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Morphological, molecular and clinical assessment of different Anisakis species infecting horse Mackerel *Trachurus trachurus* from South Mediterranean

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ABSTRACT

This study aimed to identify and characterize the most common Anisakis larvae infecting Atlantic horse Mackerel (*Trachurus trachurus*) in South Mediterranean basin; based on morphological and advanced molecular characterization. Also, the linkage between prevalence of *Anisakis* spp. larvae and some biological variables including Mackerel age and sex were also investigated. Moreover, the clinical intensity of larvae infection in different organs of infected Mackerel was assessed. Atlantic horse mackerel collected from the southern Mediterranean shores were found to be infected with third stages larvae (L3) of Anisakis species. The Anisakis larvae were found encapsulated as coiled tightly in different fish organs and uncoiled freely in the abdominal cavity of infected *T. trachurus*. These larvae were categorized into 3 types of *Anisakis* spp. as *A. simplex* type1; *A. pegreffii; Anisakis simplex* / *pegreffii* hybrid based on their morphological and molecular characterizations. Subsequently, the phylogenetic analysis of ITS region of different investigated larvae confirmed the identification of collected *Anisakis* spp. The remarkably high intensity of *Anisakis* spp. larvae in Mackerel gonads warns of possible future deleterious impacts on the growth, development and sustainability of Mackerel fisheries at the south Mediterranean coasts.

INTRODUCTION

The Atlantic horse mackerel *Trachurus trachurus* **(Linnaeus, 1758)** is an important fish which is widely distributed throughout the tropical and subtropical aquatic environments worldwide. Mackerel belongs to Perciformes, genus Trachurus and the family Carangidae **(Karaoglu and Belduz, 2011)**. *T. trachurus* is very delicacy and commercially important fish harvest for fishermen and consumers in North African region. Horse mackerel is a

migratory and schooling species **(Yankova *et al.* 2009)**. The juveniles feed on zooplankton while the adults feed on Eggs, larvae, squids and small fish **(Yankova *et al.* 2009)**. Such feeding behavior could be the main initiating factor to their infection with helminths during different stages of life cycle. Moreover, their long-life span is another detrimental factor increasing their susceptibility to helminths infections. Anisakids larvae could be recovered from different species of Mackerel. Therefore, eating Mackerel was the primary cause of human Anisakiasis worldwide **(Abdelsalam *et al.* 2020).**

Anisakis is one the most important zoonotic nematodes infecting marine fishes worldwide **(Abdelsalam *et al.* 2020)**. This group of nematodes is commonly named Anisakids and belongs to the super-family: Ascaridioidea, family: Anisakidae and Raphid-ascarididae **(Morsy *et al.* 2015; Arafa *et al.* 2019).** *Anisakis simplex* is a zoonotic parasitic nematode infecting more than 200 fish species worldwide **(Eissa *et al.* 2015; Eissa *et al.* 2018; Abdelsalam *et al.* 2020).** Therefore, the infection of fish with anisakid nematodes has posed critical health threats to marine fish consumers, especially when raw or under-cooked infected fish was consumed by humans **(Sakanari and McKerrow, 1989).** The pathogenesis of *A. simplex* larvae depends upon its ability to penetrate the mucous membrane of the digestive organs such as throat and stomach **(Sakanari and McKerrow, 1989).** In human they might migrate to different vital organs causing misdiagnosed tumor like masses. The clinical symptoms of the human anisakiasis include nausea, diarrhea; vomiting epigastric pain, and fever, which may occur from 1 to 12 h after infection. *A. simplex* also induces both gastric and allergic symptoms **(Audicana *et al.* 2002).** Therefore, anaphylactic shock could be expected when human re-infected with anisakis even following cooking **(Eissa *et al.* 2018).**

Anisakis spp. possess complex life cycles utilizing number of hosts throughout the course of their life including intermediate hosts such as crustaceans, euphausiid, cephalopods, and fish and final hosts such as cetaceans. The most common stage present in fishes is L3 or third larval stage. The final hosts get the infection by engulfing infected fish and infected intermediate hosts. In the final host, the Anisakis larvae rapidly grow to the larval stage 4 (L4) and then to adults **(Pozio, 2013)**. The adult of Anisakis exist in the gastrointestinal tract of marine mammals and cetaceans **(McClelland, 2002).**

The Anisakiasis status in south Mediterranean basin remains indistinct due to lack of epidemiological studies in fish and human. Few studies concerning the incidence of Anisakidae in the Mediterranean Sea fishes were performed in Egypt **(Morsy *et al.* 2017; Abou Zaid *et al.* 2018)**. In addition, the ichthyoparasitological studies are still poorly investigated in horse mackerel in south Mediterranean basin. The aim of this study is to characterize the most common anisakids species infecting Atlantic horse Mackerel in South Mediterranean basin; based on morphological and advanced molecular characterization. In addition, the impact of age and sex of horse mackerel on the prevalence of Anisakis larvae was also investigated. Finally, the intensity of larvae infection in different organs of infected fish species was studied.

