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# Occurrence of larval ascaridoid nematodes in the Argentinean short-finned squid *Illex argentinus* from the Southwest Atlantic Ocean (off Falkland Islands)

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ABSTRACT

The Argentinean short-finned squid (*Illex argentinus*) is an oceanic, neritic species widely distributed off the east coast of South America, representing the most abundant commercially exploited squid species in these waters. Despite the great commercial importance of Argentinean short-finned squid as a food resource, and as frozen product exported to Europe, the presence of zoonotic anisakid nematodes, especially in the mantle of the squid, is poorly known. The occurrence and site of infection of larval ascaridoid nematodes in 70 *I. argentinus* caught off the Falkland Islands were investigated. Squids were examined using the UV-Press method. In total, 30 nematodes were detected in the viscera and mantle. According to morphology, 27 were third-stage larvae ($L_3$) belonging to genus *Anisakis*, while three were $L_3$ assigned to *Hysterothylacium*. *Anisakis pegreffii* ($n = 27$) were identified by sequence analysis of the mtDNA *cox2* and the partial EF1 α-1 region of nDNA genes; *Hysterothylacium aduncum* ($N = 3$) were identified by sequence analysis of the ITS rDNA region. These findings represent the first molecular identification of *A. pegreffii* and *H. aduncum* in *I. argentinus*. Both prevalence ($P = 15.7\%$) and abundance ($A = 0.39$) of infection with *A. pegreffii* were low, and even lower values of infection were recorded for *H. aduncum* ($P = 2.1\%$, $A = 0.04$). Only 3 out of 70 (4.3%) squids hosted *A. pegreffii* larvae in the mantle. Larvae infecting viscera were coiled and mainly attached to outer surface of visceral organs. Mantle-infecting larvae were situated in the posterior half. Thus, these results suggest that – although low - the risk of acquiring anisakiasis from consumption of raw, marinated and/or undercooked short-finned squid products still exists.

## 1. Introduction

The Argentinean short-finned squid, *Illex argentinus* (Castellanos, 1960), is the most abundant commercial squid species in the Southwest (SW) Atlantic Ocean (Arkhipkin et al., 2015).

It is an oceanic, neritic and highly migratory ommastrephid species, widely distributed along the Patagonian shelf and slope, occurring from 22°S to 55°S (Haimovici et al., 1998, 2014; Hatanaka, 1988). Largest accumulations of this species were observed on the shelf of the Northwest of the Falkland (Malvinas) Islands and on the shelf edge at 45°–47°S (Haimovici et al., 1998). This catching area supports one of the most important squid fisheries of the world (Haimovici et al., 1998). *Illex argentinus* is a short lived (approximately one year of lifespan) and fast-growing species with a long and muscular mantle, which may reach 33 cm in length (Roper et al., 1984).

In the 1970s, the species was part of the by-catch of the hake trawl fishery, and total annual catches were low, i.e. < 5900 t in 1977 (Brunetti, 1990). In the 1990s, a large-scale fishery started on the Argentinean and Uruguayan shelf, around the Falkland Islands and in international waters along the Patagonian slope, where catches of squid species by trawlers and jiggers of several countries increased rapidly (Haimovici et al., 1998). *Illex argentinus* was reported as the most abundant squid species in the SW Atlantic (Haimovici et al., 1998), but as the resource is currently overexploited, Argentina has suspended the fishery since June 2017 (http://www.fao.org/in-action/globefish/market-reports/resource-detail/en/c/1071597). Argentinian export of *I. argentinus* consists almost entirely of frozen squid products, mostly directed to European (mainly Spain and Italy) and Japanese markets

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(Anderson, 2003; CBI ministry foreign affairs, 2018).

To date, only a few epidemiological studies of ascaridoid nematodes (e.g. *Anisakis* spp. and *Hysterothylacium* spp.) in *I. argentinus* from the SW Atlantic Ocean exist, reporting generally low prevalence and abundance levels (Gonzàlez and Kroeck, 2000; Haimovici et al., 2014; Nigmatullin and Shukhgalter, 1990; Santos, 1992; Sardella et al., 1990; Threlfall, 1970). However, none of the ascaridoid species were molecularly identified and information on anatomical infection site of the larvae in the squid host was not provided.

