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Spatial variation of larval ascaridoid nematode (Nematoda: Chromadorea: Ascaridoidea) infections in the Black Sea anchovy (*Engraulis encrasicolus*)

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Abstract: This study evaluates the Black Sea anchovy population infected by parasitic Ascaridoid Nematode larva in the Southern Black Sea. It assesses the larva's effect on fish body condition, energy stored in the body, and anchovy growth. For this, anchovies sampled on July 10–31, 2018, from 30 different stations were examined, and 11.8% of the 825 anchovies were found infected. The observed infection rate was higher on the western side of the study area, suggesting that this rate may be used as a biomarker for the western stock. Age and size of the anchovies play a significant role in the prevalence. Both of the fish well-being indicators (condition and hepatosomatic index) were lower in the parasitized fish. It may support the view that the larva weakened its paratenic host against its predator to facilitate its transfer to a definite host. The observed low infection rate, which is not considered an economic threat to fisheries, is linked to the warm-affinity of the anchovy. Moreover, considering the trophic role of the anchovy in the ecosystem, it is very likely that the infestation rate at the upper trophic levels is higher than that of anchovy. Therefore, a better understanding of the life cycle of the Nematodes in the Black Sea, their hosts in their life cycle, and the consequences of climate change is essential.

Key words: Ascaridoid nematode, parasite, fish health, black sea anchovy, southern Black Sea

1. Introduction

The Black Sea anchovy, *Engraulis encrasicolus* L. 1758, is a planktivorous, small pelagic fish species that make up the most substantial portion, more than 59% of the total catch of Turkey (Düzgüneş et al., 2014). Both in economic and ecological aspects, it is a very precious stock for the Black Sea. However, like others, this stock exposes various natural and anthropogenic drivers throughout their lives and generations. Environmental factors, food availability, diseases, pollution, and some other biological elements like parasite infections are some of the parameters that eventually affect the fish condition and cause an economic loss for the fish market. (Dobson and May 1987; Özer et al., 2015; Ferrer-Maza et al., 2016; Gücü et al., 2018). For the Black Sea anchovy case, any factor plays a critical role in the stock health, especially in their summer feeding season. They gain their condition for their reproductions and winter migrations (Shulman and Love, 1999; Gücü et al., 2018).

Parasites, although disregarded much, play a notable role in marine ecosystems. They show great diversity in life cycle strategies and their hosts (Klimpel and Rückert, 2005). Even though, in the Black Sea, the life cycle of members of the Ascaridoidea superfamily in their intermediate, paratenic, and eventually definitive hosts are not clearly defined, in general, it is known that these parasites use almost all living marine organisms, from small invertebrates to mammals, as hosts at their different life stages (Marcogliese, 1995).

At the adult stage, they reside in the digestive tracks of their host, which are usually located at a high trophic level. The eggs delivered in the feces of the host, embryonated in the water (L2), hatch spontaneously and become free-swimming ensheathed larva (L3). In this free-living stage, they are very susceptible to environmental changes (Mackenzie, 1999). In the course of events, they are known to be ingested by their intermediate or paratenic hosts. Crustaceans are the typical intermediate hosts, while small fish prey either the larva directly or the infested preys (Marcogliese, 1995). The L3 stage larva ingested by a fish is activated in the stomach, penetrates the stomach wall, moves into the peritoneal cavity or the organs such as the liver, and resides there until the host is preyed on by a larger organism (Buchmann and Mehrdana, 2016).

The studies on the effect of Ascaridoid Nematode larva on fish stress that the larva at the L3 stage does not exploit the resources of the host by feeding on them. However, it is also reported that they might reduce the fitness of the

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carrier (Buchmann and Børresen, 1988). It is also underlined that understanding the host-parasite relationship based on condition and liver indexes without considering the variability that may arise from the availability of food in the environment is difficult (Buchmann and Mehrdana, 2016). Although this last point is a very accurate determination, one exception could demonstrate the effect of larva on the fitness of small pelagic fishes. As these school-forming fish are exposed to the same ecological condition, a comparison of the infected and noninfected fish through such indices may be helpful to demonstrate the effect of parasite infection. With this motivation, in this study, we test our hypothesis of the parasite presence might have an adverse impact on the body condition, energy storage and the growth of the Black Sea anchovy population.

