The accuracy of visual inspection for preventing risk of *Anisakis* spp. infection in unprocessed fish

María Llarena-Reino[1, 2], Ángel F. González[1], Carlos Vello[3], Luis Outeiriño[3] and Santiago Pascual[1]

[1] Ecobiomar. Instituto de Investigaciones Marinas de Vigo. CSIC. Eduardo Cabello 6, 36208, Vigo, Spain.
[2] Present Address: Centro de Estudos do Ambiente e do Mar (CESAM) & Departamento de Biologia. Universidade de Aveiro. Campus de Santiago, 3810-193 Aveiro, Portugal.
[3] Comercial Hospitalaria-Grupo 3. Torrente Ballester 4, bajo, 36204, Vigo, Spain.

Corresponding author: María Llarena-Reino[1, 2]
Ecobiomar. Instituto de Investigaciones Marinas de Vigo. CSIC. Eduardo Cabello 6, 36208, Vigo, Spain.
mariallarena@iim.csic.es. Phone: 0034986231930, Ext.182; Fax: 0034986 292762.

ABSTRACT

The importance of the zoonoses caused by L3 Anisakidae larvae lies in the repercussion that this parasite exerts on food safety and quality. EU legislation recommends fish operators to do visual inspection of the whole fish abdominal cavity and gut to control the risk of visible parasites, thus ensuring that no contaminated fish reach the consumers. The accuracy of the above visual inspection method should fall on a well-tested statistical significance between the number of observable parasites in the abdominal cavity and the number of parasites in the edible part of the fish (i.e., musculature). The aim of this study was to analyse this statistical significance, and the efficacy of the washing practice to remove *Anisakis* spp. from gut. To carry out this work, 322 fresh individuals of *Micromesistius poutassou* and 230 of *Scomber scombrus* were necropsied within 12 hours and 48 hours post-capture. Then, descriptive statistics, correlation and regression analyses were used to evaluate the significant statistical relationship between the number of anisakid larvae found in the gut and musculature of both fish species. Additionally, livers and gonads of 25 fresh specimens of *Merluccius merluccius* were vigorously washed under tap water, and examined under stereomicroscope looking for *Anisakis* spp. larvae. Results evidenced the low efficiency of visual inspection of gut parasites as a commonly recommended method for predicting nematode larvae in the flesh of fish. Therefore, a direct-invasive inspection of musculature is stressed as the only criteria with scientific merit for accurately detecting contaminated fishes by anisakids. Moreover, fresh European hake liver and gonads showed at least one larva remained inside the tissue after washing vigorously under tap water. Results suggested that critical control points at Hazard Analysis Critical Control Point (HACCP) programmes should be reviewed to improve the risk of anisakid-induced allergies and gastrointestinal anisakiasis among consumers.

KEYWORDS

Anisakis spp. larvae; fish; gut; musculature; parasites; significant statistical relationship

1. INTRODUCTION

Anisakids are marine cosmopolitan parasites highly prevalent in wild fish stocks of commercial interest species. They are usually found in high amount in the third larval stage on the gut cavity and sometimes on the belly flaps too, during fish inspections (Abollo et al, 2001). These parasites are recognized as

human health hazard responsible for emergent zoonoses called anisakiasis, causing gastro-allergic disorders in consumers and occupathional-asma in fish-farming workers (Plessis et al, 2004; Nieuwenhuizen et al, 2006).

In the transborder Euroregion Eixo Atlantico (NW Iberian peninsula), the traditional *escandallo* or inspection procedure, is a rapid and reliable sensory method largely used in the seafood industry to ensure the quality of fishery products and to make commercial trade more confident. The above inspection method follows an internationally used protocol which should guarantee the safety of inspected seafood products. In fact, at the Euroregion, some international companies inspect and evaluate the risk of these biological contaminants by managing these inspections in retail chains, certifying customers that no prohibited contaminants are in fact present at the critical control points from the fishery to the plate. EU legislation (Commission Regulation EC No 2074/2005; EC 853/2004 rev.) pointed out that visual inspection of the whole fish abdominal cavity (including liver, gonad and egg mass) should be done by fish operators to control the risk of visible parasites, thus ensuring from the catch to the plate that no contaminated fish reach the consumer.

The accuracy of a visual inspection method in the fish industry largely depends on the training and skills of inspectors (Levsen et al, 2005), but mostly on a well-tested statistical significance between the number of observable parasites free or encysted in the abdominal cavity and surrounded organs, and the number of parasites in musculature or edible part of the fish. The later is especially important when expending untreated fresh fish products (e.g., coastal fish), because no prophylactic processes have been carried out to kill *Anisakis* spp. larvae or inactivate their somatic and metabolic antigens during harvest and distribution, making the final consumer manage the hazard.

