PAPER

LARVAL STAGES OF *SULCASCARIS SULCATA* (NEMATODA: ANISAKIDAE) IN SCALLOPS FROM THE NORTHERN ADRIATIC SEA: IMPLICATIONS FOR SEAFOOD CONTROL AND SURVEILLANCE

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ABSTRACT

A survey of fresh great Mediterranean scallops (*Pecten jacobaeus*) and frozen queen scallops (*Aequipecten opercularis*) collected from the northern Adriatic Sea demonstrated the presence of nematode larvae that were morphologically and molecularly identified as *Sulcascaris sulcata*. The presence of this nematode in the northern Adriatic Sea has been described in the loggerhead sea turtle, which is the final host, but it was only recently described in the intermediate molluscan host. Affected scallops present rust-brown lesions in the adductor muscle. In this study, necroscopic and histopathological lesions are described in affected scallops. Furthermore, the detrimental effects of this parasite on the commercialization of scallops under the current European legislation concerning the hygiene of seafood products are evaluated.

Keywords: emerging parasites, food legislation, nematode, RFLP, scallop, *Sulcascaris sulcata*

1. INTRODUCTION

Sulcascaris sulcata (RUDOLPHI, 1819) is a nematode parasite of sea turtles that belongs to the Anisakidae family. This nematode was first described by COBB (1930) in *Pecten* spp. in North Carolina and was named *Paranisakis pectinis*. The adult stage of *S. sulcata* has been recorded in loggerhead sea turtles (*Caretta caretta*) and green sea turtles (*Chelonia mydas*) in the Mediterranean and Caribbean Seas and in the South Atlantic, western Atlantic, and western Pacific oceans (LICHTENFELS *et al*., 1978; LESTER *et al*., 1980; BERRY and CANNON, 1981; WERNEK *et al*., 2008; SANTORO *et al*., 2010). In the western Atlantic, *S. sulcata* has also been recorded in Kemp's Ridley turtles (*Lepidochelys kempii*) (GREINER, 2013). Since 1998, *S. sulcata* infection has been extensively investigated in *C. caretta* from the Adriatic Sea (MANFREDI *et al*., 1998; GRAČAN *et al*., 2012; SANTORO *et al*., 2019; MARANGI *et al*., 2020).

Intermediate hosts for *S. sulcata* have been identified from natural or experimental infections in several mollusc species, including both bivalves and gastropods, as reviewed in SANTORO *et al.* (2020). The occurrence of larval stages of *S. sulcata* in molluscs from the Mediterranean basin has been only recently described in *Mytilus galloprovincialis* from the Tyrrhenian Sea (SANTORO *et al*., 2020) and in two pectinidae scallops, *Pecten jacobaeus* and *Aequipecten opercularis,* from the northern Adriatic Sea (MARCER *et al*., 2020).

Adults of *S. sulcata* reside in the gastric lumen of sea turtles, which become infected by consuming parasitized molluscs (BERRY and CANNON, 1981). As adult nematodes undergo sexual reproduction, eggs are released into the water column in the faeces (BERRY and CANNON, 1981; DEARDOFF, 1989). Nematode larvae progress through the first two stages of development (L1 and L2) in the water column/benthos and enter marine molluscs through inhalation in the siphon, where L3 larvae grow and moult into the L4 stage (BERRY and CANNON, 1981; DEARDOFF, 1989), appearing as tubular worms coiled within a sheath. These larvae encapsulate in the adductor muscle, which exhibits brown, rust or yellow-coloured lesions or cysts with an elongated shape (LICHTENFELS *et al*., 1978; BERRY and CANNON, 1981; DEARDOFF, 1989).

From a food hygiene perspective, the colour of the L4 larvae can be whitish and difficult to identify in the adductor muscle, although its presence is easily detectable because of the rust–brown lesions created by the encysted nematode. This pigmentation has been associated with the bivalve's response to the excretory/secretory liquid produced by the worm (DEARDOFF, 1989).

