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Larval migration of the zoonotic parasite *Anisakis pegreffii* (Nematoda: Anisakidae) in European anchovy, *Engraulis encrasicolus*: Implications to seafood safety

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ABSTRACT

Anisakid nematodes belonging to the species *Anisakis pegreffii* are distributed in a wide variety of fishes from the Mediterranean Sea and they are known to cause the human zoonosis anisakiasis. The present study investigated, for the first time, the response of *A. pegreffii* larvae (identified to species level by allozymes and mtDNA *cox2* sequence analysis) to the storage temperature of European anchovies, *Engraulis encrasicolus*. The larval motility of *A. pegreffii* was studied in 1300 fish specimens, captured from a highly infested area (FAO 37.2.1, 43°8′N, 14°16′E), maintained under different temperatures (2 °C, 5 °C, 7 °C), and examined at different time intervals (immediately after fishing, 24 h, 48 h and 72 h). Parasitological analysis was carried out with the UV-press method. The results showed that the increase of infection values with *A. pegreffii* in the fillets of anchovies was statistically positively related to the increase of the temperature (at 5 °C and 7 °C) and time of storage (after 24 h, 48 h, and 72 h). Accordingly, a significant statistical correlation between the increasing of the worm burden in the fillets and a decreasing of *A. pegreffii* in the viscera was observed. In contrast, those fish constantly maintained at 2 °C showed no statistically significant variation in infection either in the viscera or the fillets, after 24, 48 and 72 h. In the same batches of anchovies, larvae of the non-zoonotic nematode parasite *Hysterothylacium aduncum* (identified to the species level by ITS rDNA sequences analysis) were found, but they were never observed infecting the musculature of the anchovies. Our results suggest that temperature plays an important role in the *post-mortem* motility of *A. pegreffii* larvae in anchovies. In addition, the presence of *A. pegreffii* in the fillets inspected immediately after their capture indicates that *intra-vitam* migration may also occur. As a consequence, the importance of the adoption of rules to prevent human anisakiasis, as the deep freezing to −20 °C for 24 h, was underlined.

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1. Introduction

Anisakis species are marine parasites, with crustaceans as first intermediate hosts, fish and squids as second intermediate/paratenic hosts, and definitive hosts being mainly cetaceans (Mattiucci & Nascetti, 2006, 2008). Larvae of *Anisakis* spp. commonly infect the viscera and musculature of many teleost fish species (Mattiucci & Nascetti, 2008). The fish acquires infective larvae by ingesting an infected first intermediate host. The larva subsequently penetrates the intestinal wall, and then it may encyst in a coil on the surface of the internal organs, and/or migrate into the musculature. This migration occurs both when fish are alive (i.e., *intra-vitam* migration) (Cipriani et al., 2014; Karl, 2008; Karl, Meyer, Banneke, Jark, & Feldhusen, 2002; Quiazon, Yoshinaga, & Ogawa, 2011; Smith, 1984) and after death (i.e., *post-mortem* migration) (Hauck, 1977; Smith & Wootten, 1975). However, data regarding the migration of *Anisakis* spp. larvae from viscera into the musculature of fish intermediate/paratenic hosts are scanty and the results are often controversial (Roepstorff, Karl, Bloemsma, & Huss, 1993; Smith & Wootten, 1975).

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On the other hand, data on the distribution of larval *Anisakis* spp. in the musculature, thus concerning the edible parts of the fish, are of crucial importance, since larval *Anisakis* spp. are etiological agents of human anisakiasis (Van Thiel, van Kuipers, & Roskam, 1960). Indeed, over the last 30 years, there has been an increase in reported cases of human anisakiasis throughout the world (Mattiucci et al., 2013, and citations therein). Therefore, deepening the knowledge on the presence of larvae in the edible part of fish represents a keystone to define the risk assessment for humans to contract the zoonotic diseases. The scientific opinion on risk assessment related to the presence of parasites in seafood products by the Panel on Biological Hazards (European Food Safety Authority, EFSA, 2010) stated: "… based on scientific evidence it is not clear when, under what conditions and in which fish species, *post-mortem* migration of *Anisakis simplex* larvae occurs …".

European anchovy *Engraulis encrasicolus* (Linnaeus, 1758) is a coastal marine pelagic species, forming large schools. It is a principal target species for commercial fisheries in Europe. In Italy, *E. encrasicolus* is the most fished species by weight (25% of the total catches) and the second one by value (Lleonart, 2005; Lleonart & Maynou, 2003). Much of this anchovy catch goes to local markets where it is distributed and sold as fresh fish. *E. encrasicolus* is a small pelagic fish and, therefore, it is allowed to be landed, stored and sold directly to the consumer as "ungutted". In Italy, anchovy is the main fish species consumed raw after home preparation (in lemon or vinegar, according to the regional/local traditional receipe, i.e., "marinated anchovies"). In Italy the consumption of these raw marinated anchovies, not previously frozen at −20 °C for 24 h (recommended by health guidelines), represents the main human risk of the fish-borne zoonosis, anisakiasis. Several cases of human anisakiasis documented so far in Italy are associated with the consumption of raw marinated anchovies (Mattiucci et al., 2011, 2013). *Anisakis pegreffii* is implicated as causing human anisakiasis, either gastric (D'Amelio et al., 1999; Fumarola et al., 2009; Umehara, Kawakami, Araki, & Uchida, 2007) intestinal (Mattiucci et al., 2011) and gastro-allergic anisakiasis (GAA) (Mattiucci et al., 2013).

Third-stage larvae of *Anisakis* spp. have been reported in anchovies from the fishing grounds of the Mediterranean Sea (Cavallero et al., 2015; Ciccarelli, Aliventi, Di Trani, & Semeraro, 2011; De Liberato et al., 2013; Meloni et al., 2011; Mladineo, Simat, Miletić, Beck, & Poljak, 2012; Piras et al., 2014; Serracca et al., 2014) and European Atlantic waters (Rello, Adroher, Benítez, & Valero, 2009). However, despite the bulk of data reporting *Anisakis* spp. larval infestation in anchovies from some fishing grounds, there is no detailed information regarding the site of infection by larval *A. pegreffii* in that fish species, especially in the fillets. Furthermore, there has been no analysis of *intra-vitam* infection of the host and/or *post-mortem* migration by *Anisakis* spp. larvae, from the viscera to the flesh, of anchovies.

The aim of the present work was to ascertain the distribution of *Anisakis* spp. larvae in the viscera and musculature of European anchovy, in order to: *i)* quantify the *intra vitam* migration and document the *post-mortem* migration of the zoonotic parasite, *A. pegreffii* in fish stored at different temperature conditions and at various time intervals; *ii)* provide epidemiological data both in viscera and musculature of the anchovy to assess the human infection risk posed by *A. pegreffii*.