The double aim of this work was (1) to study the existence of a statistical significance between gut parasites and muscular parasites, and (2) to evaluate the efficiency of the washing practice to remove *Anisakis* spp. from gut, in order to evaluate the accuracy of the current legislation.

2. MATERIAL AND METHODS

Commercial lots of 322 fresh individuals of the blue whiting *Micromesistius poutassou* and 230 of Atlantic mackerel *Scomber scombrus*, caught in the western Iberian Sea (ICES division IXa), were necropsied within 12 hours and 48 hours post-capture. The time passed after capture, the number of fishes in each lot and the ranges of total length and total weight for both species are showed in Table 1. The heads and tails were removed from each fish, and the remaining musculature was separated into the hypaxial (ventral) and epaxial (dorsal) regions following the horizontal septum. The nematodes were isolated by digestion from the whole gut and from the fish musculature, according to CODEX STAN 244-2004 rev. Sixteen variables were recognized and defined to compare the number of *Anisakis* spp. larvae, taking into account fish species, fish body region and time from capture to necropsies (Table 2). Descriptive statistics for parasite counts including the mean, median, mode, variance, skewness, kurtosis, a box-whisker graph and a Kolmogorov-Smirnov test were calculated. Correlation and regression analyses, were also used to evaluate the significant statistical relationship between variables, regarding the number of *Anisakis* spp. larvae found in the gut and musculature (epaxial, hypaxial and total musculature, separately) of both fish species. Spearman correlation coefficient (r), t (N-2) and p-level values (for statistical significance) only were specified for pairs of variables which revealed correlation between variables. When necessary, anisakid counts were logarithmic transformed to normalize the data (Rózsa et al, 2000).

Moreover, demographic values of infection for *Anisakis* spp. larvae were determined specifically for gut, epaxial and hypaxial region, and total musculature at both fish species. The terms prevalence, mean intensity and mean abundance of infection were used as defined in Bush et al (1997) and Rózsa et al (2000).

Additionally, a commercial lot of 25 fresh individuals (250-300 mm sized) of the European Hake *Merluccius merluccius,* was necropsied 12 hours post-capture. Fresh liver and gonads were vigorously washed under tap water. Then, both organs were examined under stereomicroscope looking for the presence of *Anisakis* spp. larvae, and infected tissues were processed for histological sections following standard protocols.

3. RESULTS

Descriptive statistics for anisakids counts in both fish species showed that any of the *Anisakis* spp. count combining variables did not follow a normal distribution (Kolmogorov-Smirnov Test <0.05) (Table 3; Fig. 1). Table 3 shows demographic values (prevalence, mean intensity and mean abundance) of infection attributable to *Anisakis* spp. larvae, calculated specifically for gut, epaxial and hypaxial region, and total musculature at both fish species. These values clearly evidenced higher infection in gut than in musculature, and larger values of worm burdens in hypaxial region than in epaxial musculature, in all cases. Other results in the same table suggested that an increased mean, median and variance of *Anisakis* spp. larvae in the gut of *Micromesistius poutassou* at 48 hours post-capture led increments in the mean, median and variance of these parasites in the hypaxial region and at the total musculature in the same group of fishes. This tendency was not observed in the rest of the fish lots analized (*Micromesistius poutassou* at 12h, *Scomber scombrus* at 48h and at 12h).

Every pairs of variables were analysed by Spearman Rank Order Correlations (Table 4). The results revealed that the worm burden in the total musculature was more correlated to the parasites present at hypaxial musculature (r values between 0.92-0.98) than at epaxial region, which gaves lower significant rates (r =0.18-0.38) at 12h and 48h post-capture in both fish species. Moreover, there was a positive relationship (r=0.25-0.51) between gut and total muscular worm burdens at *Micromesistius poutassou* at 48h and *Scomber scombrus* at 12h and at 48h. The positive relationship between gut and musculature in *Micromesistius poutassou* at 48h was significantly higher specifically at hypaxial muscular region (r=0.52) than at epaxial muscle (r=0.21). As well for *Scomber scombrus* at 12h, the same positive relationship was higher at hypaxial muscle (r=0.34) than at epaxial (no significant correlation). However, *Scomber scombrus* at 48h did not give interesting values of correlation between anisakids in gut and hypaxial or epaxial musculature. These were two of the eight remaining pairs (including all variables not showed in Table 4) that presented an absence of strength between the variables compared in each pair (at $p<0.05$). This fact also occurred, for example, when comparing the number of parasites in the gut of blue whiting, with the parasites in the musculature (any of regions) at 12 hours post-capture. Even the number of parasites at both regions of the musculature had no correlation between them. Equally, Atlantic mackerel at 48 hours post-capture showed no associations in the number of parasites comparing epaxial and hypaxial musculature.

