International Journal of Food Microbiology 339 (2021) 109021

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Anisakis simplex (s.s.) larvae (Nematoda: Anisakidae) hidden in the mantle of European flying squid *Todarodes sagittatus* (Cephalopoda: Ommastrephidae) in NE Atlantic Ocean: Food safety implications

Paolo Cipriani [a,*], Marialetizia Palomba [b], Lucilla Giulietti [a], Miguel Bao [a], Simonetta Mattiucci [b], Arne Levsen [a]

[a] Department of Contaminants and Biohazards, Institute of Marine Research (IMR), Nordnes, Bergen, Norway
[b] Department of Public Health and Infectious Diseases, Section of Parasitology, Sapienza - University of Rome, Italy

ARTICLE INFO

Keywords:
Anisakiasis
Zoonotic parasite
Molecular identification
Cephalopod
Epidemiology
mantle's tissue

ABSTRACT

Few reports exist upon the occurrence and localization of zoonotic anisakid nematodes in *T. sagittatus*, especially in the mantle of the squid. The occurrence and site of infection of larval anisakids in 98 *T. sagittatus* caught West off St. Kilda, NE Atlantic Ocean, were investigated. Squids were examined for anisakids using the UV-Press method. In total, 689 nematodes were detected in the viscera and mantle. According to morphology, all the larvae (L_3) were assigned to genus *Anisakis*. Diagnostic allozymes and mtDNA *cox*2 sequence analysis permitted to genetically identify all larvae as *Anisakis simplex* (s.s.) (N = 100). Overall prevalence (P = 81%) and mean intensity (mI = 8.6) of infection with *A. simplex* are provided. Most of the larvae present in the mantle cavity were embedded in the stomach wall or attached in the outer layer of the stomach and caecum (49%). Over a third of squids (37%) hosted *A. simplex* (s.s.) larvae in the mantle. A novel schematized representation of larvae distribution in the mantle is provided, showing where they were mostly located. According to the results obtained, the risk of anisakiasis associated with consumption of raw or undercooked *T. sagittatus* should be considered.

1. Introduction

The flying squids belonging to the Family Ommastrephidae, are commercially valuable cephalopod species, occurring throughout the oceans. Members of this family have an important role in marine food-webs, and they represent key trophic elements in marine communities (Clarke, 1996). *Todarodes sagittatus* (Lamarck, 1798) is a very abundant and widespread ommastrephid pelagic squid that lives over the continental slope and oceanic waters (Dunning and Wormuth, 1998).

Todarodes sagittatus is thought to be an opportunistic predator, consuming mainly a variety of pelagic and mesopelagic fish, Crustacea and other cephalopod species (Breiby and Jobling, 1985; Joy, 1990; Piatkowski et al., 1998; Quetglas et al., 1999; Wiborg et al., 1982). The diet of the squid (Quetglas et al., 1999), together with its autecology, suggest that *T. sagittatus* may have mostly a pelagic and mesopelagic habit (Lordan et al., 2001), undergoing diel vertical migration in the water column, and seldomly also feeding on benthic preys (Breiby and Jobling, 1985).

Interest in cephalopods has increased considerably in the last decades, because they represent a valuable target for fisheries, retain a high market value, and constitute an important food resource for human consumption (Gestal et al., 2019). Species of the Ommastrephidae family are the most important commercial fishery among cephalopods (Vieites et al., 2019). Commercial catches of Ommastrephidae are thought to be composed mainly of *Illex coindetii*, *Illex argentinus*, *Todaropsis eblanae*, *Todarodes pacificus*, *Todarodes sagittatus*, and even if reports of commercial catches requests data by species, most landings are still identified only to family level (Gestal et al., 2019; ICES, 2019). *Todarodes sagittatus* has been caught for centuries as a bycatch in southern European trawl fisheries and exploited in specific jigging fisheries of some northern countries, especially in Norway (Borges and Wallace, 1993). The total catch in 2017 was 1759 t. (FAO, 2020). The species is mostly exploited in the Mediterranean Sea by Italy (1005 t.) followed by Spain (230 t.), and in the North East Atlantic mainly by Spain (411 t.), United Kingdom (76 t.) and Norway (29 t.) (FAO, 2020). *Todarodes sagittatus* is currently considered an underexploited species

* Corresponding author at: Institute of Marine Research (IMR), P.O. Box 1870 Nordnes, 5817 Bergen, Norway.
E-mail address: paolo.cipriani@hi.no (P. Cipriani).

https://doi.org/10.1016/j.ijfoodmicro.2020.109021
Received 16 May 2020; Received in revised form 11 November 2020; Accepted 6 December 2020
Available online 11 December 2020

(Vieites et al., 2019).

According to the UN Food and Agriculture Organization reports (FAO, 2016, 2018), the use of squid for human consumption is extensive worldwide. The main consumers and importers of squid are Spain, Italy and Japan (Gestal et al., 2019; Vieites et al., 2019). In European countries, cephalopods are most commonly consumed cooked, and squid-based products are commercialized fresh, refrigerated, frozen, canned, salted or dried (Gestal et al., 2019). In Japan, squids are a traditional and valuable food resource, consumed in a large quantity, mainly raw, as sashimi and sushi (Nagasawa and Moravec, 1995).

Despite an increasing commercial significance of cephalopods in the EU and not EU- markets, and the numerous European recommendations (EFSA, 2010) on the risk of parasites in fishery products issued in recent years, little is known about the presence of pathogens in cephalopods (Gestal et al., 2019). Identifying pathogens and the resulting diseases is one of the main requisites in 'assessment of health and welfare' of animals, as suggested in the Directive 2010/63/EU (Gestal et al., 2019). Cephalopods are intermediate, paratenic, or definitive hosts of a range of parasites with different life cycle strategies (Gestal et al., 2019). They occupy an ecological niche that makes them vulnerable to infection by specific groups of parasites, which are transmitted to the definitive host, namely fish, marine mammals, or birds (Gestal et al., 2019). Among the various parasites of cephalopods, ascaridoid nematodes, comprehending some potentially zoonotic species, have a remarkable importance.

The genus *Anisakis* includes so far nine species of heteroxenous parasites of marine organisms, with crustaceans as first intermediate hosts, fishes and squids as intermediate and/or paratenic hosts, and cetaceans as definitive hosts (reviewed by Mattiucci et al., 2018). In fish and squids, the third larval stage larva (L_3) of *Anisakis* spp. can reside encysted on the visceral organs (Abollo et al., 2001; Gestal et al., 2019), or into the fish flesh or squid mantle (Cipriani et al., 2019). *A. simplex* (s. s.) is the most frequently reported *Anisakis* species in the NE Atlantic, with its distribution comprising coastal and pelagic marine realm extending above the Gibraltar Strait until Northern tip of the Arctic (Mattiucci et al., 2017a).