2. Materials and methods

2.1. Fish sampling and parasitological analysis

A total of 1300 anchovies, *E. encrasicolus*, were obtained from the same fishing area (Central Adriatic Sea – 43°8′N, 14°16′E) and the same fisherman, between October and December 2014 (respectively the 27th of October, the 18th of November, and the 2nd of December) (Table 1). The full sample was divided into three batches of anchovies to run three different experiments. The fish was obtained over a period of three months in order to follow the infection levels over a period included in the same season, and minimize any seasonal variation. The fishing locality was chosen as known to have high levels of infection by *A. pegreffii.* This was determined during an ongoing parasitological survey we are conducting for *Anisakis* spp. larvae in *E. encrasicolus* from different basin waters of the Mediterranean Sea. Anchovy samples were selected to eliminate any effect of host size/age variation (Poulin, 2000) on *Anisakis* infection parameters, obtaining batches of fish of the same size range. A *t*-test was run to compare the size of the 13 batches (each comprising 100 fish), which evidenced any statistically significant differences in length between them. Mean abundance and prevalence values of *Anisakis* spp. show considerable variation, even in fish from the same stock; to compensate for this (Karl et al., 2002), a large sample number (usually about 100 per sample group) was required to ensure statistical significance. In consequence, directly on receipt after landing, the fish were divided into subsamples, each of 100 specimens. A batch of 100 fish from each daily sample was immediately frozen, after landing, at −20 °C, thus representing the "zero condition" for each experiment (see below), while the other subsamples were shipped in refrigerated boxes surrounded by ice (constantly maintained at 2 °C by a refrigerated truck), directly to the Parasitology Laboratory of "Sapienza-University in Rome". Once at laboratory, they were immediately placed in different storage conditions, as reported in Table 1. The storage temperature was monitored during transport using a data logger, and it remained constant at 2 °C for the refrigerated samples, and −20° for the frozen samples.

2.2. Experiments to study the larval migration within the fish

To detect any temperature dependent migration of larvae within *E. encrasicolus*, three experiments were carried out on batches of fish, held at different temperatures and periods, as reported in Table 1 and described below:

2.2.1. Experiment no. 1

This first experiment included the parasitological examination of the three fish samples (N = 100 × 3), refrigerated at the same temperature (2 °C) in drained polystyrene boxes, and examined in three different time intervals since their capture (i.e., 24 h, 48 h and 72 h) (Table 1). Thus, the first batch of 100 fish kept at 2 °C was

Table 1
Number (N), total length, and storage conditions (temperatures and time intervals) of the different batches of the 1300 anchovies *Engraulis encrasicolus* analyzed in the study. Parasitological examination of the fish occurred after specified periods and temperatures of storage.

	N	Fish total length	Storage conditions
Exp no. 1	100	14.32 ± 0.78 (12.00–16.80)	Immediately frozen (−20 °C)
2 °C	100		2 °C for 24 h
	100		2 °C for 48 h
	100		2 °C for 72 h
Exp no. 2	100	14.49 ± 0.64 (13.00–16.50)	Immediately frozen (−20 °C)
5 °C	100		5 °C for 24 h
	100		5 °C for 48 h
	100		5 °C for 72 h
Exp no. 3	100	14.06 ± 0.66 (12.50–16.50)	Immediately frozen (−20 °C)
7 °C	100		7 °C for 24 h
	100		7 °C for 48 h
	100		7 °C for 72 h
Exp no. 4	100	14.30 ± 0.65 (13.00–15.50)	14 °C for 4 h
14 °C			

examined after 24 h from its fishing, the second sample was checked 48 h after the fish activity, and, finally, the third one after 72 h. The residual liquid in each box/batch was sieved and the recovered larvae were counted and stored.

2.2.2. Experiments no. 2 and no. 3

The same procedure described in experiment no. 1 was repeated for 3 batches (N = 100 for each) of the second subsample stored at 5 °C (Exp no. 2), over the same periods (24 h, 48 h and 72 h) (Table 1).

The same procedure was repeated for 3 batches of the third subsample, all stored at 7 °C (Exp no. 3) over 24 h, 48 h and 72 h as previously (Table 1). The latter two temperatures chosen for the experiments typify temperatures commonly used in domestic refrigerators (respectively, at 5 °C and 7 °C).

2.2.3. Experiment no. 4

100 anchovies were kept at 2 °C until arrival at the laboratory, when the batch was held at 14 °C for 4 h to simulate the stocking conditions of a direct market seller. Fish were boxed with ice, and constantly monitored with the aid of a "time-lapse" camera during this interval to directly observe any larval movement outside the fish. The parasitological examination then followed similarly to the procedures described above.

Finally, the three samples frozen at −20 °C, representing the "point zero" of each experiment (N = 100 × 3), were analyzed, following to the same parasitological procedures reported above.

2.3. Parasitological procedures for larval detection in the viscera and musculature of anchovies

All 1300 anchovies in the study were measured (total length) to the nearest 0.1 cm before being subject to parasitological examination. The mean length of the three batches of anchovies were 14.32 ± 0.78 (12.00–16.80) cm in the first sample, 14.49 ± 0.64 (13.00–16.50) cm in the second and 14.06 ± 0.66 (12.50–16.50) cm in the third (Table 1).

The fish were washed to remove any nematodes that moved on the external body surface. The wash water was collected and sieved to capture any larvae present. The anchovies were then gutted and the viscera of each fish were removed for examination, first by eye, and then by dissecting microscope. The musculature ("butterfly fillets") and viscera were then placed in individual plastic bags in preparation for the UV-press method of larval inspection.

The UV-press method for viscera and fillet inspection was applied as described by Karl and Leinemann (1993) and later updated and modified by Karl and Levsen (2011). In addition, to monitor any dispersion of migrating larvae in the transport boxes, the residual liquid with the stored fish was sieved. After sieving, any worms present were counted and identified to genus level, by optical microscopy, to discriminate between larval nematodes of the genus *Anisakis* from those belonging to the genus *Hysterothylacium*.

The parasitological inspection of each 100 specimen batches was led by the same two operators, and routinely completed within 3 h.

2.4. Genetic identification of larval nematodes by multilocus allozyme electrophoresis (MAE) and sequences analysis of mtDNA cox2 gene

Nematodes obtained from the parasitological analysis were washed in physiological saline and stored at −50 °C until genetic/molecular identification.

A subsample of at least 500 *Anisakis* spp. larvae, randomly selected from each of the four experiments, was identified to species level by means of multilocus allozyme electrophoresis (MAE). Each single specimen was crushed in distilled water, and a small amount of homogenized larva was taken to be sequenced at the mtDNA *cox2* gene. Standard horizontal starch gel electrophoresis was performed for those allozyme loci proven to be diagnostic for *Anisakis* spp. (see Mattiucci et al., 2014; Mattiucci & Nascetti, 2006; Mattiucci et al., 1997). The staining procedures used have been reported in detail by Mattiucci et al. (1997). Isozymes were numbered in order of decreasing mobility from the most anodal one. Allozymes were identified by numbers indicating their mobility (in mm, standardized conditions) relative to the most common allele, designated as *100*, found in the reference population (i.e., *A. pegreffii* from the Mediterranean Sea). The statistical significance of departures from the Hardy–Weinberg equilibrium was estimated using the $\chi 2$ test. Genetic analysis was performed using BIOSYS-2 software (Swofford, Selander, & Black, 1997). In addition, a number of the *Anisakis* spp. larvae first identified by allozymes (i.e., 40 specimens), were sequenced at the mtDNA *cox2* gene. The total DNA was extracted, using the cetyltrithylammonium bromide method (CTAB), from 2 mg of homogenized tissue from each single nematode (Valentini et al., 2006). The mitochondrial cytochrome *c* oxidase subunit II (*cox2)* gene was amplified using 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′) (Nadler & Hudspeth, 2000) spanning the mtDNA nucleotide position 10,639–11,248, as defined in *Ascaris suum* [GenBank X54253]. The PCR (polymerase chain reaction) was carried out using the following conditions: 94 °C for 3 min (initial denaturation), followed by 34 cycles at 94 °C for 30 s (denaturation), 46 °C for 60 s (annealing) and 72 °C for 90 s (extension), followed by final extension at 72 °C for 10 min (Valentini et al., 2006).

