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Research Article

First Report of *Ligula intestinalis* (Cestoda: Pseudophyllidea) in *Barbus ercisianus* (Cypriniformes: Cyprinidae) from the Nemrut Crater Lake, Turkey

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Abstract: Van Basin has a rich geographical structure in terms of water resources and endemic fish species. In this study, *Ligula intestinalis* parasite, which is the host of *Barbus ercisianus* living in Nemrut Crater Lake, was recorded for the first time. The parasite was described molecularly as well as morphologically. In this context, a primer set was designed for molecular identification for the 28S rRNA gene region of *L. intestinalis*. Real-Time PCR results with the designed primers gave positive results in all parasite samples. The results were confirmed by a single peak (87) in the HRM analysis.

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Nemrut Krater (Turkey) Gölü'nde Yaşayan *Barbus ercisianus* (Cypriniformes: Cyprinidae) Balık Türünde *Ligula intestinalis* (Cestoda: Pseudophyllidea)'in İlk Bildirimi

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Anahtar Kelimeler

Sestodlar,
Balık hastalıkları,
Balık parazitleri,
Real-Time PCR

Öz: Van Gölü Havzası su kaynakları ve endemik balık türleri açısından zengin bir coğrafi yapıya sahiptir. Bu çalışmada Nemrut Krater Gölü'nde yaşayan *Barbus ercisianus*'ta konakçı olan *Ligula intestinalis* parazitinin ilk kez kaydı verilmiştir. Parazit, morfolojik tanımlamanın yanı sıra moleküler olarak da tanımlanmıştır. Bu bağlamda, moleküler tanımlama için, *L. intestinalis*'in 28S rRNA gen bölgesini hedef alan bir primer seti tasarlanmıştır. Tasarlanan primerler ile gerçekleştirilen Real-Time PCR sonuçları tüm parazit örneklerinde pozitif sonuç vermiştir. Elde edilen sonuçlar, HRM analizinde tek bir pik (87) ile doğrulanmıştır.

1. Introduction

Fisheries is an important sector with a high-income revenue because it is a good source of food, provides significant employment, and has a vital export product status. Due to its climatic and geographical structure, Turkey has the appropriate facilities for the cultivation of many aquaculture products in seas and freshwaters (Önalan, 2016). Since fish are grown in closed areas such as ponds and cages, and fish stock density is higher than it should be, fish diseases are an essential problem in aquaculture. This leads to unsuitable aquaculture conditions and, consequently, increased susceptibility to infections (Sakai, 1999). The Van Lake Basin, a closed basin, is rich in both plant and animal species (Şekercioğlu et al., 2011; Elp et al., 2016; Adızel et al., 2017; Demirkuş et al., 2018; Toyran et al., 2018). Also, there are many ponds and streams in the basin such as natural lakes of Van, Erçek, Nazik, Nemrut, Aygır, Arin and the dam lakes of Sarımehmet, Koçköprü, Zenek, Morgedik (Cetinkaya, 1993). All of the naturally distributed fish species in these water resources, such as *Barbus ercisianus, Alburnus tarichi, Alburnus timarensis, Capoeta kosswigi,* and *Oxynoemacheilus ercisianus,* are endemic (Karaman, 1971).

B. ercisianus is taxonomically one of the members of the Cyprinidae family and was reported as Data Deficient (DD) in the IUCN red list category (Elp et al., 2016). *B. ercisianus* shows the distribution in the Zilan and Deliçay river basins and Nemrut Crater Lake (Şen et al., 2018). Its body is generally long and covered with cycloid scales. The mouth is ventrally positioned, and there are two double whiskers around it. There is no distinct carina on the back, while there are dark spots on fins and throughout its body. *B. ercisianus* usually inhabits fast-flowing, pebbly and sandy streams and feeds on species such as Daphnia, Gammarus, and Diaptamus (Geldiay and Suleyman, 2009). Temporary white spots on the body surface of males in the reproduction period are called tubercles.

