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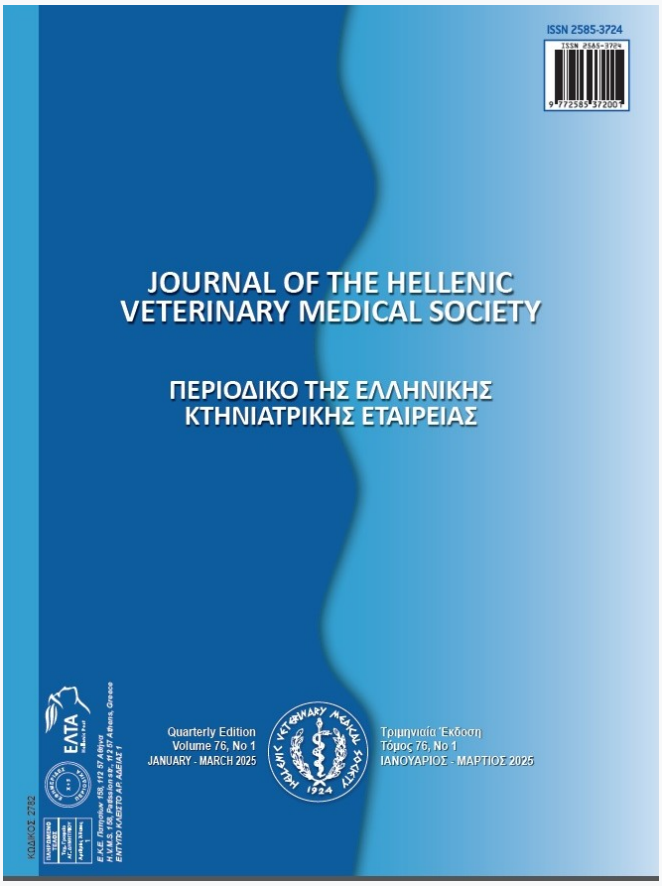

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Research article
Ερευνητικό άρθρο

Warm Water Strawberry Disease (WWSD) Outbreak in Cultured Rainbow Trout

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ABSTRACT: This study reports the first observation of warm-water strawberry disease in market-sized rainbow trout when the water temperature is above 15°C between April and September for 2 years in Turkey. Diseased fish with hemorrhagic inflammatory skin lesions were obtained from 4 different rainbow trout farms located in Muğla, Aegean Region of Turkey where the outbreak occurred. Microbiological, molecular, histopathological and scanning electron microscopy (SEM) examinations were performed to determine the causative agent of the disease, but no particular microbial growth nor positive nested PCR results were obtained from the samples. In contrast, a rod-shaped coccoid bacterium was observed in SEM views of inflammatory skin samples. Severe lymphocytic infiltration was determined in both the epidermis and the dermis by histopathological examination. The etiology of this skin disease is still uncertain; however, the disease continues to spread in the region where most of the rainbow trout production occurs with economic loss in relation to inevaluable market size fish. It is important to observe the effects of the disease to prevent spread between farms and determine appropriate protection and control strategies.

***Keywords*:** Rainbow trout; Skin disease; Skin lesions; Warm water strawberry disease

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INTRODUCTION

Global aquaculture production has been increasing along with high consumption rates, and Türkiye is one of the main producers of farmed fish, especially rainbow trout (*Oncorhynchus mykiss*). There are several skin conditions such as bacterial coldwater disease (*Flavobacterium psychrophilum*), columnaris disease (*Flavobacterium columnare*), and furunculosis (*Aeromonas salmonicida*) that bring significant economic damage in rainbow trout farms however, new emerging skin diseases of rainbow trout have become a major threat for Europe and Mediterranean aquaculture recently (Schmidt et al., 2018).