MATERIALS AND METHODS

2.1. Fish Sampling

Two hundred and forty (240) specimens of Atlantic horse Mackerel were haphazardly collected from local fishermen and retail markets along the southern Mediterranean coasts. The fishes classified into 131 male and 109 females. The collected fishes also; classified according to its age into (3-Month-old: 84; 4 months - old: 95; 5 - months old: 15; 6 months -old: 46).

The collected samples were transported in ice boxes to the laboratories of Aquatic Animal Medicine and Management Lab (AAMML) at the Faculty of Veterinary Medicine, Cairo University. The collected fish were cleaned with saline solution (0.9%) for cleansing out the fish from sea weeds, mud and other contaminants. The examined fishes were opened from left side to expose all organs according to **Eissa *et al.* (2018); Attia *et al.* 2021.** Abdominal cavity and internal organs were examined carefully for presence of any anisakids nematodes larvae either free or encapsulated. Intestinal tract was longitudinally opened throughout the entire length and examined with a dissecting microscope for detection of *Anisakis* spp.. The clinical and postmortem finding of infected fish with anisakids nematodes were recorded and photographed.

2.2. The impact of age and sex of infected fish on the prevalence of *Anisakis* spp.

Intensity was assessed through counting the number of *Anisakis* larvae in each examined organ.

2.3. Identification of the collected nematoda

The collected larvae either encapsulated or free; were rinsed with phosphate buffered saline (pH 7.2) and kept for relaxation at 4 °C. after that, they were cleared in lactophenol and mounted with glycerin jelly then examine under stereomicroscope with optical digital USB camera connected computer **(Abdelsalam *et al.* 2020)**. The anisakids larvae were identified based on the standard morphological criteria described by **Eissa *et al.* (2018) and Abdelsalam *et al.* (2020);** utilizing the morphological characteristics of the lips surrounding the anterior end, the shape of the esophageal ventriculus; the presence or absence of a boring tooth, the shape of the postanal tail and its mucron or spine. All taken measurements were displayed in millimeters. Another fully identified anisakid nematodes (identification was done under dissecting microscope) were preserved in absolute ethanol (**Farjallah *et al.* 2008; Quiazon *et al.* 2009; Attia *et al.* 2021a**) for molecular characterization.

2.4. Molecular characterization of the collected anisakids larvae

Briefly, the procedures combined the standard PCR for amplification of Internal Transcribed Spacer using the following primers NC2: (5'-TTAGTTTCTTTTCCTCCGCT-3') and NC5(5'-GTAGGTGAACCTGCGGAAGGATC ATT-3') as described by **Zhu *et al.* (1998),** and **Abdelsalam *et al.* (2020).** The ITS PCR amplicons were excised from the gel, and the DNA was extracted from the gel using GF-

1 AmbiClean kit (Vivantis, Malaysia) and sequenced using cycle sequencing PCR reaction with Big- Dye_ Terminator v3.1 Kit (AB-Applied Bioscience), then sequenced in four-capillary ABI PRISM_ 3100-Avant Genetic Analyzer. The chromatogram files were displayed and manually edited using ChromasPro version 2.1.6 software (Technelysium, Australia). Subsequently, BLAST search on NCBI (http://www.ncbi.nlm.nih.gov/pubmed) and Clustal W multiple sequence alignment was applied for the examined consensus sequences to compare with the previously published sequences (**Mahmoud *et al.* 2021)**. Phylogenetic analysis of obtained sequences was performed using the neighbor-joining method with 1000 bootstrap replications of MEGA 7 according to **(Kumar *et al.* 2016; Attia *et al.* 2021b).** *Anisakis berlandi* was chosen as the out group.