While the adductor muscle itself shows no signs of quality loss, its aesthetics may cause an economic problem in the scallop market, which has been involved in raw seafood consumption, a trend that has spread in the last several years. Traditionally cooked molluscs, such as pectinid, are now increasingly consumed raw. Therefore, the gross lesions caused by *S. sulcata* in edible molluscs may be perceived as unacceptable quality defects.

The aim of this study is to describe the macroscopic and histological appearance of the lesions caused by *S. sulcata* in the intermediate hosts *P. jacobaeus* and *A. opercularis* collected in the northern Adriatic Sea. Implications regarding the commercialization of scallops under the enforcement of European legislation on seafood safety are also considered.

2. MATERIALS AND METHODS

2.1. Sampling and necroscopic observation

A total of 54 wild-caught great Mediterranean scallops (*P. jacobaeus*) and 10 frozen queen scallops (*A. opercularis*) from the northern Adriatic (FAO zone 37.02.01) were collected between 2017 and 2019 by the Public Health Authority (Local Veterinary Services, Venice) at the wholesale fish market and were submitted to the IZSVe laboratory. Using a dissecting microscope, researchers evaluated the specimens for the presence of rust-brown lesions and *S. sulcata* larvae. All organs were observed with a focus on the adductor muscle and were dissected with parallel cuts to evaluate deep lesions in the muscular mass. Dissected organs (visceral mass, gonad, adductor muscle, and mantle) were artificially digested in a sodium chloride-pepsin solution (0,85% sodium chloride with pepsin added to a concentration of 10 mg/L) according to JACKSON *et al*. (1981). The pH of the solution was adjusted by adding HCl 6N, obtaining a final value of pH 2.0, before incubation using a magnetic stirrer at 37°C for 24 h.

2.2. Morphological examination and viability of the larvae

Larval length and morphology were assessed with a Leitz Diaplan light microscope (Leica, UK) and compared with the identification keys proposed by BERRY and CANNON (1981). Digital images and measurements were obtained using an integrated LEICA MC170HD (Leica, UK) camera and LAS 4.5.0 (Leica, UK) software. The viability test was performed by transferring the larvae in a Petri dish with saline solution at room temperature (20°C) for 2 h to verify any spontaneous movement and reaction to tactile stimulation.

2.3. Biomolecular analysis

DNA extraction was performed on 20 whole larvae using the QIAamp® DNA Mini Kit according to the manufacturer's instructions. The amplification of the entire rDNA fragment comprising ITS1, 5.8S, and ITS2 was performed with the forward primer NC5 (5′ -GTA GGT GAA CCT GCG GAA GGA TCA TT-3′) and the reverse primer NC2 (5′ -TTA GTT TCT TTT CCT CCG CT-3′) (ZHU *et al*., 2001) following the protocol of MARCER *et al.* (2020). PCR amplicons (~1050 bp) were digested with 2 restriction enzymes, *HhaI* and *HinfI,* as described by D'Amelio *et al.* (2000). DNA restriction fragments were evaluated by electrophoresis on a 2% w/V agarose gel stained with ethidium bromide at 4,5/6 V/cm for 40 min. Another four Anisakidae species (*Anisakis physeteris*, *A. pegreffii*, *A. sensu stricto,* and *Pseudoterranova* spp.) and a Raphidascarididae (*Hysterothylacium aduncum*) were included in the restriction fragment length polymorphism (RFLP) analysis for comparison. Subsequently, amplification of the *cox2* mtDNA gene (measuring 609 bp) was performed with the primers 211F (5′ -TTT TCT AGT TAT ATA GAT TGR TTY AT-3′) and 210R (5′ -CAC CAA CTC TTA AAA TTA TC-3′) (GARBIN *et al*., 2011; MARCER *et al*., 2020); the amplicons were sequenced and compared with the GenBank database using BLAST software. For further details, see the work of MARCER *et al*. (2020).

