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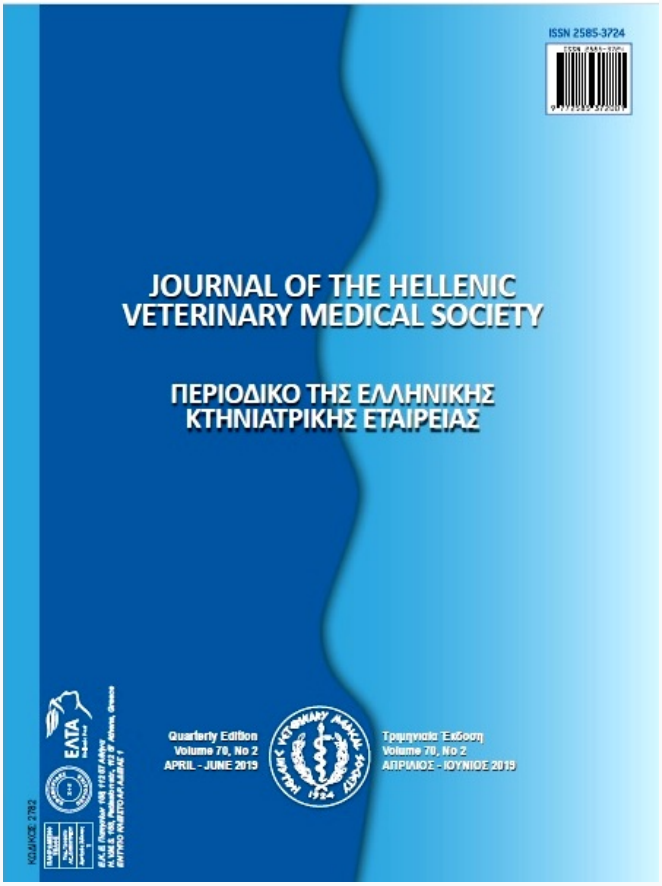

First Report of Saprolegnia parasitica from a Marine Species: Gilthead Seabream (Sparus aurata) in Brackish Water Conditions

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Research article
Ερευνητικό άρθρο

First Report of *Saprolegnia parasitica* from a Marine Species: Gilthead Seabream *(Sparus aurata)* in Brackish Water Conditions

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ABSTRACT. Saprolegniosis is a serious fungal disease that mostly affects freshwater fish species and eggs. It has a cotton wool-like appearance on the body of amphibians, crustaceans and several fish species. Infected gilthead seabream (*Sparus aurata*) were subjected to clinical, microbiological, parasitological and pathological investigation. On the infected skin samples, grey-white cotton-like patches, erosion of the skin and scale affusion were detected. Lesions covered the whole body of *S. aurata* in the advanced stages. Bacterial growth and parasitic symptoms were not observed in microbiological examination. Microscopic examination showed hyphaes carrying cysts that were long and branched. In scanning electron microscopy overviews fungal zoospores were observed. In histopathological observations of sections of skin, erosive-ulcerative dermatitis and mycelium of *Saprolegnia parasitica* were seen in the muscle tissue. Gene sequence-based identification found *Saprolegnia parasitica*. *S. parasitica* has not until now been detected in *S.aurata*. The low salinity of the brackish water is believed to be the predisposing factor of Saprolegniosis in sea bream in this case.

Keywords: Fish disease, fungal disease, *Saprolegnia parasitica*, *Sparus aurata*

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INTRODUCTION

Saprolegniosis is an acute infection and a worldwide mycotic freshwater disease affecting fish. It is widely distributed and all freshwater fish and eggs are susceptible to this infestation (Gaikowski et al., 2003). It is indicated in amphibians, crustaceans and several fish species : (channel catfish (*Ictalurus punctatus*) (Howe et al., 1999); cultured pike (*Sander lucioperca*) (Willoughby, 1985); elver (*Anguilla rostrata*) and suckers (*Catostomus commersonii*) (Roberts, 1989)) and especially in salmonids, tilapia species and carps ((*Oncorhynchus kisutch*) Hatai and Hoshiai, 1993; (*Salmo trutta*) Pickering and Christie, 1990 ; (*Tilapia nilotica*) Zaki et al., 2008; (*Carassius auratus*) Parra-Laca et al., 2015). It has also caused serious production losses (Alderman and Polglase, 1985; Post 1987). In Scottish, Norwegian and Chilean salmon hatcheries and farms in particular, Saprolegnia infections have been reported as causing losses of more than 10% (Langvad, 1994; Phillips et al., 2008; Van den Berg, 2013).