The genus *Anisakis* includes species of heteroxenous parasites of marine organisms, with crustaceans as first intermediate hosts, fishes and squids as intermediate and/or paratenic hosts, and mainly cetaceans as definitive hosts (reviewed in Mattiucci et al., 2018). In fish and squid, the third larval stage (L3) of *Anisakis* spp. commonly resides encysted on the visceral organs. Some larvae may migrate from the visceral cavity into the fish flesh or squid mantle, thus posing a potential human health risk. Aspects on general *Anisakis* life cycle and transmission ecology have been recently reviewed (Mattiucci et al., 2018). *A. pegreffii* is the most frequently reported *Anisakis* species in the southern Argentine Sea, dominated by sub-Antarctic waters, and can be considered as representatives of southern region of the South-West Atlantic (Hernández-Orts et al., 2015; Lanfranchi et al., 2018; Mattiucci and Nascetti, 2007). Indeed, data on their distribution in the edible parts (mantle, in the case of squids), together with infection levels in the viscera, are of great importance, since the L3 of some *Anisakis* species is the etiological agent of a zoonotic disease known as human anisakiasis.

Anisakiasis may occur if live larvae are accidentally ingested when eating raw, marinated or undercooked parasitized fish/squid products (Audicana and Kennedy, 2008; Daschner et al., 2012; Nieuwenhuizen, 2016). Among the nine *Anisakis* species which have been described and genetically characterized to date (Mattiucci et al., 2014, 2018), only *A. simplex* sensu stricto and *A. pegreffii* have been recognized as zoonotic species, potentially causing human anisakiasis (Fumarola et al., 2009; Guardone et al., 2018; Lim et al., 2015; Mattiucci et al., 2011, 2013, 2018; Mladineo et al., 2016; Umehara et al., 2007).

The genus *Hysterothylacium* currently consists of > 70 species and is considered one of the largest of the ascaridoid genera parasitizing fish (Shamsi, 2016). Species belonging to this genus have also been found in *I. argentinus* from the Southwest Atlantic Ocean (Nigmatullin and Shukhgalter, 1990) and other squid species, such as *I. coindetii* (Picó-Durán et al., 2016) and *T. sagittatus* (Angelucci et al., 2011) in the Mediterranean Sea.

The aim of the present study was to detect larval ascaridoid nematodes in a sample of *I. argentinus* caught off the Falkland Islands, in order to: i) identify larval nematodes to species level using morphological and molecular methods; and ii) determine parasitic infection levels and the anatomical infection sites in the squid host.

## 2. Materials and methods

### 2.1. Squid sampling

In total, 70 specimens of *I. argentinus* were obtained from a catch fished by angling off the East coast of Argentina/Falklands (FAO 41 area) in February 2018 (Fig. 1). The squids were immediately frozen and shipped to the laboratory of the Institute of Marine Research (IMR) in Bergen, Norway, to be analysed for contaminants and biohazards as part of a routine veterinary border control.

### 2.2. Parasitological analysis

After thawing, all specimens were measured (mantle length) to the nearest 0.5 cm before inspection for parasitic nematodes. Since not all squids were intact, the mantle length of six specimens had to be estimated by aligning them with similarly sized individuals on the measuring board. Total body weight was not reported because several squids lacked body parts such as tentacles or arms.

Following evisceration, viscera and mantle of each squid were placed in separate plastic bags, flattened to 1–2 mm thick layers in a hydraulic press at 8 bar 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). The larvae 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 identified to genus level using bright field microscopy and by following the diagnostic keys according to Berland (1961).

*Anisakis* larvae were identified to species level using a multi-marker genotyping approach (mtDNA *cox2* and EF1 α-1 nDNA genes sequence analyses). 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 (*cox2*) gene, PCR amplification was performed using the primers 211F (5′-TTTTCTAGTTATATAGATTGRTTYAT-3′) and 210R (5′-CACCAACT CTTAAAATTA TC-3′) (Mattiucci et al., 2014). Polymerase chain reaction (PCR) was carried out according to the procedures provided by Mattiucci et al. (2014). The sequences obtained at the mtDNA *cox2* 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).