2.4. Histological examination

Portions of the adductor muscle that contained encysted larvae were fixed in Carson's fixative for 24 h, dehydrated and embedded in Paraplast® applying standard histological protocols (HOWARD *et al*., 2004). Sections measuring 3 μm were stained with Harris's haematoxylin and eosin-floxin. Slides were observed with a Leitz Diaplan microscope at 40-1000X magnification.

3. RESULTS AND DISCUSSION

3.1. Necroscopic observation

Upon visual examination, affected scallops presented rust-brown to orange/brown singular or multifocal areas with elongated shapes (measuring 2-7 mm in length and 1-3 mm in width) on the surface of the adductor muscle. Nearly all lesions were observed along the exterior edge of the adductor muscle (Fig. 1A) on the opposite side of the digestive gland. Not all the lesions that were observed contained encysted nematodes.
Nematode larvae were observed in 35 out of 54 *P. jacobaeus* with an overall number of 277 larvae, while in *A. opercularis,* only 1 specimen out of 10 was infested by two nematodes. The number of parasites recorded for each specimen ranged between 1 and 10 nematodes.
The artificial digestion method, which was performed after carefully checking the scallops by visual examination, did not indicate the presence of additional larvae.
The presence of fewer nematode larvae compared with the number of brownish lesions has been explained by the relocation and re-encystment of the larvae in the muscle as their growth progresses (RUDDERS *et al*., 2019).

3.2. Morphological examination and viability of the larvae

The morphology of the larvae and their dimensions (9-21 mm in length, 0.5 mm in width) were consistent with *S. sulcata* specimens in the L4 larval stage (BERRY and CANNON, 1981). Larvae showed a well-differentiated oesophageal ventriculum that was characterized by shrinkage of the proximal junction with the oesophagus, absence of lateral diverticula and a rounded flask-like distal fund (Fig. 1D); in some specimens, cranial development of an intestinal caecum was visible as a rudimental sac lateral to the ventriculum.
The lack of ventricular appendix and noticeable intestinal caecum differentiate *S. sulcata* from the genera *Hysterothylacium, Contracaecum, Phocascaris and Pseudoterranova,* while its ventriculum morphology differentiates it from the genus *Anisakis* (Fig. 1E). All the nematodes evaluated were viable with the exception of 2 larvae in frozen *A. opercularis*.

3.3. Biomolecular analysis

Biomolecular analyses confirmed the species identification of *S. sulcata*. Consensus sequences of the *cox2* gene displayed 100% nucleotide similarity to the *S. sulcata* sequence (HQ328505) available in the GenBank database; see also MARCER *et al.* (2020).