The sequences obtained at the mtDNA *cox2* for those larval specimens analyzed in the present study were compared with those already obtained for the same gene in the species *A. pegreffii* and *A. simplex* sensu stricto, and with respect to the other species of *Anisakis.* Therefore, the following mtDNA *cox2* sequences of *Anisakis* spp., retrievable from GenBank, were used for the identification of those larval specimens previously identified by allozymes: *A. simplex* (s. s.) (DQ116426), *A. pegreffii* (JQ900761), *Anisakis berlandi* (KC809999), *A. typica* (DQ116427), *A. ziphidarum* (DQ116430), *A. nascettii* (FJ685642), *A. physeteris* (DQ116432), *A. brevispiculata* (DQ116433) and *A. paggiae* (DQ116434).

In addition, a subsample of 20 specimens of *Hysterothylacium* spp. were identified to species level by sequences analysis of the internal transcribed spacers (ITS rDNA) region sequence. PCR amplification was performed using the primers NC5 (5′-GTAGGTGAACCTGCGGAAGGATCATT-3′) and NC2 (5′-TTAGTTTCTTTTCCTCCGCT-3′), with the procedure reported in Zhu, D'Amelio, Paggi, and Gasser (2000). PCR amplification conditions of 94 °C for 5 min (initial denaturation), followed by 30 cycles at 94 °C for 30 s (denaturation), 55 °C for 30 s (annealing), 72 °C for 30 s (extension) and a final elongation step at 72 °C for 5 min (Zhu et al., 2000). The sequences obtained were analyzed by Genbank Blast software and aligned with previously characterized ITS of the Raphidascarididae family by ClustalX (Thompson, Gibson, Plewniak, Jeanmougin, & Higgins, 1997).

2.5. Statistical analysis of the epidemiological data

The parasitic infection levels with *Anisakis* spp. and *Hysterothylacium* spp. larvae in the anchovies were given as: prevalence (P, %) with confidence limits (Clopper-Pearson), mean abundance (A) with bootstrap confidence limits, and mean intensity with range of larvae reported, following previous report (Bush, Lafferty, Lotz, & Shostak, 1997; Reiczigel, 2003; Rózsa, Reiczigel, & Majoros,

2000). They were estimated by using the Software Quantitative Parasitology Qpweb, implemented for the web (Reiczigel & Rozsa, 2005). The statistical significance of the differences observed in the prevalence and mean abundance values of the infestation by *Anisakis* spp. larvae observed in the various experiments were assessed by Fisher's exact test and Bootstrap *t*-test, respectively, using the Software Quantitative Parasitology Qpweb (Reiczigel & Rozsa, 2005). The statistical significance of the differences observed in the relative proportions of *Anisakis* spp. larvae in their different localization (viscera and musculature) was assessed by the χ^2 test (Yates corrected). Differences were considered significant when $p < 0.05$.

3. Results

3.1. Identification of Anisakis spp. and Hysterothylacium spp. larvae

All the *Anisakis* spp. larvae collected (N = 8228) from the anchovies examined parasitologically, were first assigned to the larval type I on the basis of those morphological characters available at the genus level by microscopy (*sensu* Berland, 1961). A subsample (N = 500) of randomly selected *Anisakis* spp. larvae was identified to the species level by means of allozyme diagnostic loci. According to the alleles observed at the diagnostic loci, i.e., *Sod-1*^{100}, *Adk-2*^{100}, *PepB*^{100}, *PepC-1*^{100}and *PepC2*^{100}, the 500 *Anisakis* type I larvae were assigned to the species *A. pegreffii*. In addition, some of the specimens (N = 40) of *A. pegreffii,* previously identified by diagnostic allozymes, were sequenced at the mitochondrial *cox2* gene (mtDNA *cox2*). As recommended by Venter et al. (2004), sequence similarities that had a GenBank Blast match of 97% were accepted as taxon identification. Following this procedure, the 40 samples of *Anisakis* spp. sequenced were identified as *A. pegreffii*, matching 100 or 99% with the sequences previously deposited in Genbank, under the species *A. pegreffii* (Mattiucci et al., 2013, 2014; Valentini et al., 2006), thus confirming the results obtained with the allozymes.

Besides those larval specimens of *Anisakis*, specimens (N = 661) of the raphidascaridid nematodes belonging to the genus *Hysterothylacium* spp., were recovered in syntopy with *Anisakis* spp. larvae in the viscera of the anchovies inspected. However, they were never found infecting the fish fillets. A subsample (N = 20) of *Hysterothylacium* spp. was assessed by genetic-molecular identification, (sequences analysis of the ITS region of rDNA) to determine species. The sequences obtained (936 bp) from the 20 specimens of the raphidascaridid nematode collected from the inspected anchovies matched at 99% with the ITS rDNA sequence of *Hysterothylacium aduncum* deposited in GenBank (JQ934878) (Vardić Smrzlić, Valić, Kapetanović, Kurtović, & Teskeredžić, 2012).

The mtDNA*cox2* sequences obtained in this work from *A. pegreffii* larvae were deposited in Genbank under the accession numbers KP979764, KP979765 and KP979766, while the ITS sequences of *H. aduncum* larvae have the accession numbers KP979761, KP979762 and KP979763.

3.2. Infection levels with A. pegreffii larvae

3.2.1. Infection of the "control" frozen batch of anchovies

Data on prevalence (P) and mean abundance (A) of infection by *A. pegreffii* larvae, and their relative proportions in different host sites of infection in the anchovies examined in the present study according to different temperatures and time intervals (as detailed in Table 1) are given in Table 2.

The comparison of the infection levels by *A. pegreffii* larvae between the three frozen batches, each comprising 100 anchovies, and considered as "control" samples for the experiments no.1, no. 2 and no. 3, did not show any statistically significant variation in values either of prevalence or mean abundance (Table 2). Indeed, the prevalence showed similar values being, P = 96.0, P = 87.0, P = 94.0, respectively. In addition, the mean abundance levels were all similar, i.e., A = 7.61 A = 7.04, A = 6.46, respectively, in the three subsamples fished from the same area but at different times. In addition, out of the total worm burden of *A. pegreffii* detected, the percentage of *A. pegreffii* infecting the fillets and the viscera of the "control" anchovies was similar, representing respectively, 96.5%, 95.7%, and 95.7% localized in the viscera *versus* the 3.5%, 4.3%, and 4.3% of larvae detected in the musculature (Table 2). Thus, no statistically significant differences were found between the relative proportions of the larvae detected in viscera and fillets in the three frozen samples (Table 2) (being always $p > 0.05$).

