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RESEARCH ARTICLE

Mullus barbatus L. and *Mullus surmuletus* L. from western Mediterranean waters (SE Spain) are infected by *Hysterothylacium fabri*, but not by zoonotic nematodes. Possible impact on fish hosts[#]

Manuel Morales-Yuste | Jesús López-Valverde | Natalia Sánchez-Fernández | Jesús Veiga | Mario Garrido | Francisco Javier Adroher | Rocío Benítez

Departamento de Parasitología, Facultad de Farmacia, Universidad de Granada, Granada, Spain

Correspondence
Francisco Javier Adroher and Rocío Benítez, Departamento de Parasitología, Facultad de Farmacia, Universidad de Granada, 18071 Granada, Spain.
Email: fadroher@ugr.es and rbenitez@ugr.es

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Abstract

In order to know whether there is a risk of anisakiasis (or anisakidosis) by consumption of fish of the genus *Mullus* from the western Mediterranean Sea, which are appreciated for their quality, an epidemiological survey was carried out to evaluate the occurrence of zoonotic or potentially zoonotic nematodes in *M. barbatus* and *M. surmuletus*. Although the presence of the third larval stage (L3) of anisakids (*Anisakis* and *Contracaecum*) has been previously described in these fish, the results showed the absence of anisakids and the presence, never in muscle, of L3 and L4 of raphidascaridids of the genus *Hysterothylacium*, molecularly identified as *H. fabri*. Phylogenetic analysis groups them into the Mediterranean Sea clade, far from individuals isolated in the Pacific Ocean. Prevalence was slightly higher, but not significant, in *M. barbatus* versus *M. surmuletus* (72.3% vs 60.0%), but mean intensity (MI) and mean abundance (MA) parameters were approximately twice as high in *M. barbatus* as in *M. surmuletus* (MI 5.8 vs 2.8, $p = .001$; MA 4.2 vs 1.7, $p < .001$). The presence of the parasite seems to have different effects on these two sympatric species. In *M. barbatus* it seems to affect their growth, as it appreciably reduces the value of allometry coefficient in infected fish (2.78 vs. 2.18). On the other hand, in *M. surmuletus* the infection significantly ($p < .04$) affects the Fulton's condition factor, an indicator of the health status of the fish. It can be concluded that the ingestion of these fish by the people poses negligible risk of anisakiasis, but the consumer should continue to be urged to follow the rules of prevention against this illness.

KEYWORDS
Mullidae, Food safety, Public health, Anisakidae, Raphidascarididae, Spain

Manuel Morales-Yuste and Natalia Sánchez-Fernández are contributed equally to this work.

[#]Note that throughout the manuscript the authors use the term 'red mullets' in the plural to refer collectively to the two species of the genus *Mullus* surveyed. When used in the singular, they refer only to *Mullus barbatus* (red mullet). Not to be confused with mullets, family Mugilidae.

1 | INTRODUCTION

Mullus barbatus Linnaeus, 1758, commonly known as red mullet, and *Mullus surmuletus* Linnaeus, 1758, also called striped red mullet or surmullet, are two commercially valuable fish species belonging to the family Mullidae. Both species are found in the eastern Atlantic Ocean, ranging from the British Isles to Senegal, as well as in the Mediterranean and Black Sea. Despite being commonly caught at depths between 30 and 100 m, they can be found on the continental shelf—dwelling in sandy, muddy and gravelly bottoms—at depths ranging from 10 to 300 m, where they mainly fed on small benthic crustaceans, worms and molluscs (Froese & Pauly, 2024a, 2024b).

Several studies have documented the presence of anisakid larvae in both species (mostly *Anisakis* and to a lesser extent *Contracaecum*) throughout the Mediterranean Sea, but not in the Black Sea (e.g. Akmirza, 2013; Barcala et al., 2018; Carreras-Aubets et al., 2010; Goffredo et al., 2019; Pulleiro-Potel et al., 2015). As a result, consuming of these fish with *Anisakis*, mainly, may pose a potential risk of anisakiasis/anisakidosis (both infection and allergy) to the consumer (Adroher-Auroux & Benítez-Rodríguez, 2020). Larvae and adults of *Hysterothylacium* (e.g. Ferrer-Maza et al., 2015; Gelen & Pekmezci, 2023; Hristovski et al., 1995; Janiszewska, 1949; Petter & Maillard, 1988)—formerly in the family Anisakidae, now in the family Raphidascarididae—have also been detected throughout the Mediterranean Sea. Although they are unlikely to cause digestive disturbance (only one human case of *H. aduncum* occurrence in association with abdominal pain and diarrhoea has been reported (Yagi et al., 1996)), they should be monitored because of the possibility of an unsightly consumer rejection and the potential for *Anisakis*-like allergens in these nematodes, which may lead to an *Anisakis*-like allergic reaction (Cavallero et al., 2020; Fernández-Caldas et al., 1998; Valero et al., 2003).

The aim of this work was to evaluate the presence of anisakids, which cause anisakiasis in humans, and raphidascaridids in highly valued fish of the genus *Mullus* marketed in Granada (Spain) and originating from the western Mediterranean Sea (Levante fishing area). The presence of anisakids in commercial fish is frequent, so regular surveys to assess the prevalence and intensity of these parasites in fish are necessary, following the recommendations of the EFSA expert group (EFSA-BIOHAZ, 2010; EFSA-BIOHAZ Panel et al., 2024), as a main method for food safety and to predict the risk of human disease caused by these nematodes. In addition, we explored whether the health or the growth of these fish could be affected by the presence of *Hysterothylacium*, as some previous studies have suggested for these nematode species (Ferrer-Maza et al., 2015; Morales-Yuste et al., 2022; Vuić et al., 2022). Finally, the phylogenetic position of the isolated *Hysterothylacium* larvae within the parasite species identified was investigated.