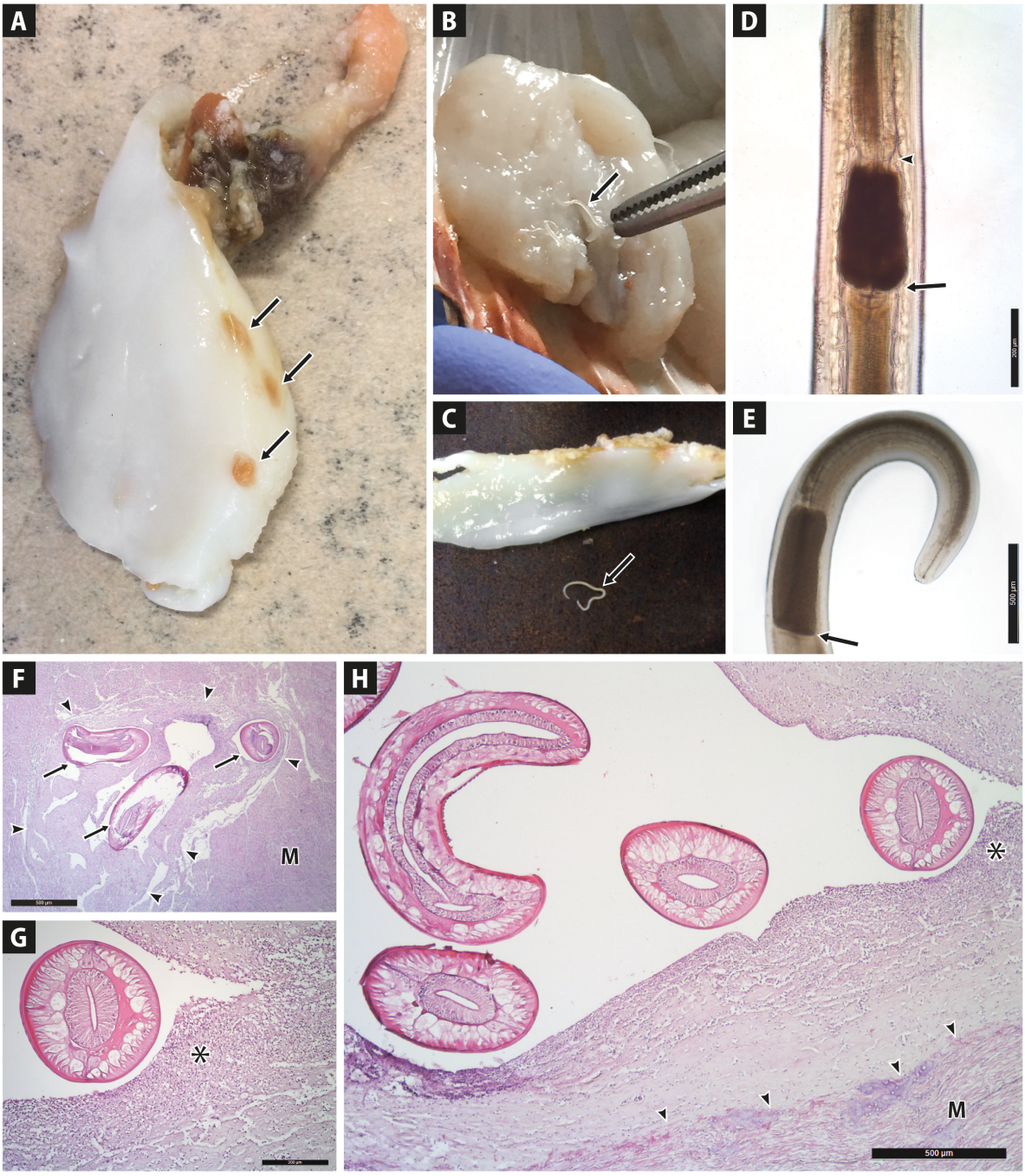

Figure 1. A) Cross-section of the adductor muscle of *Pecten jacobaeus*, showing on its surface rust-brown spots (arrows) containing encysted nematodes; B and C) cream-white *Sulcascaris sulcata* larvae (arrows); D) microscopic observation of the oesophageal ventriculum of *S. sulcata* larvae, shrinkage of the proximal junction with the oesophagus (arrowhead), rounded flask-like distal end of the ventriculum (arrow); E) comparison with the ventriculum of *Anisakis simplex* showing blunt distal fund (arrow); F) histological observation of encysted and coiled *S. sulcata* (arrows) with the external margin of the connective sheath highlighted (arrowheads), normal adductor muscle (M)(4X); G) higher magnification of a cross-section of the nematode, showing haemocytic infiltration (*) (25X); H) connective sheath (encapsulating *S. sulcata*, haemocytic infiltration (*) is present in the inner layer, deposition of small pigmented granules is observed at the periphery of the connective capsule (arrowheads), normal musculature (M) (10X).

All 20 restriction profiles obtained with RFLP were analogous. Restriction with *HhaI* produced three fragments measuring approximately 100 bp, 250 bp and 400 bp; conversely, restriction with *HinfI* produced only one fragment of 500 bp. The restriction profile of *S. sulcata* appeared different from those of the other evaluated *Anisakidae* species (Fig. 2). RFLP results were also compared with the restriction profiles of other species: *Anisakis simplex/pegreffii, A. paggiae, A. typica, A. brevispisculata, A. ziphidarum, A. sp.* A and *Contracaecum rudolphii* according to D'AMELIO *et al.* (1999) and ZHU *et al*. (2000). The profile of *S. sulcata* did not match any of these species.

