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Infection process, viability and establishment of *Anisakis simplex s.l.* L3 in farmed fish; A histopathological study in gilthead seabream.

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ABSTRACT

Anisakis spp. (Nematoda, Anisakidae) are parasites known by their economic and health impacts, as their L3 larval stages infect a variety of fish species, many of them commercial species, sometimes causing zoonotic episodes due to consumption of raw or undercooked fish. The aim of this study is to determine the infection process and the potential impact of *A. simplex s.l.* L3 on gilthead seabream (*Sparus aurata* L.), one of the most important fish species in Mediterranean aquaculture, by periodic histological monitoring of the infection process. For this, fish were experimentally infected with *A. simplex s.l.* L3 and periodically analysed for L3 larvae, collecting samples at different time points (hours post ingestion, hpi): 3, 6, 12, 18, 24, 36, 48, 72, 96, 120, 144, 168 and 192, up to 6 months post infection (mpi). All samples were observed under a stereomicroscope and later fixed for histological examination. *A. simplex s.l.* L3 were only found on the visceral surface and mesenteric tissue, but never free or encapsulated in muscle. Chronological events were found to occur faster than those reported in previous studies. They were first observed 6 hpi in the coelomic cavity, being present up to 48 hpi. While the earliest evidence of fibrocytes surrounding *A. simplex s.l.* L3 larvae were observed at 18 hpi, complete spiral encapsulation occurred by 72 hpi. Alive parasites were observed up to 6 mpi. Although the infection of gilthead seabream by *Anisakis* spp. larvae is feasible, it seems unlikely, especially in aquaculture given the hygienically controlled feeding systems. In the event of infection, the transmission would be unlikely due to the poor condition in which specimens of *Anisakis* spp. are found. Furthermore, since no larvae were detected in the fish's muscle, human infection seems improbable.

1. Introduction

Third larval stages of the nematode *Anisakis* spp. (Ascaridoidea, Anisakidae) are commonly found encapsulated in the muscle and connective tissue of fishes for human consumption (Rahmati et al., 2020). Humans become accidental hosts by ingesting the larvae in raw, undercooked, or inadequately processed fish. In humans, the parasites can remain alive in the gastrointestinal mucosa or even invade the visceral mesenteries, causing diarrhoea, vomiting and abdominal pain as the most common symptoms (Adroher-Auroux and Benítez-Rodríguez, 2020). The release of antigens in the areas surrounding the parasite can cause allergic reactions that can lead to anaphylaxis in hypersensitized patients (Alonso-Gómez et al., 2004). *Anisakis* spp. are therefore an important issue of public health concern, especially in geographic areas where the fish consumption is high (Bao et al., 2019; Rahmati et al., 2020).

Many fish species commonly consumed by humans have been found infected by these nonspecific parasites (Gibson et al., 2005; Aibinu et al., 2019). Despite the wide range of hosts for these nematodes, there are other potential hosts that have not been reported to host the pathogen, either because they are less susceptible to this parasite or because they are not part of food webs that include infected intermediate or definitive hosts (Balbuena and Raga, 2009). Aquaculture-reared fish is considered the safest alternative when it comes to consumption of raw or lightly processed fish, because of the controlled environment and the regulated feeding strategies (pathway of parasitisation in fish) (Fioravanti et al.,

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2021). Farmed fish are fed with dry feed, which hardly has any risk of infection with the parasite (APROMAR, 2012). Allergists recommend consumption of species with lower risk to *Anisakis*-allergic patients, either because they are less susceptible to *Anisakis* spp., or because they live in habitats where the parasite is not present. For this reason, farmed species are also recommended by allergists to avoid allergic reactions (García et al., 2005). Moreover, *Anisakis* spp. are generally transmitted among pelagic paratenic hosts, therefore coastal species such as gilthead seabream (*Sparus aurata* L.) are considered less likely to be infected (Pita et al., 2002; Guardone et al., 2020).

However, these parasites can affect aquaculture through alternative infection paths based on ingestion of encapsulated larvae in invertebrates trespassing the cages or free L3 larvae in the water (Køie, 2001). In addition, some species, such as bluefin tuna (*Thunnus* spp.), are also fed pieces of fish, likely creating a new infection route for farmed fish, if bait is not properly handled. Gilthead seabream is one of the main fish species in Mediterranean aquaculture and along with European seabass, *Dicentrarchus labrax* (L.), one of the first species in terms of production. The global production of gilthead seabream in 2019 was about 228,576 tonnes, with an estimated 252,406 tonnes in Europe and Mediterranean countries and 13,521 tonnes in Spain. Seabream aquaculture accounts for 94.3% of the total sales of this species in the market, and only a small fraction reaches Spanish fishing ports as catch (836 tonnes in 2018) (APROMAR, 2020).

According to some surveys, such as those by Peñalver et al. (2010) and Fioravanti et al. (2021), *Anisakis* sp. in gilthead seabream and European seabass was not present in European aquaculture facilities. Nevertheless, some *A. pegreffii* Campana-Rouget & Biocca, 1955 were found in *D. labrax* from aquaculture facilities (Cammilleri et al., 2018), and other anisakid, *Contracaecum* sp., in farmed and wild *S. aurata* (Salati et al., 2013; Guardone et al., 2020). The high non-specificity, as well as the existing possibility of infection by other routes, suggests that these results are the outcome of the low probability of infection and not to the non-susceptibility of gilthead seabream. In fact, *A. pegreffii* has been described on the mucosa of experimentally infected gilthead seabream, proving wrong the non-susceptibility of this species to *Anisakis* spp. (Marino et al., 2013).