2 | MATERIALS AND METHODS

2.1 | Host and parasites

In the city of Granada, 82 red mullets were purchased from fishmongers. The fish were caught from the Levante fishing area (SE Spain) in the western Mediterranean Sea (FAO area 37.1.1) by professional fishermen using bottom otter trawls at depths of approximately 30–100 m. The landing ports were Calpe, Mazarrón and Águilas (Figure 1). After landing, the fish are auctioned at the fishmarket and distributed to several marketing centres in Spain, including Granada. Fish were acquired in autumn 2022 and spring 2023 as they became available. Fish were kept cold with ice flakes from the capture to arrival at the laboratory in less than 24 h.

FIGURE 1 Map of Iberian Peninsula. Area of investigation (Levante fishing area) showing ports where red mullets were landed (•) and the fishmarkets sampled (o). Vertical and horizontal lines delimit the Levante fishing area.

Once in the laboratory, fish were identified to species level and measured to 0.1 cm accuracy and weighed to 0.01 g accuracy. From the total of 82 red mullets used in this study, 47 were identified as *M. barbatus* and 35 as *M. surmuletus* (Aguirre Villaseñor, 2000; Fischer et al., 1987). The Fulton's condition factor (CF) was calculated according to the formula $CF = 100\,W/L^3$ (Fulton, 1904; Nash et al., 2006), where *W* is the total weight of the fish and *L* its total length. It is generally considered that the relationship between weight and total length of the fish is cubic. In this sense, growth follows a potential curve of the type $y = a \cdot x^b$ in which the exponent *b* is 3 if the growth is isometric and different from 3 if it is allometric (Froese, 2006).

Each fish underwent an individual necropsy procedure where the digestive tract, viscera and musculature were separated, and thoroughly examined with a lighted magnifying glass for macroscopic nematodes. Next, in order to detect any hidden nematodes, viscera and musculature were digested separately with an aqueous solution containing 10 g/L commercial pepsin and 5 mL/L commercial hydrochloric acid (modified from Smith and Wootten (1975)), at pH ~2.0, at 37°C, with gentle agitation, for 1 and 2 h, respectively. Digested tissues were carefully re-examined to detect possible nematodes.

In all cases, the detected nematodes were washed with saline solution (NaCl, 0.9% w/v), examined under stereo and/or light microscopy for identification of morphological characters and morphologically classified to genus and, when possible, species level (Petter & Maillard, 1988; Tedesco et al., 2018). Every larvae were then placed individually in a properly labelled eppendorf and stored at −20°C until use.

2.2 | Genetic identification

From the total *Hysterothylacium* larvae collected, a subset of 37, 26 from *M. barbatus* and 11 from *M. surmuletus*, were subjected to molecular identification by PCR-RFLP (polymerase chain reaction-restriction fragment length polymorphism). For this, DNA was extracted from each larva with the RealPure kit, following the manufacturer's instructions. The rDNA fragment of the ITS1-5.8-ITS2 sequence was then amplified using NC5 (forward) and NC2 (reverse) primers according to Zhu et al. (1998). For PCR, a previously described procedure was followed (Buzo-Domínguez et al., 2021; Molina-Fernández et al., 2015). Amplification was performed using the following programming: one cycle of 94°C for 5 min, 60°C for 30 s, 72°C for 90 s; 35 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 60 s; and a final cycle of 94°C for 30 s, 60°C for 30 s and 72°C for 5 min, then cooled and stored at 4°C until use. From the PCR, an amplicon of about 1000 bp was obtained and subjected to RFLP, in which two restriction enzymes, *AluI* and *TaqI* (FastDigest, Thermo Scientific), were used individually at a final concentration of 0.5 U/μL and at temperatures of 37 and 65°C, respectively, for 10 min. For the separation of the digestion fragments, the digestion result was subjected to electrophoresis on 3% agarose gel and revealed ethidium bromide, showing a characteristic species banding for each restriction enzyme (Kijewska et al., 2002; Tedesco et al., 2018). For better recognition, two controls were used in each gel, one from *H. aduncum* and one from *H. fabri*, corresponding to *Hysterothylacium* species previously described morphologically in commercial fish of the genus *Mullus* from the western Mediterranean Sea (Ferrer-Castelló, 2015; Ferrer-Maza et al., 2015). For sequencing purposes, eight PCR products, one from L3 and three from L4 of each fish species, were selected, purified and sequenced in both directions using the facilities of the STAB-VIDA company (Lisbon, Portugal). For comparative purposes, one L4 of *H. fabri* isolated from *Citharus linguatula* from the Gulf of Cádiz, NE Atlantic Ocean (Figure 1; Morales-Yuste et al., 2022) was also processed. DNA sequences were edited and assembled to obtain the final consensus sequences based on both sequencing directions using Geneious v2023.2.1, which were compared with previously published data using BLAST tools (NCBI, National Center for Biotechnology Information). Furthermore, 115 additional sequences from the studied species were obtained from GenBank, and one sequence of *Contracaecum osculatum* (JX467663) was used as outgroup. Sequenced larvae and sequences obtained from Genbank were aligned by the ClustalW algorithm, and phylogeny was inferred by maximum likelihood. The best-fit nucleotide substitution model was selected based on Akaike Information Criteria (Posada & Buckley, 2004). The best-fit nucleotide substitution model was 'Transversion model' (TVM + G + I) which was used for the phylogeny analysis. Maximum likelihood phylogenetic analyses were performed using the functions *pml* and *optim.pml*. To improve the topology search, a stochastic rearrangement was chosen when optimizing the phylogenetic trees, together with the gamma ratio and the proportion of invariant sites (log-likelihood = −6423.996; gamma shape parameter = 0.22; the proportion of invariant sites = 0.26; nucleotide frequencies: $a = 0.23$, $c = 0.20$, $g = 0.29$, $t = 0.28$). To estimate the topology support, bootstrapping based on 1000 replicates was used (Felsenstein, 1985). Phylogenetic analyses were run with R software (R Core Team, 2013), using *Biostrings* (Pagès et al., 2021) to read the sequences, *msa* (Bodenhofer et al., 2015) to do the alignment, *ape* (Paradis & Schliep, 2019) and *phangorn* 2.7.1 (Schliep, 2011) to build the phylogeny, and *ggplot2* (Wickham, 2016) to explore the phylogenetic tree. The sequences generated in this study have been deposited in GenBank under accession numbers OR899265–OR899273.