Simple linear regression analysis of gut vs. muscular anisakids for both species, showed no significant relationship between the number of parasites in the gut cavity and those in any other region of the musculature (Table 5). This absence of statistical significance was the observed pattern every case, except for the SSG48 - SST48 pair, the only one that evidenced a causal relationship between them.

Otherwise, examination of liver and gonads from fresh European hake showed high demographic values of *Anisakis* spp. infection (Table 6; Fig. 2A-C). After washing vigorously under tap water most *Anisakis* spp. larvae were removed but in all cases at least one larva remained inside the tissue. These larvae usually corresponded with deeply embedded parasites or older capsules which were observed in histological sections (Fig. 2D-F).

4. DISCUSSION

Results suggested the low efficiency of visual inspection of gut parasites as a commonly recommended method for predicting nematode larvae in the flesh of fish. In fact, association does not imply predictability. It is feasible that by counting many parasites in gut someone can have an idea that many parasites are in fact infecting the fish musculature, but it is not easy to predict how many parasites will be found there. This implies that in absence of anything better for fish operators, correlation matrices are useful but not enough to ensure a robust statistical predictable value to infer muscular anisakids based on the evidence of gut parasites. This is the case of blue whiting, which none significant relationship between parasites in gut and flesh regions was determined in, after linear regression analyses. Furthermore, in the best case (e.g., in the Atlantic mackerel inspected at 48 hours) the amount of variability in the dependent variable, *number of muscular parasites*, explained by the predictor variable, *number of gut parasites*, was less than 8% (as estimated by the R^2). Bussmann et al (1979) studied blue whiting as well, from different geographical sampling areas and seasons. He reported linear regression analyses with significant positive associations ($p<0.05$) between the number of parasites in gut, hypaxial musculature and epaxial flesh, based in not normalized data. However, as some other authors recommends, raw data of the frequency distribution does not work well, and a good alternative to proceed is the log transformation (log[x+1]) before calculating the mean (Rózsa et al, 2000). In addition, different geographical sampling areas and seasons could influence on relationships between sites of infestation (Bussmann et al, 1979).

In relation to demographic values of infection obtained from the biological data, comparing prevalences at both species with the same hours post-capture, higher percentages of parasites in blue whiting than in Atlantic mackerel were noticed (for 12h and 48h post-capture). Mean intensity comparisons revealed four clearly different degrees of infection. At least for the four main groups of fishes that this study revised, the order of the regions according to their degree of infection (from highest to lowest) coincided the same; (1º) gut cavity, (2º) total musculature, (3º) hypaxial musculature and (4º) epaxial musculature. In all cases, mean intensity of hypaxial muscles influenced very strongly on total musculature. The highest values of *Anisakis* spp. larvae in hypaxial or in total musculature were obtained at the group with the highest worm burden value in gut (*Micromesistius poutassou* at 48h post-capture). At the same time, the lowest intensity of worms at epaxial region was found at the group with the lowest number of parasites in gut. Both facts may have been due to three factors: the distance from epaxial region to gut, the proximity of hypaxial musculature to gut, and the larvae migration that can occurs intra vitam or subsequently to host dead. Many factors can explain the possibility and timing (intra vitam or post-mortem) of anisakid migrations from fish gut to the flesh, mostly related to physiological trade-off of parasites, to ecological and immunological factors operating in living fish, or to the biochemical post-mortem changes which occurred in autolysed fish (Karl, 2008). Recently, Scientific Opinion on risk assessment of parasites in fishery products by the Panel on Biological Hazards (European Food Safety Authority, EFSA, 2010) stated that “based on scientific evidences it is not clear when, under what conditions and in which fish species, post-mortem migration of *A. simplex* larvae occurs”. In summary, these appreciations evidence different proportions of infection that can be found in fishes depending on the anatomical region. But these proportions that may be considered as “stages of infection” can fluctuate more or less, if comparing different fish species. The observation of both types of parasites (intra-vitam and post-mortem) inhabiting fish musculature, emphasized that in case of significant regression values for a given fish species, the predictive model only would be workable to infer muscular anisakids in fish inspections, if preliminary epidemiological data for that target commercial fish is available. This data would provide the penetration rate (the ratio of the number of larvae detected in the muscle to the total number of larvae detected under various holding conditions), from the abdominal cavity into the muscle of the fish. The establishment of this epidemiological monitoring programme would also allow the standardization of inspection methodology including sampling size in each commercial species, following the current artificial digestion protocol by CODEX STAN 244-2004 rev. These issues are not defined in legislation and represent a source for uncertainty in hazard analysis during fish inspections. Moreover, other edible fish parts such as gonads and liver remain contaminated with *Anisakis* spp. after gutting and washing gut vigorously under tap water which clearly does not accomplish with legislation.