RESULTS

3.1. Clinical findings

The examined fish were either apparently normal fish or those showing various clinical findings (from mild to severe lesion) such as hemorrhages in different fins, eroded fins, and detached scales **(Fig. 1).**

3.2. Postmortem findings

The infected fish had several lesions in different organs as congestion in stomach, intestine, pyloric caeca and gonads. Visceral adhesions and presence of live nematodes in the organs was the most common postmortem findings**.** In addition, the anisakids larvae were encapsulated and coiled on the serosal layers of internal organs or lie free in the abdominal cavity of infected fish.

3.3. Identification of *Anisakis* species larvae (L3)

The 3rd stage larvae of *Anisakis* spp were collected either free or encapsulated in a creamy colored capsule; the larvae were found in most organs of fish as liver; intestine; abdominal cavity. The body is cylindrical and non-segmented but had striation on the cuticle **(Table 1)**. The collected *Anisakid* spp. were identified as: *Anisakis pegreffii*; *Anisakis simplex* and *Anisakis simplex / pegreffii hybrid.*

The length of ventriculus is the main distinguishing point between *A. pegreffii* and *A. simplex* whereas; in *A. pegreffii* 0.48- 0.79 (0.68 ± 0.27) while in *A. simplex* 0.85- 1.7 (1.28 ± 0.54). The ventriculus length in the hybrid was very short in comparison with the previous collected anisakids 0.25- 0.54 (0.26 ± 0.18). All measurements were recorded in **Table 1**. All *Anisakis* spp. larvae were cylindrical in shape, their bodies were attenuated at the two ends, the length and width of the whole body of the three recorded species present in **Table 1**. The anterior end is surrounded by inconspicuous lips, with presence of boring tooth. The larva has esophagus composed mainly of two parts, glandular-ventriculus part and the muscular part with an oblique junction in esophago-intestinal part. The posterior extremity of the body is provided with short mucron; all measurements of the three species recorded in **Table 1**.

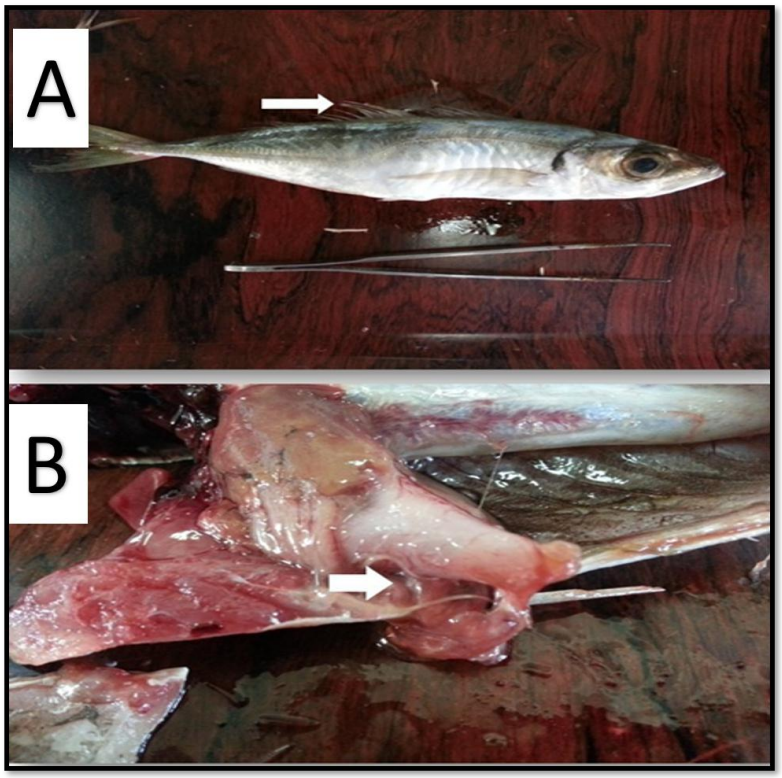

Fig. 1. Clinical finding and postmortem examination of Atlantic horse Mackerel; A: showing erosions in dorsal fin and above the lateral line(referred by arrow) in Atlantic horse Mackerel. B: Mild congestion in stomach, intestine, pyloric and severe congestion in musculature due to Anisakid nematoda which referred by arrow. (Anisakid nematoda referred by arrow).

Table 1. Morphological criteria of third stage larvae (L3) of *Anisakis* species reported in Atlantic horse mackerel caught from Mediterranean shores.