Since the L3 of some *Anisakis* species is the etiological agent of a zoonotic disease known as human anisakiasis, data on their distribution, epidemiology and localization in the fish or squid hosts are of great importance. Anisakiasis may occur when live larvae are accidentally ingested when eating raw, marinated or undercooked parasitized fish/squid products (Audicana and Kennedy, 2008; Bao et al., 2019; Daschner et al., 2012; Mattiucci et al., 2018; Nieuwenhuizen, 2016). Among the nine *Anisakis* species which have been morphologically described and genetically characterized to date (Mattiucci et al., 2014, 2018), only *A. simplex* (s.s.) and *A. pegreffii* have been so far recognized as zoonotic species (D'Amelio et al., 1999; Daschner and Cuéllar, 2020; Fumarola et al., 2009; Lim et al., 2015; Mattiucci et al., 2011, 2013, 2017b, 2018; Mladineo et al., 2016; Roca-Geronès et al., 2020; Umehara et al., 2007).

Epidemiological studies of ascaridoid nematodes in *T. sagittatus* and other ommastrephid species are scanty and based on few squid specimens. So far, *A. simplex* (s.s.) was detected in *T. sagittatus* fished in Spanish NE Atlantic waters (Abollo et al., 2001; Mattiucci et al., 1997; Pascual et al., 1996), *A. physeteris* and *A. pegreffii* in *T. sagittatus* originating from southern Mediterranean (Ionian Sea) (Costa et al., 2012); *A. pegreffii* in *T. sagittatus* from Central Mediterranean Sea (Mattiucci and Nascetti, 2008) and in *T. sagittatus angolensis* fished off South African coast (Mattiucci et al., 1997); whereas, *Anisakis* type II larvae (presumably *A. physeteris*) in central Mediterranean Sea (off Sardinia) (Angelucci et al., 2011). Third stage larvae of anisakid nematodes in cephalopods are generally reported as free in the mantle cavity or "encapsulated in flat spirals in the sheath of connective tissue surrounding the mantle muscle, mesenteries, gonads and internal wall of the stomach" (Abollo et al., 2001). None of these *Anisakis* larval specimens were detected encysted inside the mantle tissue of *T. sagittatus*. More recent evidence revealed that L3 of *Anisakis* can penetrate the mantle muscular tissue of squids, as observed for *A. pegreffii* larvae in *Illex argentinus* (Cipriani et al., 2019). Being the mantle a conspicuous edible part of the host, consuming it raw, marinated or undercooked poses a potential human health risk, as demonstrated by recent cases of human anisakiasis associated with consumption of raw squid in Japan (Furuya et al., 2018; Ogata et al., 2015; Tamai and Kobayashi, 2015).

The aim of the present study was to investigate the occurrence, tissue distribution and species composition of larval anisakid nematodes in *T. sagittatus* caught off the St. Kilda islands, NE Atlantic Ocean, in order to: i) identify larval nematodes to species level using morphological and molecular methods; ii) determine parasitic infection levels; iii) define the anatomical infection sites in the squid host.

2. Materials and methods

2.1. Squid sampling

In total, 98 specimens of *T. sagittatus* were obtained as bycatch during a commercial trawl fishing cruise aimed to fish blue whiting (*Micromesistius poutassou*) by trawling West off St. Kilda islands (N58°04′ W09°40′), in the NE Atlantic Ocean (FAO 27 area, Division VI a) in May/June of 2018 and April 2019 (Fig. 1). The squid species was determined according to the diagnostic keys provided in Guerra (1992). The squids were inspected on-board the commercial fishery and research vessel MS Kings Bay (Institute of Marine Research cruise no. "Kings Bay" 2018843). All specimens were measured (mantle length) to the nearest 0.5 cm before inspection for parasitic nematodes. Total body weight was not reported because several squids lacked body parts such as tentacles or arms, that were damaged during catch.

2.2. Parasitological analysis

Squids were first examined for the presence of anisakids by plain visual inspection, followed by standard UV-press method procedures (Levsen et al., 2018). Each specimen was cut along the ventral mid-line of the mantle, viscera removed.

Squids stomachs were emptied before pressing them, to avoid counting the larvae that could be free within the digestive tract (present in the stomach content). Mantle was visually inspected by naked eye for eventual visceral larvae accidentally left on the surface during manipulation. Subsequently, the mantle was positioned flat open in a triangle shape, in order to map the infection site of larvae into it. Viscera, mantle and stomach content of each squid were placed in separate plastic bags, flattened to 1–2 mm thick layers in a hydraulic press at 8 bar, frozen for $\geq$24 h, and subsequently checked for nematodes under a 366 nm UV-light source (dead nematodes fluoresce when irradiated by UV-light) (Karl and Leinemann, 1993; Karl and Levsen, 2011; Levsen et al., 2018; Pippy, 1970). Nematodes recovered from squids were counted, washed in saline solution, and stored at −70 °C for further morphological and molecular identification.

2.3. Morphological and molecular identification of larval nematodes

The recovered nematodes were morphologically assigned to genus level using bright field microscopy and by following the diagnostic keys according to Berland (1961).

A subsample of 100 *Anisakis* spp. larvae randomly selected between the ones detected in viscera and mantle of all infected squids, were identified to species level using a multi-marker genotyping approach, consisting of three diagnostic allozymes loci (Mattiucci et al., 2014) and mitochondrial cytochrome *c* oxidase II (mtDNA *cox*2) gene sequence analyses. The diagnostic allozyme loci (*Adk-2*, *Pep C-1* and *Pep C-2*) were analyzed according to established procedures (see Mattiucci et al., 2014) on 100 *Anisakis* spp. larvae. The total DNA was extracted from ≈2 mg of homogenized tissues from each specimen, using the DNeasy® Blood and Tissue Kit (QIAGEN® GmbH, Hilden, Germany). For sequencing the mitochondrial cytochrome C oxidase subunit II (*cox*2) gene, PCR