2. Materials and methods

A total number of 825 anchovies were collected from the southern half of the Black Sea, from 30 stations shown on the map (Figure 1a), using the midwater trawl on the board of RV Bilim-2 of the Middle East Technical University. The sampling was carried out within a limited period in summer (between July 10 and 31, 2018).

The parasitological examination was an additional activity of the research project. The total length (cm) of each individual was measured, and they were weighed before dissection. The internal organs and body cavity were examined visually for the presence of Nematode larva. The gutted fish were weighed again. The otoliths of the individuals were removed for age determination.

For regional comparisons, the southern Black Sea was divided into two, as used in the definition of the fishing area; the stations located on the west of 35°E longitude was devoted to "western" and the others to "eastern."

Le Cren's relative body condition index (Le Cren, 1951) was calculated to test whether the conditions of the anchovies infested by Nematode larva are different from noninfested anchovies (Lloret et al., 2014).

Considering that the parasites were absent in the anchovies smaller than 9 cm, to eliminate the size dependency of the indices, comparisons were made using only the individuals larger than 9 cm.

The index was calculated by applying the following equation:

$$Kn_i = W_i/aL_i^b ,$$

where Kn is the relative body condition, W_i is the gutted weight (g), and the L_i is the total length (cm) of the ith sample; and a and b are the slope and the intercept of the weight-length relationship representing the entire dataset.

Hepatosomatic index, which was considered as an indicator of the energy storage in the fish, was calculated as follows:

$$HSI_i = LW_i / W_i \text{ x } 100 ,$$

where HSI is the hepatosomatic index, W_i is the gutted weight (g), and the LW_i is the Weight of the liver of the ith sample.

The growth rates of the anchovies were compared using the von Bertalanffy growth function that fitted to each population in question (i.e. infested males, infested females, noninfested males, and noninfested females) applying general-purpose optimization function, optimum of R to minimize the difference between the observed and modeled values. The resulting curves were compared statistically.

2.1. Statistical analysis

A series of modifications were applied to the data to distinguish the effect of the parasite from others. Firstly, to eliminate the variations in the indicators linked to the time of the year (due to varying physiological states), only the fish sampled within the same period were used in the study. Secondly, it was assumed that the fish sampled in each trawl operation consisted of members of the same "group" and that the mean of the well-being indicator was not different within the group. In the third step, each group (data from trawl hauls) was mean-centered to minimize the difference among the groups consisting of different schools and having different feeding histories.

During the statistical analysis, infection and the location were set as two separate factors, and considering the unbalanced nature of the data set, a type III ANOVA was applied to the data modified through the steps given above. Consequently, the null hypothesis was that there is no difference in the means of the factors and no interaction between them.

Statistical evaluations were done under R studio, using *TropFishR*, *LambertW*, *car, plyr,* and *ggplot2* libraries of R.

3. Results

3.1. State of Ascaridoid Nematode infestation in the Black Sea anchovy

Out of 825 anchovies examined, 98 individuals were infested by the Ascaridoid Nematode, indicating a total infestation rate of 11.8% in the southern part of the Black Sea during the spawning season. The rate was as high as 55.6% at a sampling point (Figure 1a). The percentages changed significantly (Z-test; $P < 0.05$) on the western side of the study area (35°E Longitude) and increased to 16%. In the east, the rate of infestation in the population was only 4%. As shown in Figure 1a, where the temperature and prevalence of parasites are depicted together, the stations with the highest infection rate were found in the colder areas. When tested statistically, a significant negative correlation was observed between two variables ($P < 0.01$).