L. intestinalis Linnaeus, 1758 (Cestoda: Pseudophyllidea) is a tapeworm species with a three-host life cycle in freshwater habitats. After completing the larval development in two intermediate hosts, they mature primarily in the intestine of bird species (Stefka et al., 2007).

In this study, the identification of *L. intestinalis* infestation in the *B. ercisianus* species from the Nemrut Crater Lake was carried out for the first time.

2. Material and Methods

2.1. Study area

The study was conducted between September 2018 and February 2019. SAMUS 725 MS brand electroshock device was used to sample fish in the Nemrut Crater Lake (Figure 1). In this study, 11 fish were sampled. Infestation was observed in 6 of the fishes. It was observed that infestation at the sampled fish level could have a prevalence of more than 50%. The lake is located in the east of Turkey, west of the Van Lake, 13 km from Tatvan district center and 25 km from Ahlat district of Bitlis province. The lake is the largest volcanic lake in Turkey and the second largest in the world. The maximum depth of lake has been determined as 176 m and is located at an altitude of 2247 m. The lake has a surface area of 12.52 km² and a total water volume of 1.25 km³ (Kuluöztürk and Doğru, 2015; Kurttaş and Tezcan, 2018). Fishes taken from the sampling area were brought to the Van Yüzüncü Yıl University, Fisheries Faculty, Aquarium Unit on the same day and kept in an aquarium at 13 ºC water temperature until the parasite identification was carried out. Morphological and molecular studies of parasites in fish samples were carried out at Van Yüzüncü Yıl University, Fisheries Faculty, Department of Fish Diseases.

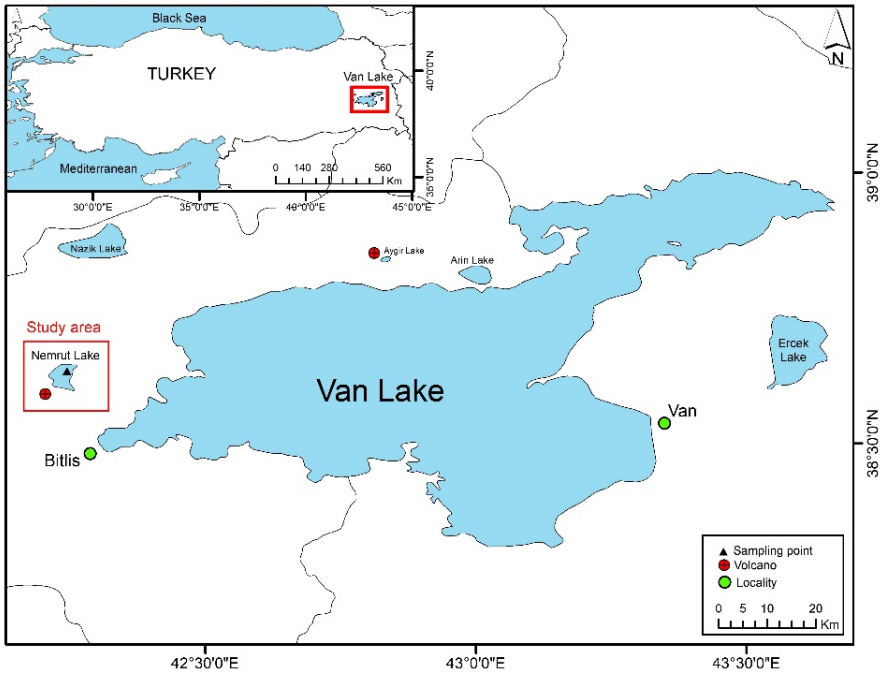

Figure 1. Map of the study area in Van Lake Basin.

2.2. Determination of the Duration of the Parasite to Leave the Host

Following the death of the infected fish, the duration of the parasite to leave the host was determined by keeping the fish in an aquatic environment and a dry environment. A quarantine environment was created that provided the same conditions as the temperature at which the fish were kept in the aquarium.

Similarly, a 13 ºC external environment was set for another infested fish following its death. The procedure was carried out on a glass petri dish in a cooled incubator (Barrow et al., 2019).