It usually starts with cotton wool-like growth on the head and dorsal fin region then spreads all over the body as focal patches (Abdel-Aziz et al., 2002; Bangyakkun et al., 2003; Osman et al., 2008; Roberts, 2012). In particular, cool and warm water fish eggs are reported to be at risk because their incubation temperatures are generally the same or near the optimum temperature range for zoospore growth (Gaiokowski et al., 2003). Stress factors such as abrasions, poor water quality, malnutrition, overcrowdedness, handling, spawning or any bacterial or parasitic infections lead to the presence of this mycotic disease (Noga, 1993; Pickering, 1994; Hussien et al., 2010). Temperature stress, especially cold temperatures, block immune system activity and reduce defenses against invading disease organisms, causing osmotic stress and mortality (Knights and Lasee, 1996; Hussien et al., 2010). It has been claimed that the final stages of this infection cause impairment of osmoregulation, failure of the respiratory system and, in some cases, of organs (Pickering and Willoughby, 1982; Van den Berg et al., 2013).The oomycete pathogen usually establishes itself focally, invading the stratum spongiosum of the dermis and then extending laterally over the epidermis, eroding it as it spreads. A relatively superficial invasion of the dermis rapidly leads to fluid imbalance and peripheral circulatory failure (shock) due to an inability to maintain blood circulation (Roberts, 2012).

In aquaculture, Saprolegniosis is observed mostly as *Saprolegnia parasitica* in numerous freshwater fish species (Van den Berg et al., 2013). *S.parasitica* was isolated from seabass (Cook and Unwin, 1985, press communication) and meagre (Abou El-Atta and Saleh, 2010) in brackish water conditions. Saprolegnia isolates were found not to develop any sexual stage in in vitro cultures and therefore cannot be identified (Grandes et al., 2000). The difference in radial growth rate, some biochemical characteristics and variation in the esterase isoenzyme pattern are used for the identification of some saprolegnia isolates (Beakes andFord 1983; Hatai et al., 1990; Welsh and McClelland, 1990), but moleculer methods have recently been used to characterise this infection (Van den Berg et al., 2013).

Formalin is the only drug currently approved by FDA for treatment as it has a strong effect and is relatively cheap. It is recommended to use 150-300 mg L^{-1} for the ova and fish (Marking et al., 1994; Taylor Francis et al., 1994). Malachite green was previously used to control Saprolegniosis, but it is now banned because of its carcinogenic, mutagenic and teratogenic characteristics (Gaiokowski et al., 2003). In addition, hydrogen peroxide has been used against Saprolegniosis but is not recognized by the FDA (Barnes et al., 1998; Howe et al., 1999; Gaiokowski et al., 2003).

In the present study, *Saprolegnia parasitica* was isolated from gilthead seabream (*Sparus aurata)*. In general, Saprolegniosis has been reported from fresh water fish species, especially in rainbow trout eggs in Turkey, but in the present water conditions, the fish were in brackish water, which is believed to be the reason for this first observation from sea bream.

MATERIALS AND METHODS

Fish

Infected gilthead seabream *(Sparus aurata)* from the university ponds at Katip Çelebi University Fisheries Research and Training Center which had a fungal infection were studied. A total of 48 fish weighing nearly 100 g were examined during the infestation. Fish were subjected to clinical, microbiological, parasitological and pathological investigation.

Water

During the outbreak, the water parameters of the ponds were monitored. Temperature, salinity, oxygen and pH parameters were determined to be 10 ºC, 3.93‰, 10.07 mg/L and 7.7, respectively.

Microbiological examination

External examination was conducted on the skin, abdomen, fins, scales, and internal organs such as the gills, kidney, spleen, intestine and liver were also investigated. Bacteriological examination was carried out according to Austin and Austin (2007). Bacterial isolates from the kidneys and spleen of infected fish were streaked on Tryptic Soy Agar (TSA, Oxoid) and Tryptic Soy Agar supplemented with 5% defibrinated sheep blood (BTSA) and incubated at 25 °C for 72 hours.

Pure cultures were provided by inoculation of the samples through taking a small tuft of mycellum from the fish skin and grown on Sabouraud Glucose Agar (SGA) and Malt Extract Agar (MEA) at 21 ºC for 3-4 days. Inoculation occured in a Haier Bio-Medical Biological Safety Cabinet. After incubation, unstained and colored examination was conducted with an Olympus BX53 Light Microscope. Colorization of the fungus was conducted with Giemsa staining (Arda, 2006) in order to identificate the fungi samples. Identification was performed according to Dvarak and Atanoesk (1969) both from wet samples from skin ulcers and growth on SGA and MEA (Dvarak and Atanoesk, 1969).