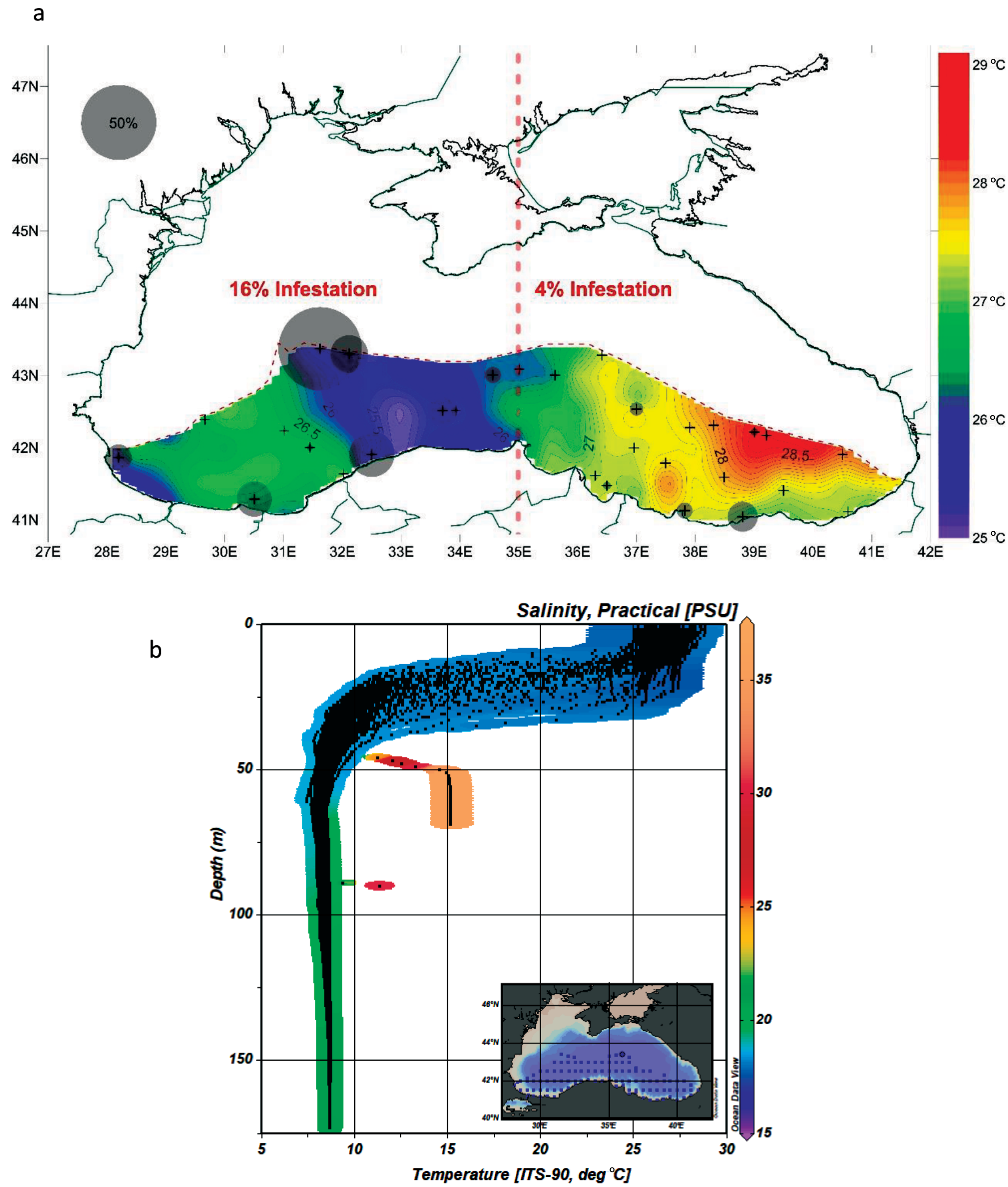

Figure 1. (a) Trawl (+) and CTD* (x) stations (for in situ conductivity, temperature, and depth measurements), and the prevalence of parasites (% infestation) in the sampling stations. (b) Thermocline profile of the CTD stations.
*a package of in situ electronic instruments that measure conductivity, temperature, and depth.