Despite the difficulty of transmitting these species in aquaculture systems, it is essential to know the real infection potential of *Anisakis* spp. in gilthead seabream via food intake. This will help us to consider prophylactic measures to keep seabream in its "*Anisakis*-free" condition in aquaculture. Therefore, the main objective of this work is to determine the infection success of *Anisakis* spp. in gilthead seabream by (i) histologically monitoring the infection process and parasite migration within the fish, and (ii) determining the chronology and infection success. Special interest has been placed to study the infection of the musculature, as it is the main part destined for human consumption.

2. Materials and methods

2.1. Fish and parasite collection and identification

A total of 44 juvenile specific-pathogen-free (SPF) gilthead seabreams (mean body weight ± SD (range): 30.2 ± 7.5 (27.0 – 42.1) g) were supplied by a hatchery in Burriana (Castellón, Spain), and maintained in 3000 L tanks in sea water (37‰) at 21 – 22 ℃ at the installations of SCSIE (Central Support Service for Experimental Research, University of Valencia) until their use in different experimental assays.

Anisakis sp. third larval stage (L3) were extracted from blue whiting *Micromesistius poutassou* (Risso, 1827), from extractive fishing of Rías Bajas (Galicia, Spain, 42° 15'N 8° 45'O), acquired in commercial markets of Valencia (Spain). *Anisakis* spp. larvae were found encapsulated in the mesentery or already excapsulated. Then, larvae were observed in saline solution under a binocular stereomicroscope (Leica MZ APO), and carefully excapsulated with a fine needle, when necessary. Only larvae that showed high activity/vitality and undamaged cuticle were selected. Excapsulated larvae were placed in 25-cell-well plates (2 cm^3) containing saline solution used in experimental infections. Ascaridoid nematodes of the genus *Hysterothylacium* (Raphidascarididae) were also found but discarded for the experimental study.

The morphological examination confirmed that all anisakids were *Anisakis simplex sensu lato.* Larvae were classified in vivo according to three lengths: < 0.5 cm (hereafter, "small"); 0.5–1.5 cm (hereafter, "medium"); > 1.5 cm (hereafter, "large"); the majority of L3 of *Anisakis* spp. found in the blue whitings were large-sized worms. To reduce the morphological variability of the larvae, only large *Anisakis* spp. L3 were used for experimental infections. To explore the genetic variability of the worms, 24 large-sized worms were preserved in 100% ethanol for molecular analyses, three of them extracted from three *S. aurata* six months after the experimental infection. Additionally, one small and three medium-sized worms were also preserved in 100% ethanol for molecular studies. For DNA extraction, one small, three medium and 24 large *Anisakis* spp. specimens were dissolved in 400 µL TNES-urea buffer (10 mM Tris-HCl, pH 8, 125 mM NaCl, 10 mM EDTA, 0.5% SDS and 4 M urea) with overnight digestion using 50 µg mL^{-1} proteinase K at 55 °C. Following a phenol-chloroform protocol for DNA extraction (Holzer et al. 2004), DNA was resuspended in 50–100 µL RNAse/DNAse-free water and left to dissolve overnight in the fridge.

Polymerase chain reaction amplifications (PCRs) were conducted with a programmable thermal cycler (LifePro, Bioer) in a final volume of 10 µL, containing ~0.5 units of Taq-Purple DNA polymerase (Top-Bio), the related 10 × buffer $MgCl_2$ (15 mM), 0 dNTPs (10 mM), 0.5 µM of each primer and approximately 100 ng of template DNA. The mitochondrial Cytochrome c Oxidase subunit 2 (COX2) was amplified and sequenced using the PCR primer combination 210 (5 '- CACCAACTCTTAAAATTATC – 3') and 211 (5 '- TTTTCTAGTTATATAGATTGRTTTAT – 3') (Nadler and Hudspeth, 2000). Cycling conditions consisted of initial denaturation at 94 ºC for 3 min, followed by 34 cycles of 94 ºC for 30 s, 46 ºC for 1 min s, 72 ºC for 90 s, and a final extension at 72ºC for 10 min. DNA amplicons were visualized with a 1% agarose gel in TAE buffer, then purified using the Gel/PCR DNA Fragments Extraction Kit (Geneaid Biotech Ltd., USA) and commercially sequenced (Seqme, Czech Republic). Contiguous sequences of COX2 were assembled and inspected for errors using Geneious® v1.1 (Biomatters, Auckland, New Zealand) and blasted in GenBank for similarity with previously published records. The obtained sequences of the large-sized specimens were compared with others previously published for *A. pegreffii* and *A. simplex* (Rudolphi, 1809) (Valentini et al., 2006; Mattiucci et al., 2014; Cipriani et al., 2018). Seven specimens revealed 98.60–99.27% similarity (KY565560.1) and 98.95–99.63% similarity (MW324554.1) to *A. pegreffii*; and 17 specimens revealed 98.25–99.44% similarity (DQ116426.1) and 98.77–99.82% similarity (KC810003.1) to *A. simplex*. *Anisakis pegreffii* specimens showed up to 1.08% (0–6 bp) differences between them over 553–573 bp, and up to 1.45% (0–8 bp) differences for *A. simplex* specimens over 536–573 bp. Interspecific differences ranged between 4.04% and 5.29% (22–30 bp). All nematodes extracted from experimental infections belonged to *A. pegreffii*. Small size larvae revealed 99.81% similarity over 539 bp (MN624205.1) to *A. pegreffii* and medium size larvae revealed between 99.83% and 100% similarity over 595 and 574 bp with *A. simplex* (KC810003.1). Nine of the paraffin-embedded tissues of experimentally infected *S. aurata* were used for DNA extraction following the modified procedures described by Mattiucci et al. (2011), however, no sequences could be extracted.

