be related to the absence of differences in the glucose analysis, suggesting that the plasma lactate increase could have been enhanced by a reduction of the cortisol-induced gluconeogenesis (Philp et al. 2005, Teles et al. 2005). However, we also have to consider that lactate can be a response to anoxia and severe gill epithelial damage by *A. ocellatum* (Lawler 1980, Noga 2012) acting in hypoxia signaling and collagen deposition processes. This is in agreement with the histopathological analysis and the data obtained by Moreira et al. (2017) in an experiment with *A. ocellatum* infestation in gilthead seabream regarding the overexpression of proteins related to wound healing and neoplasia, and could also be a consequence of the mucus production associated with *A. ocellatum* infestation, reported by Paperna et al. (1980), that can affect the exchange of oxygen and potentiate the anoxia state in the host.

The fish gills are covered by an epithelium that can be influenced by many endocrine factors and that shows characteristic changes in fish exposed to stressors. In addition, direct tissue damage caused by parasite grazing as observed in the histopathological analysis and indirect epithelial effects can compromise the integrity of the gill epithelia during a parasitological infestation. These effects may disrupt hydromineral balance and can be assessed by measuring blood ion levels and the activity of key ionoregulatory NKAs in gills (Nolan et al. 1999b). Gill NKA activity is related to the capacity of this osmoregulatory organ for excretion of excess ions in a hyperosmotic environment (Marshall & Bryson 1998, Cádiz et al. 2015) and for ion intake in a hypo-osmotic environment (Lin et al. 2004). This mechanism is controlled by several hormones, such as cortisol (mobilization of energy) or AVT (regulatory effects on several molecular components of chloride cells) (Cádiz et al. 2015). For the gill NKA activity, we have observed a significantly lower activity ($p < 0.05$) in the fishes exposed to *A. ocellatum*. This is not in

agreement with several authors that reported changes in the epithelial structure of skin and gills and elevation in the activity of gill NKA in species exposed to controlled numbers of caligid copepods (Bowers et al. 2000, González et al. 2015). However, those are not gill ectoparasites, hence the decrease of NKA activity in the gill during an *A. ocellatum* outbreak could be due to the fact that gill NKA activity is proportional to the number of chloride cells (Laiz-Carrión et al. 2003). Because infestations of *A. ocellatum* provoke inflammation, necrosis and damage to the gill of the fishes (Lawler 1980, Noga 2012) and the histopathological analysis of the gills reports several changes in the gill structure, a decrease in chloride cells could have happened as reported for example by Adams & Nowak (2003) for amoebic gill disease in *S. salar*. This could explain the drop of gill NKA activity observed in the *A. ocellatum* experimental infection.

The osmoregulatory impairment, inflammation, necrosis and damage to fish gills as a result of infestations of *A. ocellatum* could induce changes in the osmolytes and consequently alterations in plasma pH and osmolarity (Noga 2012, Lawler 1980). Therefore, some differences in these values were expected for fish infested by *A. ocellatum*, as reported by González Gómez et al. (2016) in *S. salar* facing low abundance infestation of *C. rogercresseyi*, or González et al. (2015) in *S. salar* infested by *C. rogercresseyi*. Nevertheless, pH and osmolality values did not show any significant differences ($p < 0.05$) between Control and Infection tanks, even if osmoregulatory levels were in the range of the values obtained by Sangiao-Alvarellos et al. (2005) for *S. aurata*. The total protein levels also did not show significant differences ($p < 0.05$) between Control and Infection tanks. The absence of changes in this indicator, together with pH and osmolality could suggest that the fish are not affected by a possible osmoregulatory failure (Bowers et al. 2000), even if these were expected. These observations could be in accordance to Bayne & Gerwick (2001), who reported that the high cortisol levels control the blood osmolality and pH by regulating the balance of blood potassium and sodium ions coming from the higher production of other metabolites and proteins in response to stress (González et al. 2016a). That could explain why, even in an *A. ocellatum* outbreak, we could not observe differences in the plasma pH, osmolality and total proteins. This absence of differences was also observed in osmolality levels of *Trypanoplasma borreli*-infected carp *Cyprinus carpio* L. (Meyer et al. 2002) and in pH and total protein levels of *C. rogercresseyi*-infected Atlantic salmon (González et al. 2016a).

One possible interpretation of these results is that the observed gill lesions caused by the parasite *A. ocellatum* induced a stress response in the fish (Landsberg et al. 1998), which would explain the higher amount of cortisol in the Infection tanks in relation to Control tanks after 18 h. The cortisol possibly induced the metabolization of glucose and subsequently lactate to cope with the higher energy requirements of the organism under stress (Soengas et al. 2007, Olsen et al. 2008). At the time of sampling, the lactate levels were higher in Infection tanks, and there were no significant differences in the glucose levels between experimental groups, which could indicate a depletion of the glucose levels in the organism and the activation of the glycolysis metabolic pathway (Soengas et al. 2007).

The severe epithelial damage observed in this experimental infestation by the parasite *A. ocellatum* can also induce anoxia and osmoregulatory impairment in the infected organism (Lawler 1980, Noga 2012). However, with possible osmoregulatory impairment, there should be changes in the pH, osmolarity and total protein levels in fish plasma. This was not observed in our experiment, although there was a decrease in gill NKA activity possibly due to the tissue inflammation and necrosis (Laiz-Carrión et al. 2003). Nevertheless, because cortisol has also a function in the regulation of the plasma homeostasis (Bayne & Gerwick 2001), it could explain the absence of significant differences in pH and osmolarity between treatments observed at the time of sampling.

CONCLUSIONS

This study presents clear evidence of physiological stress and osmoregulatory problems associated with infestation by *A. ocellatum*. However, further work must be performed to fully understand the physiological reactions of fish to *A. ocellatum* outbreaks, which could help for the development of new approaches to manage this disease.

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