There are different characterizations of rainbow trout skin disorders, most of which have unknown etiology. Similar conditions have been described as strawberry disease (SD) (Lloyd et al., 2008; Lloyd et al., 2011), warm water strawberry disease (WWSD) (St-Hilaire and Jeffery, 2004), cold water strawberry disease (CWSD) (Verner- Jeffreys et al., 2008), and red mark syndrome (RMS) (Metselaar et al., 2010; Sasani et al., 2016; Cecchini et al., 2017; Galeotti et al., 2017; von Gersdorff Jorgensen et al., 2019; Galeotti et al., 2021a, 2021b). Some of the available literature has shown a correlation between skin lesions and the presence of bacteria (Ferguson et al., 2006; Takeuchi et al., 2021). Oidtmann et al. (2013) explained that *Flavobacterium psychrophilum* (*F. psychrophilum*) or Rickettsia-like organism (RLO) are suggested as the causative agents of the CWSD and RMS which are observed below the water temperature of 15°C. Currently, RLO has been identified as a Midichloria-like organism (MLO) (Metselaar et al., 2022) and RMS cases have been associated with MLO in rainbow trout (Oh et al., 2019; von Gersdorff et al.,2019; Metselaar et al., 2020; Galeotti et al., 2021a, 2021b; Orioles et al., 2022a) however the relationship between the causative agents and the disease is elusive.

The causative agent of SD is still unknown (Sandoval et al., 2016) and in some research it was associated with bacterial agents such as *F. psychrophilum* (Ferguson et al., 2006), *Piscirickettsia salmonis* (Fryer et al., 1992) or the presence of *Rickettsia*-like organism (RLO) (Lloyd et al., 2008; Metselaar et al., 2010). However, isolation of the RLO from infected fish with strawberry disease or red mark syndrome has not been possible, which is the main problem in working with intracellular pathogens that require cell lines (Metselaar et al., 2010). Although the histological examination has been declared essential to define the progression of the disease so far (Metselaar et al., 2022). WWSD, US rash, and US strawberry disease were described with unknown etiology and separated by temperature differences and histopathological changes (Oidtmann et al., 2013). Isolation difficulties cause unsuccessful or inconsistent results in providing the etiologic agent with traditionally accepted methodologies as a fulfillment of Koch's postulates (Lloyd et al., 2011).

This study is the first detailed investigation of WWSD with molecular, histopathological examination, and SEM views among cultured rainbow trout from the Aegean region, Turkey, where most of the production is carried out. The disease has spread in the area between high-tonnage rainbow trout producers with some adverse economic conditions, such as decreasing the market value of the fish and even sometimes not being able to evaluate the fish based on pathological disorders. Understanding this serious disease that continues to cause concern in the region is essential to demonstrate significant effects on fish and among producers to evaluate the proper sanitation and treatment protocol and ensure health assistance.

MATERIALS AND METHODS

Fish samples

The outbreak was observed in rainbow trout farms between 2019 and 2020, especially during the warm season (between May and September) in the southern Aegean region. 4 different farms were monitored in the same area. Each production unit uses the same water body one after another, but supplies their own fry in their hatcheries.

Recently dead diseased fish samples of 300-350 g with multiple ulcerated skin swellings were determined, observed, and obtained from each farm, then clinical, parasitological, pathological, and microbiological examinations were carried out according to Austin and Austin (2007).

Microbiological and molecular analyses

To isolate the causative agent of the disease, fish were dissected and isolates of skin lesions, spleen, liver, and kidneys were streaked on Tryptic Soy Agar (TSA, Oxoid) and Tryptic Soy Agar supplemented with 5% defibrinated sheep blood (BTSA) and Cytophaga agar (Anacker and Ordal, 1959; Austin and Austin, 2007). TSA and BTSA plates were incubated at 21°C for 24-48 h, while Cytophaga agar plates were incubated at 21°C for 7 days and 10°C for 10 days.

The nested PCR method was carried out for the sensitive detection of the causative agent directly from infected tissue samples. After dissection, skin lesions, liver, and spleen samples were preserved in 95% ethanol prior to use. The extraction of genomic DNA and the 16S rRNA PCR amplification was carried out according to McCarthy et al. (2005) EubB (5‘-AGAGTTTGATCMTGGCTCAG-3‘) and EubA (5‘-AAGGAGGTGATCCANCCRCA-3‘) 16S rRNA universal primers were used in the first round of amplification. 16S rRNA primers specific to *Piscirickettsia salmonis* (PS2S 5‘-CTAGGAGATGAGCCCGCGTTG-3‘ and PS2AS 5‘-GCTACACCTGCCAAACCACTT-3‘) were specified for the second round. Band screening of the PCR products was observed by gel electrophoresis. The quality and density of the isolates were determined by Thermo Scientific Nanodrop 2000 (USA).