2.2. Experimental infection of S. aurata with Anisakis simplex s.l

A fixed dose of ten *Anisakis simplex s.l.* L3 specimens was administered into the pharynx and oesophagus of each gilthead seabream using a 0.4 cm wide plastic syringe with a blunt. Larvae were, mixed, with a moist paste prepared with commercial pelleted feed (Skretting D2). Fish were previously anesthetized with MS222 (tricaine methanesulfonate,

0.03% solution buffered in seawater; Sigma-Aldrich) and later reanimated in a recovery bath containing clean, oxygenated seawater. They were then maintained in 3000 L tanks with constant filtration and aeration (37‰ salinity, at 21–22ºC). Fish were experimentally infected and periodically analysed for L3 larvae for eight days. No remains of rejected or vomited larvae were observed neither in recovery tanks nor in the bottom and surface filters of the maintenance tanks (filters cleaned twice a week). Samples were collected at different hours post ingestion (hpi), 3, 6, 12, 18, 24, 36, 48, 72, 96, 120, 144, 168, 192, and up to 6 months post infection. Fish were killed with an overdose of MS222 and all samples were observed under the stereomicroscope and later fixed for their histological examination. Muscle tissue from all samples was analysed for infection with *A. simplex s.l.* L3 by enzymatic digestion using the methodology described in Bier et al. (2009).

2.3. Histological examination and SEM

The viscera were preserved for histological processing and fixed in 4% saline formaldehyde. After fixation, samples were rinsed with distilled water, dehydrated with a series of ethanol at increasing concentrations (with variable duration according to sample size/thickness) and sequential baths of xylene followed by a xylene/paraffin bath (v/v). Samples were then embedded in melted paraffin (melting point 56ºC) in three successive baths. After paraffin inclusion and preparation of the blocks, 5 μm thick histological sections were obtained using a microtome (Leica RM 2125RT), stained with hematoxylin and eosin and mounted in Entellan®, Merck. For the follow-up of the infection process, the histological sections were observed with light microscopy (Leica DMR) for the presence of *Anisakis* sp., including encapsulated and excapsulated individuals, as well as evidence of remnants of old infections. In cases where the evidence of encapsulation was observed, the nematodes capsule thickness was measured using the Image J software programme, avoiding oblique sections of the capsule in order to standardize measurements of the thickness.

To investigate potential injuries in the *Anisakis* sp. cuticle caused by the infection process, worms found within the coelomic cavity of 6-month infected fish were fixed in 2.5% glutaraldehyde, dehydrated in an alcohol series (80–100%), critical-point dried, covered with palladium-gold and examined under a Hitachi S-4800 scanning electron microscope (Hitachi High Technologies Co Ltd, Tokyo, Japan).

3. Results

3.1. Infection rates

The overall prevalence of *Anisakis simplex s.l.* L3 in *S. aurata* was 54.6% (n = 44) and the prevalence of the 19 fish analysed 6 months post infection was 63.2%, with a mean intensity of 1.5 parasites per infected fish and a maximum number of four parasites in one fish. Of the 440 worms administered to fish, 45 were found encapsulated in fish viscera (10.2% recovery rate); however, several worms showed relevant internal and external damage (see *Infection Process*).

3.2. Infection process

Fresh and histological observations showed the course of infection of the free larvae within the digestive lumen until their encapsulation in the coelomic cavity (mesentery and serosa of the liver), mostly in spiral shape (Fig. 1). No larvae were found in muscle tissue, peritoneum, or skin. At 3 hpi, L3 were already found in the digestive tract (Fig. 2A); some unbroken worms were found even up to 48 hpi within the stomach. Remains of the cuticle of digested worms were often observed within the digestive tract up to 24 hpi (Fig. 2a'). At 6 hpi, *Anisakis simplex s.l.* L3 larvae were found for the first time in the outer part of the digestive tract, on the visceral mesentery (Fig. 2B), with the first evidence of fibrocytes surrounding the parasite at 18 hpi (free in the coelom, not attached to a host tissue) (Fig. 2C). At this moment, severe infiltrate composed of eosinophils, heterophils, few macrophages and mast cells were also observed around the parasite. No damage to the host intestinal mucosa was observed during the experiment. There were also extravasated erythrocytes (haemorrhage) along with inflammation (Fig. 3A). An imprint of *A. simplex s.l.* L3 on the liver was macroscopically visible at 24 hpi, whereas the worms were encapsulated and partially adhered to the mesenteries only after 36 hpi (Fig. 2D). After 72 hpi, some larvae were spirally encapsulated (Fig. 2 E–H). From 120 hpi until the end of the experiment, several worms were found fully encapsulated within the mesenteries or visceral fat. At 120 hpi, areas with hypereosinophilic core and karyorrhectic debris admixed with degenerated eosinophils and