2.3 | Epidemiological study and statistical analysis

The infection parameters prevalence, mean intensity and mean abundance as defined by Bush et al. (1997) were determined. The free software Quantitative Parasitology 3.0 (Reiczigel et al., 2019; Reiczigel & Rózsa, 2005), based on the theoretical framework developed by Rózsa et al. (2000), was used for their calculation and statistical comparison in order to address notoriously left-skewed

parasite frequency distributions. Prevalence was compared using Fisher's exact test whilst mean intensity and mean abundance were analysed with a two-sample bootstrap *t*-test employing 20,000 replicates. Additionally, the length, weight and CF of *Hysterothylacium*-uninfected and infected hosts were compared by Student's *t*-test.

To assess the impact of infection status (parasitised vs. non-parasitised) on the correlation between fish weight and fish length, separate ANCOVA analyses were carried out for each species. In these analyses, weight served as the response variable, while infection status (as a factor), length (as a covariate) and their interaction were considered as explanatory variables. Moreover, in order to evaluate the influence of parasitism on hosts, we conducted multiple Gaussian Generalised Linear Models (GLMs). These models incorporated abundance, prevalence and intensity as response variables, while hosts' length, weight and Fulton's condition factor were considered as explanatory variables. Finally, to examine the impact of the season on the association between parasite abundance and host fish length, weight and CF, we conducted several ANCOVAs, including these terms as covariates and seasons as a factor. Significant interactions between covariates and factors suggest that the relationship between abundance and covariates varies across seasons. Outlier detection was performed using the chi-squared test. All statistical analyses, except for those mentioned earlier, were conducted in R software (R Core Team, 2013) using the *car* (Fox & Weisberg, 2019) and *outliers* (Komsta, 2022) packages. Differences were considered significant when $p \leq .05$.

3 | RESULTS

3.1 | Fish hosts

A total of 47 *Mullus barbatus* and 35 *M. surmuletus* from the Levante fishing area on the Mediterranean coast of south-eastern Spain were examined (Figure 1). The mean total length (L) ± SD (standard deviation) for *M. barbatus* was 16.3 ± 0.8 cm, while the mean total weight (W) ± SD was 49.93 ± 7.42 g, and the mean condition factor (CF) ± SD was = 1.140 ± 0.084. Similarly, these parameters for *M. surmuletus* were 16.0 ± 1.2 cm, 50.47 ± 16.12 g and CF = 1.189 ± 0.171 (Table 1).

Figure 2 displays the growth curves for weight and length along with the corresponding potential equations, of the type $y = a \cdot x^b$, for both fish species. The data show that the growth of *M. barbatus* has an allometrically negative value ($b < 3$) while *M. surmuletus* has an allometrically positive value ($b > 3$) (Froese, 2006).

To evaluate the impact of *Hysterothylacium* parasitism on the growth of two species of red mullets, the growth curves of infected and non-infected fish were compared. Results are presented in Figures 3 and 4, illustrating the effects of parasitism on *M. barbatus* and *M. surmuletus*, respectively. Both fish species show a decrease in the allometry coefficient *b*. However, this decrease is remarkable ($\Delta b = 0.8$) and marginally significant ($p < .07$) for the former, whereas it is not significant for the latter.

TABLE 1 Descriptive and epidemiological parameters of *Hysterothylacium fabri* in *Mullus barbatus* and *M. surmuletus* coming from the western Mediterranean Sea.

Parameters	*Mullus barbatus*	*Mullus surmuletus*
Number of fish	47	35
Total length ± SD (cm) Range	16.3 ± 0.8 13.9–18.4	16.0 ± 1.2 13.3–18.4
Weight ± SD (g) Range	49.93 ± 7.42 35.21–74.94	50.47 ± 16.12 21.34–89.21
Condition factor ± SD Range	1.140 ± 0.084 0.996–1.390	1.189 ± 0.171 0.761–1.532
Prevalence (%) (CI 95%)	72.3 (57.4–84.4)	60.0^{ns} (42.1–76.1)
Mean intensity (range) (CI 95%)	5.82 (1–21) (4.76–7.47)	2.76* (1–11) (2.14–3.38)
Mean abundance (CI 95%)	4.21 (3.19–5.66)	1.66* (1.09–2.26)

Note: Prevalence = 100·N/F, mean intensity = A/N, mean abundance = A/F; where F is the total number of fish, N is the number of infected fish and A is the number of larvae. Comparison of *Hysterothylacium* prevalence between the two *Mullus* species studied using Fisher's exact test showed non-significant statistical differences (ns). However, a statistical comparison of mean intensity and mean abundance by a two-sample bootstrap *t*-test (with 20,000 replicates) showed high significance in both cases (*$p \leq .001$). The parameters of total length, weight and condition factor were not compared as these are two different fish species with their own biological characteristics.
Abbreviations: CI, confidence interval; SD, standard deviation.

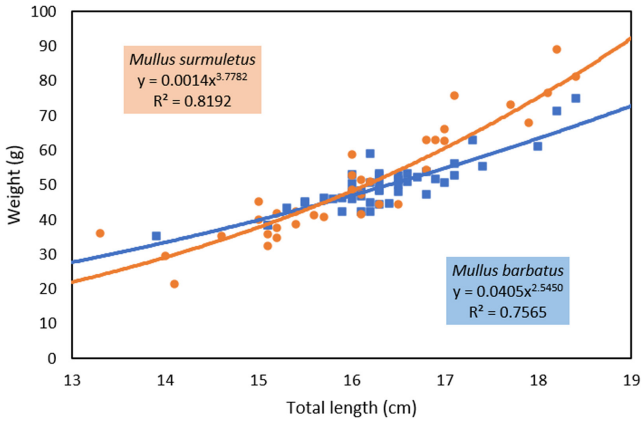

FIGURE 2 Potential weight–length relationship ($y = a \cdot x^b$) in *Mullus barbatus* ($n = 47$; blue square) and *M. surmuletus* ($n = 35$; orange circle). *M. barbatus* ± SD (standard deviation): exponent $b \pm 0.2176$, coefficient $a \pm 0.0246$. *M. surmuletus* ± SD: exponent $b \pm 0.3135$, coefficient $a \pm 0.0012$.