3.2.2. Infection of the anchovies in experiment no. 1

In the three batches of 100 anchovies stored at 2 °C and examined after 24 h - 48 h - 72 h, no significant variation of *A. pegreffii* prevalence or mean abundance in the viscera of the fish was observed, (Table 2). The only exception was the difference between the overall prevalence values (reported as "Total" in Table 2) observed in the "control" samples (frozen after fishing) and the sample inspected after 72 h, where a slight decrease in percentage of anchovies infected was found (P = 96.0 in "control" sample *versus* P = 88.0 at 72 h, respectively; $p = 0.03$) (Table 2). Moreover, the mean abundance of *A. pegreffii* infection in the musculature increased slightly with each time interval (Tables 2 and 3); this increase, however, resulted statistically significant for both P and A values just when comparing the immediately frozen "control" batch with the fish examined after 72 h (Tables 2 and 3).

Concerning the infection in the fish viscera, a statistically significant decrease of the prevalence (P) value of *A. pegreffii* was recorded only between the "control" batch and that inspected 72 h after fishing (P = 95.0 in "control sample", *versus* P = 85.0 at 72 h, respectively; $p = 0.03$) (Table 3).

In addition, the total number of *A. pegreffii* collected in the host infection sites (i.e., both viscera and musculature) at different intervals before examination, did not vary significantly (Table 2). Similarly, comparisons of the relative proportions of larvae counted in fish viscera and musculature after different time intervals did not show any statistical significant variation (p values obtained by χ^2 test always resulted > 0.05) (Table 2). Finally, fewer larvae were recovered after the sieving of storage liquid, than those collected with the same procedure, in the experiments no. 2 and 3 (see below and Table 2).

3.2.3. Infection of the anchovies in experiments no. 2 and 3

The results obtained in experiments no. 2 and 3, carried out on anchovies maintained under different storage temperatures, (i.e., 5 °C and 7 °C, respectively) (Table 1) did not show significant differences in the worm burden (Table 2), confirming the overall homogeneity of the samples. There was any significant difference between couples of P and A values observed at the two storage temperatures (5 °C and 7 °C) compared at various time intervals. In addiction, any significant difference of relative proportions was recorded between parasite burden in viscera and musculature at the two storage temperatures (5 °C and 7 °C) after the various time intervals (Table 4). Indeed, similar observations in those estimates by *A. pegreffii* in both viscera and musculature of anchovies were achieved during the two experiments (Table 2). In both the experiments, while the prevalence and mean abundance of the infection with *A. pegreffii* decreased significantly in the viscera of the anchovies, at the increasing of the storage time (i.e., after 24 h, 48 h and 72 h) (Tables 2 and 3), a significant increment in infection of the flesh of the same fish (both P and A) was recorded (Tables 2 and 3). Thus, while the relative proportions of *A. pegreffii* over the

Table 2
Prevalence (P, %), mean abundance (A) and mean intensity (Im and its range) of *Anisakis pegreffii* in *E. encrasicolus* collected in each batch of fish examined in the three experiments and stored in different conditions. Number of total larvae (N_{Tot}) and their relative proportions (%) in different tissues are also given.

	Total (viscera + musculature)				Viscera				Musculature				Storage liquid		N_{Tot}
	P	A	Im (range)	N_{Tot}	P	A	Im (range)	N_{Tot} (%)	P	A	Im (range)	N_{Tot} (%)	N_{Tot} (%)		
Exp no.1															
2 °C															
Immediately frozen −20 °C	96.0 (90.1–98.9)	7.61 (6.35–9.29)	7.91 (0–45)	761	95.0 (88.7–98.4)	7.34 (6.22–8.99)	7.71 (0–45)	734 (96.5)	22.0 (14.3–31.4)	0.27 (0.17–0.39)	1.23 (0–3)	27 (3.5)	0 (0.0)		761
2 °C 24 h	91.0 (83.6–95.8)	7.48 (6.45–8.84)	8.22 (0–26)	748	90.0 (82.4–95.1)	7.14 (6.11–8.49)	7.93 (0–26)	714 (95.1)	28.0 (19.5–37.9)	0.34 (0.23–0.47)	1.21 (0–3)	34 (4.5)	3 (0.4)		751
2 °C 48 h	88.0 (80.0–93.6)	6.94 (5.71–8.71)	7.89 (0–40)	694	87.0 (78.8–92.9)	6.48 (5.30–8.21)	7.45 (0–40)	648 (91.0)	33.0 (23.9–43.1)	0.46 (0.32–0.61)	1.39 (0–4)	46 (6.0)	24 (3.3)		718
2 °C 72 h	88.0 (80.0–93.6)	6.30 (5.14–7.68)	7.16 (0–35)	630	85.0 (76.5–91.4)	5.81 (4.78–7.23)	6.84 (0–33)	581 (80.0)	38.0 (28.5–48.3)	0.49 (0.36–0.63)	1.29 (0–3)	49 (7.0)	93 (13)		723
Exp no.2															
5 °C															
Immediately frozen −20 °C	87.0 (78.8–92.9)	7.04 (5.86–8.51)	8.09 (0–33)	704	87.0 (78.8–92.9)	6.74 (5.57–8.17)	7.75 (0–31)	674 (95.7)	25.0 (16.9–34.7)	0.30 (0.20–0.42)	1.20 (0–3)	30 (4.3)	0 (0.0)		704
5 °C 24 h	81.0 (71.9–88.2)	5.42 (4.36–6.60)	6.69 (0–22)	542	76.0(66.4–84.0)	5.00 (4.09–6.16)	6.58 (0–21)	500 (66.5)	34.0(24.8–44.2)	0.42 (0.30–0.56)	1.23 (0–3)	42 (5.6)	210 (27.9)		752
5 °C 48 h	75.0 (65.3–83.1)	2.81 (2.30–3.42)	3.75 (0–15)	281	60.0 (49.7–69.7)	2.12(1.70–2.65)	3.53 (0–11)	212 (33.3)	47.0 (36.9–57.2)	0.69 (0.52–0.88)	1.47 (0–8)	69 (10.8)	356 (55.9)		637
5 °C 72 h	79.0 (69.7–86.5)	2.56 (2.08–3.12)	3.24 (0–14)	256	61.0 (50.7–70.6)	1.65 (1.25–2.16)	2.70 (0–14)	165 (26.7)	51.0 (40.8–60.1)	0.91 (0.69–1.14)	1.78 (0–5)	91 (14.7)	363 (58.6)		619
Exp no.3															
7 °C															
Immediately frozen −20 °C	94.0 (87.4–97.8)	6.46 (5.12–7.99)	6.63 (0–44)	646	93.0 (86.1–97.1)	6.18 (4.95–7.90)	6.61 (0–43)	618 (95.7)	20.0 (12.7–29.7)	0.28 (0.17–0.42)	1.40 (0–3)	28 (4.3)	0 (0.0)		646
7 °C 24 h	79.0 (69.7–86.5)	4.52 (3.64–5.57)	5.72 (0–25)	452	77.0 (65.7–84.8)	4.21 (3.33–5.23)	5.47 (0–24)	421 (67.6)	27.0 (18.6–36.8)	0.31 (0.21–0.42)	1.15 (0–2)	31 (5.0)	171 (27.4)		623
7 °C 48 h	65.0 (54.8–74.3)	3.34 (2.45–4.51)	5.14 (0–24)	334	63.0 (52.8–72.4)	2.54 (1.88–3.46)	4.03 (0–18)	254 (40.4)	36.0 (26.6–46.2)	0.80 (0.55–1.13)	2.22 (0–8)	80 (12.7)	295 (46.9)		629
7 °C 72 h	75.0 (65.3–83.1)	3.15 (2.56–3.99)	4.20 (0–20)	315	64.0 (53.8–73.4)	1.98 (1.55–2.67)	3.09 (0–17)	198 (29.8)	57.0 (46.7–66.9)	1.17 (0.92–1.51)	2.05 (0–6)	117 (17.6)	350 (52.6)		665