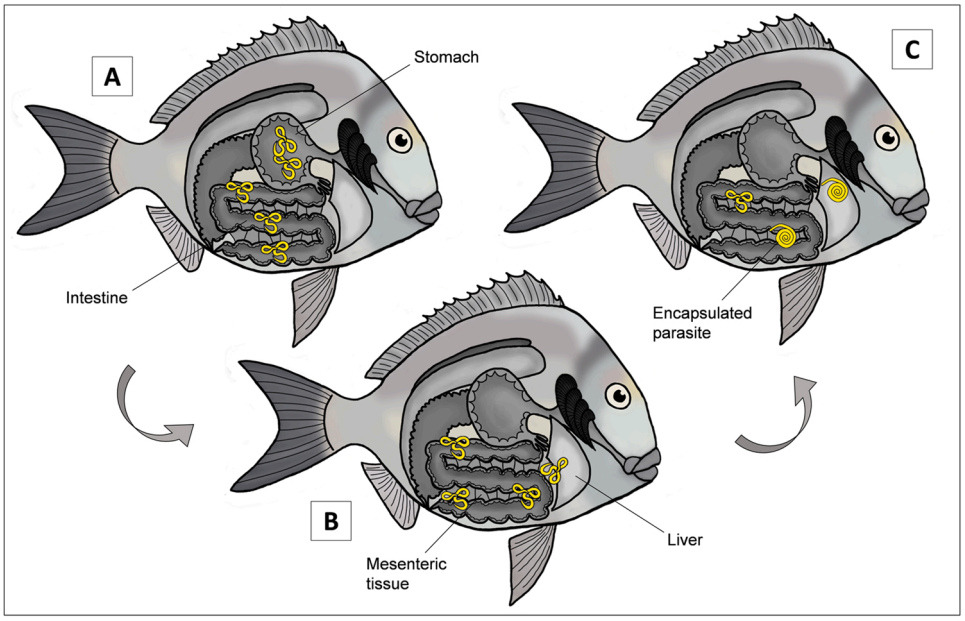

Fig. 1. Development of the infection process of *Anisakis simplex s.l.* L3 in *Sparus aurata*. A) 3–6 h post infection (hpi): worms can be found in the digestive tract; B) 6–48 hpi: worms are in the digestive tract as well as in the coelomic cavity; C) 48–144 hpi: worms are found encapsulated in mesenteries and spiral capsule are formed (72 hpi).