3.2 | Parasites identification

Thirteen third-stage (L3) and 243 fourth-stage larvae (L4) of *Hysterothylacium fabri* (Rudolphi, 1819) Deardorff & Overstreet, 1980 were morphologically identified (Petter & Maillard, 1988; Tedesco

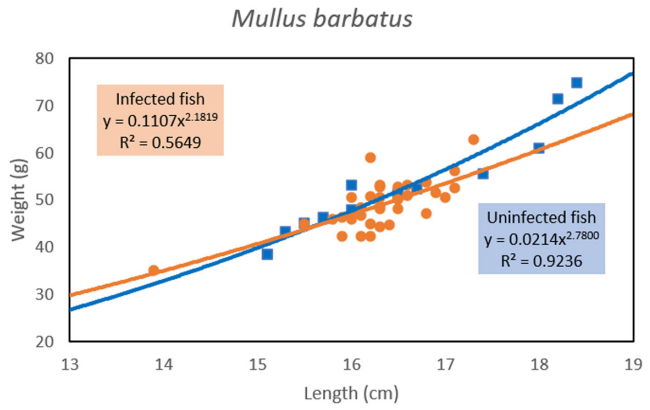

FIGURE 3 Potential weight–length relationship ($y = a \cdot x^b$) in *Hysterothylacium*-infected ($n = 34$; circle) and non-infected *Mullus barbatus* ($n = 13$; square). Infected ± SD: exponent $b \pm 0.3436$, coefficient $a \pm 0.1061$. Uninfected ± SD: exponent $b \pm 0.2509$, coefficient $a \pm 0.0151$.

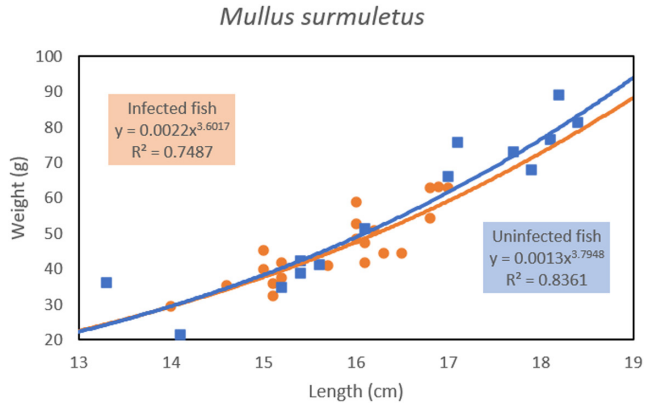

FIGURE 4 Potential weight–length relationship ($y = a \cdot x^b$) in *Hysterothylacium*-infected ($n = 21$; circle) and non-infected *Mullus surmuletus* ($n = 14$; square). Infected ± SD: exponent $b \pm 0.4905$, coefficient $a \pm 0.0030$. Uninfected ± SD: exponent $b \pm 0.5034$, coefficient $a \pm 0.0019$.

et al., 2018) from the examined red mullets. Larvae were discovered in the visceral cavity of the fishes, but none were found in their musculature or digestive tract. Some L3 were observed moulting into L4. One of these moulting larvae was seen passing through the stomach wall. All fish showing some L3 also showed L4. By host species, nine L3 (in eight fish) and 189 L4 were retrieved from the 34 infected *M. barbatus*. Of these, 26 (4 L3 + 22 L4 from 23 different hosts) were identified using PCR-RFLP. Of the 21 infected *M. surmuletus* 4 L3 (in four fish) and 54 L4 were recovered, 11 of them (1 + 10 from 9 different hosts) were subjected to PCR-RFLP identification. All processed larvae were confirmed as *Hysterothylacium fabri* (Rudolphi, 1819) Deardorff & Overstreet, 1980 (data not shown).

Sequencing worked successfully for the nine specimens assayed, obtaining consensus sequences of 782–938 base pair. All the parasite specimens, including the one from the Gulf of Cádiz (NE Atlantic), are identical (100% of identity) to several sequences of *H. fabri* from Tunisia (i.e. MF134409 or KX592169). The phylogenetic analysis (Figure 6) shows two major highly supported clades (bootstrap-value = 95.3%). One of them (Figure 6, group 4) includes mostly sequences from the Pacific Ocean isolated from East Asia (China and South Korea), but also two sequences from Brazil, one from Turkey and one from unknown origin. The other one includes sequences from the Mediterranean Sea isolated from Tunisia, Italy, Turkey and Spain. All of the sequences obtained in this study group in the Mediterranean Sea clade, were mixed among the other sequences. The phylogenetic tree also shows some smaller groups of sequences from China which diverges from the main Pacific Ocean clade.

3.3 | Epidemiological parameters

From the 82 red mullets examined 54 were parasitised with an intensity ranging from 1 to 21 larvae. Table 1 shows the prevalence, mean intensity and mean abundance of *H. fabri* in both *Mullus* species. *Mullus barbatus* exhibits a statistically significant higher mean intensity ($p = .001$) and mean abundance ($p < .001$), both more than twice the values observed in *M. surmuletus*.

Data separated by infection status can be seen in Table 2. Although the biological data of uninfected fish are higher than those of infected fish, no statistical differences were found, except for *M. surmuletus* weight ($p < .05$). Additionally, no significant relation between any of the infection parameters (prevalence, intensity and abundance) and with length or weight fish was detected. Yet, CF is influenced by infection status in *M. surmuletus* ($F_{1, 32} = 4.42$; $p = .04$; CF uninfected: 1.27 ± 0.05, CF infected: 1.16 ± 0.03), after one outlier removal (CF = 0.761; chi-squared test for outliers = 9.41, $p = .002$). Moreover, when the *M. surmuletus* data are separated by season (autumn vs. spring), it is observed that both the abundance-length and the abundance-weight relationships are season-dependent (ANCOVA season-length, $F_{1, 31} = 5.77$, $p = .023$; ANCOVA season-weight $F_{1, 31} = 4.35$, $p = .045$), being positive during spring and negative for autumn (Figure 5). No effect of season was found for *M. surmuletus* CF, nor for *M. barbatus* length, weight, or CF. Finally, the epidemiological parameters of the sampled fish are not dependent on the port of landing within the study area (Levante fishing area; Table 3).