total worm burden decreased in the viscera of anchovies, it increased in the fish musculature, progressively with time, between storage and examination (i.e., from the immediately frozen "control" sample until to 72 h) (Table 2, Fig. 2). When comparing the relative proportion of larvae detected in viscera and musculature of the fish, statistical significant differences were observed in both experiments between the sample considered as "control" and the fish inspected after 48 h ($p = 0.0008$) and 72 h ($p < 0.0001$) (same p values for experiment no.2 and experiment no.3, respectively).

Accordingly, the total number of *A. pegreffii* collected in the musculature of the fish was very high in both experiments no. 2 and 3 after 48 and 72 h (Table 2 and Fig. 2). In addition, a huge increase of larvae collected after sieving the residual liquid throughout the storage time intervals was found in both the experiments no. 2 and 3 (Table 2).

No significant differences ($p > 0.05$) in prevalence and mean abundance between viscera and musculature were recorded between the "control" batch frozen 2 h after fishing and the batch stored for 24 h at 5 °C and (same result between the "control" batch and the sample inspected after 24 h stored at 7 °C - experiment no. 3) (Table 3). On the contrary, significant increases in both prevalence and mean abundance were reported between the "control" batch and the batch inspected after 48 h, between the "control" batch and the one stored for 72 h, between 24 h and 72 h, while a significant increase in mean abundance only was recorded between the batches examined at 24 h and 48 h after capture (Table 3).

Concerning the fish viscera, a significant decrease of *A. pegreffii* P and A was recorded between the "control" batch *versus* the batch inspected after 48 h ($p < 0.0001$), "control" vs 72 h (both P and A, $p < 0.0001$), 24 vs 48 h (for P and A respectively, $p = 0.02$ and $p < 0.001$), 24 vs 72 h (for prevalence and mean abundance, respectively, $p = 0.03$ and $p < 0.0001$), while a significant decrease only in mean abundance values was reported between the "control" batch *versus* that held for 24 h ($p = 0.03$) (Tables 2 and 3).

3.2.4. Infection levels in anchovies from experiment no. 4

Statistically significant differences between the "control" batch of 100 anchovies immediately frozen after capture, and the subsample of 100 anchovies of the same batch stored 4 h at 14 °C (Exp no. 4), were recorded in the prevalence and mean abundance of *A. pegreffii* both in viscera and flesh (Table 5). There was a significant increase in both prevalence and mean abundance of *A. pegreffii* in the flesh of the fish held for 4 h at 14 °C (respectively $p = 0.013$ and $p < 0.0001$), while a significant decrease was reported for both prevalence and mean abundance of the same parasite species in the viscera of the 100 fish (respectively $p = 0.006$ and $p < 0.0001$). The relative proportions of *A. pegreffii* detected in viscera, flesh, and residual liquid changed respectively from 96.5%, 3.5% and 0.0%–32.2%, 11.6%, 56.2% after the anchovies were stored 4 h at 14 °C, with a statistical significant difference between the recorded values ($p > 0{,}0001$) (Table 5, Fig. 2c and d).

3.2.5. Comparison of infection levels in batches of anchovies from the different experiments

Tables 3 and 4 shows grids with the statistical comparison of the infection levels (P and A) of the pair of batches from the different experiments, stored at different temperatures for different time intervals.

3.2.6. Infection levels of H. aduncum larvae

H. aduncum infection parameters in batches of anchovies held at different temperatures and times are reported in Table 6. No *H. aduncum* were detected in fish flesh in any of the experiments. No significant differences ($p > 0.05$) were observed in prevalence or

Table 3
Statistical comparison of the prevalence (P) and mean abundance (A) values of the infection with *Anisakis pegreffii* larvae in the musculature and in the viscera of *Engraulis encrasicolus* stored at three different temperatures (i.e., 2 °C, 5 °C, and 7 °C), between the four time intervals (i.e., immediately frozen, 24 h, 48 h and 72 h).

			Musculature						Viscera					
			Immediately frozen −20 °C		24 h		48 h		Immediately frozen −20 °C		24 h		48 h	
			P	A	P	A	P	A	P	A	P	A	P	A
Exp no. 1	Immediately frozen	P	–						–					
Stored at 2 °C	−20 °C	A												
	24 h	P	ns		–				ns		–			
		A		ns						ns				
	48 h	P	ns		ns		–		ns		ns		–	
		A		ns		ns				ns				
	72 h	P	*		ns		ns		*		ns		ns	
		A		*		ns		ns		ns		ns		ns
Exp no. 2	Immediately frozen	P	–						–					
Stored at 5 °C	−20 °C	A												
	24 h	P	ns		–				ns		–			
		A		ns						*				
	48 h	P	*		ns		–		***		*		–	
		A		***		*				***		***		
	72 h	P	***		*		ns		***		*		ns	
		A		***		***		ns		***		***		ns
Exp no. 3	Immediately frozen	P	–						–					
Stored at 7 °C	−20 °C	A												
	24 h	P	ns		–				*		–			
		A		ns						*				
	48 h	P	*		ns		–		***		*		–	
		A		***		ns				***		*		
	72 h	P	***		***		*		***		ns		ns	
		A		***		***		ns		***		***		ns

P = significance level calculated by Fisher's exact test of differences between Prevalence values.
A = significance level of differences between mean Abundance values calculated by Bootstrap 2-sample t-test.
$^{***}p < 0.001$, $^{*}p < 0.05$, ns = not significant.

mean abundance of *H. aduncum* between the batches of anchovies stored at 2 °C and inspected after differing periods (i.e., "control" sample, 24 h, 48 h and 72 h) (Table 6).

On the contrary, in the anchovies stored at 5 °C, a significant decrease in mean abundance of infection by *H. aduncum* was found between the batch considered as "control" (immediately frozen) with that one analyzed after 48 h ($p = 0.004$) and with the other one after 72 h ($p = 0.008$) (Table 6).

Also in the case of *H. aduncum*, an increasing number of larvae were observed migrating from the viscera to the surface of anchovies, and into the residual liquid draining into the boxes containing the anchovies, throughout the whole storage period (Table 6). Accordingly, while the total number of *H. aduncum* increased in the "storage liquid" with time, their relative percentages decreased in the viscera (Table 6).