Scanning electron microscopy (SEM) images

The fixation and preparation of the samples for scanning electron microscope (SEM) scans were processed according to Kashi et al. (2014). After careful dissection of representative tissues, 2.5% gluteraldehyde was used in 0.1 M sodium cacodylate buffer for fixation, followed by postfixation in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer. Then, a standard-graded ethanol dehydration process was performed. Tissue samples were sputtered with gold (QUORUM Q150 RES), examined and digital images were captured using Carl Zeiss 300 VP SEM at the Izmir Katip Çelebi University Central Research Laboratory.

Histopathology

Skin, muscle, liver, and gill tissue samples were taken from rainbow trout samples with clinical signs of the disease and fixed in 10% neutral buffer formalin. After standard ethanol dehydration protocols, tissue samples were processed for embedding in paraffin and then sectioned into 5μm thickness. The samples were deparaffinized in xylene and hematoxylin-eosin

Figure 1. Warm-water strawberry disease lesions in rainbow trout individuals

(H&E) staining was processed to examine the tissues under the light microscope (Olympus CX22RFS1).

RESULTS

The outbreak occurred at >15°C, especially between April and September in the Aegean region of Turkey. Single or multiple skin lesions around the abdomen and the base of the fin were the main pathological signs of the disease. The superficial lesions were usually hemorrhagic with desquamations and central ulceration (Fig. 1).

No pathogen growth was observed on TSA, BTSA media, including the suspected growth of Flavobacterium on Cytophaga agar. While DNA extraction from the affected tissues was successful, 16s rRNA PCR nor *Piscirickettsia salmonis* specific nested PCR products were deteded. However, nested PCR results were unable to identify *Piscirickettsia salmonis* in this case. The SEM overviews showed that rod-shaped coccoid bacteria were present in the inflammatory skin samples of diseased fish (Fig. 2).

Histological changes in examined tissue samples have confirmed the effect of the disease agent. The structure of the epidermis and the stratum compactum showed degenerative changes in the lesion sections; the inflammatory response was extended to muscle tissue. Intense lymphocytic infiltration was observed between the scales and in the remaining epidermis. In many cases, intense basophil infiltration (dermatosis) was observed in the epidermis. This appearance was accompanied by muscle differentiation and lymphocytic infiltration in the dermis. The liver tissue was also affected; the morphology was altered by syncytial hepatocytes. Infected gill lamellae were accompanied by rupture of the vacuole where lymphocytic infiltration and degenerated lamellae associated with gill proliferation were observed (Fig. 3).