All the *Anisakis* spp. larvae, previously identified by mtDNA *cox2* gene, were sequenced at the elongation factor (EF1 $\alpha-1$ nDNA) nuclear gene. The EF1 $\alpha-1$ nDNA was amplified using the primers EF-F ( 5′-TCCTCAAGCGTTGTTATCTGTT-3′) and EF-R (5′-AGTTTTGCCACTA GCGGTTCC-3′) (Mattiucci et al., 2016). The PCR conditions and procedures followed those reported in Mattiucci et al. (2016). The sequences obtained for the EF1 $\alpha-1$ nDNA gene for the larval specimens were compared with those previously deposited in GenBank, at the diagnostic positions (i.e. 186 and 286) as previously detailed (Mattiucci et al., 2016).

In addition, the larval specimens of *Hysterothylacium* spp. were identified to species level by sequence analysis of the internal transcribed spacers (ITS rDNA) region. DNA was extracted using the same procedure reported above for *Anisakis* spp. PCR amplification was performed using the primers NC5 (5′-GTAGGTGAACCTGCGGAAGGAT CATT-3′) and NC2 (5′-TTAGTTTCTTTTCCTCCGCT-3′), according to the procedures reported in Zhu et al. (2000). All sequences obtained were compared with the Raphidascarididae sequences previously deposited in GenBank.

### 2.4. Epidemiological data analysis

Quantitative infection assessment focused on nematode prevalence and abundance, separately for viscera and mantle of the squids. The epidemiological parameters considered were: prevalence (P, %) with confidence limits (Clopper-Pearson), abundance (A) with range of infection (min – max) and mean intensity (mI) with standard deviation ( ± SD). Spearman rank test was run to assess the relationship between squid mantle length and *Anisakis* spp. larval abundance, using Statistica 13.1.