4. Discussion

Two parasite species were identified in *E. encrasicolus* from the central Adriatic Sea: *A. pegreffii* and *H. aduncum*. *A. pegreffii* occurred both in viscera and flesh of the examined anchovies; while, *H. aduncum* was found only in the viscera. Of the two species, *A. pegreffii* is a zoonotic parasite, and it is, indeed, responsible for gastric, intestinal and gastro-allergic anisakiasis in humans (Mattiucci et al., 2013). *H. aduncum* has not been implicated in human infection.

E. encrasicolus populations from the central Adriatic basin of the Mediterranean Sea were chosen for this study because of their known high rate of infection by *A. pegreffii* (Cipriani, pers. com.).

The results obtained in this study revealed that both *intra vitam* migration and *post-mortem* migration of *A. pegreffii* larvae occur from the viscera to the flesh of *E. encrasicolus* (Table 2). The *intra vitam* migration was quantified with the worm burden detected in the musculature of the fish examined shortly after capture, thus likely representing the amount of *A. pegreffii* larvae that moved from the visceral cavity to the flesh of the fish in life. To support this hypothesis, it was found that larval numbers in the musculature of the 300 "control" anchovies in the three experiments, frozen immediately after capture, had constant and low prevalence and parasite burden (Tables 2 and 3).

Indeed, the relative proportions of *A. pegreffii* in the musculature was on average 4.0% of the total larvae collected *versus* the frequency of 96.0%, on average, of worms found in the body visceral cavity and/or embedded in the visceral organs of the fish host (Table 2). Furthermore, it was observed that almost all the *A. pegreffii* larvae detected in fish examined immediately after catch (the frozen "control" batch) were in the encapsulated spiral shape, i.e., as coiled larvae (Fig. 1a and b). This finding suggests that those larvae were in a latent phase in their paratenic/intermediate fish host. On the other hand, our observations suggest that "*post-mortem*" migration or, generally, the movements of *A. pegreffii* larvae, are dependent on the fish storage temperature (Table 2, Fig. 2). It was observed that at both 5 °C and 7 °C storage temperature, while the mean abundance of the infection of *A. pegreffii* decreased in the viscera, the same parameter increased in the musculature, proportionally to the time of storage (Fig. 2, Table 4). Indeed, the fish maintained at 2 °C showed a slight variation in the mean abundance values recorded both in fish visceral cavity and flesh during the complete storage interval (from 24 h until 72 h) (Table 3, Fig. 2).

It was also observed that some *A. pegreffii* larvae moved from the viscera and through the muscle, to emerge on the fish skin (Fig. 1c and d). Consequently, some larvae were found also in the residual

Table 4
Statistical comparison of the prevalence (P) and mean abundance (A) values of the infection with *Anisakis pegreffii* larvae in the musculature and in the viscera of *Engraulis encrasicolus* at three time intervals (i.e., 24 h, 48 h and 72 h) between the three different storage temperatures (i.e., 2 °C, 5 °C, and 7 °C).

			Musculature				Viscera			
			2 °C		5 °C		2 °C		5 °C	
			P	A	P	A	P	A	P	A
24 h	2 °C	P	–				–			
		A								
	5 °C	P	ns		–		*		–	
		A		ns				*		
	7 °C	P	ns		ns		*		ns	
		A		ns		ns		*		ns
48 h	2 °C	P	–				–			
		A								
	5 °C	P	ns		–		***		–	
		A		ns				***		
	7 °C	P	ns		ns		***		ns	
		A		*		ns		***		ns
72 h	2 °C	P	–				–			
		A								
	5 °C	P	ns		–		***		–	
		A		*				***		
	7 °C	P	*		ns		*		ns	
		A		*		ns		***		ns

P = significance level calculated by Fisher's exact test of differences between Prevalence values.
A = significance level of differences between mean Abundance values calculated by Bootstrap 2-sample t-test.
$^{***}p < 0.001$, $^{*}p < 0.05$, ns = not significant.

storage liquid of the anchovies (Fig. 1d). On the other hand, the emergence of some *Anisakis* larvae through natural orifices and from muscle through the skin of anchovies *post-mortem* has also been previously observed (Rello et al., 2009). Data on the relative frequencies of the distribution of larvae between viscera, flesh, and storage residual liquid are reported in Tables 2–4 and Fig. 2.

According to the data obtained in the present work, it can be hypothesized that some physical or chemical cues associated with the increase of the temperature, may be responsible in stimulating the motility of those coiled *A. pegreffii* larvae, both in the viscera and fillets, during the life-span of the fish host. This temperature-dependent motility of the *A. pegreffii* larvae was even more evident when the fish were stored at high temperature (14 °C). In less than 4 h, the larvae completely changed their location (Table 5, Fig. 1d), and a significant number of worms had emerged only 2 h after being exposed at 14 °C (Fig. 1c).

However, the information regarding the movement of *Anisakis* larvae from viscera into the flesh is controversial. Some authors, such as Smith and Wootten (1975) reported that: "… A large scale migration of Anisakis larvae from the viscera to the flesh of the herring occurred in both our experiments so that almost 20 per cent of the total worm burden was present in the flesh after 37 h". On the contrary, Roepstorff et al., (1993) observed that no migration of *Anisakis* larvae in the flesh of herrings occurred when the fish was kept over a range of temperatures. Later, Karl et al. (2002) described a significant increase of the nematode burden into the flesh of fishes stored "ungutted" in ice. However, those experiments were carried out on larger fish species and the parasite species infecting those fish was likely to be *A. simplex* (s. s.), this based on