Histopathological examination

For histological examination, infected tissues of the skin with muscles, gills, liver and kidney were fixed in 10% buffered formalin after the necropsy. The tissue was then processed routinely and prepared into paraffin blocks. The blocks of the tissues were cut to 5 μm thickness and stained with Haematoxylin and Eosin (H-E) and periodic acid schiff (PAS) and examined under a light microscope (Culling et al., 1985).

Scanning electron microscopic examination

Samples were sputtered with gold by QUORUM Q150 RES and examined in a Carl Zeiss 300 VP scanning electron microscope in the Central Research Laboratory, Izmir Katip Celebi University.

Molecular Identification

DNA isolation was conducted using the GeneMATRIX Tissue and Bacterial DNA Purification Kit. For PCR amplification of the 5.8S rRNA gene, ITS1 and ITS4 primers were used. Amplified products of the template DNA were sent to the Macrogen direct sequencing service (Macrogen, Holland) for sequence determination. Samples were sequenced in two directions from opposite strands and the results were compared. Sequences were then checked with the BLASTN 2.6.1. database.

RESULTS

Infected fish were found to be stunned while swimming near the surface of water, with a loss of balance and reduced feed intake during the outbreak. The mortality rate was calculated to be 15% in that period. For treatment, formalin (containing approximately 37% formaldehyde by weight) was used to control the infection following a 30 min exposure at 2 ml/L every other day. On the infected skin samples, grey-white cotton-like patches, erosion of the skin and scale affusion were shown (Figure 1). Mycelial growth was detected especially on the head, eyes, gills, fins and body surface. In advanced stages lesions covered the whole body of *S. aurata* (Figure 2). The results of bacteriological and parasitological studies showed that neither pathogenic bacterial growth nor parasites were present in infected fish.

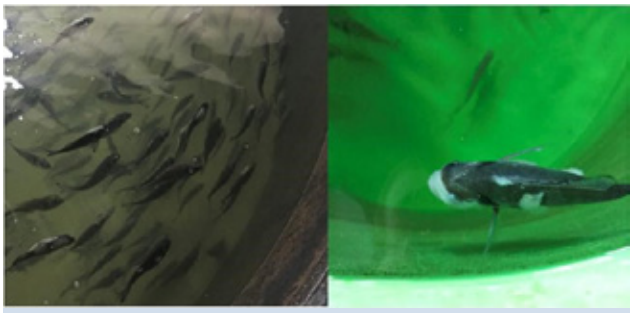

Figure 1. Infected gilthead sea bream (*Sparus aurata)* in unversity ponds.

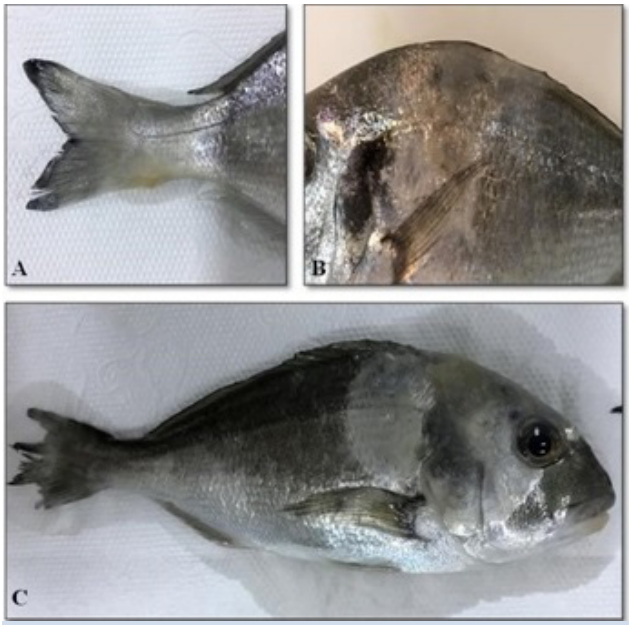

Figure 2. Infected gilthead sea bream (*Sparus aurata*). On the skin, grey-white cotton like *Saprolegnia parasitica* mycelium. A. On the pedincul. B.Dorsolateral. C. Dorsolateral, caudal and on the head localized.