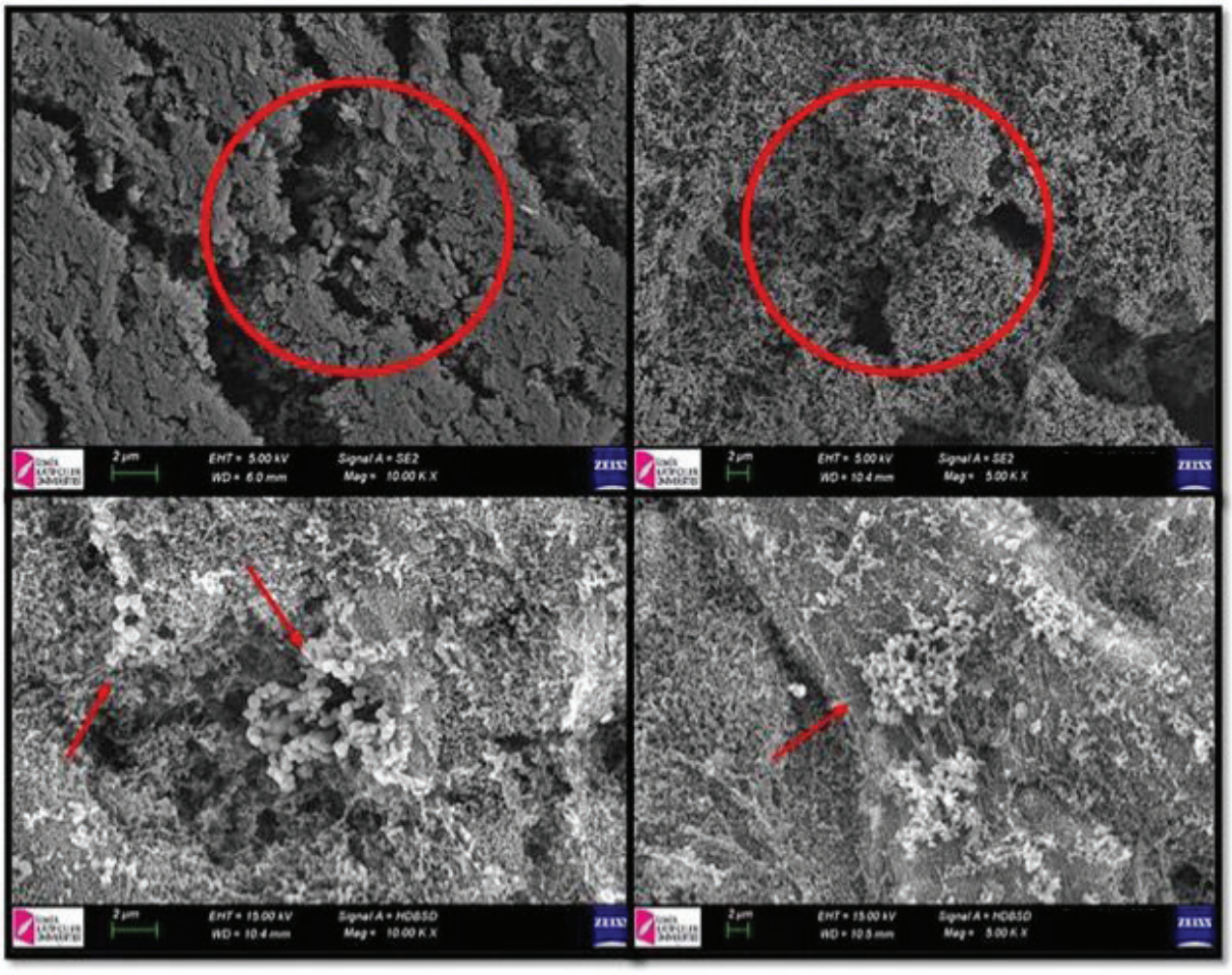

Figure 2. Rod-shaped coccoid bacteria in inflammatory skin samples of infected fish