A significant difference is found in favor of the young individual when the rate of infection is compared with regards to the age of the individuals. While the parasite among older individuals appears to be more common, none of the younger individuals at the age of 0 has an Ascaridoid Nematode. The percentage of individuals hosting parasites appears to increase with age. The rate, which was 9.8% in age-class 1, has risen to 37.5% in age class 4 (Table

Table 1. Infestation ratios of the anchovy samples by age

Age	Infested #	Non-Infested #	% Infested	Infested Rate	Non-Infested Rate
0	0	91	0	0	1.02
1	13	119	9.8	1.07	1.01
2	32	104	23.5	1.01	0.99
3	10	10	50	0.97	0.99
4	3	3	50	0.94	0.94

Table 2. ANOVA (Type III), displaying the effect of parasites and location on the body condition (Kn) of the adult anchovies

Response: Kn	**Sum Sq**	**Df**	**F value**	**Pr(>F)**
(Intercept)	0.0043	1	1.0307	0.3105
Parasite	0.0339	1	8.2085	0.0044**
Location	0.0496	12	1.0015	0.4463
Parasite: Location	0.0483	12	0.9750	0.4718
Residuals	1.9280	467		

1). Similarly, the individuals smaller than 9 cm are free of parasites, and as the size of the individual increases, the infestation rate increases accordingly (Figure 2).

A slightly lesser percentage of the male anchovies (10.7%) hosted the parasite when compared with female anchovies (14.0%). This difference, however, is not statistically significant, indicating that the gender of the samples does not affect the rate of infestation (Z-test; $P > 0.05$).

3.2. The impact of infestation on the anchovies

The relative condition of parasite-bearing anchovies larger than 9 cm was estimated as 0.986, which is significantly lower than the condition of the noninfected anchovies [Kn = 1.005; ANOVA (type III), $P < 0.05$; Table 2].

The hepatosomatic index, however, displayed bimodal distribution originating from significant variability in the sex-associated liver size. The index was, therefore, compared to each sex separately (HIS of infested male: 2.63, female: 1.04; noninfested male: 2.48, female: 1.03). There was a difference in the HSI of individuals hosting parasites with those of parasite-free anchovies [ANOVA (type III), $P < 0.05$; Table 3].

Sex has a significant impact on the size of an individual anchovy [ANOVA (type III), $P < 0.05$; Table 4], females being larger, while the presence of parasites does not make a significant difference on length. Additionally, the interaction between the total length (growth in lengths) and the age and parasite of the individual samples was evaluated (Table 5). And for each age class, it was not be found a statistically significant difference between the growth of the infected and uninfected anchovy (ANOVA, $P > 0.05$; Table 5).