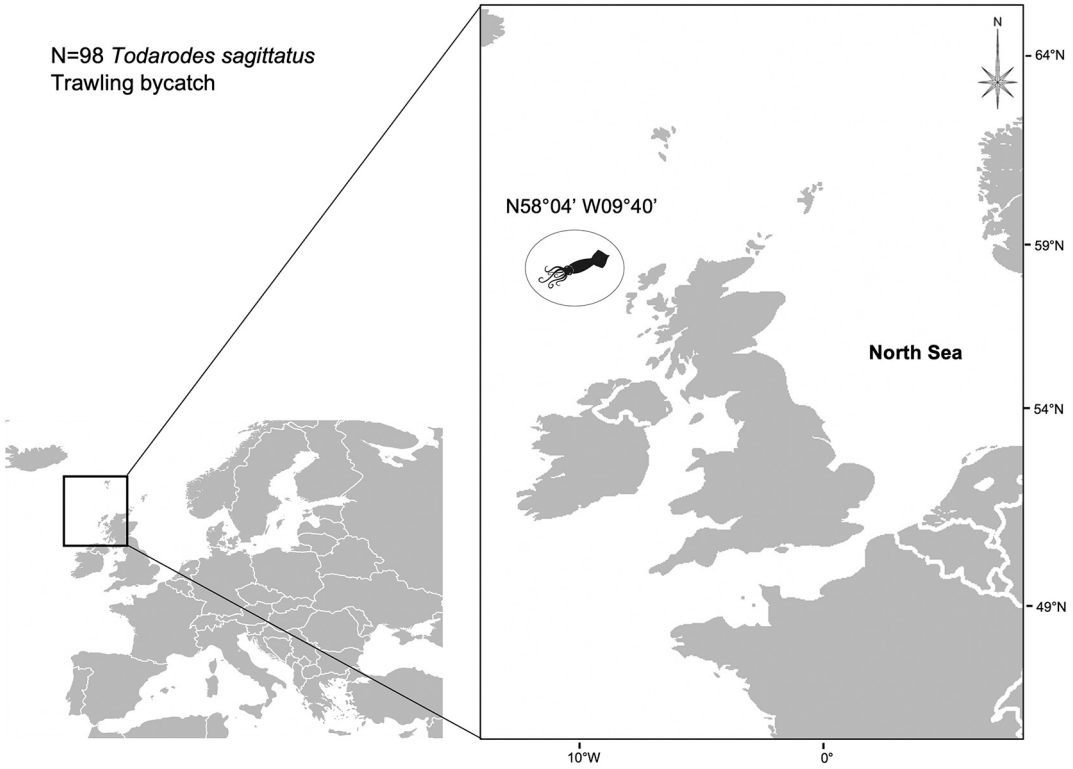

Fig. 1. Sampling locality of 98 specimens of *Todarodes sagittatus* fished as bycatch trawling West off St. Kilda islands (N58°04′ W09°40′), in the NE Atlantic Ocean (FAO 27 area, Division VI a) in May/June of 2018 and April 2019.

amplification was performed using the primers 211F (5′-TTTTCTAGTTATATAGATTGRTTTYAT-3′) and 210R (5′-CACCAACTCTTAAAATTATC-3′) (Nadler and Hudspeth, 2000). Polymerase chain reaction (PCR) was carried out according to the procedures provided by Mattiucci et al. (2014). The sequences obtained at the mtDNA *cox*2 for the larval nematodes were compared with those already obtained for the same gene in our previous works and deposited in GenBank: *A. simplex* (s.s.) (DQ116426), *A. pegreffii* (JQ900761), *A. berlandi* (KC809999), *A. typica* (DQ116427), *A. ziphidarum* (DQ116430), *A. nascettii* (FJ685642), *A. physeteris* (DQ116432), *A. brevispiculata* (DQ116433) and *A. paggiae* (DQ116434).

2.4. Epidemiological data and statistical analysis

Quantitative infection assessment focused on nematode prevalence and abundance in squids was carried out separately for viscera and mantle. The epidemiological parameters considered, calculated using Statistica 13.1, were: prevalence (P, %), mean abundance (mA), and mean intensity (mI) with standard deviation (±SD) and range of infection (min–max) (according to (Bush et al., 1997). Correlation between squid length and values of the parasites abundance in the viscera, mantle, and overall, were tested by means of a Spearman rank test using Statistica® 13.3.0 (TIBCO Software Inc., CA, USA).

3. Results

3.1. Morphological and molecular identification

A total of 689 nematode larvae were collected in the viscera and mantle of the examined squids. Based on basic diagnostic morphological characters, all larval ascaridoid nematodes were recognized as *Anisakis* third-stage larvae (L3) showing larval Type I characters (sensu Berland, 1961).

According to the alleles observed at the diagnostic loci, i.e., *Adk-2*^{105}, *Pep C-1*^{90} and *Pep C-2*^{96} (Mattiucci et al., 2014), all *Anisakis* type I larvae of the randomly selected subsample (N = 100) were assigned to the species *A. simplex* (s.s.). The mtDNA *cox*2 nucleotide gene sequences (563 bp) obtained from the 100 larvae of the same subsample, matched 99% with *A. simplex* (s.s.) sequences previously deposited in the GenBank. Three sequences of *A. simplex* (s.s.) recovered from *T. sagittatus* were deposited in GenBank under these accession numbers: MW082624, MW082625, MW082626.

3.2. Epidemiology and site of infection

Anisakis simplex (s.s.) larvae were detected both in the mantle cavity encapsulated outside the internal organs, and inside the muscular mantle tissue of the squids (Figs. 2 and 3). Larvae positioned over visceral organs were coiled, mainly attached to outer surface of the stomach, mesenteries and alimentary tract (Fig. 2). No larvae were observed free in the stomach contents, thus recently acquired through diet.

Mantle-infecting larvae were mostly situated in the posterior half of the mantle adjacent to the squid hosts' digestive tract organs (especially, stomach) (Fig. 3). Some of the larvae were completely embedded in the muscular tissue, whereas few others were attached on the internal surface of mantle, but firmly adhering on the mantle tissue, only removable ripping the tissue with tweezers.

Data on infected squid number, prevalence (P), mean abundance (mA), and mean intensity (mI) of *A. simplex* (s.s.) larvae at different sites of infection (viscera and mantle) are given in Table 1. Concerning the larvae present in the viscera, the 54.4% of larvae were detected on the surface of stomach (seldom in the stomach wall), while the rest 45.6% of them were attached to viscera in the mantle cavity.

Spearman's correlation coefficient between *A. simplex* (s.s.) abundance in mantle, and mantle length was weakly positive, but not significant ($r = 0.106$, $p = 0.299$). Conversely, Spearman's correlation coefficient between mantle length and *A. simplex* (s.s.) abundance in