4 | DISCUSSION

Previous studies performed in the Mediterranean basin have reported infection by different ascaridoid nematodes both in *Mullus barbatus* and *Mullus surmuletus*: *Hysterothylacium* sp. (L3), *Hysterothylacium aduncum* (L3, L4 and adults), *Hysterothylacium fabri* (L3, L4 and adults), *Contracaecum* sp. (L3) and *Anisakis pegreffii* L3 (Barcala et al., 2018; Carreras-Aubets et al., 2010, 2012; Ferrer-Maza et al., 2015; Hristovski et al., 1989, 1995; Janiszewska, 1949; Orecchia & Paggi, 1978; Petter & Maillard, 1988; Pulleiro-Potel

TABLE 2 Descriptive parameters of *Mullus barbatus* and *M. surmuletus* coming from the western Mediterranean Sea by infection status with *Hysterothylacium fabri*.

Fish species	Parameters	Non-infected	Infected
Mullus barbatus	Number of fish	13	34
	Total length ± SD (cm) Range	16.5 ± 1.2 (15.1–18.4)	16.3 ± 0.6^{ns} (13.9–17.3)
	Weight ± SD (g) Range	52.29 ± 11.29 (38.56–74.94)	49.04 ± 5.23^{ns} (35.21–62.88)
	Condition factor ± SD Range	1.160 ± 0.169 (1.047–1.296)	1.132 ± 0.089^{ns} (0.996–1.390)
Mullus surmuletus	Number of fish	14	21
	Total length ± SD (cm) Range	16.4 ± 1.6 (13.3–18.4)	15.8 ± 0.8^{ns} (14.0–17.0)
	Weight ± SD (g) Range	56.87 ± 21.22 (21.34–89.21)	46.21 ± 10.07* (29.48–63.05)
	Condition factor ± SD Range	1.233 ± 0.214 (0.761–1.532)	1.159 ± 0.133† (0.942–1.435)

Note: Comparison by Student's *t*-test between infected and non-infected fish: (ns) not significant; (*)$p < .05$; (†) when the condition factor (CF) outlier of one *M. surmuletus* (CF = 0.761; confirmed by chi-square test for outliers, $p = .002$) is removed, a clear statistical difference ($p < .04$) is observed in the condition factor between infected and non-infected hosts.

Abbreviation: SD, standard deviation.

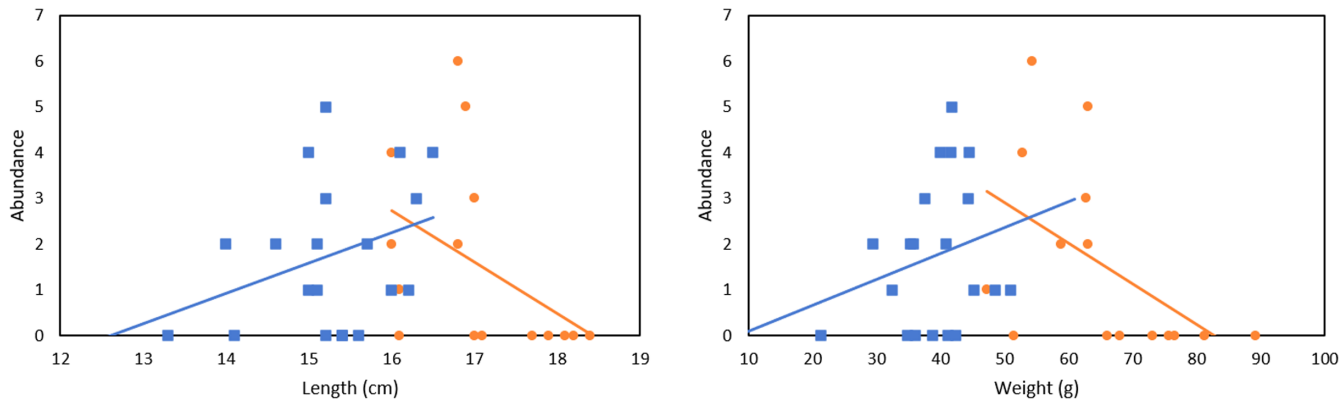

FIGURE 5 Effect of season (spring: blue squares; autumn: orange circles) on the relationship between *Hysterothylacium fabri* abundance in *Mullus surmuletus* versus host fish length and weight. Left: Abundance-Length plot: spring slope + 0.66, autumn slope −1.12. Right: Abundance-Weight plot: spring slope +0.06, autumn slope −0.09.

et al., 2015; Salati et al., 2013; Sardo et al., 2019; Schleicherová et al., 2023; Tedesco et al., 2018). *Anisakis* L3 type I and *Anisakis simplex* s.l. (L3) have also been reported in Italian waters, so both are probably *A. pegreffii* (Angelucci et al., 2011; Manfredi et al., 2000), the only species of *Anisakis* L3 type I found in *Mullus* in these waters. The occurrence of *Hysterothylacium reliquens* (larvae) in *M. barbatus* and *Dujardinascaris* spp. (adults) in *M. surmuletus* has also been reported (Hassan, 2019; Ramdani et al., 2022). These nematodes are usually found in the digestive tract, on the viscera or in the mesentery of these fish, but *Anisakis* larvae (Goffredo et al., 2019; Pulleiro-Potel et al., 2015) and *Hysterothylacium* larvae (Debenedetti et al., 2013) have occasionally been found in the musculature. However, in the present study, only L3 and L4 of *H. fabri* were found in the visceral cavity of both *Mullus* species, but not in the musculature or digestive lumen. To the best of our knowledge, only three reports with molecular identification of *Hysterothylacium* spp. in red mullets have been published worldwide (Gelen & Pekmezci, 2023; Şimşek et al., 2021; Tedesco et al., 2018), and the current study is the first in *Mullus* spp. from FAO area 37.1.1.