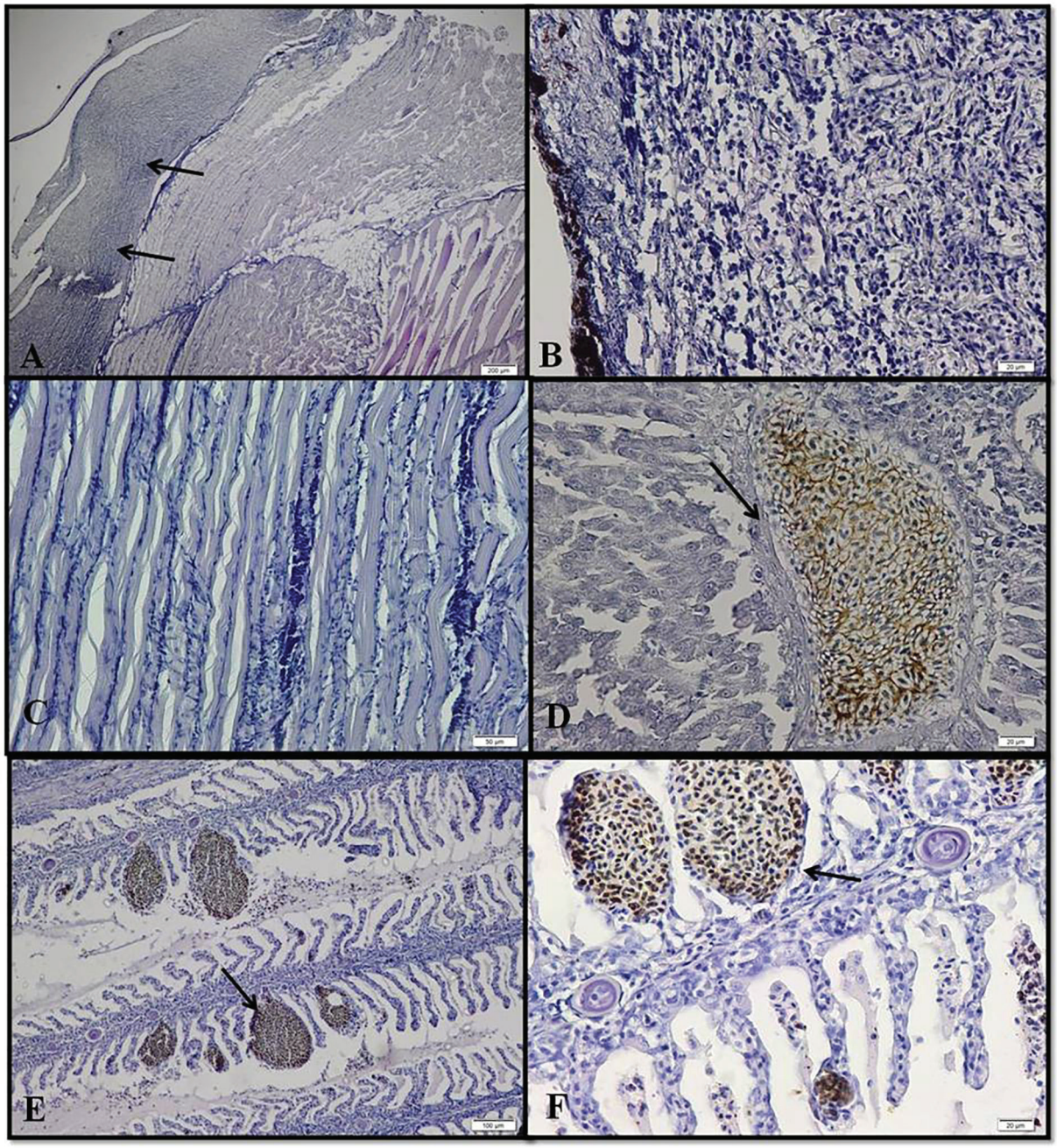

Figure 3. A - Intense lymphocytic infiltration between the scales and the epidermis, B- Intense basophilic infiltration in the epidermis, C - Muscle differentiation and lymphocytic infiltration in the dermis, D - Syncytial hepatocytes in liver tissue, E, F - Lymphocytic infiltration and degenerate lamellae associated with gill proliferation

DISCUSSION

The disease mainly affects market-sized fish in the 15-18°C temperature range with the description of clinical signs such as diffuse, large, multifocal and hemorrhagic lesions that appear in several centimeters (Austin and Austin, 2007). This pathological disorder may not lead mortality during the production period, but inflammatory skin lesions cause problems in the marketing and processing of fish. If the lesions heal, there remains a notable patch on the surface of the skin (Fig. 1). This situation affects the quality of the carcass and reduces the value of the product.

Kubilay et al. (2014) reported the first observation of red mark syndrome in marketable size rainbow trout in the Mediterranean region of Turkey with hemorrhagic dermatitis, focal or multifocal oval-shaped bright red lesions, and the case was observed between December and February when the water temperature

was 11°C. Similarly, Sasani et al. (2016) stated Red Mark Syndrome in cultured rainbow trout in Iran when the water temperature was below 15°C. The bright red, ulcerative, or non-ulcerative multifocal appearance was displayed in macroscopic observations in the dorsal and ventral regions, rather than on the head or fins. Metselaar et al. (2010) reported the association between Red Mark Syndrome, Strawberry Disease, and *Rickettsia*-like organism in diseased rainbow trout individuals in the USA. Furthermore, Lloyd et al. (2011) indicated a positive correlation between *Rickettsia*-like organism and swelling skin lesions such as Strawberry disease in rainbow trout. Verner-Jeffreys et al. (2008) diagnosed multifocal raised lesions on flans in rainbow trout as Cold Water Strawberry Disease or Red Mark Syndrome. Similarly a strawberry disease case was reported from farmed Chilean rainbow trout when the water temperature was 10°C and 11°C from 400 g of fish with gross skin lesions around the flanks and red spots with a diameter of up to 3 cm (Sandoval et al., 2016). The characteristics of these skin disorders of rainbow trout were documented by Oidtmann et al. (2013) along with discrimination points of the four conditions: Red Mark Syndrome, Warm Water Strawberry Disease, US Rash and US Strawberry Disease according to the temperature range, spread of the injuries on the body, affected area of the body, appearance of the individual injuries, whether or not the scales are affected and the inflammatory response of the skin layers. Based on the temperature range, the description of the clinical signs of the lesions, and the histopathological characteristics, the results of this study reflect the epidemiology of WWSD. In this study similar pathological findings were observed and the case was identified as WWSD for the first time in the Aegean region of Turkey, which has the highest rainbow trout production in the country.