Microbiological results

Isolation of *Saprolegnia parasitica* was conducted on SGA and MEA. In four days complete growth was observed.

Microscopic examination of *Saprolegnia parasitica* from native and colored samples is shown in Figures 3 and 4. The hyphae carrying the cysts are shown to be long and branched. Fungal zoospores from the scanning electron microscopy overviews are displayed (Figure 5).

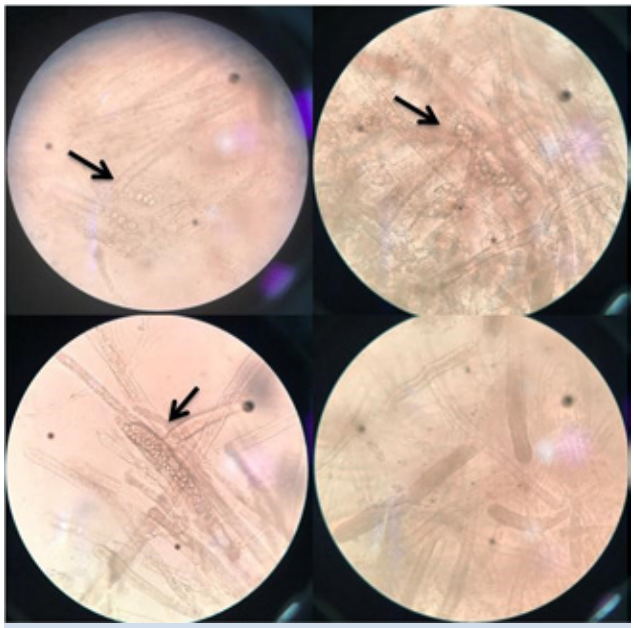

Figure 3. Unstained images *Saprolegnia parasitica* that shows zoospormatogoniums (arrows) (x400).

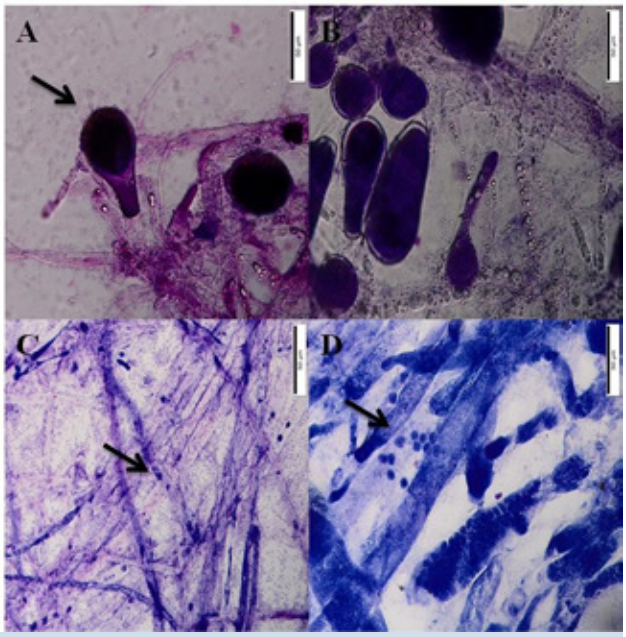

Figure 4. *Saprolegnia parasitica*. A-B. Zoospormatogoniums (arrow). C-D. Primer spormatogoniums (arrows) (Giemsa, x 200).