2.3. Morphological Identification

The outer surfaces of the infested fish were disinfected with 70% ethanol. The time of death, the length and weight values of the collected fish, the number of parasites, the length and weight values of the parasites were determined. The number and length of parasites taken from each fish according to their abdominal images and fish size during necropsy of fish samples were examined by a SMZ 745T Stereo zoom microscope (Dabara et al., 2020).

2.4. DNA Isolation

L. intestinalis samples obtained from necropsy were exposed to cell lysis at a rate of 50 ms sec-1 for 3 minutes (TissueLyser, Qiagen). Then, total DNAs were isolated using the Qiamp DNA mini kit in the QIACube device in accordance with the manufacturer's instructions. The DNA purity was measured by a nano spectrophotometer (Thermo) (Pinchi et al., 2013).

2.5. Primer Synthesis

The primer sets designed for Real-Time PCR were obtained using the 28S rRNA gene region of *L. intestinalis* with access No. KY552819.1 on the NCBI website. The forward primer sequence was synthesized as Li-F-5'-AGATGCCACTGT-TTCTCG-CAC-C-3', while the reverse primer sequence was synthesized as Li-R-5'-AAT-GCA-CGC-CTT-TCC-AAC-GAC-C-3'. The melting temperatures of the designed primers were determined to be 62.1 ºC for both forward and reverse primers.

2.6. Real-Time PCR

PCR master mix was prepared with DNA molecules obtained from the parasite samples (2.0 µl), forward (1.0 µl) and reverse (1.0 µl) primers, RT2 SYBRGreen qPCR master mix (10.0 µl) and DNase-RNase free water (11.0 µl). The cycle process of the PCR protocol comprised pre-denaturation at 95 °C for, after 10 min, 95 °C for 45 sec, 62.1 °C for 30 sec and 72 °C for 45 sec and the cycle was repeated 45 times. The final elongation was carried out for 7 min at 72 ºC (Hoseinifar et al., 2017). Following the PCR procedure, the HRM procedure was performed from 50 ºC to 99 ºC, using a Rotorgene Q, 5Plex (Qiagen) with 0.01 ºC sensitivity.

2.7. Data analysis

Positive and negative results were evaluated using the Ct (The cycle threshold value) values of sigmoidal curves obtained from Real-Time PCR (Threshold: 0.5).

3. Results

Following the necropsy procedure performed under aseptic conditions, it was seen that external morphological properties of the samples obtained from the fishes were similar to those of the typical *L. intestinalis* (Figure 2). The parasite's significant presence was observed in the abdominal cavities (Figure 3) of infested fish taken from the sampling area.