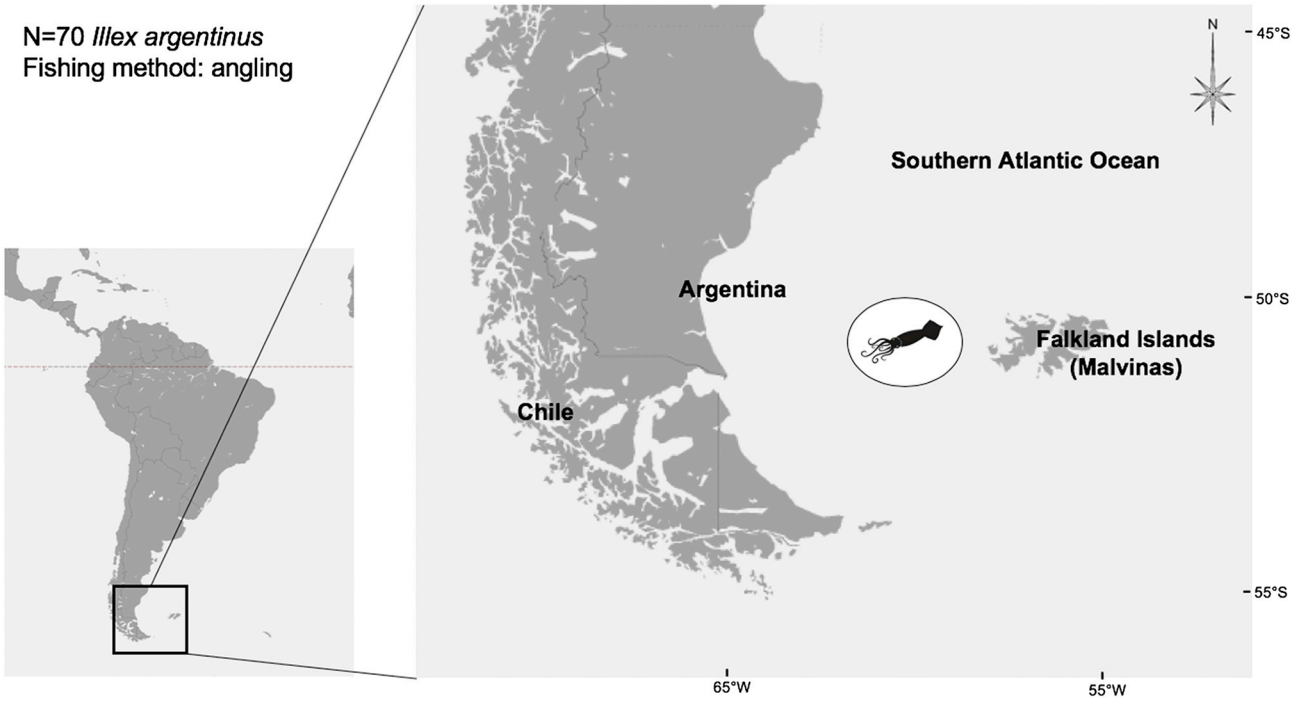


**Fig. 1.** Sampling locality of 70 specimens of *Illex argentinus* fished by angling off the East coast of Argentina/Falklands (FAO 41 area) during February 2018.

## 3. Results

### 3.1. Morphological and molecular identification

A total of 30 nematode larvae were detected in the viscera and mantle of the examined squids. Based on basic diagnostic morphological characters, 27 out of 30 (90%) larval ascaridoid nematodes were recognized as *Anisakis* third-stage larvae ($L_3$) showing larval Type I characters (sensu Berland, 1961), while the other 3 specimens (10%) were identified as $L_3$ belonging to genus *Hysterothylacium* (Ascaridoidea, Raphidascarididae, Nematoda).

According to the obtained sequences at the mitochondrial *cox2* gene and those at the EF1 α-1 of nDNA gene, the 27 *Anisakis* larvae analysed were assigned to species *A. pegreffii*.

The mtDNA *cox2* sequences obtained (563 bp) matched at 99% with the mtDNA *cox2* sequences of *A. pegreffii* obtained in previous works and deposited in GenBank.

The EF1 α-1 of nDNA subunit partial gene (409 bp) sequences also identified the larvae as *A. pegreffii*, according to the two diagnostic nucleotide positions described previously at that locus (Mattiucci et al., 2016), when compared with the sequences previously deposited in GenBank.

For each molecular marker analysed (mtDNA cox2 and EF1 α-1 nDNA genes), three sequences of *A. pegreffii* from *I. argentinus* were deposited in GenBank (accession numbers, respectively: MK598051, MK598052, and MK598053; MK598054, MK598055, and MK598056).

Based on the ITS rDNA sequence analysis, the 3 specimens of *Hysterothylacium* sp. were identified as *Hysterothylacium aduncum*. The sequences obtained (871 bp) matched at 99% with the ITS rDNA sequence of *H. aduncum* deposited in GenBank.

Two ITS rDNA gene sequences of *H. aduncum* larvae from *I. argentinus* were deposited in GenBank (accession numbers: MK580822 and MK580823).

### 3.2. Epidemiology and site of infection

*Anisakis pegreffii* larvae were detected in the viscera and mantle of the squids. Larvae infecting viscera were coiled, mainly attached to outer surface of the stomach and alimentary tract. Mantle-infecting larvae, embedded in the muscular tissue, were situated in the posterior half of the mantle covering the squid hosts' visceral organs.

Data on infected squid number, prevalence (P), abundance (A), and mean intensity (mI) of *A. pegreffii* larvae at different sites of infection (viscera and mantle) are given in Table 1. The overall prevalence recorded for *A. pegreffii* was $P = 15.7\%$ (11 infected out of 70), with a mean intensity of 2.45 (Table 1). The prevalence in the mantle was $P = 4.3\%$, and the three infected *I. argentinus* hosted a single larva each. The relative proportion of *A. pegreffii* larvae by infection site was 88.9% of larvae in the viscera and 11.1% in the mantle.

Spearman's correlation coefficient between *A. pegreffii* numbers and mantle length was weakly positive but non-significant ($r = 0.180$, $p = 0.135$).

All *H. aduncum* ($N = 3$) were detected in the mantle cavity among visceral organs of the squid host, showing low prevalence ($P = 2.9\%$) (2 infected out of 70), and low abundance (A = 0.04).