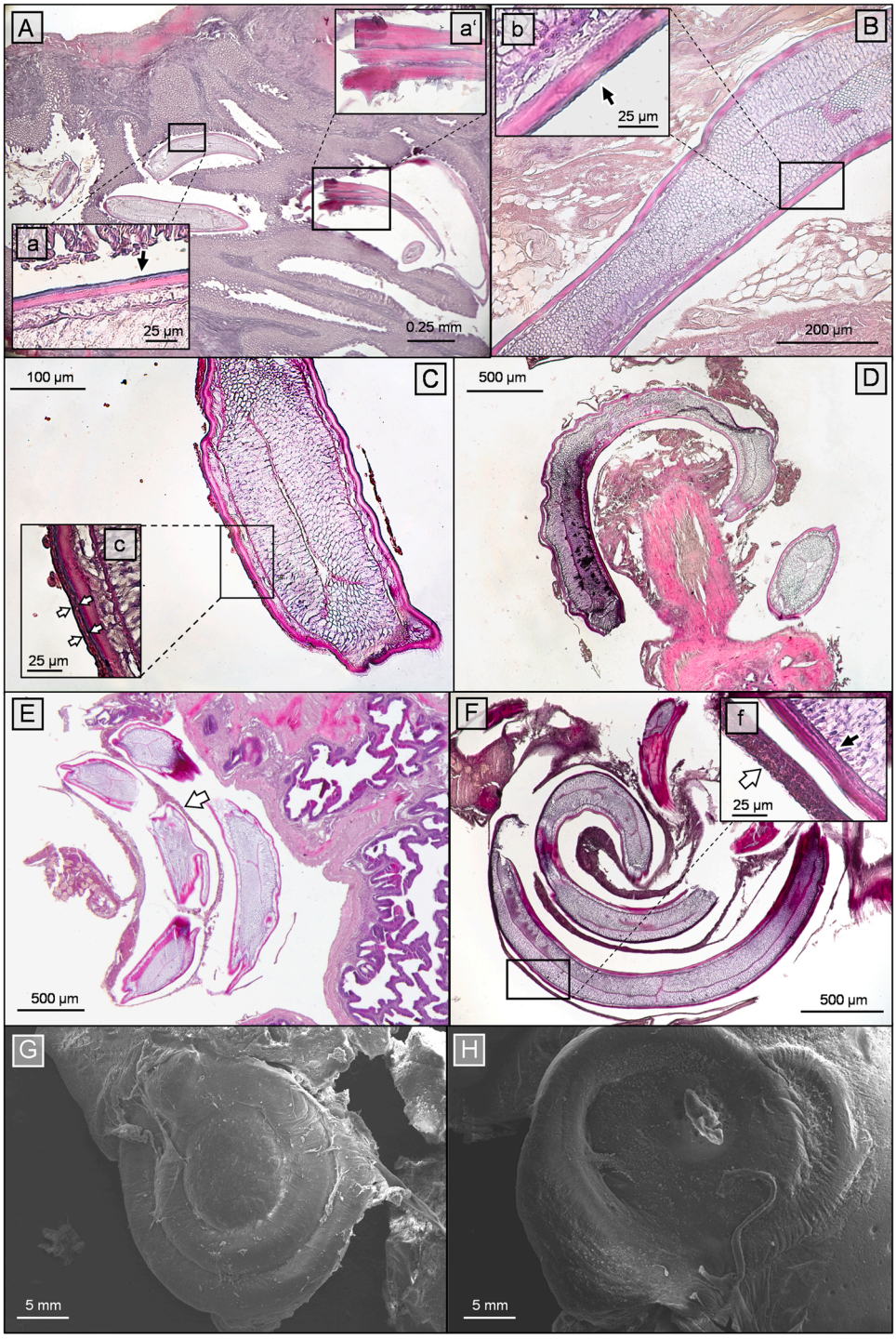

Fig. 2. Infection process of *Anisakis simplex s.l.* L3 in *Sparus aurata*. A–F: H&E stained paraffin histological sections. A) *A. simplex s.l.* L3 in the stomach, 3 hpi, showing details of the parasite at pharynx level (a) and cuticle fragments (a'); B) first evidence of *A. simplex s.l.* L3 free in coelomic cavity, 6 hpi, with detail of the parasite without capsule, close to the intestine (b); C) early host capsule of *A. simplex s.l.*, 18 hpi, free in the mesenteric tissue, showing details of the fibrocytes surrounding the parasite (c); D) first evidence of *A. simplex s.l.* L3 partially adhered to the mesentery, 36 hpi; E) first record of *A. simplex s.l.* L3 spiral encapsulated, 72 hpi; F) late spiral capsule, 144 hpi, with detail of the capsule (f). G & H: scanning electron microscope images. G) spiral capsule in mesenteric tissue, 6 mpi; H) spiral capsule in liver, under the serosa, 6 mpi. The white arrow shows the capsule layer, while black arrows show the parasite cuticle.

heterophils (eosinophilic granuloma) were observed (Fig. 3F). The necrotic foci were surrounded by epithelioid macrophages, eosinophils, few lymphocytes, and mast cells, with absence of haemorrhage. At 3 mpi, there was no mesenteric damage with absent inflammation, granulomas, and haemorrhages (Fig. 3B). Viable encapsulated larvae were observed up to 6 mpi, at which time one worm was found encapsulated on the caecum serosa. Most of the parasites were found within spirally shaped capsules, in a few others the spiral was partial (6.2%). Capsules that increased in thickness from 4.4 (18 hpi) to 7.0–23.9 µm (144 hpi) were recorded in 13 out of 16 samples, without further thickening of the capsules thereafter (Fig. 2 C–F).

About 25% of the larvae examined (15/58), either in early or late stages of infection, showed perforations on the cuticle (Fig. 3 C–E), often related to local alterations and disorganisation of the digestive tract (see damaged pharynx of *Anisakis* sp. in Fig. 3C–D). In some cases, some parasite capsules showed granulomas at different levels (Fig. 3G), even in spiral shape capsules (Fig. 3H). At 6 mpi, larvae were located in the coelomic cavity, mostly in the mesentery and liver serose.

4. Discussion

Anisakid infections are one of the most common health problems associated with fish consumption (Gómez et al., 2003; Balbuena and Raga, 2009). The L3 of these parasites are highly nonspecific (meaning