Morphological characters	***Anisakis simplex***	***Anisakis pegreffii***	***Anisakis simplex/ pegreffii hybrid***
Total body length	17.9–23.6 (18.6 ± 1.5)	11.10 – 26.78 (17.24 ± 11.08)	9.00 – 17.5 (12.5 ± 6.01)
Maximum body width	0.55–0.9 (0.75 ± 0.45)	0.38 – 0.60 (0.47 ±0.155)	0.25 – 0.56 (0.37 ±0.21)
Distance of nerve ring to anterior end	0.4 – 0.55 (0.47 ± 0.07)	0.20 – 0.31 (0.25 ± 0.07)	0.19 – 0.23 (0.21 ± 0.02)
Length of esophagus	1.7- 3.9 (2.95 ± 0.55)	1.04 – 2.11 (1.57 ± 0.75)	0.56 – 1.75 (0.98 ± 0.84)
Length of ventriculus	0.85- 1.7 (1.28 ± 0.54).	0.48- 0.79 (0.68 ± 0.27)	0.25- 0.54 (0.26 ± 0.18).
Width of ventriculus	0.25 – 0.38 (0.29 ± 0.10)	0.12 – 0.27 (0.18± 0.10)	0.12 – 0.20 (0.15 ± 0.05)
Length of tail	0.15 – 0.19 (0.16 ± 0.07)	0.05 – 0.12 (0.07 ± 0.04)	0.04 – 0.09 (0.06 ± 0.03)
Length of mucron	0.029– 0.040 (0.034 ± 0.04)	0.02 – 0.03 (0.024 ±0.007)	0.01 – 0.03 (0.02 ± 0.014)

Data expressed as min- max (average ± SE) mm.

3.4. Sequence analysis of ITS region

An approximately 1000 bp fragment of the ITS region of Anisakis larvae type I was yielded by using the universal primer NC5 and NC2. Four purified PCR products of ITS regions were directly sequenced to identify the species. The accession numbers of ITS regions of *Anisakis spp*. larvae were MW507186, MW507187, MW507188 and MW507189. The accession numbers (MW507186 and MW507187) revealed 99.88% identity with *A. pegreffii* (LC536533-LC536532-Japan), with one nucleotide insertion in the Egyptian samples. On the other hand, the accession number (MW507188) exhibited 99.88% identity with *A. simplex* (MT516319-Spain) and (LC536534-Japan), with one nucleotide differences. While (MW507189) showed 99.88% identity with *Anisakis simplex/ Anisakis pegreffii* hybrid (KF032056-Turkey) and (JN005768-Portugal), with one nucleotide differences.

Remarkably, the neighbor-joining phylogenetic tree of amplified sequences of A. pegreffii, A. simplex, and A. simplex x A. pegreffii hybrid were grouped with known sequences of the relevant sequences of anisakidae species and separated from each other. The clade of present A. pegreffii sequences grouped with A. pegreffii from Japan, Thailand, China and Spain. On the other hand, A. simplex sequence in this study grouped with A. simplex from Japan, Spain, Turkey, Portugal, Germany and Turkey. Furthermore, A. simplex x A. pegerffii hybrid sequence grouped with A. simplex x A. pegerffii hybrid from Turkey, Germany, and Portugal. **(Fig. 2).**

3.5. Impact of Atlantic horse mackerel sex on the prevalence of *Anisakis* spp.

The percentages of infected females (26%) were relatively higher than males (21%). The total number of Anisakis larvae in female fish was 973 while the number was 615 in males **(Table 2)**. Statistical analysis indicated that there were significant differences in total count of 3rd stage larvae among examined fishes between male and female seasons at probability ratio Pr= 0.04.

3.6. Impact of age factor on the prevalence of *Anisakis* spp. infection

The 240 fish samples were categorized according to age into 3, 4, 5 and 6 years. The statistical analysis indicated that there was a significant difference (Pr= <0.0001) age / infection level. This obviously indicated that the older fish got the higher number of larvae in their bodies. In the 3 years old examined fish, a total of 5% of fish sample has third stage larvae. In the 6 years old fish, the prevalence of third larval stage was 56 % which is remarkably higher than the 3 years age category. **(Table 3)**.