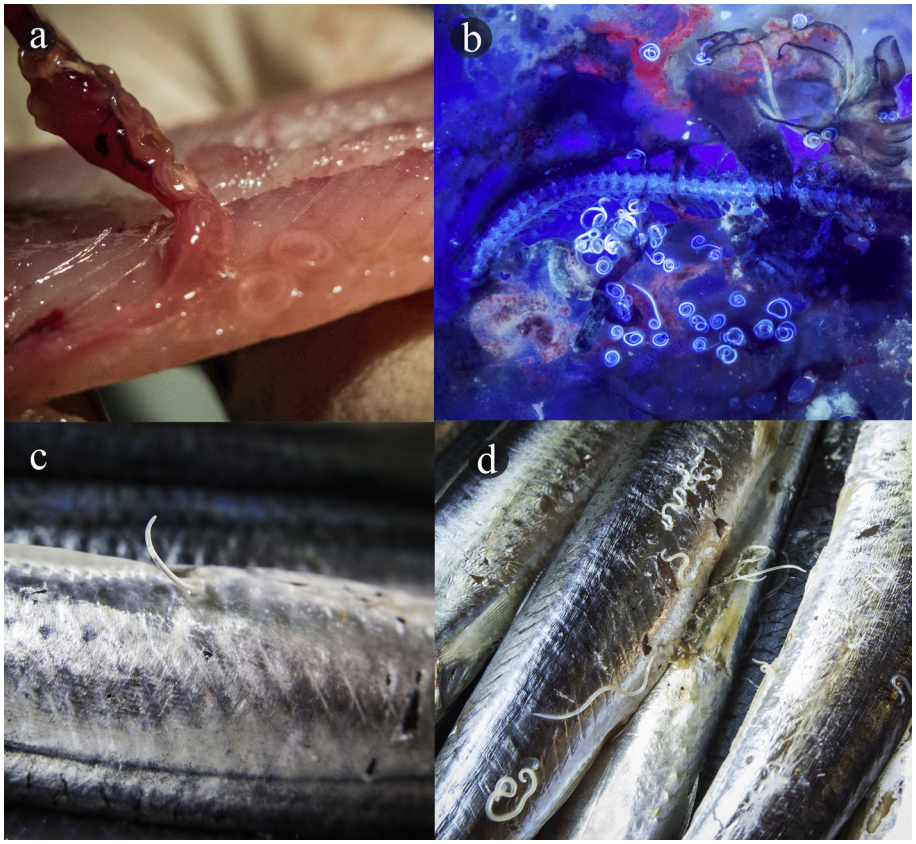

Fig. 1. Macrophotographs showing localization of *A. pegreffii* larvae in *E. encrasicolus* a): coiled larvae on the surface of the visceral organs and on the belly ventral fillet of the fish; b): coiled larvae of *A. pegreffii* in the viscera of a heavily infected fish, viewed with the UV light; c): Larvae of *A. pegreffii* migrating out of *E. encrasicolus*, as seen during experiment no. 3; d): Several specimens of larval *A. pegreffii* crawling out from *E. encrasicolus* after 4 h at 16 °C.

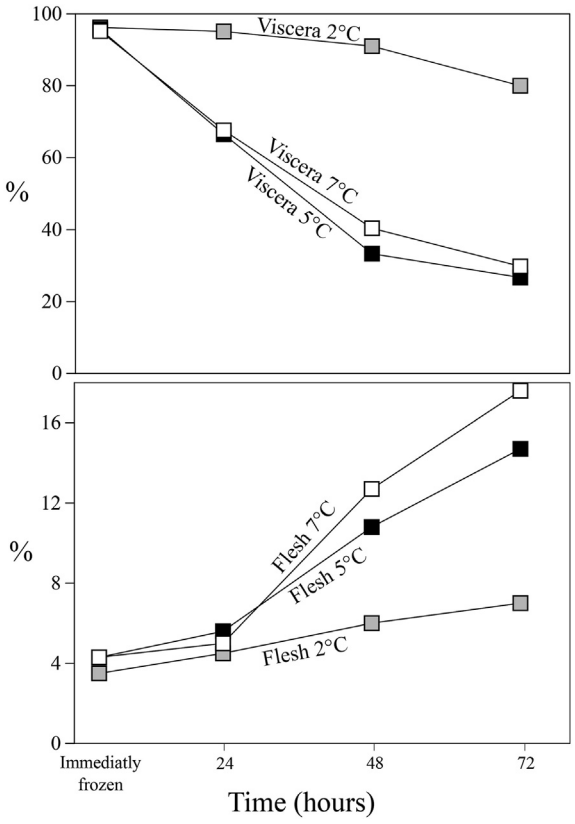

Fig. 2. Relative frequencies of the distribution of *A. pegreffii* larvae between viscera and flesh of *E. encrasicolus*, at different fish storage temperatures (2 °C, 5 °C, and 7 °C) and at different time intervals (immediately frozen "control" batch, 24 h, 48 h and 72 h).

the known *Anisakis* sp. occurring in the reported fishing grounds of those fish species.

The present study is the first attempt to evaluate a possible correlation of *A. pegreffii* larval *post-mortem* migration with the temperature and time of storage of a fish species, *E. encrasicolus*. Our results, achieved in two of the performed experiments, are congruent with the results obtained in 1975 in herring (Smith & Wootten, 1975), despite the host fish and *Anisakis* species being different. Also, it was recently reported that different *Anisakis* species show a different capacity for migration into the flesh of other fish hosts, such as the European hake, *Merluccius merluccius* (Cipriani et al., 2014), mackerel, *Scomber scombrus* (Suzuki, Murata, Hosaka, & Araki, 2010), and as seen in experimentally infected fish species, such as the rainbow trout and olive flounder (Quiazon et al., 2011). Indeed, data so far acquired also suggest that *A. pegreffii* and *A. simplex* (s. s.) differ in their site of infection in European hake *M. merluccius*, with *A. pegreffii* showing a lower propensity to invade the flesh (Cipriani et al., 2014). It has been also observed, in experimental infections, that at high temperatures (generally >25 °C), *Anisakis* spp. larvae are able more likely to migrate from the viscera to the flesh; however, a lower capacity to migrate was observed for *A. pegreffii* compared with *A. simplex* (s. s.) (Quiazon et al., 2011).

It has been also suggested that the propensity of *Anisakis* spp. larvae to migrate to the flesh could be related to differences in the nature [such as fatty acid content (Smith, 1983)] of the flesh in various fish species. The coiled habit (Fig. 1a and b) assumed by larvae in small fish hosts like anchovies appeared different from the coiled/embedded larvae seen in larger fish species, such as the European hake, *M. merluccius*, or the blue whiting, *Micromesistius poutassou*. In those fish species, *Anisakis* spp. larvae were more encapsulated and embedded in the surfaces of visceral organs, showing a thicker capsule, and maintaining the coiled position even if exposed at higher temperatures (Cipriani, pers. com.).

Factors causing excapsulation and migration of *Anisakis* larvae after death of the host fish are unknown, but presumably relate to physico-chemical changes in the viscera (Smith & Wootten, 1975). A direct observation of how larvae are disposed in the fish host in the present study showed that almost the 100% of larvae recovered were coiled when fish were inspected shortly after capture (Fig. 1b: macro-photograph of a visceral content under UV light of one specimen of "control" samples in our experiments, i.e., anchovies immediately frozen after capture), but they tended to become very active and able to migrate as soon as the storage temperature increased. Temperature appears to be the most important variable determining this change. This is not surprising since according to Smith & Wootten, 1975 -"… a rise in temperature might be expected to cause increased activity since the third stage larvae of Anisakis *complete their life-cycle in warm-blooded mammals …*". Recently, it was also suggested that the level of mRNA expression of the heat shock protein (Hsp90) of *A. pegreffii* did not change significantly under cold shock (−4 °C) (Chen, Cheng, Grabner, Chang, & Shih, 2014). In cold stress experiments, carried out by those authors, the larvae remained alive, even if they were less active. In contrast, after the larvae were incubated at 37 °C for 24 h, they showed increased motility (Chen et al., 2014).

Despite jeopardizing food safety, in Italy and in other Mediterranean countries, anchovies are landed, stored and sold as fresh "ungutted" fish, due to their small size. Indeed, the gutting of anchovies on board immediately after their fishing, would be an extremely time consuming procedure for fishermen, especially when taking into account the high number of specimens of *Engraulis encraulis* caught at every fishing activity. Thus, considering the observations on the *post-mortem* migration, as presented in the present paper, maintenance of the fish at <2 °C from capture until consumption, could reduce larval migration from the viscera

Table 5
Prevalence (P,%), mean abundance (A), number of larvae (N_{Tot}) with relative proportions and statistical significance, of *Anisakis pegreffii* observed in two samples of *Engraulis encrasicolus*, *i.e.* the "control" batch (N = 100, frozen immediately after fishing activity) and the batch (N = 100) stored 4 h at 14 °C (simulating the conditions of a direct fish market seller).