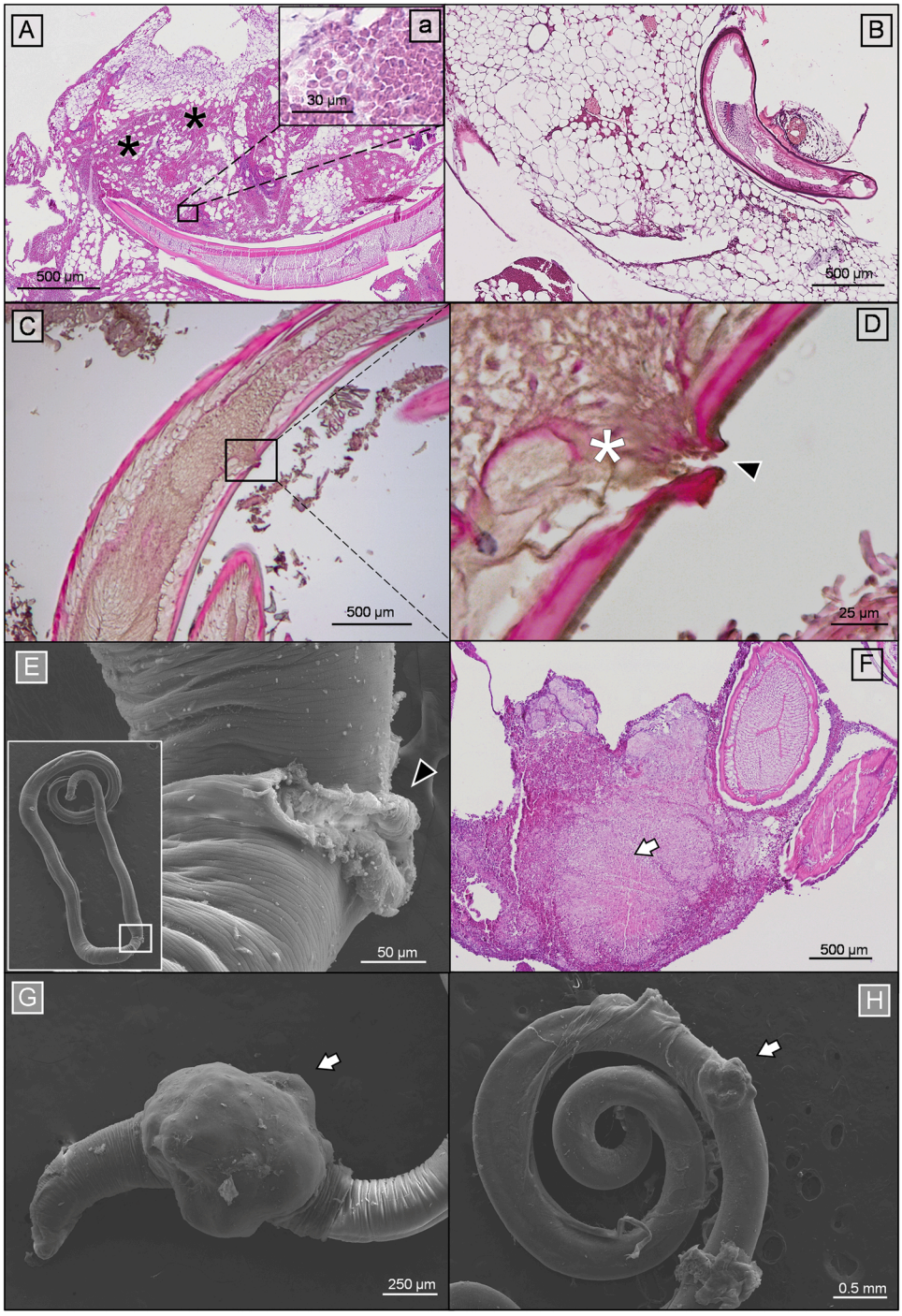

Fig. 3. Damage in parasites and hosts related to *Anisakis simplex s.l.* L3 infection in *Sparus aurata*, H&E paraffin histological sections (A–D, F) and scanning electron microscope images (E, G–H). A) severe inflammatory infiltrate composed of heterophils, eosinophils, and few macrophages and haemorrhage, including detail of the area (a) (18 hpi); B) undamaged infected mesentery (3 mpi); C) *A. simplex s.l.* L3 in the digestive cavity with pierced cuticle (18 hpi); D) detail of C, damaged area of the parasite; E) *A. simplex s.l.* L3 with a perforation in the cuticle (6 mpi); F) section of granuloma enclosing capsule of *A. simplex s.l.* L3 (6 mpi); G) granuloma enclosing capsule of *A. simplex s.l.* L3 close to its pharynx (6 mpi); H) damaged spirally encapsulated *A. simplex s.l.* L3 with granuloma enclosing capsule at intestine level (6 mpi). Black arrowheads show perforations of the cuticle; white arrows show granulomas; white asterisks show disorganised parasite pharynx; black asterisks show haemorrhages.

paratenic invertebrate and fish hosts) and the large number of marine species susceptible to these parasites (Davey, 1971) as well as the increasing number of cases (Mattiucci et al., 2018) reported worldwide evidence the ubiquity and broad infectivity of these species. Previous experimental infections with *Anisakis* sp. larvae challenging different fish species have shown that different anisakid species have different host infection sites (Buchmann and Mehrdana, 2016). However, even if not all *Anisakis* species appear to be equally infective and not all hosts are equally susceptible, research involving experimental infections usually identify only to genus level or to any of the *Anisakis* L3 species complex. In this study, both species found in the donor blue whiting, *A. pegreffii* and *A. simplex,* belong both to the type I species complex. Nevertheless, Quiazon et al. (2011) indicated that there are differences between these two species in terms of infection sites and host specificity, with *A. simplex* tending to migrate into fish muscle, suggesting that the ability of *A. pegreffii* to infect the muscle is reduced; nevertheless, Cipriani et al. (2015) demonstrated that *A. pegreffii* could infect fish muscle. Therefore, experimental infections with both species could result in overlooking/misplacing the different effects that each species could cause in fish. In this study, all sequences extracted from experimentally infected *S. aurata* (N = 3) were identified as *A. pegreffii*, but we cannot ensure that all infections were made with this species, as the total pool of sequences mostly corresponded to *A. simplex*. Molecular identification from paraffin embedded parasites has been unsuccessful, however, it is still recommendable to explore the genetic variability of the *Anisakis* species employed in experimental studies, even if several

studies refer to particular *Anisakis* species not including genetic analyses. Accomplishing this is also relevant from a sanitary perspective, as species such as *A. simplex* have been suggested as potentially more infective to humans due to their potential to survive gastric acids and penetrate human tissue (Arizono et al., 2012).

The number of larvae which successfully infected fish in this study was very low (10% recovery rate) despite the relatively large parasite burdens supplied. However, the percentage of infected gilthead seabream was relatively high (55%). There are no previous studies showing the infection success rates of *Anisakis* spp. in *S. aurata* under experimental conditions. However, Santamarina et al. (1994) reported similar results in trout, i.e., 50% of infection rate and 9% recovery rate 55 days after oral infection. The higher infection levels reported in wild fish may be related to the higher number of worms per prey item, as well as to the high number of encounters with the infected preys throughout the host's life (Gómez et al. 2003; Pontes et al. 2005), exponentially increasing the chances of infection over time. *Anisakis* spp. are found in many fish species worldwide, but the worms are usually located in the digestive tract, not necessarily meaning that they achieve a successful infection, as observed in this work.