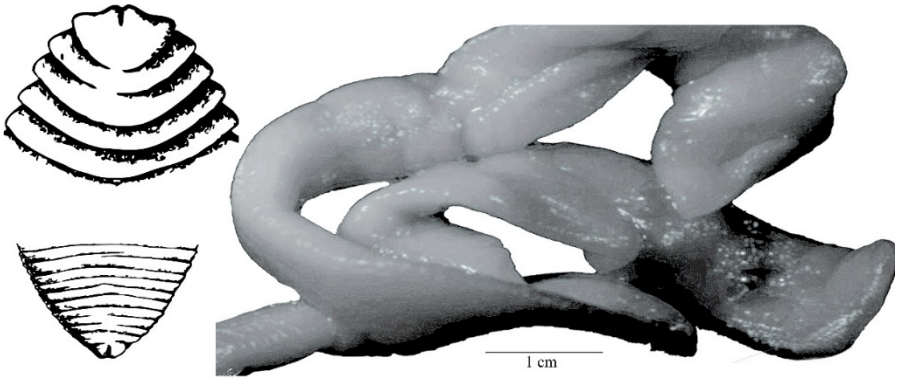

Figure 2. Image of *L. intestinalis* collected from *Barbus ercisianus.*

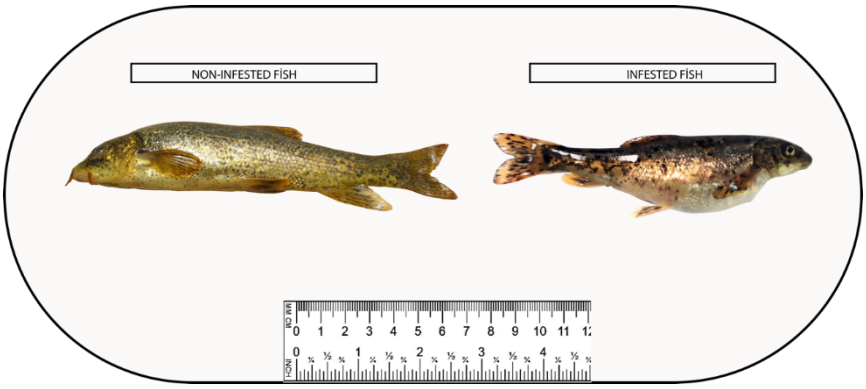

Figure 3. Healthy and infected individuals and abdominal cavity differences.

The average weight of the infested fish (6 of 11) was calculated as 2.75 ± 0.01g, and the average weight of the parasites was determined to be 0.2 g (Figure 4). The highest parasite weight was 0.3 g, whereas the lowest was 0.17 g. While eight parasites were removed from the most massive 3.46 g fish, three parasites were removed from the lightest 2.04 g fish. The length of the parasites ranged from 6 to 12 cm (Figure 5), while the length of the fish ranged from 5.5 to 8 cm. Images of sampled fish and parasites obtained after necropsy are given in Figure 6 and Figure 7.