The individuals of *H. fabri*, including the one from the Gulf of Cádiz (NE Atlantic), are closely related to those from the Mediterranean Sea studied by other authors (Costa et al., 2018; Simsek et al., 2018; Tedesco et al., 2018), which are clearly differentiated from the ones from the Pacific Ocean (Figure 6). The differentiation of *H. fabri* into two major clades has been previously reported (Tedesco et al., 2018), but with fewer sequences. To address whether isolation

TABLE 3 Epidemiological parameters of *Hysterothylacium fabri* in *Mullus barbatus* and *M. surmuletus* coming from the western Mediterranean Sea by port of landed.

Fish species	Parameters	Calpe	Mazarrón	Águilas
Mullus barbatus	Number of fish	27	20	–[a]
	Prevalence (%) (CI 95%)	74.1 (53.7–88.9)	70.0 (45.7–88.1)	–
	Mean intensity (range) (CI 95%)	6.00 (1–21) (4.50–8.75)	5.57 (1–11) (4.14–7.07)	–
	Mean abundance (CI 95%)	4.44 (3.00–6.74)	3.90 (2.45–5.45)	–
Mullus surmuletus	Number of fish	10	10	15
	Prevalence (%) (CI 95%)	60.0 (29.1–85.0)	80.0 (44.7–96.3)	46.7 (21.3–73.4)
	Mean intensity (range) (CI 95%)	2.17 (1–3) (1.50–2.50)	2.75 (1–5) (1.50–3.75)	3.29 (1–6) (2.00–4.43)
	Mean abundance (CI 95%)	1.30 (0.50–2.00)	2.20 (1.00–3.20)	1.53 (0.60–2.67)

Note: Prevalence = 100·*N*/*F*, mean intensity = *A*/*N*, mean abundance = *A*/*F*; where *F* is the total number of fish, *N* is the number of infected fish and *A* is the number of larvae. Comparison of *Hysterothylacium* prevalence between landed ports using Fisher's exact test showed statistically non-significant differences. Similarly, a pairwise statistical comparison of mean intensity and mean abundance using a 2-sample bootstrap *t*-test (with 20,000 replicates) showed statistically non-significant differences.

Abbreviation: CI: confidence interval.

[a]Only striped red mullet (*M. surmuletus*) were collected from the port of Águilas.

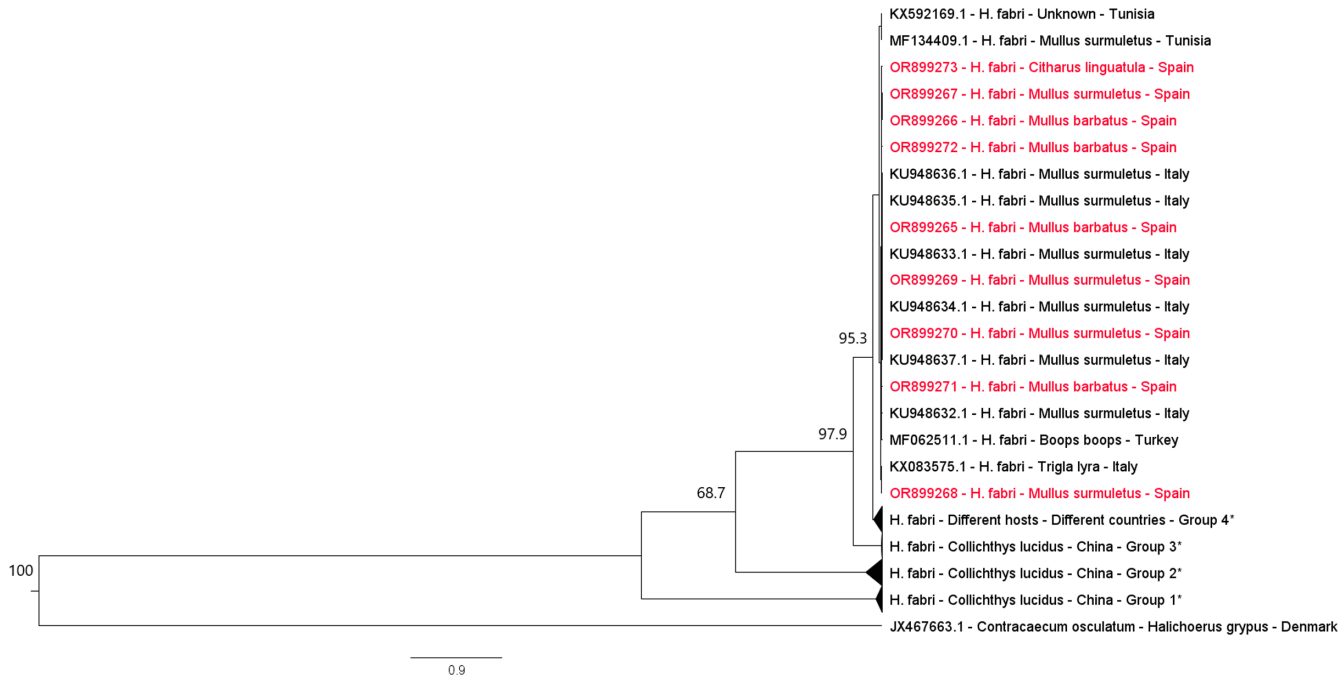

FIGURE 6 Phylogenetic tree based on ITS1-5.8-ITS2 sequences, with maximum likelihood topology, of *Hysterothylacium fabri* from Spain, together with sequences from GenBank. The bootstrap values are only shown on the main clades. Samples sequenced in this study are highlighted in red (OR899265-OR899273). (*) Details of the sequence groups are summarised in Table S1.

by distance may promote genetic differentiation within this species, further research of sampling across a wide gradient of distances and genetic structure analysis with the genetic markers optimized to this type of analysis will be essential (Teske et al., 2018).

