First case of chronic systemic spironucleosis in Freshwater Angelfish (*Pterophyllum scalare* Schultze, 1823) in Türkiye

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ABSTRACT

This study aimed to identify the cause of sustained mortality in freshwater angelfish (*Pterophyllum scalare* Schultze, 1823) kept at an ornamental fish production facility in Türkiye. Parasitological, bacteriological and histopathological examination were performed on moribund hybrid angelfish individuals. The moribund fish had haemorrhaged eyes, darkened skin, scale loss, ascites and false faeces. A pale liver, splenomegaly and a thinning of the intestinal wall were observed internally. The parasitological examination revealed flagellated protozoan endoparasite *Spironucleus* sp. in the intestine. A number of histopathological changes were observed including lipid degeneration in the liver, hemosiderin deposits as well as granulomas in the spleen, a large number of mast cells in the lamina propria of the intestine and enteritis. Numerous *Spironucleus* parasites were seen in the intestinal wall. According to physiological and biochemical tests, the bacterial isolates obtained from the visceral organs of some fish were identified as *Citrobacter freundii*. After oral metronidazole treatment, with a dose of 50 mg/kg fish daily for 5 days, a decrease in fish mortality and resumed feeding were noted. A chronic spironucleosis, which systemically affects fish by penetrating the intestinal mucosa, was identified as the cause of this sustained mortality in freshwater angelfish.

The total global value of ornamental fish trade is 15 billion US$ and deals with more than two billion live ornamental fish mostly from freshwater (21). Ornamental fish farming, which started out as a private enterprise in Türkiye, has now become a major industry. Freshwater angelfish (*Pterophyllum scalare* Schultze, 1823), originally imported from Amazon River of Brazil and Peru, Colombia, French Guiana and eastern Ecuador, are the most important commercial cichlid species (7, 16). Freshwater angelfish adults can reach 15 cm in length and females can lay up to 1000 eggs. Individuals are considered monogamous because they tend to mate with a single partner (29). These fish require good water quality, so care must be taken to avoid large fluctuations in conditions and partial water changes must be made regularly. The majority of reported disease outbreaks are either caused by systemic iridovirus (22) or protozoan parasite *Spironucleus* infections (14).

Diplomonad flagellates of the genus *Spironucleus* have been reported to cause serious systemic infections in both aquaculture and wild fish since the first systemic spironucleosis outbreak was reported in Norwegian Atlantic salmon (*Salmo salar*) in 1989 (13, 30). *Spironucleus salmonicida* (basonym *S. barkhanus*) has been identified in grayling (*Thymallus thymallus*), brown trout (*Salmo trutta*), Arctic char (*Salvelinus alpinus*) and *Oncorhynchus namaykush* as the causative agent of spironucleosis (8, 9, 25). *Spironucleus salmonis* was reported in brook trout (*Salvelinus fontinalis*) (18), Chinook salmon (*O. tshawytscha*) (11), rainbow trout (*O. mykiss*) (1, 2). *Spironucleus torosus* was reported in Atlantic cod (*Gadus morhus*), haddock (*Melanogrammus aeglefinus*) (18), burbot (*Lota lota*) (24). *Spironucleus elegans* has been detected in *Titurus alpestris* and freshwater angelfish (12). *Spironucleus vortens* was found in freshwater angelfish (15), ide or orfe (*Leuciscus idus*)

(26) and discus (*Symphysodon discus*) (17). They can cause cellular damage in the intestinal tract of fishes with severe infections (13, 15, 17).

In Türkiye, *Capillaria* sp. (28), hexamitiasis (10) and edwardsiellosis (27) were previously reported in angelfish. The purpose of the present study was to identify the cause of sustained mortality in freshwater angelfish kept at an ornamental fish production facility in Türkiye.