Research on experimental infections with *Anisakis* spp. is scarce: while most studies report the presence and numbers of anisakid species in surveys of wild or cultured fish (Gibson et al., 2005; Peñalver et al., 2010), very few describe the chronological details of parasite infection. Nevertheless, there are some investigations that address *post-mortem* processes in fish associated with *Anisakis* L3, mostly exploring larvae migration into fish muscle and motility at different temperatures and time intervals (Smith and Wootten, 1975; Cipriani et al., 2016). Experimental studies on *A. simplex* and *A. pegreffii* have previously been conducted in other fish species to investigate infection success and microhabitat selection (Quiazon et al., 2011, Bahlool et al., 2012).

Our results show that alive larvae of *Anisakis simplex s.l.* L3 can be found within the lumen of the digestive system up to 48 hpi, surpassing the time lapse described by Marino et al. (2013), who found larvae in the stomach lumen of gilthead seabream up to 15 days after the challenge. The same survival rate of larvae within the lumen was reported for *D. labrax,* using identical infection procedure (orogastric infection with no food, Macri et al., 2012). The infection procedures of Macri et al. (2012) and Marino et al. (2013) differ from those of the present study. The previous authors inserted the parasite directly with a cystoscope sheath, whereas here the parasites were inserted into the stomach with a syrinx with food. However, the long period of survival of *Anisakis* sp. in the digestive lumen reported by these authors also contrasts with other papers. Other experimental studies in which trout was infected with *A. simplex s.l.* reported a short period of time when the larvae stayed within the lumen, showing that larvae can enter the coelomic cavity in about 2 hpi (Wootten and Smith, 1975), which is consistent with our histological results (6 hpi).

Regarding the chronology of encapsulation, data on *A. simplex s.l.* in rainbow trout (Santamarina et al., 1994) showed the first coiling of the nematode at 21 dpi, considerably later compared to our results (72 hpi). Other studies have reported variable encapsulation times in salmonids (31–36 dpi, Santamarina et al., 1994; 21–28 dpi Bahlool et al., 2012). Coiling and encapsulation are highly different from our findings in *S. aurata,* which show an early capsule at 18 hpi, and a fully coiled and encapsulated worm at 72 hpi. The encapsulation chronology may vary among hosts (even intra-specifically), and moreover, this cellular response to larvae is thought to be one of the stimuli that triggers parasite coiling (Larsen et al., 2002). In addition, Santamarina et al. (1994) obtained their results based on gross pathology, and at earlier stages the fibrocyte layer is probably detectable only by histological examination. In this study, the fibrocyte layer was observed to progressively thicken up to 24 μm (144 hpi), and the capsule apparently stops growing. As a note of caution, although the flat spiral shape of the capsules is considered as diagnostic for *Anisakis* spp. (Berland, 2003), some larvae were found encapsulated and non-coiled at 6 mpi, which could lead to misidentifications with naked eye.

The third stage larvae of the anisakid *Contracaecum osculatum* (Rudolphi, 1802) (encapsulated in cod liver) can survive in captive cod for over three years and immediately regain activity if released from the host material (Buchmann, 2012). However, there is limited information on the longevity of *Anisakis* spp. in experimental infections of fish. In this study, even at a relatively low infection rate, histological follow-up showed that several L3 of *Anisakis* type I can settle and survive within *S. aurata* for a long period (at least six months). Long-term studies with gilthead seabream would be needed to evaluate potential risks for transmission and infection in aquaculture. Experimental infections of fish from Mediterranean cultures with *A. pegreffii* confirmed the presence of the parasite up to 60 days after infection (*Dicentrarchus labrax* and *S. aurata*; Macrì et al., 2012, Marino et al., 2013, respectively). Longer lifespans have been reported for *Anisakis* sp., e.g., 33 weeks in *Morone saxatilis* (Walbaum, 1792) (Moronidae), however, the authors stated that the larvae were somehow degraded (Moser et al., 1958). Other studies suggested that *A. simplex* could survive up to three years in *Clupea harengus* L. and up to two years in *Gadus morhua* L. (Smith, 1984, Hemmingsen et al., 1993, respectively). Our study indicates that the presence of parasites in the fish does not necessarily mean a successful infection, as several encapsulated parasites were found to be severely injured despite being appropriately established. Wootten and Smith (1975) attempted to reinfect trout (*Oncorhynchus mykiss* (Walbaum, 1792) and *Salmo trutta* L.) with apparently healthy larvae recovered from experimental infections in the same hosts, but none of them succeeded in reinfection. Very low reinfection rates have been also observed by Díez et al. (2022), who infected seabass with *Anisakis* sp., using parasitised liver of European hake (*Merluccius merluccius* (L.)), and finding only a 0.0021% recovery rate.

Although L3 have a great capacity for survival, some of the encapsulated nematodes were extremely damaged during the experimental infection, likely associated with unnoticed damaged provoked by the handling of the larvae or by natural processes inherent to ingestion and digestion in fish. The feeding behaviour and structural features of the fish's pharyngeal cavity might cause the observed damage to the nematode's cuticle, as it provides a physical barrier that prevents successful infections. The survival of these damaged parasites is thus probably compromised, and the unsealed cuticle leaves the worm exposed to the digestive process during further infections. Further studies need to be conducted to understand the mechanisms of fish feeding and their effects on anisakids.