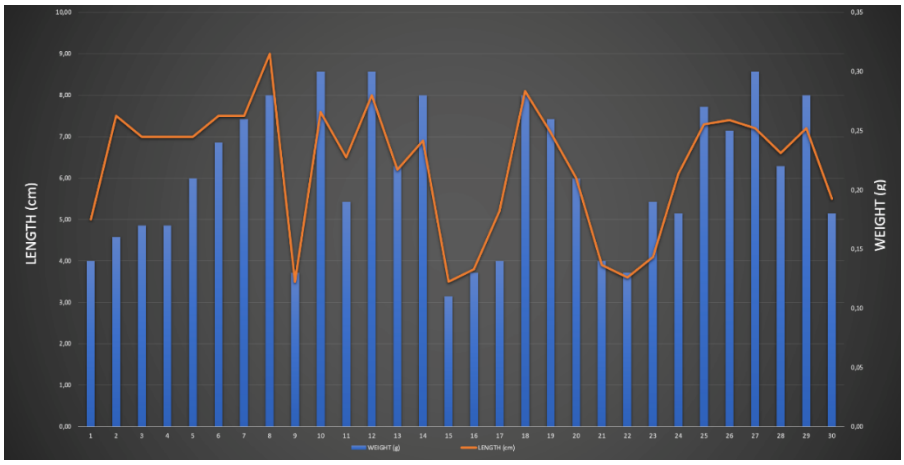

Figure 4. The length and weights of the *L. intestinalis.*

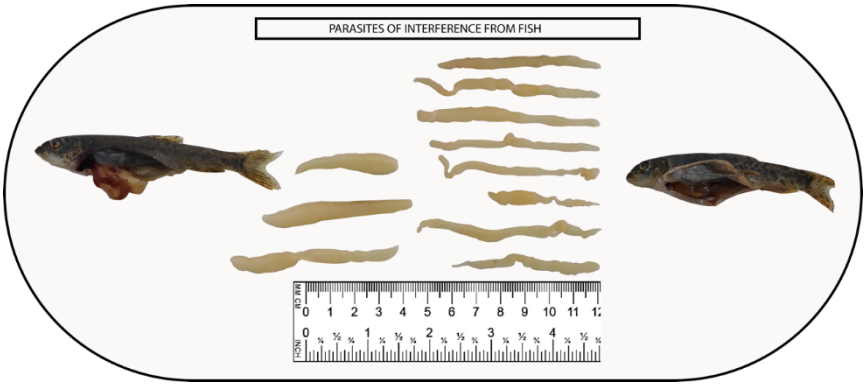

Figure 5. The lengths of some *Ligula intestinalis* in *Barbus ercisianus.*

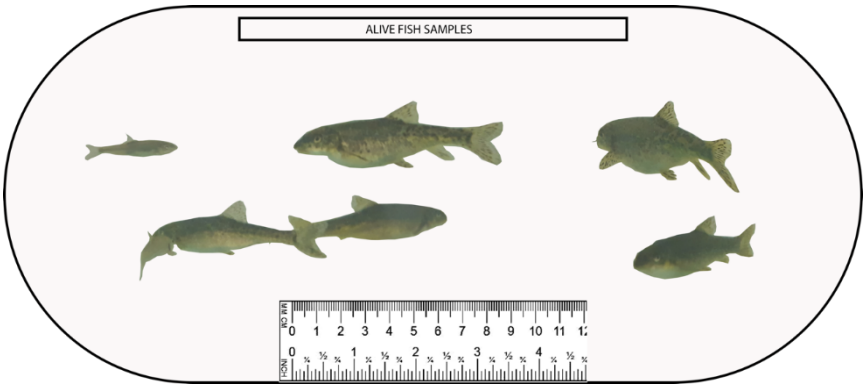

Figure 6. Image drawings of some the sampled fish in the aquarium.

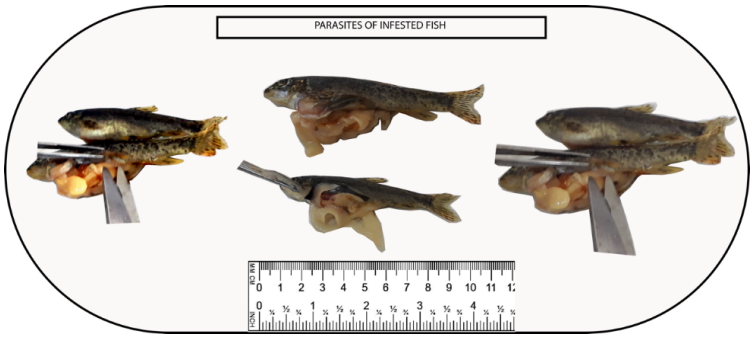

Figure 7. Some necropsied fish samples and their parasites.

It was observed that the symptomatic fish sampled in the natural environment received feeds administered, and their movements in water slowed down. Two of these fish died on the first day of the observation. These fish were kept at the same temperature as the water temperature of the Nemrut Crater Lake. One of the fish was kept in the petri dish to determine the duration of the parasite to leave the host, while another was taken into the aquarium with the same conditions, and the duration of the parasite to leave the host was determined. It was observed that the parasite movements became limited, and the duration of the parasites to leave the host increased due to the decrease in the water content of the fish and drying as macroscopic observations. It was determined that the parasite left the host after 80 min in the aquatic environment and after 130 min in the dry environment.

As a result of PCR performed with DNA obtained from the *L. intestinalis* agent, all of the samples yielded sigmoidal curves and positive graphs. The samples in which the master mix, water, and primers were used separately yielded negative results, and it was observed that the compounds used in the study did not give false-positive results. The image obtained in the PCR is given in Figure 8.