3.7. The intensity of *Anisakis* spp. larvae (L3) in different fish organs

A total number of 1588 of larvae were collected from 55 infected fish out of 240 examined Atlantic horse mackerel samples. A total of 299, 264, 221, 100, 19 and 685 were collected from stomach, intestine, pyloric caeca, muscles, abdominal cavity and gonads respectively. The total number of Anisakis larvae was much higher in ovaries 438 than testis 247. All five organs (gonads, stomach, intestine, pyloric caeca and abdominal cavity) were infected with L3 stage of *Anisakis* species at different intensities and there

was significantly difference (Pr = <0.0001). The highest intensity of infection was in ovaries (28%).

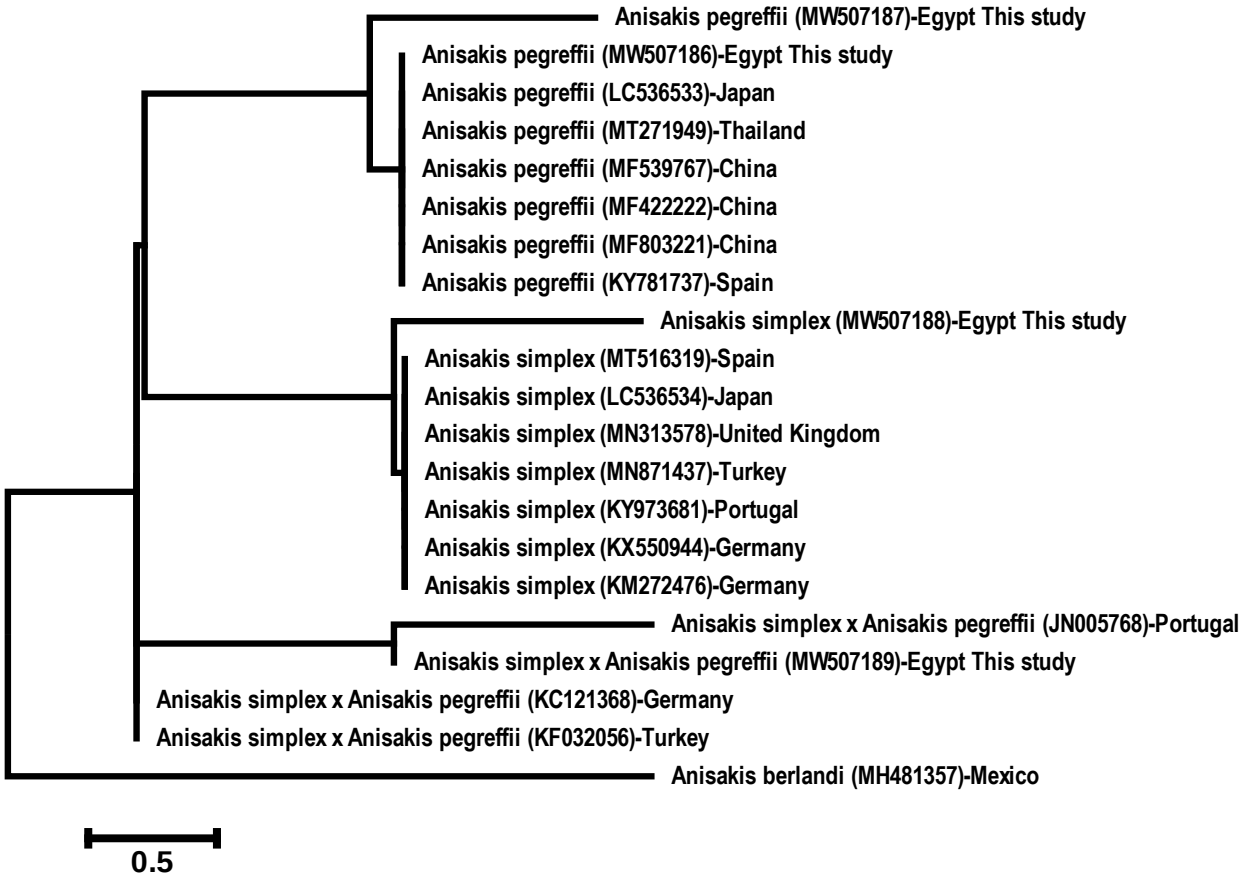

Fig. 2. Neighbor-Joining Phylogenetic tree showed the comparative analysis of ITS region sequences of *Anisakis simplex*, *A. pegreffii* and *A. simplex* x *A. pegreffii* hybrid identified in this study with other closely related *Anisakis* spp.