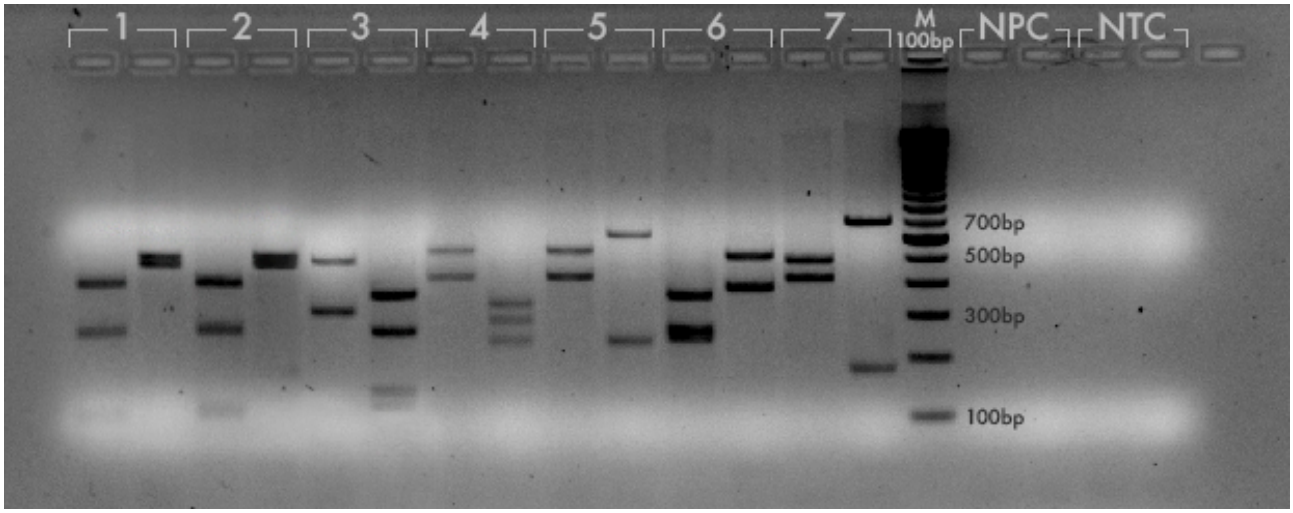

Figure 2. Restriction fragment length polymorphism (RFLP) patterns of the rDNA region spanning ITS-1, the 5.8S gene and ITS-2 with two restriction enzymes (*HhaI, HinfI*). Lanes: 1 and 2, *Sulcascaris sulcata*; 3, *Anisakis physeteris*; 4, *A. pegreffii*; 5, *A. sensu stricto*; 6, *Hysterothylacium aduncum*; *7*, *Pseudoterranova* spp.; M, marker (*Bio-Rad* 100-bp molecular ruler); NPC, negative process control; NTC, negative template control.

3.4.Histological investigation

Microscopic observation of the affected adductor muscle indicated moderate haemocytic infiltration (Fig. 1G-H) and intense fibrotic reaction around the encysted larvae, which resulted in the encapsulation of the parasite (Fig. 1F). The muscle tissue around the connective capsule did not appear to be affected (Fig. 1F-H). The presence of light brown pigmented granules in the outer layer of the connective sheath around the larvae was observed, as reported by DEARDOFF (1989).

3.5. Regulation overview

European legislation concerning consumer protections for fishery and aquaculture products permits the consumption of raw products (finfish and cephalopods) only if previously frozen or properly treated with other systems of proven efficacy against the parasites, except for fish farmed with special guarantees (Reg. EU 1276/2011, amending Annex III to Regulation EC 853/2004). No reference is made to other aquatic animals, such as bivalve molluscs.