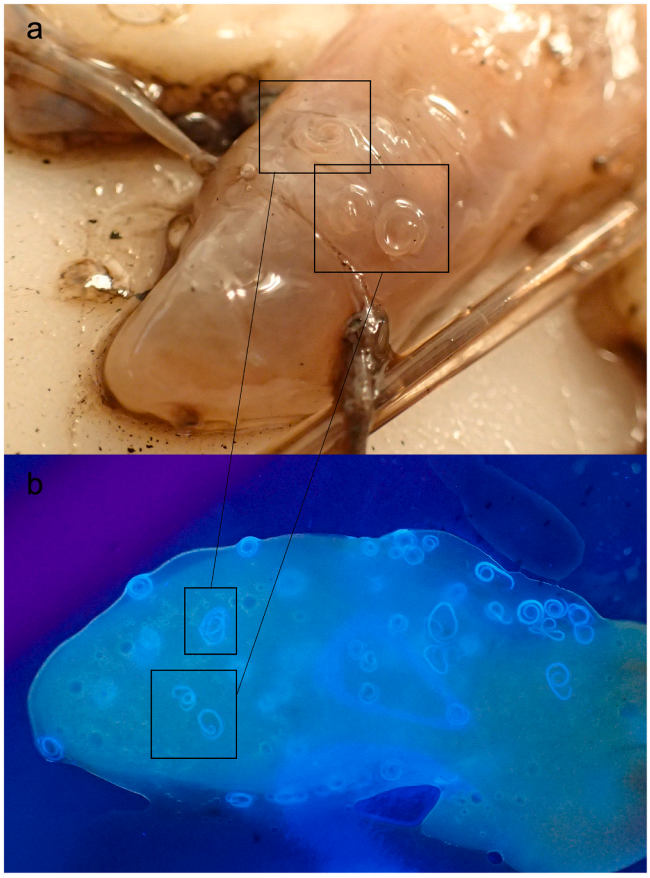

Fig. 2. Close up view of *Todarodes sagittatus* caecum with visible L_3 *Anisakis simplex* (s.s.) larvae. Above, picture took during squid dissection. Below, the same organ after being pressed and visualized under UV light.

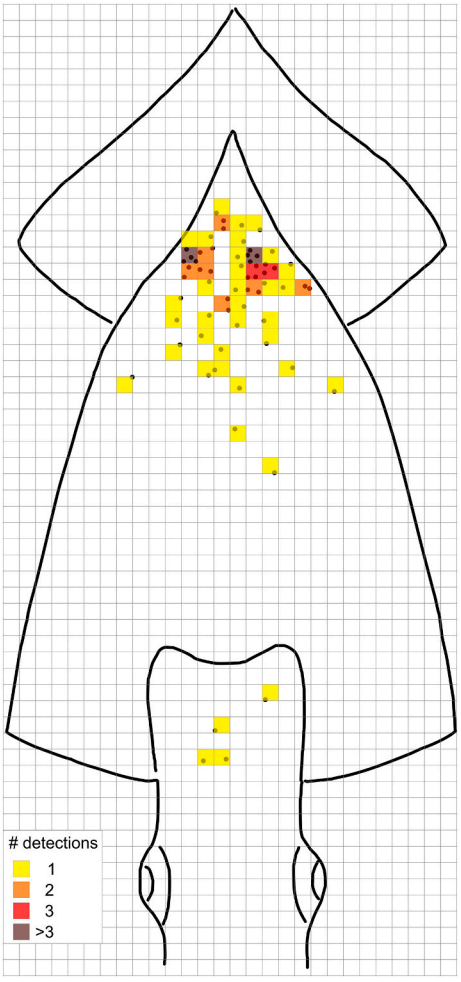

Fig. 3. Sketch of *Anisakis simplex* (s.s.) larvae distribution in *Todarodes sagittatus* mantle. The colors are associated to the abundance of larvae detected in each single cell. Warmer colors indicate higher abundances of larvae.

viscera, and overall (i.e. viscera and mantle), resulted positive and statistically significant (respectively r = 0.394, $p < 0.001$ and r = 0.393, $p < 0.001$) (Fig. 4).

4. Discussion

In the present study, data on occurrence and distribution of *A. simplex* (s.s.) infecting *T. sagittatus* from West off St. Kilda islands (NE Atlantic Ocean) are presented. The findings represent the first molecular detection of the zoonotic nematode *A. simplex* (s.s.) in the tissue mantle of *T. sagittatus*.

The overall infection level of *A. simplex* (s.s.) in *T. sagittatus* in the present study (P = 81% mI = 8.58) were higher than previous findings of anisakid nematodes in the same host species originating from other localities of the NE Atlantic Ocean and from the Mediterranean Sea. Pascual et al. (1996) conducted an epidemiological study on a wide range of cephalopod species fished in several fishing grounds off Galicia (NW Spain), and reported the presence of *A. simplex* B (former name of *A. simplex* (s.s.)) in the visceral cavity of 22 out of 65 *T. sagittatus* specimens examined (P = 34%). In the same geographical area, Abollo et al. (2001) reported 34% prevalence and 7.55 mean intensity of *A. simplex* (s.s.) in 70 *T. sagittatus* (mean mantle length 333 mm). The authors inspected the squid mantle by using peptic digestion method, but they did not detect *Anisakis* larvae in this tissue. Regarding epidemiological data of nematodes in *T. sagittatus* from the Mediterranean Sea, only 4 out 60 specimens fished in the Ionian Sea and South of Sicily resulted infected with *Hysterothylacium* sp., *A. pegreffii* and *A. physeteris* (Costa et al., 2012). Squids were visually inspected, and no larvae were detected in the mantle. Further, Angelucci et al. (2011) reported the presence of *Anisakis* type II and *Hysterothylacium* sp. in a small batch of 5 *T. sagittatus* obtained from a catch off Sardinia (central Mediterranean Sea).

Table 1
Anisakis simplex (s.s.) infection in *Todarodes sagittatus* (N_o = 98; mean length 248 ± 46 mm, min 150 mm, max 395 mm) collected off the NE Atlantic off St. Kilda coast (FAO 27 area): number of infected squids, prevalence (P, %), mean abundance (mA), mean intensity ($_m$I) with standard deviation (±SD) and range (min-max). Number of total larvae (N_{LTot}) and their relative distribution (%) in different sites of infection are also given.

	N_o of infected squids	P (%)	mA	$_m$I (±SD) (min-max)	N_{LTot} (%)
Overall	79	80.6	6.92	8.58 ± 12.27 (1–68)	689
Mantle	36	36.7	0.65	1.78 ± 1.57 (1–8)	64 (9.3%)
Viscera	78	79.6	6.38	8.01 ± 12.18 (1–68)	625 (90.7%)

Some studies on the parasite load of ommastrephid squids from the North Pacific Ocean, most referring to Japanese waters, reported the presence of *Anisakis* spp. in *Todarodes pacificus*. In a really large sample size of 2153 *T. pacificus*, a 3.2% prevalence of *A. simplex* (s.l.) was observed by Takahara and Sakurai (2010). In the same squid host, Nagasawa and Moravec (1995) detected "*A. simplex*" larvae, without details on the epidemiology. Unfortunately, no genetic identification