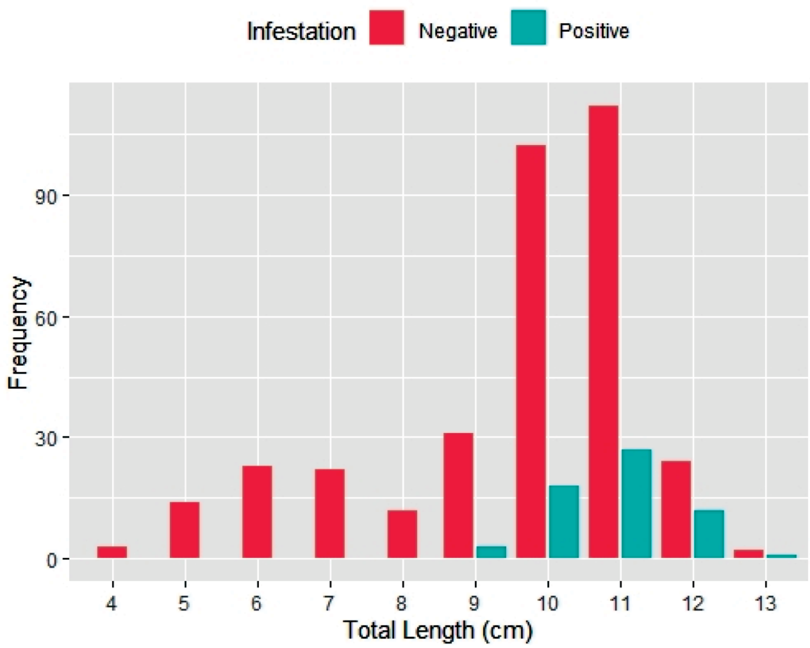

Figure 2. Number of infested (positive in blue) and noninfested (negative in red) anchovy samples by length.

Table 3. ANOVA (Type III), displaying the effect of parasites and location on the hepatosomatic index (HSI) of the adult anchovies

	Sum Sq	Df	F value	Pr(>F)
(Intercept)	0.115	1	0.6959	0.4046
Parasite	0.659	1	3.9739	0.0468*
Location	1.226	12	0.6165	0.8288
Parasite: Location	2.646	12	1.3302	0.1975
Residuals	79.063	477		

Table 4. ANOVA (Type III), displaying the effects of Age, Sex, and Parasites on the total lengths (TLs) of anchovies

Response: TLs	Sum Sq	Df	Mean Sq	F value	Pr(>F)
Scaled (Age)	0.00	1	0.0000	0.0000	1.00000
Sex	5.71	1	5.7120	5.8049	0.01644*
Parasite	0.58	1	0.5779	0.5873	0.44394
Residuals	386.71	393	0.9840		

Table 5. ANOVA (Type II), displaying the age and parasite effects on the growth (TL in cm)

Response: TL	Sum Sq	Df	F value	Pr(>F)
Parasite	0.14	1	0.1637	0.6859
Age	0.01	1	0.0117	0.9140
Parasite: Age	0.08	1	0.0870	0.7681
Residuals	432.89	490		

4. Discussion

The taxonomy of the Nematode observed in the Black Sea anchovy has not been settled yet. For the Black Sea anchovy, two genera of Ascaridoidea superfamily Nematode infection have been reported before. One is *Hysterothylacium aduncum* (Gayevskaya et al., 2010; Tepe and Oğuz, 2013; Matishov et al., 2014; Akmirza, 2016) the other is *Contracaecum aduncum* (Kamburov and Danilevski, 1969; Shchepkinai, 1985; Chashchin, 1996). The recent genetic studies results showed that the *Hysterothylacium* sp. belongs to the Raphidascarididae family, specified separately from the Anisakidae family where *C. aduncum* still belongs. (Keskin et al., 2015; Li et al., 2018; Pekmezci et al., 2019). Therefore, to be able to stay on the safe side in the current study, Nematode larva was used at a level of superfamily name. These challenges also illustrate that further investigations for the accurate taxonomy of the parasitic fish Nematodes are needed for the Black Sea anchovy.