**Table 1**
*Anisakis pegreffii* infection in *Illex argentinus* (N = 70) collected off the East coast of Argentina/Falklands (FAO 41 area): number of infected squids, prevalence (P, %) with confidence limits (Clopper-Pearson), abundance (A), mean intensity ($_mI$,) with standard deviation ( ± SD) and range (min-max). Number of total larvae ($N_{LTot}$) and their relative proportions (%) in different sites of infection are also given.

| | N of infected squids | P (%) | A | $_mI$ ( ± SD) (min-max) | $N_{LTot}$ (%) |
|---|---|---|---|---|---|
| Overall | 11 | 15.7<br>0.080 - 0.264 | 0.39 | 2.45 ± 2.58<br>(1 - 9) | 27 |
| Mantle | 3 | 4.3<br>0.009 - 0.120 | 0.04 | 1.0 ± 0.00<br>(1 - 1) | 3<br>(11.1%) |
| Viscera | 9 | 12.9<br>0.061 - 0.230 | 0.34 | 2.67 ± 2.69<br>(1 - 9) | 24<br>(88.9%) |

## 4. Discussion

Despite the importance of *I. argentinus* as a food resource in the SW Atlantic Ocean, and as frozen product exported to various European markets, the presence of zoonotic anisakid nematodes, especially in the flesh of the squid, has only been poorly investigated. To date, a few epidemiological reports on anisakid nematodes in *I. argentinus* exist (Gonzàlez and Kroeck, 2000; Nigmatullin and Shukhgalter, 1990; Sardella et al., 1990; Santos, 1992; Threlfall, 1970), but none of the studies carried out molecular identification, nor provided details on the specific anatomical infection site of the larvae in the squid host.

In the present study, data on occurrence and distribution of genetically identified larvae of *A. pegreffii* and *H. aduncum* infecting *I. argentinus* from the greater Falkland Islands fishing area (SW Atlantic Ocean) are presented. The findings represent the first molecular identification of *A. pegreffii* and *H. aduncum* in *I. argentinus*. Furthermore, this is the first report of *A. pegreffii* larvae in the mantle tissue of this squid species.

The overall infection level of *A. pegreffii* in *I. argentinus* from Falkland waters was low when compared to previous reports of anisakids in this host species from other areas of the SW Atlantic Ocean. For example, Gonzàlez and Kroeck (2000) reported 100% prevalence and 21.64 mean intensity of *Anisakis* sp. in *I. argentinus* fished in the San Matìas Gulf (Argentina). However, it must be noted that the squids sampled by Gonzàlez and Kroeck (2000) were considerably larger (ML range 230–380 mm) than the present specimens (ML range 150–230 mm) (further details in relation to the accumulation pattern of *Anisakis* spp. with host length are discussed below). Previously, in the same geographical area, three larval nematodes referred to as *Anisakis* spp. were reported in a batch of 142 specimens of *I. argentinus* purchased in a marketplace and examined for helminth parasites (Threlfall, 1970). Nigmatullin and Shukhgalter (1990) reported the presence of nematodes belonging to two anisakid species, *A. simplex* (s. s.), and *Anisakis* spp. type I in *I. argentinus* caught in the western South Atlantic, with 12.9% prevalence for both species. However, reliable identification of *Anisakis* L3 to species level can only be achieved by molecular/genetic means (Mattiucci and Nascetti, 2008; Mattiucci et al., 2018). Thus, it is not clear which *Anisakis* species Nigmatullin and Shukhgalter (1990) actually detected. Along the North Patagonian coast, Sardella et al. (1990) reported 13.2% prevalence for *Anisakis* sp. in 68 specimens of *I. argentinus*, ranging 200–300 mm in mantle length.