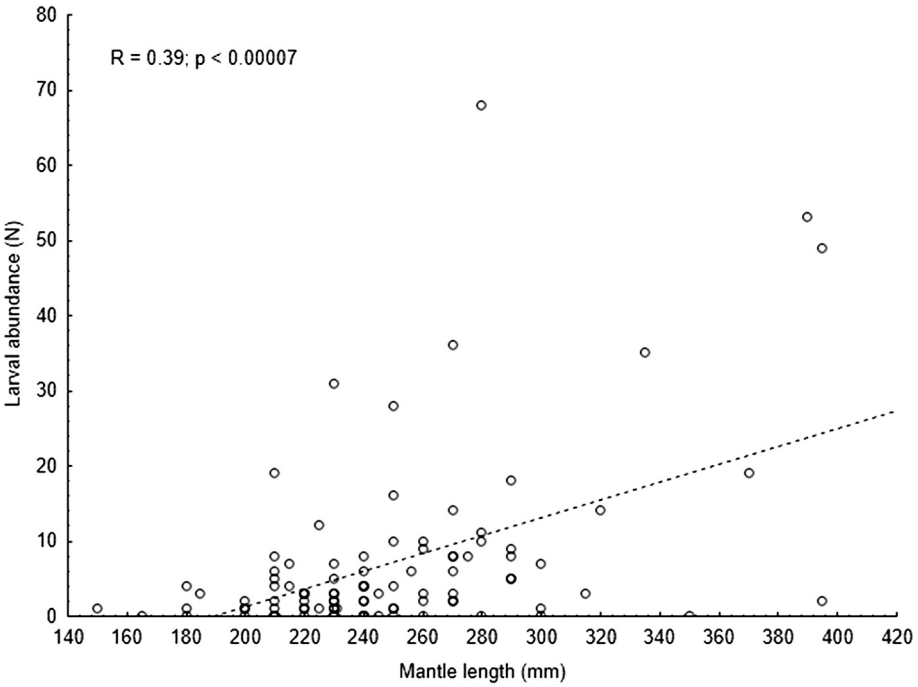

Fig. 4. Spearman's correlation coefficient tested by means of a Spearman rank test, between *A. simplex* (s.s.) abundance in *T. sagittatus* mantle, and mantle length.

was provided in support of these observations, and larval stages of the *A. simplex* (s.l.) complex cannot be identified on the basis of morphological traits.

Several of the aforementioned studies were based on a visual approach methodology, and candling, for the detection of helminth parasite in squid. This methodology, as detailed by Levsen et al. (2005) for fish, can lead to an underestimation of the total burden of anisakid parasites, and consequently loss of information on the larvae present in certain organs/tissue of the host, mostly in the muscular tissue. The presence here reported of several larvae of *A. simplex* (s.s.) in the mantle of *T. sagittatus*, and the higher prevalence recorded, could be partially explained by the efficiency of UV-press method in detecting *Anisakis* larvae in the squid mantle. The efficiency of this method used to detect *Anisakis* spp. larvae in fish hosts (Gómez-Morales et al., 2018), and also in squids, as *I. argentinus* (Cipriani et al., 2019) is described in Levsen et al. (2018), Gómez-Morales et al. (2018) and Cipriani et al. (2019), respectively.

Besides this methodological aspect, the higher parasite burden of the squid examined in this study could be addressed to ecological drivers, such as the squid population of origin, their feeding habits and prey availability (discussed further), their migratory patterns, the year of capture (owing to the short life span of squids), or even large scale environmental changes occurring currently in NE Atlantic area (see Levsen et al., 2020). Considering that the geographically closest previous parasitological study on *T. sagittatus* in this ocean district was carried out in 2001 (Abollo et al., 2001), the overall scanty data on parasite in ommastrephids so far available, and the short life span of these voracious predators, it is difficult to propose any explanation supporting the higher parasite burden found in the present study with respect to the past surveys.

The mean length of the 98 specimens of *T. sagittatus* was 248 ± 46 mm (min 150 mm, max 395 mm) (Table 1). According to an age/size relationship of *T. sagittatus* proposed by Rosenberg et al. (1981), squid with a size ranging from 150 mm to 395 mm could have an age ranging from 220 to 365 days.

In host-parasite systems such as ommastrephid squid, the helminths usually survive the whole host-life cycle (Hochberg, 1990). Morsan et al. (1999) reported a similar trend, with *Anisakis* sp. larvae prevalence and abundance showing an increment with squid age. According to histopathological studies, a typical cell mediated immune response to anisakid nematodes was observed on cephalopod tissue (Ford, 1992; Gestal et al., 2019), resulting in an encapsulation of the parasite as immune strategy to avoid parasite migration (Gestal et al., 2019). Thus, the positive and statistically significant correlation ($r = 0.394$, $p < 0.005$) between *T. sagittatus* mantle length and *A. simplex* (s.s.) overall abundance (viscera and mantle) (Fig. 4) can be explained by a biological accumulation of larvae through diet during the life span of the squid host. Ecological and behavioral traits of the cephalopod life cycle (namely: deep range, bathymetry, mantle size, potential fecundity and vagility, feeding behavior) appear to be important determinants of parasite richness (González et al., 2003). Furthermore, larval accumulation seems to be facilitated by the longevity of *Anisakis* spp. larvae, which may stay alive for long periods or even over fish host's lifetime (Hemmingsen et al., 1993; Køie, 2001; Smith, 1984). *Todarodes sagittatus* is an opportunistic predator, in upper level in the trophic web of marine ecosystems, feeding mostly on pelagic and mesopelagic fish species of a certain size (Joy, 1990; Lordan et al., 2001; Piatkowski et al., 1998; Quetglas et al., 1999). A particularly important prey of *T. sagittatus* in NE Atlantic seems to be represented by myctophids *Maurolicus muelleri*, *Micromesistius poutassou* and *Argentina* sp. (Piatkowski et al., 1998; Lordan et al., 2001). Some observations of the stomach content of *T. sagittatus* here examined, showed the presence of fish scales and residuals probably traceable to small mesopelagic myctophids. Furthermore, *T. sagittatus* were sampled as bycatch of *M. poutassou*, and myctophids were also spotted in the same catch. These observation on squid feedings seems in accordance with findings reported by Lordan et al. (2001). Furthermore, the finding of *A. simplex* (s.s.) in some myctophid spp. and *M. muelleri* (Bao et al., paper in preparation), and a high prevalence and abundance of *A. simplex* (s.s.) in *M. poutassou* in the same fishing ground (personal observation; Levsen et al., 2018), suggest that both those myctophids species and *M. potassou* could represent a source of *A. simplex* (s.s.) infection for *T. sagittatus*.