The variability in the number of infected fish, among other things, is associated with temperature in several ways. It is shown that the eggs of *Hysterothylacium aduncum,* kept at 5 ˚C under laboratory conditions, could survive for five months (Balbuena et al., 1998). An increase in the ambient temperature from 1.9 ˚C to 24.3 ˚C decreases the incubation time (the number of days until hatching of eggs of *Anisakis simplex* B) significantly (from 7481 to 3 days; Brattey and Clark, 1992). Due to the same reason, it is postulated that climate change may cause an increase in parasites belonging to this family in the polar regions (Rokicki, 2009). The rise in temperature also affects the survival rate of the larva. The larva that can survive up to 131 days at low temperatures (2–11 ˚C) either cannot survive at temperatures above 13 ˚C (Højgaard, 1998) or their survival time is shortened dramatically (Measures, 1996). This cold water affiliation of the larva will eventually hinder or significantly reduce the chances of the larva being detected and preyed on by a potential intermediate host in the warm waters. When the heterogeneity in the prevalence observed in the Black Sea is considered with regards to temperature (Figure 1a), the number of infected fish was higher on the cold west than on the warm east. However, it will not be meaningful to explain the 12% difference of prevalence with only 1.3 ˚C of difference in the sea surface temperature alone.

From another point of view, on the Northwestern of the Black Sea, it is well-known that the pollution rate is higher due to the anthropogenic pollutant and nutrient inputs throughout the big rivers. But, Lafferty (1997) had emphasized this physically measured information does not show how it may affect ecological life. Accordingly, parasite prevalence may be one of the important parameters to demonstrate it. For instance, eutrophication, thermal effluent, and oils are critical pollution parameters that increase the Nematode abundance (Lafferty, 1997; MacKenzie, 1999). This higher prevalence rate in the western side might show the pollution indicator potential of the parasite and the parasite prevalence in fish. However, it should be considered that different parasitic taxa respond differently to the varying types of pollution. Therefore, knowing the exact taxa of the parasite, its life cycle characteristics, and its susceptibility to the environment is very important to know. Yet, it could be a point to start for future pollution investigations. Moreover, this regional difference seems to be a helpful feature that can be taken into account in the discrimination of eastern and western anchovy stocks in the Black Sea. Indeed, Chashchin (1996) suggested using the rate of infection to separate the Azov and the Black Seas anchovies whose overwintering ground overlap.

The demographic structure of the host population is also known to affect the level of prevalence. Higher infestation rate at longer and older fish, which was also observed in this study (Table 1), is a typical pattern that has already been noted earlier in the Black Sea (Hennig, 1974; Chashchin, 1996; Rello et al., 2009). Totoiu and Patriche (2018) drew attention to a sharp decrease of infestation at younger fish and indicated that the parasites were observed in all anchovies larger than 11 cm, while in the smaller size groups, the rate decreased to 40%.

The absence of L3 larva in 0 age class or the individuals below 9 cm may be attributed to several reasons. One such is associated with the diet of the anchovies. A similar situation was observed in cod in the Baltic Sea. While Cods smaller than 30 cm carry no or very few larva, a significant percentage of the population larger than 38 cm was infected with this parasite (Zuo et al., 2016). It is believed that this difference was related to the ontogenetic diet shift of the cod. The sprat acts as a transfer host of Anisakid Nematodes, and while it is an essential prey for cod, it is not found in the diet of Cods smaller than 30 cm long. Similar changes in the diet of the Black Sea anchovy have also been reported (Mazlum et al., 2017; Sağlam and Yıldız, 2019). This difference is thought to be caused by the very sharp vertical temperature gradient in the Black Sea occurring in the summer. The intense thermocline layer observed in Figure 1b forms a strong barrier between the species with cold and warm affiliations. The anchovy, being a thermophilic species, is, expectedly, isolated from cold-water species, like *Calanus euxinus* (Sakınan and Gücü, 2017), *Sagitta setosa* (Besiktepe and Unsal, 2000; Mutlu, 2006), *Pseudocalanus elongatus* (Yıldız and Feyzioğlu, 2014) in summer. These cold-water species are reported as intermediate and/or paratenic hosts of Ascaridoid Nematodes in the Black Sea (Gaevskaya et al., 2010). In the Black Sea ecosystem, it is known that anchovy is a paratenic host, where L3 larva maintains its stage without undergoing further development and is accumulated. These zooplankton species listed just above are among the primary food sources of anchovy (Yıldız and Feyzioğlu, 2014), and hence, the main sources of infection on anchovy. Therefore, there can be a correlation between the higher parasite burden in longer and older fish and their time spent feeding the infected invertebrate organisms (Cipriani et al., 2018). Moreover, it is highly likely that 0-year-old anchovies in the samples, which were hatched at the end of spring when thermal stratification was formed, have not met with free-living Ascaridoid Nematode larva or with their intermediate hosts at the time of sampling. Furthermore, the notably high occurrence of young and small individuals in the length-frequency distribution of the samples may explain the low infestation rate observed in this study (Figure 2).