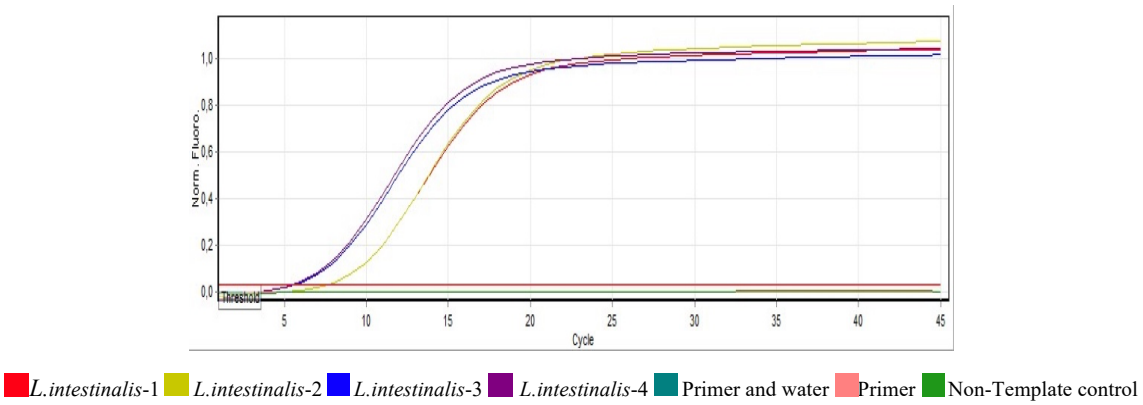

Figure 8. Real-Time PCR results of parasitic DNAs.

Following the Real-Time PCR procedure, single peaks after HRM analysis with PCR amplicons confirmed the accuracy of PCR primers and the PCR procedure (Figure 9).

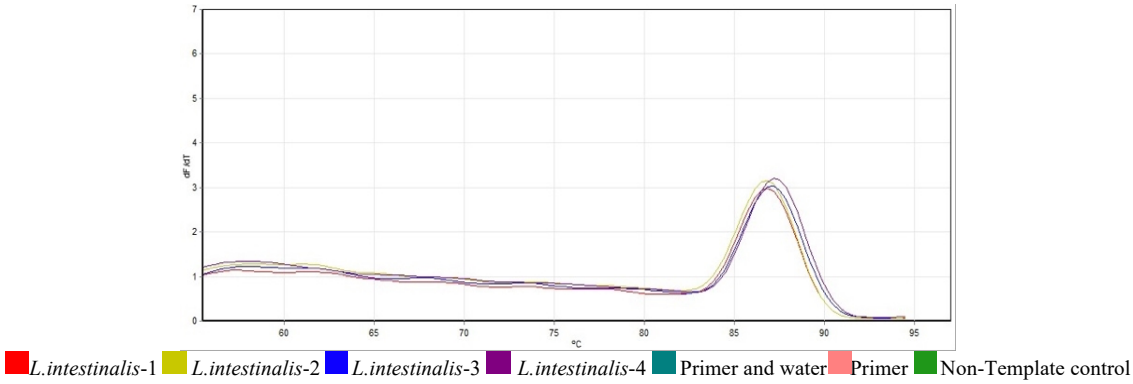

Figure 9. Image of HRM analysis with PCR amplicons.

4. Discussion and Conclusion

The word endemic is used based on climate and soil characteristics for plant and animal creatures spread in a specific region on earth. The Van Lake Basin is rich in endemic fish species. In addition to *B. ercisianus*, other fish species, including *Alburnus tarichi*, *Alburnus timarensis*, *Capoeta kosswigi*, and *Oxynoemacheilus ercisianus,* also show the distribution in the basin (Şen et al., 2018). It is essential to identify the problems encountered in terms of the protection and monitoring of these endemic species. *L. intestinalis* infestation was reported for the first time in the *B. ercisianus* and in the Nemrut Crater Lake, which requires monitoring.

L. intestinalis is a type of endoparasite that causes death in fish. This species is commonly seen in the species belonging to the Cyprinidae family. The mature ones are found in aquatic birds' intestines, while the larvae (plerocercoids) are found in freshwater fish. The first studies related to this species were done by Başaran and Kelle (1976) and Cantoray and Özcan (1975) in Turkey. Various studies were carried out on this infestation in fish in Turkey (Uzbilek and Yıldız, 2002; İnnal et al., 2007; Demirtaş and Altındağ, 2011; Koyun et al., 2015; Saç et al., 2015) and in the World (Olson et al., 2002; Bouzid et al., 2008a; Hajirostamloo, 2009; Mehraban et al., 2015).