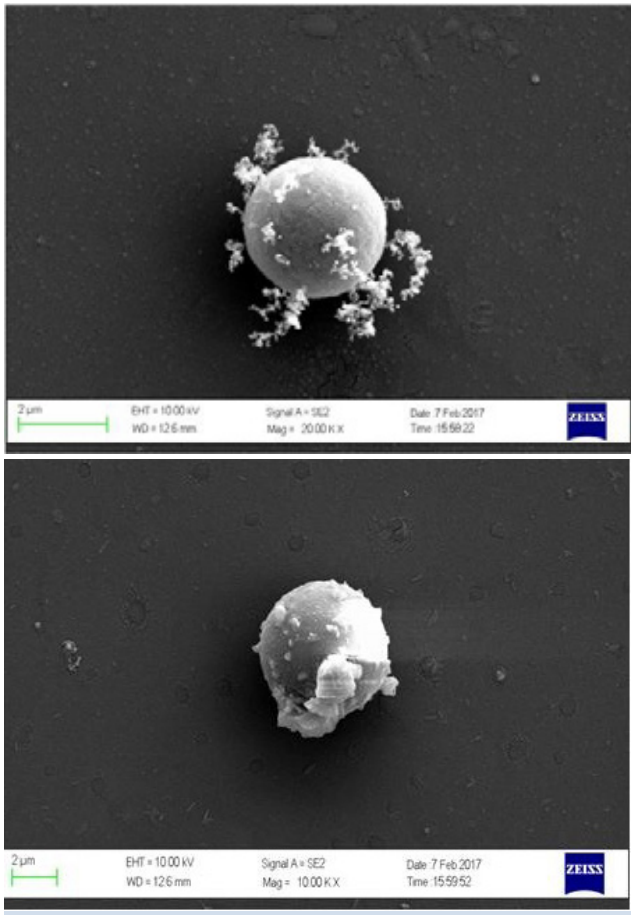

Figure 5. The zoospores of *Saprolegnia parasitica*. SEM. Bar. 2 µm scale.

Histopathological results

Erosive-ulcerative dermatitis was seen in skin sections. In epidermis and hypodermis necrosis, there were regenerations in intact epithelium. Dense filamentous mycelium was detected on the surface. This reacted positively to PAS staining. Mycelium and leukocyte infiltration were observed in dilate flake pockets (Figure 6). Liquefaction necrosis was detected in the muscles of the same area. A small number of mycelia was found among these muscles (Figure 7). Hepatocytes were moderately fatty in the liver, while histopathological findings were not found in other organs.

Molecular Identification Results

The PCR amplification of 18S rRNA gene sequence was checked in the BLASTN 2.6.1 database. The FASTA homology resulted in 100% nucleotide identity between the current isolate and *Saprolegnia parasitica* (accession number AM228725.1)

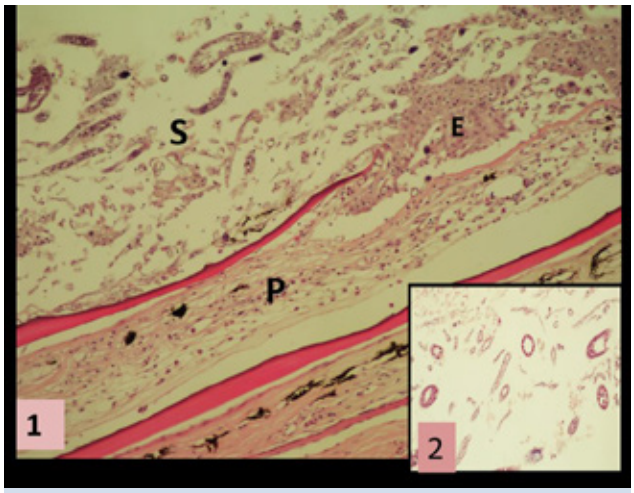

Figure 6. Intensive *Saprolegnia parasitica* hyphas and zoospores on skin surface (S). 1. Dilatation on epidermis and scale pockets (P), cell infiltration. Lateral section of zoosporagonium (H.E. x 200).

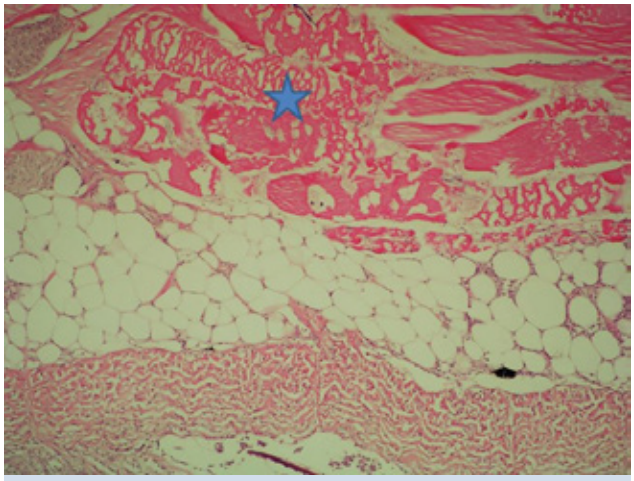

Figure 7. Liquefaction necrosis in subcutaneous muscles .(star).H.E. x100).

DISCUSSION

Saprolegniosis, which is caused by *S. parasitica*, is a worldwide disease that mostly affects freshwater fish and eggs and cause great economic losses to the aquaculture industry (Duboon et al., 2006). Countries like Norway, Ireland, Japan and the UK have reported mass mortalities based on salmon aquaculture (Bruno et al., 2011). Eggs are particularly susceptible to this infestation because of incubation systems that allow close contact and attachment of zoospores, although infertile and dead eggs are also convenient substrate for zoospore colonization (Gaikowski et al., 2003).