Our results also showed that the infection process also resulted in mild lesions in the fish. In the histological sections, haemorrhages and inflammatory response were observed associated with *Anisakis simplex s.l.* L3, mainly visible at the early stages of infection, although none of them were visible macroscopically. The inflammatory response seems to dissipate with time, and signs of haemorrhages and eosinophilic granulomas were barely present at 3 mpi. In their gastroscopic study, Marino et al. (2013) also observed damage caused by *A. pegreffii* in *S. aurata*, such as haemorrhages and irregular neoformations within the coelomic cavity, even at 60 dpi. The presence of granulomas and necrotizing coelomitis associated with *Anisakis* sp. in wild *Seriola lalandi* Valenciennes, 1833 has also been reported (Keller et al., 2011). However, according to Bahlool et al. (2012), the encapsulation process and immune response to *Anisakis* spp. differ among fish species. The histopathological observations are also consistent with previous observations on the cell types involved in anisakids. The presence of macrophages and mast cells was reported by Buchmann (2012). Our observations agree with Dezfuli et al. (2007), the first report of macrophage aggregates in an inflammatory response to a helminth infection (*Anisakis simplex s.l.*); according to the authors, this type of granulomatous response is more typical of infections with bacteria and protists. Similar observations were described by Ramakrishna et al. (1993) in rainbow trout infected with *Pseudoterranova decipiens* (Krabbe, 1878).

Wild gilthead seabream is mostly coastal and feed mainly on benthic

invertebrates, especially the younger individuals. These habitats and hosts are unusual for the typically pelagic life cycle of *A. simplex s.l.*; therefore, host-parasite co-occurrence may become difficult, explaining why no natural infections have been reported to date (Davey, 1971; Gómez et al., 2003). Some studies have confirmed that wild gilthead seabream can be infected with other nematodes belonging to the family Anisakidae (*Contracaecum* sp.) or the closely related family Raphidascarididae (*Hysterothylacium aduncum* (Rudolphi, 1802)) (Bruce et al., 1994; Mariniello et al., 2000; Salati et al., 2013; Guardone et al., 2020). Experimental infections show that *S. aurata* is a suitable host for *Anisakis* spp., and the ability of gilthead seabream to also prey on pelagic crustaceans and clupeids (*Sardinella aurita* Valenciennes, 1847; *Sardina pilchardus* (Walbaum, 1792)), especially on larger specimens (Hadj Taieb et al., 2013), makes then susceptible to infection with *Anisakis* spp. (Gibson et al., 2005) if they would eat an infected clupeid. Gilthead seabream infection observed in this study is not unexpected, due to the high nonspecificity of *Anisakis* spp. (Gibson et al., 2005). In fact, Polimeno et al. (2021) recently reported the presence of the Ani s4 allergen in extracts of both infected and cultured *S. aurata*. Even though probability of these fish to be infected is low, since extruded pellets should be free of viable entire worms, this fact is particularly important for *Anisakis*-allergic consumers, as it demonstrates that at least the parasite allergens are present in aquaculture.

5. Conclusion

This is the first histological follow-up of the infection process of *Anisakis* spp. in this fish species. Results indicate faster chronological events than those reported in previous studies (e.g. penetration, encapsulation). Aquaculture fish are assumed to be *Anisakis*-free, or at least that its parasitological risk is negligible (Castiglione et al., 2021, Fioravanti et al., 2021). Studies show that gilthead seabream from aquaculture net pens are free of *Anisakis* spp. (Peñalver et al., 2010) as they are fed with parasite-free feed, avoiding transmission via the trophic route. However, most fish can become infected if they are exposed to the parasite. Experimental studies with optimised infection methods are necessary for realistic risk assessments. In the Mediterranean, gilthead seabream can be proposed as a model for testing the infectivity of *Anisakis* spp., as they are easy to obtain, manipulate and maintain for long periods. Regarding the risk to consumers of suffering a zoonotic disease caused by a reinfection in an accidentally infected gilthead seabream from aquaculture is almost negligible, not only because of the low infection success, and the numerous damaged specimens, but also because the fish muscle of the challenged gilthead seabream was never infected with the parasite. As a general recommendation for aquaculture, special care should be taken in cultures where bait is used, as the parasites may be present in the fresh feed. Taking precautions is of vital importance to ensure that aquaculture continues to be considered infection-free production.

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Ethics approval and consent to participate

This study was approved by the Ethics Committee of the University of Valencia, authorisation procedure 2018/VSC/PEA/0172 (Dirección General de Agricultura, Ganadería y Pesca, Valencian Local Government). Gilthead seabreams were obtained from Mediterranean aquaculture hatchery destined for human consumption. The minimum number required was used to perform this study. Gilthead seabream is listed as a "least concern" species by the UICN.

CRediT authorship contribution statement

A. López-Verdejo: Writing – original draft, Methodology, Investigation, Conceptualization. **A. Born-Torrijos:** Methodology, Writing – review & editing, Supervision, Project administration. **E. Montero-Cortijo:** Data curation, Investigation. **J.A. Raga:** Project administration, Supervision, Validation. **M. Valmaseda-Angulo:** Data curation, Investigation. **F.E. Montero:** Writing – original draft, Methodology, Supervision, Conceptualization.

Conflict of interest

The authors declare that they have no conflicts of interest.

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