Table 2. Prevalence of the retrieved third stage larvae (L3) of different *Anisakis* species among examined male and female Atlantic horse mackerel.

Anisakis spp	Sex	No. infected fish	No. of retrieved larvae	Prevalence%*
	M	27	615	21
	F	28	973	26

*Represent percentage of infection in relation to the total number of collected fish.

M: Male: number of collected male fish=131

F: Female: number of collected male fish=109

Table 3. Prevalence of retrieved third stage larvae of *Anisakis* species infected in examined Atlantic horse mackerel through different ages.

Anisakis spp.	Age of collected fish	No. of infected fish	No. of larvae	Prevalence%*
Anisakis species	3	4	22	5
	4	17	407	18
	5	8	194	53
	6	26	965	56

*Represent percentage of infection in relation to the total number of collected fish.
No. of collected fishes at different age (3 Month: 84; 4 months: 95; 5 months: 15; 6 months: 46).

DISCUSSION

The Atlantic horse mackerel fish is an economically and commercially important fish harvest. Thus, studying the health, growth and fecundity of this valuable fish species is of great interest for aquatic veterinarians at Mediterranean basin. The occurrence of zoonotic nematodes is increasing among Mediterranean countries as a result of dumping of sewage directly into the Mediterranean Sea **(Fernandez-Jover *et al.* 2010).** Several *Anisakis spp.* has been documented infecting marine fishes among southern countries of Mediterranean basin including Morocco, Tunisia and Finally Libya **(Farjallah *et al.* 2008; Eissa *et al.* 2015).** *Anisakis* spp including *A. simplex, A. typica,* and *A. pegreffii*, infected several commercial fish inhabiting the Mediterranean Sea**.** Literatures from the Southern Mediterranean countries have proved that horse mackerel is commonly found to be infected with Anisakids larvae **(Manfredi *et al.* 2000; Abdelsalam *et al.* 2020).**

In the current study, the identification of anisakid recovered from horse mackerel were coincided with the criteria of *A. pegreffii* and *A. simplex complex* as described by **(Eissa *et al.* 2018; Abdelsalam *et al.* 2020).** A total of 904 *A. pegreffii* larvae, 440 *Anisakis simplex* larvae, and 244 *Anisakis simplex/ Anisakis pegreffii* hybrid larvae were identified from a total of 1588 Anisakid nematodes which were collected from 240 Atlantic horse Mackerel throughout spring and summer seasons. For those identified as *A. pegreffii,* the recorded taxonomical measures were the total length / width of the ventriculus, esophageal length, and body length were shorter than those documented for *A. simplex* and slightly larger than *A. typica* as described in table (2) **(Quiazon *et al.* 2009; Eissa *et al.* 2015).** On the contrary, those clusters assigned as "*Anisakis simplex / pegreffii hybrid* genotype. The main difference point between *Anisakis simplex* and A. *pegreffii* is the length of ventriculus, body length **(Davey, 1971; Mattiucc *et al.* 2005).** There is a clear difference was observed in the ventriculus length between *A. simplex* and *A. pegreffii*. The main criteria that discriminated between Type I and Type II Anisakis larvae was the ventriculus length whereas in type I ventriculus length (0.65–1.5 mm) while in type II

(0.52–0.75 mm); while the *A. pegreffii* the ventriculus length 0.50 – 0.78 mm but have mucron that the Anisakis type II did not have this mucron **(Quiazon *et al.* 2008).**

This study proved the importance of the combination of genetic and morphometric tools for accurate identification and differentiation between different Anisakis spp. The sequencing of ITS regions demonstrated its usefulness in detecting and distinguishing A. pegreffi from A. simplex and A. simplex x A. pegreffii hybrid. In this study, the A. pegreffii of this study grouped with other A. Pegreffii samples and separated from A. simplex and A. simplex x A. pegreffii hybrid. The present A. pegreffii, A. simplex and A. simplex x A. pegreffii hybrid displayed the highest similarity percentages with other relevant A. pegreffii, A. simplex and A. simplex x A. pegreffii hybrid, respectively. Accordingly, morphometeric and genetic evidence proved that these nematodes larvae belonging to the family Anisakidae and could be identified as A. pegreffii, A. simplex, and A. simplex x A. pegreffii infecting the Egyptian marine fishes.