Eight moribund individuals of each hybrid type (silver, zebra, koi, and black lace angelfish) were examined. Individuals that were immobile at the bottom of the aquarium and showing clinical signs were collected and necropsy was performed. The behavior of the moribund fish was monitored and the anamnesis of the epizootic was obtained from the fish farm executives. The body cavity, all internal organs, the gills, the eyes, the skin, and the fins were examined for parasites. Some visceral organs such as kidney, spleen, liver were placed on a slide with a drop of water to make a tissue squash. Along with these, the intestine and bile contents were examined under light microscope (14). Tissue samples were fixed in 10% buffered formaldehyde for histopathological processing and then tissue samples were dehydrated in ethanol series, cleared in trichloromethane and embedded in paraffin. Finally, the tissue sections were stained with haematoxylin-eosin (5). Photomicrographs were taken using a microscope with an imaging system. Swabs taken from internal organs were streaked onto Tryptic Soy Agar (TSA) and incubated at 22°C for 48h. After incubation, the bacterial isolates were examined using standard laboratory protocols and biochemical test were performed. The isolates were also evaluated for antimicrobial susceptibility using the Kirby-Bauer disc diffusion method and analyzed according to recommendations of the Clinical and Laboratory Standards Institute (3).

The moribund fish stopped feeding and had hemorrhaged eyes, darkened skin, scale loss (Figure 1a, b, c, d), ascites and false faeces. A pale liver, splenomegaly and thinning of the intestinal wall were observed internally (Figure 1 c, d). Highly motile flagellated protozoan endoparasites of the genus *Spironucleus* were found in intestines (Figure 2) and bile contents. These findings are very similar to previous reports of the intestinal form of this disease. Here in contrast, *Spironucleus* sp. was also found in the bile content. A yellowish mucoid fluid was also observed in the intestines of black lace angelfish hybrids in this study.

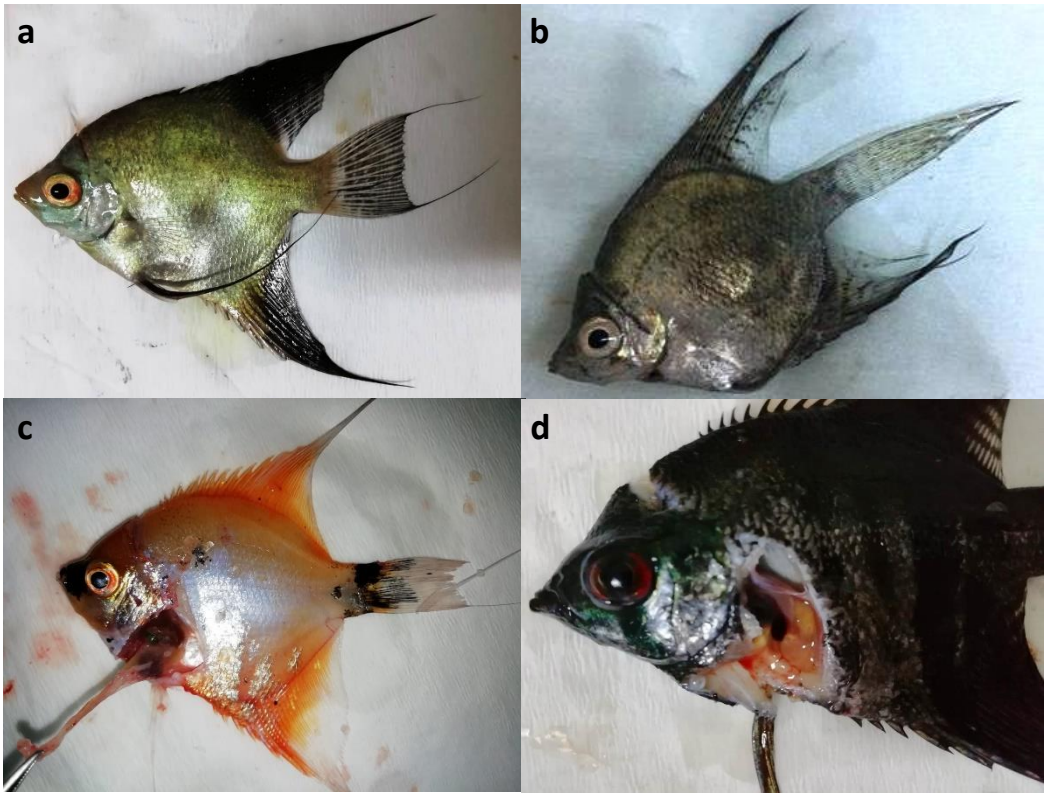

Figure 1. Moribund freshwater angelfish hybrids.
(a) Zebra angelfish display hemorrhaged eyes and scale lose
(b) Silver angelfish had dark skin pigmentations, scale lose
(c) Koi angelfish examination revealed hemorrhaged eyes, scale lose and an internally pale liver
(d) Black lace angelfish observations included splenomegaly, thinning of the intestinal wall and mucoid yellowish fluid in the intestine.