Throughout the outbreak, the water parameters of the ponds were observed. Temperature, salinity, oxygen and pH parameters were determined to be 10 ºC, 3.93‰, 10.07 mg/L and 7.7, respectively. Elatta (2013) reported a fungal disease (*Aphanomyces sp*.) in *Sparus aurata* with nearly the same salinity, 3‰. In research on Norwegian salmon hatcheries, Thoen et al (2015) claimed that water temperatures higher than 6 ºC did not result in an increase in the number of Saprolegnia spp. spores, and this was also the case in regard to oxygen pressure and water flow. Chauhan (2014) isolated some fungi species from the pond culture of *Tilapia mossambicus* and identified two of them as *Saprolegnia diclina* and *Saprolegnia parasitica*. During the study, the water temperature was recorded as 16 ± 2 ºC, pH 7.9 ± 1.6 and dissolved oxygen 6.8 ± 3.0 mg/l. These parameters are similar to those found to be suitable for Saprolegniosis reproduction in the current study.

Temperature stress may slow immune system activity and reduce the defense against pathogens (Knights and Lasee, 1996). Temperature shock and poor water quality are also inductors of Saprolegniosis (Yanong, 2003; Gieseker et al., 2006). Howe and Stehly (1998) claimed that during the winter months, fungal infections cause major losses. Bly et al (1992) explained this as the rapid decrease of the water temperature leading to immunosuppression that crosses over with the presence of fungal zoospores. In this study, the Saprolegniosis outbreak occurred after the rapid decrease of water temperature, from 13° C to 10° C, with an increase of turbidity which are also contributing factors along low salinity, in January 2017.

Saprolegniosis has been reported in rainbow trout eggs (Diler, 1992), fry (Kubilay et al., 2008) and adult individuals from different commercial companies in Turkey (Aydın and Küçükgül, 2014). Aydın and Küçükgül (2014) published results showing that in four rainbow trout farms Saprolegniosis was determined in half of these at times in the year when the water temperature was 10-14 ºC. During these periods of time mortality rates were calculated to be 8% and 13.3% as a result of these infestations. In the current study, Saprolegniosis was isolated in a marine species, gilthead sea bream, in nearly the same temperature conditions and caused 15% mortality during the outbreak.

Hussien et al (2010) observed focal greyish-white patches on the skin, fins, gills and head region of *Mugel cephalus* which were caused by the Saprolegniosis infection. Similarly, Das et al (2012) noted visible red or grey patches of filamentous mycelium in Indian major carp fingerlings. Cotton-like patches characteristically reported for Saprolegniosis on macroscopic

examination (Chauhan et al., 2014) were observed on the skin surface of all gilthead sea bream in this study. Localizations were more pronounced in the dorsal and caudal regions, as mentioned above.

Bruno et al (1999) considered Saprolegniosis as a secondary infection arising from bacterial infections, immunosuppression, parasite infestations and poor husbandry. It reduces osmoregulation and often leads to death (Pickering and Willoughby 1988). Kubilay et al (2008) reported Saprolegnia spp. from rainbow trout fry that were infected with *Flavobacterium columnare*. On the contrary, Pottinger and Day (1999) observed no secondary disease problems with the same species. In this study, neither pathogenic bacteria nor parasites were detected in gilthead sea bream during the infestation.

The primary zoosporagonias and mycelia, as described by Mueller (1992), were formed as clusters with PAS stain made from skin-scraping samples. Among these, primer zoospores in the form of free spots were also observed. The predominant clinical and histopathological feature in all cases is the extensive ulcerative lesion, which overlies a penetrating myopathy extending deep into the muscle. It has a greyish-white necrotic superficial covering of degenerating tissue and fungal hyphae (Roberts, 2012; Hussein et al., 2013). Similar findings were observed in this study, but fungal lesions reported in internal organs were not found in sea bass (Chauhan et al., 2014). Lesions and fungi were limited to the skin only. The reason for this is related to the differences between the water and the fish species.

CONCLUSIONS

S. parasitica has not yet been detected in cage farms of *S. aurata*. The reason for the absence of the infestation in cage farms is the high salinity that does not allow any zoospore growth in sea water. Brackish water salinity tends to let both marine and freshwater species exist, as it also does the zoospores of the Saprolegnia species. In this case, the low salinity parameters and the rapid decrease of water temperature caused stress and led to Saprolegniosis in sea bream.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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