The localization and distribution of *A. simplex* (s.s.) in *T. sagittatus* seems to be strictly linked to their foodborne transmission, as larvae are acquired through predation. Most of the larvae present in the mantle cavity were embedded in the stomach wall or attached in the outer layer

of the stomach and caecum (49.3%), surrounded by a thin capsule (Fig. 2). It appears that the majority of larvae penetrate the stomach wall and then ensheath over the digestive tract or move into the portion of the mantle tissue directly in contact with these organs (Figs. 2, 3). In squids, the stomach can be extremely expandable, and it serves as a storage area for the initial digestion of prey items, while the caecum is a site of absorption (Gestal et al., 2019). Thus, all larvae acquired through diet tend probably to crawl out digesting matter and penetrate initially into the mantle cavity through these two organs. Furthermore, larvae seem to overcome limited migrations inside the squid host, remaining mostly (49.3%) "nearby" the site of absorption (stomach and caecum). The schematization of the distribution of larvae detected in the mantle proposed in Fig. 3 furthermore supports this finding, showing the most of muscle invading larvae located in the anterior half of the mantle, surrounding caecum and stomach.

Since larval L3 stage of *A. simplex* (s.s.) is the etiological agent of the zoonotic disease known as human anisakiasis (D'Amelio et al., 1999; Fumarola et al., 2009; Lim et al., 2015; Mattiucci et al., 2011, 2013, 2017b, 2018; Umehara et al., 2007), the presence of larvae in *T. sagittatus*, above all in the edible mantle, might represent a risk if squid are consumed raw or poorly cooked. Even though ommastrephid squid catches are discontinuous, they assume a consistent importance in some countries. The use of squid for human consumption is extensive in Spain, Italy, and Japan, being these countries the main consumers and importers of these fish species (FAO, 2016, 2018; Gestal et al., 2019). Despite of this commercial interests, the presence of zoonotic anisakid nematodes in squids, and cephalopods in general, has been poorly investigated. The few epidemiological studies on anisakid nematodes in *T. sagittatus* report the presence of larvae in the visceral cavity, but the present study appears to be the first to report the presence of *A. simplex* (s.s.) in the mantle tissue of this squid species. The infection level of *A. simplex* in the mantle of *T. sagittatus* here reported, show that a third of caught squids (36.7%) had at least one larva in the mantle, with mean intensity 1.78, and with a single squid hosting 8 larvae. The most of mantle invading larvae were completely embedded in the muscular tissue, whereas few others were attached on the internal surface of mantle, but firmly attached to the mantle tissue. Thus, the risk of anisakiasis associated with consumption of raw or undercooked preparation should be considered. According to the larvae distribution schematized in Fig. 3, some specific portions of the squid mantle (anterior half, tissue surrounding caecum and stomach) could carry a higher number of parasites, while tentacles and other part of the edible tissue could have negligible risk of containing zoonotic agents. Applied rules to prevent zoonotic hazard by anisakid nematodes for commercialization, marketing and preparation of seafood, as recommended by EFSA (2010), should reduce the risk. Even if cephalopods in European countries are mostly consumed cooked, in Japan, indeed, a large quantity of squids are consumed fresh and raw, in sashimi and sushi preparations (Nagasawa and Moravec, 1995). In Japan the *Anisakis* risk is well known. The Japanese common squid (*T. pacificus*) is one of major fisheries resources (Nagasawa and Moravec, 1995), and its traditional consumption is considered one of the most frequent sources of anisakiasis cases in that country (Nagasawa, 1990). Several recent cases of human anisakiasis have been associated with consumption of raw squid in Japan (Furuya et al., 2018; Ogata et al., 2015; Tamai and Kobayashi, 2015).

Considering the current globalization of food preparations, and ongoing trend of consumption of raw or lightly processed seafood, getting common also in western countries, preventive measures to kill the parasite, as recommended by EFSA (2010), should be applied whenever *T. sagittatus* are to be consumed raw or lightly processed.

Declaration of competing interest

The authors declare that they have no conflicting interests

Acknowledgments

We would like to thank Alf-Tore Leine, a proud fisherman of Kings Bay SAS, for his help in sampling part of the bycatches of *T. sagittatus* that permitted this research. We thank also Dr. Gian Luca Sbaraglia for his help during squid inspection, and Dr. Simone Sammartino for his precious help in the editing of the mantle larval distribution figure.

References

Abollo, E., Gestal, C., Pascual, S., 2001. *Anisakis* infestation in marine fish and cephalopods from Galician waters: an updated perspective. Parasitol. Res. 87, 492–499.

Angelucci, G., Meloni, M., Merella, P., Sardu, F., Madeddu, S., Marrosu, R., Petza, F., Salati, F., 2011. Prevalence of *Anisakis* spp. and *Hysterothylacium* spp. larvae in teleosts and cephalopods sampled from waters off Sardinia. J. Food Prot. 74, 1769–1775.

Audicana, M.T., Kennedy, M.V., 2008. *Anisakis simplex*: from obscure infectious worm to inducer of immune hypersensitivity. Clin. Microbiol. 21, 360–369.

Bao, M., Pierce, G., Strachan, N., Pascual, S., González-Muñoz, M., Levsen, A., 2019. Human health, legislative and socioeconomic issues caused by the fish-borne zoonotic parasite *Anisakis*: challenges in risk assessment. Trends Food Sci. Technol. 86, 298–310.

Berland, B., 1961. Nematodes from some Norwegian marine fishes. Sarsia 2, 150.

Borges, T.C., Wallace, J.C., 1993. Some aspects of the fishery biology of the ommastrephid squid *Todarodes sagittatus* (Lamarck, 1798) from the Northeast Atlantic. In: Okutani, T., O'Dor, R.K., Kubodera, T. (Eds.), Recent Advances in Fisheries Biology. Tokyo University Press, Tokyo, pp. 25–36.

Breiby, A., Jobling, M., 1985. Predatory role of the Hying squid (*Todarodes sagittatus*) in north Norwegian waters. NAFO Sci. Coun. Studies. 9, 125–132.

Bush, A.O., Lafferty, K.D., Lotz, J.M., Shostak, A.W., 1997. Parasitology meets ecology on its own terms: Margolis et al. revisited. J. Parasitol. 83, 575–583.

Cipriani, P., Giulietti, L., Palomba, M., Mattiucci, S., Bao, M., Levsen, A., 2019. Occurrence of larval ascaridoid nematodes in the Argentinean short-finned squid *Illex argentinus* from the Southwest Atlantic Ocean (off Falkland Islands). Int. J. Food Microb. 297, 27–31.

Clarke, M.R., 1996. Cephalopods as prey III. Cetaceans. Philos. Trans. R. Soc Lond. (B). 351, 1053–1065.

Costa, A., Casalinuovo, F., Bonfiglio, R., Pisano, P., Sciortino, S., Di Noto, A.M., 2012. Presenza di larve di Anisakidae in Totani (*Todarodes sagittatus*) pescati lungo le coste siciliane e calabresi. XVIII Convegno Nazionale S.I.P.I. http://www.sipi-online.it/convegni/2012/atti2012.pdf (in Italian).