Experiment no.4	Viscera			Musculature			Total (viscera + musculature)		Storage liquid	N_{Tot}
	P	A	N_{Tot} (%)	P	A	N_{Tot} (%)	P	A	N_{Tot} (%)	
Immediately frozen −20 °C	95.0	7.34 (6.22–8.99)	734 (96.5)	22.0	0.27 (0.17–0.39)	27 (3.5)	96	7.61 (6.35–9.29)	0 (0.0)	761
after 4 h at 14 °C (Fish market)	74.0	2.50 (2.00–3.15)	250(32.2)	46.0	0.90(0.67–1.15)	90(11.6)	80	3.40(2.78–4.01)	436(56.2)	776
p	*	***	***	*	***	***	*	***	***	

p = significance level calculated by Fisher's exact test of differences between Prevalence values, and calculated by Bootstrap 2-sample t-test between mean Abundance values. $^{***}p < 0.001$, $^{*}p < 0.05$, ns = not significant.

Table 6
Prevalence (P,%) and mean abundance (A) values of the infection with *Hysterothylacium aduncum* larvae identified in *E. encrasicolus* recorded in three of the experiments carried out (experiments no. 1, 2 and 3).

		Viscera			Musculature	Storage liquid	N_{Tot}
		P	A	N_{Tot} (%)	N_{Tot} (%)	N_{Tot} (%)	
Exp no.1 2 °C	Immediately frozen-20 °C	27.0 (18.6–36.8)	0.41 (0.28–0.57)	41 (100.0)	0 (0.0)	0 (0.0)	41
	24 h	42.0 (32.2–52.3)	0.50 (0.37–0.63)	50 (98.0)	0 (0.0)	1 (2.0)	51
	48 h	37.0 (27.6–47.2)	0.49 (0.35–0.66)	49 (76.6)	0 (0.0)	15 (23.4)	64
	72 h	37.0 (27.6–47.2)	0.47 (0.34–0.60)	47 (61.0)	0 (0.0)	30 (39.0)	77
Exp no.2 5 °C	Immediately frozen-20 °C	32.0 (23.0–42.1)	0.48 (0.33–0.65)	48 (100.0)	0 (0.0)	0 (0.0)	48
	24 h	25.0 (16.9–34.7)	0.38 (0.24–0.53)	38 (71.7)	0 (0.0)	15 (28.3)	53
	48 h	22.0 (14.3–31.4)	0.22 (0.14–0.30)	22 (38.6)	0 (0.0)	35 (61.4)	57
	72 h	20.0 (12.7–29.2)	0.22 (0.13–0.32)	22 (40.0)	0 (0.0)	33 (60.0)	55
Exp no.3 7 °C	Immediately frozen-20 °C	32.0 (23.0–42.1)	0.52 (0.36–0.68)	52 (100.0)	0 (0.0)	0 (0.0)	52
	24 h	22.0 (14.3–31.4)	0.30(0.19–0.46)	30 (68.2)	0 (0.0)	14 (31.8)	44
	48 h	24.0 (16.0–33.6)	0.26 (0.17–0.36)	26 (43.3)	0 (0.0)	34 (56.7)	60
	72 h	18.0 (11.0–26.9)	0.21 (0.12–0.31)	21 (35.6)	0 (0.0)	38 (64.4)	59

N_{Tot} = total number of larvae collected and in percentage (%).

into the flesh. In addition to maintenance of the cold chain, rapid gutting after landing could be helpful in lowering the risk of *Anisakis* migration into the musculature. This will reduce, in turn, the probability for humans in Italy being infected by *A. pegreffii* larvae. However, a small percentage of larvae occur in the fish already at capture, as a result of *intra-vitam* migration from viscera into flesh. This partition of *Anisakis* larvae was determined in the present study as 4.0%, on average, of the total parasite burden. Nevertheless, this procedure does not eliminate of the risk of *Anisakis.* Thus, freezing the fish at −20 °C for at least 24 h before consumption of raw fish should be maintained, as stated by European Community rules.

We also wish to highlight the importance of surveying not only the viscera, but, more importantly, the flesh of the intermediate/paratenic fish hosts, when performing a parasitological survey for larval *Anisakis* spp. This should also include not only the evaluation of worm burden in the fish flesh, as the latter represents the main threat of human infection, but also its specific identification. With that aim, the present survey has shown the UV-press method to be a good alternative method for assessing numbers of larvae in the fillets, as already demonstrated (Levsen & Karl, 2014; Levsen, Lunestad, & Berland, 2005). Another advantage compared with the other widely used nematode inspection method, i.e., artificial digestion of soft tissue in an aqueous pepsin/HCl solution, is that the UV-press technique allows determination of the approximate larval infection site in both the flesh and the viscera (Levsen & Karl, 2014). For instance, this procedure allowed observation of several *A. pegreffii* larvae deeply embedded near the backbone of the host. This was evident only after significant compression of the fish visceral organs and bones. Although the digest method would have freed the worms for collection, it would have prevented observation of the worms *in situ*.

Finally, it was noted in this work that *H. aduncum* larvae, although common, occurred only in the visceral organs (Table 6). *H. aduncum* larvae do not migrate into the musculature; thus this species, being also non-zoonotic, as previously determined by Levsen and Karl (2014), has no direct effect on food safety.

5. Conclusions

The response of encysted *A. pegreffii* larvae (identified to species level by allozyme diagnostic loci and sequence analysis of a 629 bp fragment of the mitochondrial mtDNA *cox2* gene) to post capture host (*E. encrasicolus*) storage temperature has been investigated for the first time.

An increase in infection values with *A. pegreffii* in the fillets of anchovies was statistically positively related to the increase of the fish storage temperature (at 5 °C and 7 °C) and to the time of storage (24, 48, and 72 h) (Tables 2–4). Accordingly, a statistical correlation was also observed in the decreasing of the infection level by *A. pegreffii* in the viscera of the same anchovies (Tables 2–4). In contrast, those fish constantly maintained at 2 °C showed any statistically significant variation in the rate of infection both in the viscera and fillets, after 24, 48 and 72 h after the fishing activity. Finally, this work has determined that though larvae of the non-zoonotic parasite, *H. aduncum*, can co-infect *E. encrasicolus* in syntopy with *A. pegreffii,* at no time were they found in the host musculature.

Our findings suggest that the occurrence of *A. pegreffii* in the fillets of the immediately frozen anchovies, even if at lower percentage with respect to the total worm burden found, seems to indicate that also an *intra vitam* migration of larvae to the fish flesh is possible. Then, after fish capture, storage temperature plays an important role in the *post-mortem* motility of *A. pegreffii* larvae in the flesh of anchovies. This finding is crucially important for industrial storage procedures and food safety for this commercially important fish species. However, it has to be further investigated if fish host immune response, and the direct effects of the excretory/secretory products released by the *A. pegreffii* larvae in the fish musculature, could be responsible for both *intra vitam* and *post-mortem* migration of the parasite larvae.

Finally as a consequence of our findings, we emphasize the importance of the rules indicating the freezing of anchovies at −20 °C for 24 h, or at −18° for 96 h in domestic freezers (according to the Italian Ministry of Health Dispositions), before using them in culinary preparation in Italy as “marinated anchovies”, in order to prevent human anisakiasis.

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