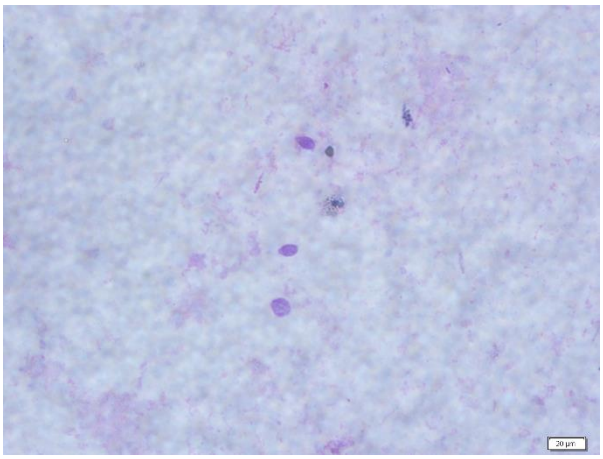

Figure 2. The flagellate protozoan *Spironucleus* sp. stained with Giemsa in intestine contents.

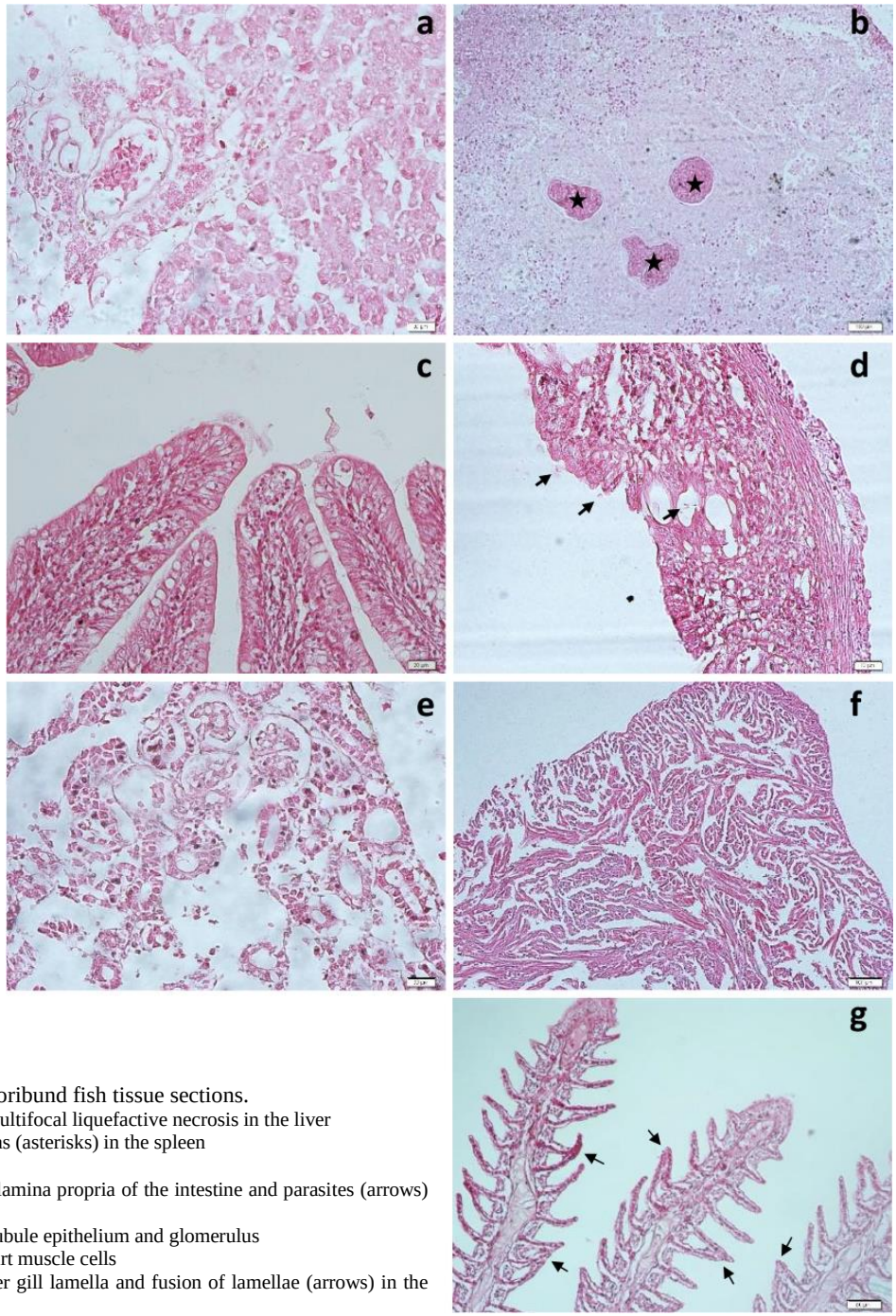

Figure 3. A photomicrograph of moribund fish tissue sections.
(a) lipid degeneration hepatocyte and multifocal liquefactive necrosis in the liver
(b) hemosiderin deposits and granulomas (asterisks) in the spleen
(c) enteritis
(d) a large number of mast cells in the lamina propria of the intestine and parasites (arrows) attached on basement membrane
(e) liquefactive necrosis in the kidney tubule epithelium and glomerulus
(f) necrosis and haemorrhage in the heart muscle cells
(g) distal tip hyperplasia in the seconder gill lamella and fusion of lamellae (arrows) in the gills.

Other observations included lipid degeneration and multifocal liquefactive necrosis in the liver (Figure 3a), hemosiderin deposits and granulomas in the spleen (Figure 3 b) and a large number of mast cells in the lamina propria of the intestine and enteritis (Figure 3c, d). These findings were similar in all examined hybrids. Numerous *Spironucleus* parasites were seen in the intestinal wall. Although it was similar to the intestinal form of the disease, a systemic infection was evident in this study. Liquefactive necrosis in the kidney tubule epithelium and glomerulus (Figure 3e), necrosis and hemorrhage in the heart muscle cells (Figure 3f), distal tip hyperplasia and fusion of lamellae in the gills (Figure 3g) were also detected. Because granuloma formation in the spleen and other findings in the internal organs resembles the systemic form of the disease, a chronic systemic spironucleosis was diagnosed in this study.

In addition to loss of appetite and excessive nervousness (1, 2), enteritis and cytoplasmic blebbing in the gastrointestinal epithelium have been reported as clinical signs of this parasitic disease (31). It has been reported in many studies that disease in farmed salmonids correlates with parasite density as well as environmental