D'Amelio, S., Mathiopoulos, K.D., Brandonisio, O., Lucarelli, G., Doronzo, F., Paggi, L., 1999. Diagnosis of a case of gastric anisakidosis by PCR-based restriction fragment length polymorphism analysis. Parassitologia 41, 591–593.

Daschner, A., Cuéllar, C., 2020. Progress in *Anisakis* allergy research: milestones and reversals. Curr. Treat. Options Allergy 1–14.

Daschner, A., Cuéllar, C., Rodero, M., 2012. The *Anisakis* allergy debate: does an evolutionary approach help? Trends in Parasitol. 28, 9–15.

Dunning, M.C., Wormuth, J.H., 1998. The Ommastrephid squid genus *Todarodes*: a review of systematics, distribution, and biology. Smithson. Contr. Zool. 586, 385–391.

EFSA, 2010. Scientific opinion on risk assessment of parasites in fishery products. EFSA J. 8, 1543.

FAO, 2016. The State of World Fisheries and Aquaculture 2016. Contribution to Food Security and Nutrition for All, Rome, p. 220.

FAO, 2018. The State of World Fisheries and Aquaculture 2018 - Meeting the Sustainable Development Goals, p. 210 (Rome).

FAO, 2020. Fishery statistical collections - global capture production. http://www.fao.org/fishery/statistics/global-capture-production/en. (Accessed 22 April 2020) (WWW Document).

Ford, L., 1992. Host defense mechanisms of cephalopods. Annu Rev Fish Disea 1, 25–41.

Fumarola, L., Monno, R., Ierardi, E., Rizzo, G., Giannelli, G., Lalle, M., Pozio, E., 2009. *Anisakis pegreffii* etiological agent of gastric infections in two Italian women. Foodborne Pathog. Dis. 6, 1157–1159.

Furuya, K., Nakajima, H., Sasaki, Y., Urita, Y., 2018. Anisakiasis: the risks of seafood consumption. Niger. J. Clin. Pract. 21, 1492–1494.

Gestal, C., Pascual, S., Guerra, A., Fiorito, G., Vieites, J., 2019. Handbook of Pathogens and Diseases in Cephalopods. Springer, p. 173.

Gómez-Morales, M., Martínez-Castro, C., Lalle, M., Otero, R., Pezzotti, P., Abollo, E., Pozio, E., 2018. UV-press method versus artificial digestion method to detect Anisakidae L3 in fish fillets: comparative study and suitability for the industry. Fish. Res. 202, 22–28.

González, Á., Pascual, S., Gestal, C., Abollo, E., Guerra, A., 2003. What makes a cephalopod a suitable host for parasite? The case of Galician waters. Fish. Res. 60, 177–183.

Guerra, A., 1992. Mollusca, Cephalopoda. In: Ramos, M.A. (Ed.), Fauna Ibérica, vol. I. Museo Nacional de Ciencias Naturales, CSIC, Madrid, p. 327.

Hemmingsen, W., Lysne, D.A., Eidnes, T., Skorping, A., 1993. The occurrence of larval ascaridoid nematodes in wild-caught and in caged and artificially fed Atlantic cod, *Gadus morhua* L., in Norwegian waters. Fish. Res. 15, 379–386.