Scallops are traditionally consumed cooked, and heat treatment rapidly inactivates *S. sulcata* (1-6 seconds at 95°C is sufficient) (RUDDERS *et al*., 2019). However, *P. jacobaeus,* a Mediterranean scallop, is increasingly consumed raw among other seafood according to current consumer eating habits (DASCHNER, 2016). No cases of *S. sulcata* infection in humans have been reported to date, although it cannot be excluded that similar to other Anisakidae, humans may act as paratenic hosts (LAUCKNER, 1983). Experimental infection trials performed in homeotherm species (chickens and cats) failed to establish L3 larvae, suggesting that infection by *S. sulcata* involves only poikilotherm sea turtles as the final host (BERRY and CANNON, 1981). *Hysterothylacium aduncum* (family Raphidascarididae), which has predatory sea fish as its final host, has recently been reported in humans and may be considered to be an exception (GONZÁLEZ-AMORES *et al*., 2017). It must be considered that L3 larvae of *S. sulcata* experimentally demonstrated a reduced viability at 37°C (3.25-6.75 h), thereby appearing unlikely to be pathogenic in humans (RUDDERS *et al*., 2019).

No information regarding possible allergic reactions caused by *S. sulcata* has been reported, unlike other Anisakidae (AIBINU *et al*., 2019). However, it should be considered in the application of European legislation ("*food business operator must ensure that the raw material or finished product undergoes a freezing treatment in order to kill viable parasites that may be a risk to the health of the consumer*") that the residual risk of *S. sulcata* to public health cannot be ruled out at present. Moreover, the ban on marketing fishery products with clearly visible parasites remains to be applied (Reg. EC 853/2004, section VIII, chap. V). Although it is difficult to establish a tolerance for the number of parasites, the presence of 3-4 larvae/scallop is immediately evident, causing a noticeable colour change in the area where the parasite is located.

The authorities supervising the presence of parasites in fishery products should consider possible accidental transfers of such parasites as *Anisakis* from a nearby parasitized fish or fillet. This transfer could occur during the handling of fresh pectinid molluscs, which are usually sold with the pulp exposed, especially when the product is placed on counters in the fish market. A simple field control with a light microscope would quickly and safely differentiate *S. sulcata* from *Anisakis* spp., considering the morphology of the ventriculum ending with a rounded fund (BERRY and CANNON, 1981).

It is advisable for food business operators to enforce, in their companies' internal quality-control systems, an evaluation of the degree of infestation of the scallops collected from different fishing sites. Both the prevalence and intensity of infestation should be considered in the evaluation, excluding products supplied from highly infested fishing sites, as provided for fish from EC Reg. no. 2074/2005, annex II, chap. II.

This report describes the presence of *S. sulcata* in pectinidae in the northern Adriatic Sea. In this area, *P. jacobaeus* and *A. opercularis* are reported as part of loggerhead sea turtle diets (LAZAR *et al*. 2011). The prevalence of the infestation recorded in *C. caretta* in the Adriatic Sea is higher than in other areas of the Mediterranean Sea, where bivalves constitute a marginal food source (SANTORO *et al.* 2019). The northern Adriatic Sea offers a large number of neritic zones for *C. caretta* foraging, where benthic invertebrates, such as scallops, are abundant (LAZAR *et al*. 2011). This trophic network could explain the abundance of *S. sulcata* in both final and intermediate hosts.

4. CONCLUSIONS

New eating habits promoting raw seafood consumption combined with the preference for local products and ecological conditions favouring the diffusion of *S. sulcata* can create new challenges for inspectors of seafood products, as well as retailers.
Scallops are collected in unclassified areas and are therefore already subject to analysis by food operators for such parameters as biotoxins, heavy metals and pathogenic microorganisms. The possibility that *S. sulcata* may also be present in the product must be considered by food business operators in their internal quality control systems (Reg. EC 853/2004, section VIII, chap. IX).

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Paper Received May 17, 2020 Accepted September 11, 2020