factors and nutritional deficiency. Symptomatically spironucleosis has been divided into two main variants; an intestinal and a systemic form. The intestinal form of this disease is benign. Lethargia, exophthalmos, ascites, and false faeces were typical both in salmonids (1, 2) and ornamental fish. Although the symptoms are not specific, the parasite has been found in large numbers in the intestine (1, 2). Intestinal spironucleosis is often associated with locomotive disorder and increased mortality. In the systemic form of spironucleosis, on the other hand, lesions in the head region have been reported and these necrotic lesions with many parasites are located symmetrically along the lateral line (30). In cichlids such as discus and angelfish, it is reported that these parasite-filled necrotic lesions in the head area are combined to form larger lesions and cause a yellow mucoid discharge. This parasite has been reported in many internal organs such as the heart, the liver and the kidney but not in the intestine in the systemic form of the disease. Atlantic salmon infected with *S. salmonicida* reveal the typical histopathology of the systemic form of this disease. These findings include severe epicarditis, large caseonecrotic areas with the formation of a granulomatous response in the kidney, liver and spleen (8, 25).

Spironucleosis causes heavy economic losses, especially in cichlids. *S. elegans* (11) and *S. vortens* (25) have so far been identified as the causative agent of spironucleosis in the posterior intestine of angelfish. Although host specificity and causing granulomatous lesions in the current study point to some particular species within the genus, it is impossible to determine *Spironucleus* species by light microscopy. It is therefore deemed necessary to use scanning electron microscopy [SEM] or transmission electron microscopy [TEM] for accurate identification of the species (8, 18, 19, 24). In this study, the parasites causing disease could only be identified to genus level.