Hochberg, F.G., 1990. Diseases of Mollusca: Cephalopoda. In: Kinne, O. (Ed.), Diseases of Marine Animals, vol. 3. Cephalopoda to Urochordata Biologisches Anstalt Helgoland, Hamburg, pp. 47–227.
ICES, 2019. Interim Report of the Working Group on Cephalopod Fisheries and Life History (WGCEPH), 5–8 June 2018, Pasaia, San Sebastian, Spain. ICES CM 2018/EPDSG:12, p. 194.
Joy, J.B., 1990. The fishery biology of *Todarodes sagittatus* in Shetland waters. J. Cephalopod Biol. 1, 1–19.
Karl, H., Leinemann, M., 1993. A fast and quantitative detection method for nematodes in fish fillets and fishery products. Arch. Leb. 44, 124–125.
Karl, H., Levsen, A., 2011. Occurrence and distribution of anisakid nematodes in grey gurnard (*Eutrigla gurnardus* L.) from the North Sea. Food Control 22, 1634–1638.
Køie, M., 2001. Experimental infections of copepods and sticklebacks *Gasterosteus aculeatus* with small ensheathed and large third-stage larvae of *Anisakis simplex* (Nematode, Ascaridoidea, Anisakidae). Parasitol. Res. 87, 32–36.
Levsen, A., Lunestad, B.T., Berland, B., 2005. Low detection efficiency of candling as a commonly recommended inspection method for nematode larvae in the flesh of pelagic fish. J. Food Prot. 68, 828–832.
Levsen, A., Svanevik, C.S., Cipriani, P., Mattiucci, S., Gay, M., Hastiee, L.C., Pierce, G.J., Bušelić, I., Mladineo, I., Karl, O., Ostermeyer, U., Buchmann, K., Højgaard, D.P., González, A.F., Pascual, S., 2018. A survey of zoonotic nematodes of commercial key fish species from major European fishing grounds—introducing the FP7 PARASITE exposure assessment study. Fish. Res. 202, 4–21.
Levsen, A., Cipriani, P., Bao, M., Giulietti, L., Mattiucci, S., 2020. Anisakiosis (*Anisakis simplex* s.l.). In: Woo, P.T.K., Leong, J.A., Buchmann, K. (Eds.), Climate Change and Infectious Fish Diseases. CABI, pp. 457–470.
Lim, H., Jung, B.K., Cho, J., Yooyen, T., Shin, E.H., Chai, J.Y., 2015. Molecular diagnosis of cause of anisakiasis in humans, South Korea. Emerg. Infect. Dis. 21, 342–344.
Lordan, C., Collins, M., Key, L., Browne, E., 2001. The biology of the ommastrephid squid, *Todarodes sagittatus*, in the north-east Atlantic. J. Mar. Biol. Assoc. U. K. 81, 299–306.
Mattiucci, S., Nascetti, G., 2008. Advances and trends in the molecular systematics of anisakid nematodes, with implications for their evolutionary ecology and host-parasite co-evolutionary processes. Adv. Parasitol. 66, 47–148.
Mattiucci, S., Nascetti, G., Cianchi, R., Paggi, L., Arduino, P., Margolis, L., Brattey, J., Webb, S.C., D'Amelio, S., Orecchia, P., Bullini, L., 1997. Genetic and ecological data on the *Anisakis simplex* complex with evidence for a new species (Nematoda, Ascaridoidea, Anisakidae). J. Parasitol. 83, 401–416.
Mattiucci, S., Paoletti, M., Borrini, F., Palumbo, M., Palmieri, R.M., Gomes, V., Casati, A., Nascetti, G., 2011. First molecular identification of the zoonotic parasite *Anisakis pegreffii* (Nematoda: Anisakidae) in a paraffin-embedded granuloma taken from a case of human intestinal anisakiasis in Italy. BMC Infect. Dis. 11, 82.
Mattiucci, S., Fazii, P., De Rosa, A., Paoletti, M., Salomone Megna, A., Glielmo, A., De Angelis, M., Costa, A., Meucci, C., Calvaruso, V., Sorrentini, I., Palma, G., Bruschi, F., Nascetti, G., 2013. Anisakiasis and Gastroallergic reactions associated to *Anisakis pegreffii* infection. Italy. Emerg. Infect. Dis. 19, 496–499.
Mattiucci, S., Cipriani, P., Webb, S.C., Paoletti, M., Marcer, F., Bellisario, B., Gibson, D.I., Nascetti, G., 2014. Genetic and morphological approaches distinguish the three sibling species of the *Anisakis simplex* species complex, with a species designation as *Anisakis berlandi* n. sp. for *A. simplex* sp. C (Nematoda: Anisakidae). J. Parasitol. 100, 199–214.
Mattiucci, S., Cipriani, P., Paoletti, M., Levsen, A., Nascetti, G., 2017a. Reviewing biodiversity and epidemiological aspects of anisakid nematodes from the North-east Atlantic Ocean. J. Helminthol. 91, 422–439.
Mattiucci, S., Paoletti, M., Colantoni, A., Carbone, A., Gaeta, R., Proietti, A., Frattaroli, S., Fazii, P., Bruschi, F., Nascetti, G., 2017b. Invasive anisakiasis by the parasite *Anisakis pegreffii* (Nematoda: Anisakidae): diagnosis by real-time PCR hydrolysis probe system and immunoblotting assay. BMC Infect. Dis. 17, 530.
Mattiucci, S., Cipriani, P., Levsen, A., Paoletti, M., Nascetti, G., 2018. Molecular epidemiology of *Anisakis* and anisakiasis: an ecological and evolutionary road map. Adv. in Parasitol. 99, 93–263.
Mladineo, I., Popović, M., Drmić-Hofman, I., Poljak, V., 2016. A case report of *Anisakis pegreffii* (Nematoda, Anisakidae) identified from archival paraffin sections of a Croatian patient. BMC Inf. Dis. 16, 42.
Morsan, E., González, R., Kroek, M., 1999. Composición poblacional y pesquería de *Illex argentinus* en el Golfo San Matías. In: Boschi, E. (Ed.), Final Report of the Project INIDEPJICA. Evaluación y monitoreo de recursos pesqueros, Mar del Plata, pp. 161–163 (in Spanish).
Nadler, S., Hudspeth, D., 2000. Phylogeny of the Ascaridoidea (Nematoda: Ascaridida) based on three genes and morphology: hypotheses of structural and sequence evolution. J. Parasitol. Res. 86, 380–393.
Nagasawa, K., 1990. *Anisakis* larvae in intermediate and paratenic hosts in Japan. In: Ishikura, H., Kikuchi, K. (Eds.), Intestinalis Anisakiasis in Japan: Infected Fish, Seroimmunological Diagnosis, and Prevention. Springler-Verlag, Tokyo, pp. 23–29.
Nagasawa, K., Moravec, F., 1995. Larval Anisakid nematodes of Japanese common squid (*Todarodes pacificus*) from the Sea of Japan. J. Parasitolog. 8, 68–75.
Nieuwenhuizen, N.E., 2016. *Anisakis* - immunology of a foodborne parasitosis. Parasite Immunol. 38, 548–557.
Ogata, M., Tamura, S., Matsunoya, M., 2015. Sonographic diagnosis of intestinal anisakiasis presenting as small bowel obstruction. J. Clin. Ultrasound 43, 283–287.
Pascual, S., Gestal, C., Estévez, J., Rodríguez, H., Soto, M., Abollo, E., Arias, C., 1996. Parasites in commercially exploited cephalopods (Mollusca, Cephalopoda) in Spain: an updated perspective. Aquaculture 142, 1–10.
Piatkowski, U., Hernández-García, V., Clarke, M.R., 1998. On the biology of the European flying squid *Todarodes sagittatus* (Lamarck, 1798) (Cephalopoda, Ommastrephidae) in the central eastern Atlantic. South African J. Mar. Sci. 375–383.
Pippy, J.H., 1970. Use of ultraviolet light to find parasitic nematodes in situ. J. Fish. Res. Board Can. 27, 963–965.
Quetglas, A., Alemany, F., Carbonell, A., Merella, P., Sanchez, P., 1999. Diet of the European Hying squid *Todarodes sagittatus* (Cephalopoda: Ommastrephidae) in the Balearic Sea (western Mediterranean). J. Mar. Biolog. Assoc. U.K. 79, 479–486.
Roca-Geronès, X., Alcover, M.M., Godínez-González, C., González-Moreno, O., Masachs, M., Fisa, R., Montoliu, I., 2020. First molecular diagnosis of clinical cases of gastric anisakiosis in Spain. Genes (Basel). 11, 452.
Rosenberg, A.A., Wiborg, K.F., Bech, I.M., 1981. Growth of *Todarodes sagittatus* (Lamarck) (Cephalopoda, Ommastrephidae) from the northeast Atlantic, based on counts of statolith growth rings. Sarsia 66, 53–57.
Smith, J.W., 1984. *Anisakis simplex* (Rudolphi, 1809, det. Krabbe, 1878): length distribution and viability of L3 of known minimum age from herring *Clupea harengus* L. J. Helmint. 58, 337–340.
Takahara, H., Sakurai, Y., 2010. Infection of the Japanese common squid, *Todarodes pacificus* (Cephalopoda: Ommastrephidae) by larval anisakid nematodes. Fish. Res. 106, 156–159.
Tamai, Y., Kobayashi, K., 2015. Asymptomatic colonic anisakiasis. Intern. Med. 54, 675.
Umehara, A., Kawakami, Y., Araki, J., Uchida, A., 2007. Molecular identification of the etiological agent of the human anisakiasis in Japan. Parasitol. Int. 56, 211–215.
Vieites, J.M., Ruiz, C.S., Fernández, F., Alonso, R.C., 2019. In: Gestal, C., Pascual, S., Guerra, A., Fiorito, G., Vieites, J.M. (Eds.), Importance of cephalopod health and welfare for the commercial sector. Springer International Publishing, Handbook of Pathogens and Diseases in Cephalopods, pp. 5–7.
Wiborg, K.F., Gjosaeter,J., Beck, I.M., Fossum, P., 1982. Squid *Todarodes sagittatus* (Lamarck) distribution and biology in northern waters, April 1981–April 1982. International Council for the Exploration of the Sea (CM Papers and Reports), CM 1982/K:30.