Contrary to the anchovy, the situation may not be as good for the cold-water species of the Black Sea, such as sprat, or for the other fish like turbot and whiting that feed on anchovy and therefore expected to have a higher level of infection due to trophic amplification. As a matter of fact, in the earlier studies conducted in the Black Sea, infections found in sprat (Avşar, 1997) and whiting (İşmen and Bingel, 1999; Özer et al., 2016) were considerably higher than the values observed for anchovy.

It has already been documented that parasites can decrease the body condition of their hosts and cause a decrease in their mobility (Shulman and Love, 1999). However, it is also known that in some cases, parasites inflict no harm to their host. The European hake in the Mediterranean (Lloret et al., 2012), in which neither condition nor the hepatosomatic indices were not affected even in the specimens hosting a large number of parasites, is an example of such cases. The effect of a parasite on its host fish is most probably linked to the life history strategy of the parasite (single-host or multihost), and more importantly, to the life stage that they spend in the host. It has been suggested that this could be an evolutionary strategy adopted by trophically transmitted multihost parasites to slow down their transfer hosts and to increase the chance of being preyed on by their definitive hosts where they reach sexual maturity. When viewed from the point of the population of the transfer species, parasite-induced mortality in the case of a high level of infestation will decrease biomass and abundance. There are several examples of this situation; the drop in the cod stocks can be given as a good example (McClelland et al., 2011; Swain et al., 2011).

However, as it is difficult to distinguish the parasite effect from other effects on fish condition in the field, it seems that the effects of Nematodes have not been adequately addressed in the literature. In this study, it is believed that the approach, sampling design, and the method applied helped remove the three major effects, namely those associated with gender, seasonality (Gücü et al., 2018), and food availability (Shulman and Love, 1999). The remaining variability in the indicators and growth rate, which was attributed to the parasite infections, suggested that the parasite in fish reduced the body condition (Table 2) and lessened the energy deposition (as inferred from HSI; Table 3). It is believed that parasites can indirectly affect their hosts by disturbing their metabolism. Shchepkinai (1985) demonstrated that parasitic nematodes caused a decrease in the level of triglycerides in the liver, red and white muscles. According to Shulman et al. (2008), it is the level of the same lipid that triggers the migration in the Black Sea anchovy. It was further postulated that fish with a high level of infestation by these parasites might not migrate as they do not deposit enough fuel to sustain their long overwintering migration (Shulman and Love, 1999).

As suggested by the analysis (Table 4), the parasites do not affect the growth of the anchovies while affecting their body condition and HSI. Anchovy is a very fast-growing fish. A significant part of its growth is achieved when the fish is 0-year-old, and at the end of the first year of their life, they reach an asymptotic size. As they are not yet met with parasites during this fast growth period, it is not unexpected to see that the Ascaridoid parasites do not affect the growth of the anchovy.

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Conflicts of interest
The authors declare that there are no competing interests.

Ethical statement
During the study, no treatment/experiment was implemented on the live animal. All sampling and laboratory work on fish have complied with the Republic of Turkey Ministry of Agriculture and Forestry animal welfare laws, guidelines, and policies as approved by the Republic of Turkey Ministry of Agriculture and Forestry Central Fisheries Research Institute, and the Middle East Technical University Animal Experiments Local Ethics Committee.

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