Spironucleosis is treated with chemicals such as dimetridazole, metronidazole, pyrimethamine, albendazole, fenbendazole, mebendazole and magnesium sulfate (23). Besides that treatment with medicinal aromatic plant extracts, including tetterwort (*Chelidonium majus*), purple coneflower (*Echinacea purpurea*), garlic (*Allium sativum*), chestnut (*Aesculus hippocastanum*), horseradish (*Armoracia rusticana*), *Bryophyllum pinnatum* (*Kalanchoe pinnata*), oregano (*Origanum vulgare*), tansy (*Tanacetum vulgare*), thyme (*Thymus vulgaris*), yarrow (*Achillea millefolium*) (20) and wormwood (*Artemisia campestris*) (4), have also yielded successful results. Diler et al. (4) reported good results when ethanol extract of the wormwood plant (*Artemisia campestris)* was applied *in vivo* (21 days) to rainbow trout juveniles in the treatment of *S. salmonis*. In another study conducted in Türkiye, Balta and Balta (1) reported that albendazole and metronidazole were the most effective *in vivo* treatments against *S. salmonis* in the rainbow trout juveniles. Metronidazole is the active compound of nitroimidazole used in the treatment of infections caused by anaerobic bacteria and protozoa, thus are prohibited from use in food-producing animals in member states in accordance with the European Union Commission Regulation No 37/2010. Since angelfish is not used for human consumption, it was recommended metronidazole treatment to the ornamental fish company officials. After oral metronidazole treatment at a dose of 50 mg/kg fish daily for 5 days, a decrease in fish mortality and resumed feeding were noted and further losses ended following parasite treatment.

Cream colored bacterial colonies were observed on TSA plates after 48h incubation at 20°C. Isolates were identified as Gram negative, motile, catalase positive, cytochrome oxidase negative, fermentative and resistant to O/129. Biochemical tests results are shown (Table 1). According to their morphological and biochemical characteristics, all isolated bacteria were identified as *Citrobacter freundii*. Likewise, in two other studies (6, 28), only the ability to degrade urea was found variable between bacterial isolates. Furthermore, we determined that all strains were erythromycin, oxytetracycline, sulfamethoxazole/trimethoprim and ampicillin resistant but sensitive to ciprofloxacin, enrofloxacin and intermediate sensitive to florphenicol (Table 2). These three antibiotics can thus be used in disease control. However, additional antibiotic treatment was not required because losses ended shortly after the treatment.

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Table 1. Morphological and phenotypical characteristics of *Citrobacter freundii*.

Morphology	B	Arginine Dihydrolase	+
Motility	+	Lysine Decarboxylase	-
Gram Staining	-	Ornithine Decarboxylase	-
Catalase	+	Degradation of Urea	V
Cytochrome Oxidase	-	ONPG	+
O/129 Resistance (150ig)	R	Esculin	-
O/F	F	Nitrate Reduction	+
Indole	-	Citrate	+
Voges Proskauer Reaction	-	Hemolysis Blood Agar	+
Methyl Red	+	TSI	+
Acid Production of			
Glucose	+	Trehalose	+
Cellobiose	+	Saccharose	+
Mannose	+	Fructose	+
Arabinose	+	Rhamnose	+
Growth on			
1,5% NaCl	+	40ºC	+

B: bacilli
+: positive reaction
-: negative reaction
F: fermentative
R: resistant
V: variable

Table 2. Antibiotic susceptibility of *C. freundii* according to disk diffusion test.

Antibiotic	**Test result**
Florphenicol	I
Oxytetracycline	R
Enrofloxacin	S
Ciprofloxacin	S
Ampicillin	R
Sulfamethoxazole/ trimethoprim	R
Erythromycin	R

I: intermediate
R: resistant
S: sensitive

In short, spironucleosis, which systemically affects fish by penetrating the intestinal mucosa, was identified as the cause of sustained mortality in freshwater angelfish. *Citrobacter freundii* was likely a secondary opportunistic pathogen associated with this case of chronic spironucleosis.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

REY and ET examined the samples and performed the lab work. REY, ET, and ST interpreted and reviewed the results. All authors contributed equally to the writing of the manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study was approved by the Istanbul University Animal Experiments Local Ethics Committee (2016).

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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