As mentioned above, at least one moulting L3 was found passing through the stomach wall of the host. Little is known about the developmental cycle of *H. fabri*, so one might consider the possibility that the moult from L3 to L4 could occur in the passage from

the stomach lumen of the fish to the visceral cavity. Due to the low number of L3 found in our study, we cannot say for sure, but it is clear that the large number of L4 found in the visceral cavity could support this hypothesis. In this sense, other authors (Gelen & Pekmezci, 2023; Tedesco et al., 2018) found L3 only in the digestive tract of *M. surmuletus* while L4 were in the serosa, surface of internal organs and visceral cavity. Therefore, *H. fabri* L3 will have histolytic enzymes that allow it to carry out this migration, as has been shown in a related species such as *H. aduncum* (Malagón, Adroher, et al., 2010; Malagón et al., 2011, 2013; Malagón, Díaz-López, et al., 2010).

The prevalence of *H. fabri* is higher in *M. barbatus* (72%) than in *M. surmuletus* (60%), despite not being statistically significant (Table 1). In the few number of studies in neighbouring areas of the western Mediterranean (Figure 1; FAO area 37.1.1) very high prevalences were found for both *M. barbatus* (>87% NE Balearic Sea coast (Carreras-Aubets et al., 2010, 2012); 63.9% eastern Balearic Sea coast (Ferrer-Maza et al., 2015)) and *M. surmuletus* (66% on the coast of Oran, Algeria (Hassani et al., 2015)). Other studies on *M. surmuletus* carried out in this FAO area reported variable prevalences of infection by *Hysterothylacium*, depending on the site of capture. Thus, in the Catalan coast, in the Levante fishing area and in the NE Alboran Sea, high prevalences were found (69.5%–88.5%), while in adjacent or close sites, prevalences are much lower, as is the case of the Gulf of Valencia (17.5%) and the NW Alboran Sea (33%) (Ferrer-Castelló, 2015). The unique oceanographic features and the distinct characteristics of the marine ecosystems could explain these varying outcomes. In this study, similar epidemiological parameters (Table 3) were detected between red mullets from Calpe port (in the northern part of the Levante fishing area) and those from Mazarrón and Águilas ports, approximately 180 km further south (Figure 1).

However, in the cited studies parasites were identified only to genus level (probably including *H. fabri* and *H. aduncum*). Although, it is not yet clear which animals act as first intermediate hosts for *H. fabri*, Arculeo et al. (1997) reported a significant correlation ($p < .01$) between the degree of infection by *H. fabri* and the frequency of amphipods in the diet of *M. surmuletus*, but no correlation with other prey. Amphipods and other crustaceans (such as copepods) are known hosts of other *Hysterothylacium* species and could therefore be good candidates to play this role also in the case of *H. fabri* (Adroher-Auroux & Benítez-Rodríguez, 2021; Luque et al., 2007; Navone et al., 1998). In this regard, Aguirre Villaseñor (2000) reports that fish smaller than 10 cm of *M. barbatus* consume more copepods than those of *M. surmuletus* and that in fish larger than 10 cm, the former consumes more amphipods than the latter. If amphipod consumption is correlated with *H. fabri* parasitism in *M. surmuletus* (Arculeo et al., 1997), it could be assumed that this correlation also exists with *M. barbatus*. Moreover, the latter, by ingesting more prey (potential hosts of *H. fabri*) than *M. surmuletus*, will probably show a higher parasite intensity and abundance, justifying our results of a mean intensity and mean abundance more than twice as high in the former as in the latter ($p \leq .001$; Table 1). This could indicate that the diet or feeding habits of *M. barbatus* facilitate a higher parasite load without significantly affecting the infection rate (prevalence). In fact, the abundance of macrobenthic invertebrates (such as amphipods, gastropods, decapods, isopods and mites) has been reported to vary significantly in relatively close marine areas along the Spanish Mediterranean coast, at least in the Levante fishing area (Sánchez-Jerez et al., 1999). This suggests that local differences in infection levels (Table 3) could be due to a different qualitative and/or quantitative composition of intermediate (Karvonen et al., 2005) and, in our case, also definitive hosts of *H. fabri*. In this context, the presence of *Uranoscopus scaber* (stargazer), a known definitive host of *H. fabri* (Tedesco et al., 2018), has been recorded in the study areas, especially associated with the muddy detrital beds (present in all the areas studied (Ministerio para la Transición Ecológica y el Reto Demográfico. Costas y Medio Marino. Protección de la Costa, 2023a, 2023b)) and the biocenosis of adjacent coastal detrital beds (which occur especially in Águilas and Mazarrón (Ministerio para la Transición Ecológica y el Reto Demográfico. Costas y Medio Marino. Protección de la Costa, 2023b)). Fish of the genus *Mullus* are also found in these areas, together with the *Posidonia* meadows where numerous marine invertebrates are abundant and form part of their diet, especially polychaetes (Aguirre Villaseñor, 2000; García-Charton et al., 2006; Jaramillo-Londoño, 2009; Ministerio para la Transición Ecológica y el Reto Demográfico, 2023). The latter has been described as transport and/or intermediate hosts for *H. aduncum* (González, 1998; Køie, 1993) and possibly also for *H. fabri*.

It is noted, especially in *M. surmuletus*, that there is no parasitism in older fish of around 2 years (>17 cm, Figure 4; Aguirre Villaseñor, 2000; Reñones et al., 1995). Although these *Mullus* species share habitat and prey, the bathymetric (Reñones et al., 1995) and trophic (Aguirre & Sánchez, 2005; Bautista-Vega et al., 2008; Labropoulou & Eleftheriou, 1997) partitioning between species may partially explain the epidemiological results. While older *M. surmuletus* could include up to 20%–25% of small fish in the diet (Labropoulou et al., 1997; Labropoulou & Eleftheriou, 1997), invertebrates (potential first intermediate hosts) such as crustaceans and polychaetes remain their primary prey in *M. barbatus* (Chérif et al., 2011; Stergiou & Karpouzi, 2002). Complementarily, other factors should be considered, such as the death and/or predation of weaker infected individuals, the enhancement of the hosts' immune response with maturity allowing parasite elimination, limitation of the parasite's life in the host, or behavioural changes of infected fish avoiding fishing grounds (Buzo-Domínguez et al., 2021; Molina-Fernández et al., 2018; Morales-Yuste et al., 2022; and references therein). In this sense, infection by *H. fabri* appears to have a differential impact on red mullets species. The infection's impact on *M. surmuletus*' CF (lower CF; $p = .04$; see footnote of Table 2) may enhance hosts' susceptibility to predation or mortality. Yet, *M. barbatus* may tolerate higher parasitic loads, as affect its growth but not CF (Figures 2 and 3, Table 2). This suggests that *M. barbatus* could act as a second intermediate host for *H. fabri* in the study area before being preyed upon by potential definitive hosts, such as *U. scaber* (Adroher-Auroux & Benítez-Rodríguez, 2021; Arculeo et al., 1997; Froese & Pauly, 2024c; García-Charton et al., 2006; Tedesco et al., 2018).