The squids showing highest *A. pegreffii* abundance were all > 200 mm (mantle length), and the largest of the presently examined specimens (230 mm ML) showed highest intensity of infection ($I_{max}$ = 9 larvae, all detected in the viscera). Although statistically non-significant (Spearman rank test $r = 0.180$, $p > 0.135$), this finding may point towards a trend of larger squid carrying more larvae than smaller squid. Morsan et al. (1999) reported a similar trend, with *Anisakis* sp. larvae prevalence and abundance showing a gradual increment with squid age. Clear patterns of significantly positive correlations between *Anisakis* spp. abundance and host size have been observed in several commercially important fish species including European hake (*Merluccius merluccius*), European anchovy (*Engraulis encrasicolus*), Atlantic herring (*Clupea harengus*), haddock (*Melanogrammus aeglefinus*), whiting (*Merlangius merlangus*) and Atlantic cod (*Gadus morhua*) (Cipriani et al., 2018a, 2018b; Gay et al., 2018; Levsen et al., 2018; Pierce et al., 2018).

The mechanisms behind the trend of many fish species to accumulate *Anisakis* spp. larvae over time seems to be closely linked to the preferred prey at different stages of the hosts' life history, and the ability of *Anisakis* spp. to survive for years in fish (Hemmingsen et al., 1993; Køie, 2001; Smith, 1984a). Thus, large piscivorous fish hosts such as European hake and Atlantic cod show typically much higher *Anisakis* spp. infection levels compared to strict plankton feeders such as anchovy, sardine and capelin (Bušelić et al., 2018; Cipriani et al., 2018a; Levsen et al., 2016, 2017). This might be linked to the fact that plankton organisms generally host *Anisakis* spp. larvae at very low prevalence (Gregori et al., 2005) compared to prey at higher trophic levels such as squid or fish. In the case of *I. argentinus*, as for many other ommastrephids, an ontogenetic shift in preferred food source occurs at around 200 mm ML (Ivanovic and Brunetti, 1994). Squid below that size feed primarily on crustaceans (amphipods and euphausiids) whereas fish and other squid become the most prevalent prey items as squid host size increases (Ivanovic and Brunetti, 1994). This might explain the considerably higher *Anisakis* sp. infection levels found by Gonzàlez and Kroeck (2000) in larger *I. argentinus* (ML range 230–380 mm) from the San Matìas Gulf (Argentina).

In addition, larval accumulation seems to be facilitated by the longevity of *Anisakis* spp. larvae, which may stay alive for extended periods or even over a given fish/paratenic host's lifetime (Hemmingsen et al., 1993; Køie, 2001; Smith, 1984b).

Of the two ascaridoid species identified, only *A. pegreffii* is zoonotic, potentially inducing gastric, intestinal or gastro-allergic anisakiasis in humans (Mattiucci et al., 2011, 2013; Mladineo et al., 2016). *H. aduncum*, however, found at very low infection level in the present squid sample, has not been recognized as pathogenic for humans. Since *H. aduncum* is primarily a parasite of fish, and hence is not adapted at any stage to the temperature conditions that prevail in the alimentary tract of mammals, it does not pose a human health risk (Levsen and Karl, 2014). Furthermore, *Hysterothylacium* larvae generally do not migrate into the fish flesh, but Picó-Durán et al. (2016) reported the presence of *Hysterothylacium* sp. larvae in the mantle of *I. coindettii*.

Although the present study revealed low prevalence and abundance levels of larval *A. pegreffii* in the mantle of *I. argentinus*, human health concerns may still arise.

Each of the three mantle-infecting larvae were situated in the posterior half of the mantle, i.e. in the hind part of the mantle clothing most of the host's alimentary system, thus roughly mirroring the most common larval infection site in the viscera. These larvae were embedded in the muscular tissue and were not readily recognizable neither by plain visual inspection (whitish larvae in whitish muscular tissue), nor candling. Only UV fluorescence permitted rapid and accurate detection of the nematodes in the mantle. However, considering the low infection level of *A. pegreffii* recorded in the mantle of the present squid samples, the risk of anisakiasis associated with consumption of *I. argentinus* within the actual size range (ML 150–230 mm) from SW Atlantic fishing areas appears to be low. However, since dishes based on raw, marinated or undercooked squid are on the menu in several regions of South America (Sardella et al., 1990), the risk of acquiring anisakiasis if squid are prepared after these regional recipes, still exists. Thus, preventive measures to kill the parasite (i.e. freezing) should be applied whenever *I. argentinus* are to be consumed raw or only lightly processed (EFSA, 2010) in order to mitigate the risk of acquiring anisakiasis.

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