Verner-Jeffreys et al. (2008) expressed a case of cold-water strawberry disease in England and Wales with no consistently identified causative infectious agent, even when different methodologies and techniques, including molecular bacteriology were applied. These results reflect those of Sandoval et al. (2016) who also identified strawberry disease without any isolated pathogenic bacteria and without positive amplification of PCR bacterial protocols from tissue samples from diseased fish. Specific antibodies against *Piscirickettsia salmonis* were tested for RMS and RLO-affected fish by Metselaar et al. (2010) and concluded that the etiological agents of RMS and SD may be the same pathogen. Oidtmann et al. (2013) described Warm-Water Strawberry Disease with an unknown etiology and uncertain incubation period. Although Koch's postulates have not proven any aetiological agents, RMS is consistently associated with the presence of Midichloria-like organisms which have been observed by transmission electron microscopy (Galeotti et al., 2017; Orioles et al., 2022b). Similarly, the rod-shaped coccoid bacteria were determined in the diseased tissue samples with SEM overviews in this study despite the ineffective isolation and negative sequence results.

The aetiology of skin diseases that appeared in rainbow trout (CWSD, WWSD, RMS, US rush, US strawberry disease) is still unclear (Austin and Austin, 2007; Verner-Jeffreys et al., 2008). Koch's postulates are needed to determine the epidemiology, case-control, and proper biosecurity procedures of the disease. Verner-Jeffreys et al. (2008) suggested the possibility of failure in the isolation or visualization of the causative agents in tissue samples that may be related to the inactivation of the pathogen that causes the inflammatory response in the late stage of the infection by the host (Verner-Jeffreys et al., 2008). Some suggestions have been made that *Flavobacterium psychrophilum* and *Rickettsia*-like organisms have been aetiological agents, but there is still no evidence of precision (Verner-Jeffreys et al., 2008; Metselaar et al., 2010; Sasani et al., 2016). The results of the current study agree with recent studies indicating that the isolation of the causal agent has been failing and, in some cases, inconsistent. More research is needed to identify and understand the aetiological agent in detail to satisfy this basic knowledge. If a significant association is asserted between these skin disorders and the agent, treatment and vaccine procedures could be developed to prevent economic loss.

The histopathological features are important in order to define a classification and as well as to understand the pathogenesis of the disease (Galeotti et al., 2021a). Histopathological findings from previous studies, are in accordance with the present study. More specifically, RMS in juvenile rainbow trout was revealed as macrophagic inflammatory infiltration in muscular tissue and hypodermis (Orioles et al., 2022b). Galeotti et al. (2021a) described three different histopathological stages of RMS disease progression and identified the morphological diagnosis as "deep chronic dermatitits associated to panniculitis and myositis, characterised by lympho-histiocytic and granulomatous reaction". Sandoval et al. (2016)

described severe dermatitis with lymphocytic infiltration in the subcutis and muscle layer of rainbow trout individuals affected by strawberry disease. Oidtmann et al. (2013) described the histopathological features of WWSD as the inflammatory response in the epidermis and dermis with lymphocytes. Furthermore, the affected epidermis noted the difference between WWSD and RMS. The disease was expressed as multifocal lymphocytic intraepithelial infiltration with extensive erosion of the epithelium and hyperplasia of goblet cells, heterophilic granulocytes, and interstitial edema in the lamellae of diseased fish (Metselaar et al., 2010). The histopathological results of the affected tissue samples in this study were in accordance with the definition of WWSD described by Oidtmann et al. (2013).

In conclusion, this is the first report of WWSD from cultured rainbow trout in the Aegean region, Turkey, which causes financial loss based on skin disorders composed of market-size fish. These initial results suggest that the disease is involved in Turkish rainbow trout farms and is spreading in the region that has high tonnage production. However, further studies are recommended to determine the causative agent and conduct challenge experiments to fulfill Koch's postulates and manage the disease on farms.

CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

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