Pathologically, Anisakis larvae utilize the triacylglycerols (TAG) deposited in fish muscle during their course of invasion/ migration. The depletion of TAG affect swimming capability of the infected fishes due to loss of energy with consequent general weakening and high liability to predation. Pathologically, the loss of the lipid deposit from the fish muscles facilitates the adhesions of visceral organs that result in granulomatous inflammatory responses with the modification of fish body shape with an ultimate poor health condition and poor growth **(Mika *et al.* 2010; Santoroa *et al.* 2010).**

The feeding behavior of horse mackerel allows them to contract larval Anisakis species from the infected mollusks and marine mussels with the early larval stages of anisakids. The early stage of Anisakis larvae developed into 3rd larval stage which can penetrate the intestinal wall to abdominal cavity and then to visceral organs with high tendency to gonads, kidney and spleen. The migration of the 3rd larval stages corrupted the gastric mucosal epithelia leading to poor digestion and absorption with ultimate poor growth rates **(Eissa *et al.* 2015).** The sex as a variable has proved an influential effect on the prevalence of *Anisakis* spp. infection among different examined Atlantic horse Mackerel. The percentage of infection was slightly higher in females (26%) than males (21%). These results relatively agreed with similar study published by **Qasim and Ayub, (2012)**. These results coincided with that of **Carvajal *et al.* (1979).** However, another study published by **Ozer, (2000)** has revealed that there are no differences in infection rate between male and female fish.

Moreover, **George-Nasciment *et al.* (1983)** have recorded that sexually mature females had significantly higher prevalence of infections with *Anisakis* spp. than had males in the Chilean Jack Mackerel ($P < 0.05$). The relative higher prevalence of nematode infections in females than males could be attributed to relative hormonal stress during the maturity and spawning periods in females. Generally, mature females are liable to diseases than males due to immunosuppressive nature of stress hormones (Corticosterols) that is known to be much higher in mature spawning females than males rendering them to be

vulnerable to infections than others **(Eissa *et al.* 2015).** Another interesting factor is the stomach fullness extent in females is much higher than males. This could allow females to apprehend larger numbers of larvae in their food than males with consequent increase in infection prevalence as well as intensity within fish organs. This hypothesis is supported by **Juras and Yamaguti, (1985)** study who declared that stomach fullness of females was higher than that of the males and immature fishes.

The prevalence of *Anisakis* spp. infection among Atlantic horse Mackerel is also variable according to the length / age of the examined fish. Previous studies have indicated that the prevalence of 3rd stage larvae of *Anisakis simplex* was highest in fishes with length 30-34 cm (6 years old) compared to other length / age categories **(Abaunza *et al.* 1995; Adroher *et al.* 1996)**. In our study the 240 examined fish samples were categorized according to age into 3, 4, 5- and 6-years categories. The statistical analysis presented a significant difference (Pr= <0.0001) considering the age / infection level variable. The results indicated that the older the fish the higher the number of larvae in their bodies and the maximum infection percentage was in the 6 years category (56 %). This higher infection prevalence could be attributed to the prolonged durations of exposure and predation along the long lifetime of such category. This hypothesis consistently agreed with that declared by **Smith, (1984)** who stated that the number of ascaridoid larvae that accumulate in marine fish is proportional with age, predatory behavior and increased feeding rates. Similar observations and explanations have been presented by **Smith and Wootten, (1978); Manfredi *et al.* (2000).**

The results also revealed that all examined five organs (gonads, stomach, intestine, pyloric ceca and abdominal cavity) were infected with L3 stage of *Anisakis* spp. at different intensity levels with noticeable significant difference (Pr = <0.0001). The highest intensity of infection was recorded in examined ovaries (28%). This result was consistent with that of **Tantanasi *et al.* (2012)** who have reported that most of L3 stage of *Anisakis* spp. retrieved from Atlantic horse mackerel were found in the gonads rather than on other organs.

CONCLUSION

In conclusion, Anisakid infection would present a vulnerable biological obstacle to the development and sustainability of Mackerel fisheries by their direct impact on growth, fertility and fecundity of both male and female fish. Moreover, their drastic impacts on intestinal, abdominal and gonadal health might be another critical factor that poses an indirect threat to the vital physiological process with consequent disruption of the fish's internal homeostasis. The sum of these biological growth, fecundity and health disruptors is an abundant decline of the mackerel fisheries through the southern coasts of the Mediterranean Sea. Ultimately, these highly invasive anisakid larvae represent a vulnerable threat to human health by their high zoonotic capability.

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