Although regular morphological appearances in the determination of parasitic infestations in fish are required, molecular methods are also frequently performed to identify subspecies accurately. In the identification studies carried out by PCR, some researchers have reported that more specific results were obtained as a result of utilizing primers designed using housekeeping genes and PCR procedures (Flisser et al., 1988; Rishi and McManus, 1988; Harrison et al., 1990). Also, it has been reported that, although the visual identification of parasitic infestations was useful, specificity was not very clear. Indeed, it has been reported that identifying two different species from the same family might be challenging. On the other hand, studies conducted using DNA have been reported to have higher specificity; however, the costs of such studies have been shown as a disadvantage (Flisser et al., 1988; Rishi and McManus, 1988; Harrison et al., 1990; Chapman et al., 1995). In this study, the agents' identifications were carried out by both external morphological forms and Real-Time PCR.

The primers from 28S rRNA gene regions were designed for molecular identification, and PCR was performed. Similarly, some researchers have stated that they carried out identification and genetic variation studies with PCR by designing specific primers and have reported that the studies using molecular methods yielded higher specificity (Gonzalez et al., 2002; Bouzid et al., 2008b). Also, in another study by (Gonzalez et al., 2004) in which the researchers compared the identifications using diagnostic methods, it was reported that the multiplex PCR method had higher specificity and sensitivity. The populations of *L. intestinalis* and its place in the ecosystem, the survival of the species in the presence of predators such as cockroaches, and the environmental factors on the chances of finding a host in such a case of the parasite were investigated. (Wyatt and Kennedy, 1989). Considering that this parasite is also carried by birds (Stefka et al., 2007), its detection for the first time in the Nemrut Region and the fact that fish species are endemic reveals the possibility of parasite transmission through migratory birds.

The average length of the parasites isolated in this study was found to be 6-12 cm. The maximum fish fork length was measured as 8 cm. Therefore, it was concluded that the parasite develops in direct proportion to the host's feeding and size. However, the growth of the parasites removed from the same fish is an obstacle to the feeding of another, leading to a consequent reduction in their length. Like the results obtained in the present study, the length of the parasites isolated ranged between 6 and 12 cm in the study investigating the *L. intestinalis* populations and their life cycle conducted by Kennedy and Burrough (1981). It was also reported that the fish infected by the parasite died in their second winter or after the summer.

It was observed that the infected fish continued to feed but were under stress compared to normal behaviour. Their movements were first sudden and then became stable. Although the abdominal cavity of the fish was filled with parasites, feed intake was thought to be due to nutritional reflexes. It can be argued that the parasites both cause stress in the body and excessively stimulate the body’s immune system. In another study, changes in the expression levels of genes in brain and liver tissues were investigated in *L. intestinalis*-infected roaches (*Rutilus rutilus*) (Boulange-Lecomte et al., 2011). The researchers reported an increase in the IGF genes associated with immunity, especially in females, by about 190%, whereas only a partial increase was observed in males compared to the uninfected samples.

In this study, *L. intestinalis* infestation was identified in September 2018. The other researcher also reported that the infestation was seen in these months (Demirtaş and Altındağ, 2011). However, based on the annual examinations according to the years, it is seen that infestation can be seen in any month of any season. Similar to the sampling period in this study, it was reported that *L. intestinalis* infestation was seen intensively in July, August, and September (Saç et al., 2015).

In fish diseases, taking prophylactic measures and treatment are of great importance. It was reported that chemicals including Acetic acid, Betadine, Bithionol, Chloramine-T, Copper sulfate, Formaldehyde, Hydrogen peroxide, Ivermectin, Levamisole, Mebendazole, Metronidazole, Niclosamide, Potassium permanganate, Praziquantel, Salt and Triclorphon were used for the treatment

of parasitic diseases in fish depending on the species and administration dose, and successful results were achieved (Kayis et al., 2009).

In conclusion, it is essential to monitor studies conducted in areas where *L. intestinalis* infestation is common, especially in areas where endemic species live. We believe that taking precautions during migration periods of birds carrying parasite infestation in closed basins and performing routine seasonal controls are very important for endemic species and parasite spread.

Acknowlegment

This study was conducted in compliance with all institutional, national, and international guidelines on the care and use of animals. This study was carried out with the permission of the Van Yuzuncu Yil University, Local Ethics Committee of Animal Experiments dated 25/10/2018 and numbered 2018/10.

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