In any case, it is necessary to verify that the season of capture does not influence these results. Statistical analyses showed that epidemiological parameters did not vary between seasons in either species (data not shown). However, descriptive data of the fish were significantly different only in the case of *M. surmuletus* (data not shown). On the other hand, statistical analyses also showed that the interaction between condition factor and abundance did not differ between autumn and spring, i.e. parasitisation and its effect on CF was not related to the season of capture (Figure 5). We believe that these data confirm the differences in CF found between *M. surmuletus* parasitised by *H. fabri* and those not parasitised by *H. fabri* (Figure 4 and Table 2).

When relating infection parameters of *Mullus* fishes, some studies count together specimens of the genus *Hysterothylacium* alone or together with anisakid larvae, so that no relationship can be established between size/age of red mullets and *H. fabri* infection. However, this has been attempted with inconsistent results (Öztürk & Yesil, 2018; Saadi et al., 2020; Serracca et al., 2013), probably because they only identify to genus level, indicating the presence of *H. aduncum* and *H. fabri* together. It is well recognized that these parameters of *H. aduncum* tend to increase with host age (Adroher et al., 1996; Rello, Adroher, & Valero, 2008; Rello et al., 2009; Ruiz-Valero et al., 1992), although not in all fish studied (Rello, Valero, & Adroher, 2008; Valero et al., 2000), but there are few data on *H. fabri* (Öztürk & Yesil, 2018). For example, in fish of the genus *Phycis* there are discrepant data showing that the prevalence of *H. fabri* increases with host size (Farjallah et al., 2006), although in other studies it is maintained in fish larger than 30 cm (Valero et al., 2006) irrespective of species. In any case, prevalence in *P. phycis* (up to 80%) is much higher than in *P. blennoides* (<10%; Farjallah et al., 2006; Valero et al., 2006). This indicates that not all fish are appropiate hosts for this parasite and that prevalence and intensity may vary depending, among other factors such as diet, on the host and the study area. In our case, as the study area is the same and trophic differences before ca. 2 years of age are scarce (they mainly consume crustaceans and polychaetes; Aguirre & Sánchez, 2005; Bautista-Vega et al., 2008; Chérif et al., 2011; El Bakali et al., 2010; Gharbi & Ktari, 1979; Labropoulou et al., 1997; Stergiou & Karpouzi, 2002), we could assume that *M. barbatus* is a more suitable host for *H. fabri*, in the sense that it survives with a higher parasite load, thus ensuring definitive host infection.

Finally, *Hysterothylacium* larvae were not found in the musculature from red mullets, so the risk of a hypothetical allergic reaction due to common allergens with or similar to those of anisakids (Cavallero et al., 2020; Fernández-Caldas et al., 1998; Valero et al., 2003), due to their phylogenetic proximity, is practically negligible, as these fish are eviscerated before consumption, at least in Spain.

5 | CONCLUSIONS

First, *H. fabri* infection appears to affect the two *Mullus* species differently despite their phylogenetic proximity and sympatry. While *M. barbatus* seems to be negatively affected in growth by decreasing its allometry coefficient, *M. surmuletus* decreases Fulton's condition factor, generally accepted as a measure of the fish's health status (Monstad, 1990). Further studies are needed to confirm that these effects are caused by *H. fabri* and that other uninvestigated pathogens are not involved.

Second, the non-detection of anisakids such as *Anisakis* spp., previously described in these hosts (Angelucci et al., 2011; Barcala et al., 2018; Carreras-Aubets et al., 2010; Goffredo et al., 2019; Manfredi et al., 2000; Pulleiro-Potel et al., 2015; Saadi et al., 2020; Salati et al., 2013), means that there is negligible risk of infection (the risk cannot be absolutely excluded in case of allergy) for the population that consumes these fish from this source. However, the population must continue to comply with preventive measures and the health authorities must continue to implement health education campaigns so that the population does not forget the good culinary practices that prevent anisakiasis, regardless of the fish consumed, by subjecting it at −20°C > 48 h or at >60°C > 1 min in the whole piece (Adroher-Auroux & Benítez-Rodríguez, 2020).

AUTHOR CONTRIBUTIONS

Manuel Morales-Yuste: Conceptualization; methodology; formal analysis; writing – original draft; writing – review and editing; supervision. **Jesús López-Valverde:** Methodology; investigation. **Natalia Sánchez-Fernández:** Methodology; investigation. **Jesús Veiga:** Data curation; writing – original draft; writing – review and editing; formal analysis. **Mario Garrido:** Data curation; writing – original draft; writing – review and editing; formal analysis. **Francisco Javier Adroher:** Conceptualization; writing – original draft; writing – review and editing; validation; funding acquisition; data curation. **Rocío Benítez:** Conceptualization; writing – original draft; writing – review and editing; validation; funding acquisition; supervision.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. The sequences of the larvae generated in this study can be consulted in the GenBank database, where they have been deposited under accession numbers OR899265–OR899273.

PATIENT CONSENT STATEMENT

Not applicable.

PERMISSION TO REPRODUCE MATERIAL FROM OTHER SOURCES

Not applicable.

CLINICAL TRIAL REGISTRATION

Not applicable.

ORCID

Manuel Morales-Yuste https://orcid.org/0000-0001-8414-5052
Jesús López-Valverde https://orcid.org/0009-0008-2203-0389
Jesús Veiga https://orcid.org/0000-0003-1140-2411
Mario Garrido https://orcid.org/0000-0001-7899-8913
Francisco Javier Adroher https://orcid.org/0000-0002-7969-6658
Rocío Benítez https://orcid.org/0000-0003-4680-1148

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