The above information should be taking into account to review critical control points at HACCP programmes to reduce the risk of anisakid-induced allergies and gastrointestinal anisakiasis among consumers. This is especially important for whole ungutted fish at local markets of the Euroregion which are stowed refrigerated and sold at the market up to 2-3 days post-capture.

5. ACKNOWLEDGMENTS

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TABLES

Species	(N)	Hours Post-capture	Total Length Range (cm)	Total Weight Range (g)
Micromesistius poutassou	163	12	21.5-28	68-172
Micromesistius poutassou	166	48	21-28.5	52-158
Scomber scombrus	166	12	27-34	123-291
Scomber scombrus	70	48	31-43	204-645

Table 1 Biological data as host sample size (N), time between capture and necropsies, and total length and weight ranges of the fish species studied for *Anisakis* spp. infection.

Variable	Species	Body Region	Hours Post-capture
MPH12	*Micromesistius poutassou*	Hypaxial Musculature	12
MPE12	*Micromesistius poutassou*	Epaxial Musculature	12
MPT12	*Micromesistius poutassou*	Hypaxial and Epaxial Musculature	12
MPG12	*Micromesistius poutassou*	Gut Cavity	12
MPH48	*Micromesistius poutassou*	Hypaxial Musculature	48
MPE48	*Micromesistius poutassou*	Epaxial Musculature	48
MPT48	*Micromesistius poutassou*	Hypaxial and Epaxial Musculature	48
MPG48	*Micromesistius poutassou*	Gut Cavity	48
SSH12	*Scomber scombrus*	Hypaxial Musculature	12
SSE12	*Scomber scombrus*	Epaxial Musculature	12
SST12	*Scomber scombrus*	Hypaxial and Epaxial Musculature	12
SSG12	*Scomber scombrus*	Gut Cavity	12
SSH48	*Scomber scombrus*	Hypaxial Musculature	48
SSE48	*Scomber scombrus*	Epaxial Musculature	48
SST48	*Scomber scombrus*	Hypaxial and Epaxial Musculature	48
SSG48	*Scomber scombrus*	Gut Cavity	48

Table 2 Sixteen variables have been established to compare *Anisakis* spp. larvae at the study, taking into account fish species, fish body region and time from capture to examination.

Variable	N	Prevalence (% ± CI)	Mean Intensity (± SD)	Mean Abundance (± SD)	Mean	Median	Mode	Variance	Skewness	Kurtosis
MPG12	163	94.47 ± 1.75	12.18 ± 14.47	11.5 ± 14.34	11.5092	7.00	3.00	205.745	3.106	12.616
MPE12	163	4.3 ± 1.5	1.71 ± 0	0.07 ± 0.16	0.02454	0.00	0.00	0.024	6.203	36.935
MPH12	163	33.13 ± 3.6	1.77 ± 1.23	0.59 ± 1	0.51534	0.00	0.00	1.078	3.055	11.216
MPT12	163	34.97 ± 3.6	1.89 ± 1.23	0.66 ± 1.04	0.53988	0.00	0.00	1.077	2.995	10.957
MPG48	166	98.79 ± 0.83	69.18 ± 92.48	68.35 ± 92.23	68.3494	40.50	31.00	8506.398	4.195	24.369
MPE48	166	12.05 ± 2.4	2.05 ± 1.28	0.25 ± 0.91	0.23494	0.00	0.00	0.835	5.638	37.846
MPH48	166	75.3 ± 3.29	7.02 ± 13.07	5.29 ± 11.72	5.24096	2.00	0.00	137.432	6.467	55.758
MPT48	166	76.5 ± 3.22	7.24 ± 13.32	5.54 ± 12.03	5.4759	2.00	0.00	144.784	6.182	51.559
SSG12	166	72.89 ± 3.38	11.55 ± 51.77	8.42 ± 44.45	8.42169	2.00	0.00	1975.942	12.007	150.355

SSE12	166	1.2 ± 0.82	1 ± 0	0.01 ± 0.11	0.01205	0.00	0.00	0.012	9.027	80.451
SSH12	166	18.67 ± 2.96	2.16 ± 1.92	0.4 ± 1.16	0.38554	0.00	0.00	1.341	4.488	24.136
SST12	166	19.28 ± 3	2.16 ± 1.96	0.42 ± 1.19	0.40361	0.00	0.00	1.418	4.297	21.786
SSG48	70	57.14 ± 5.79	4.92 ± 9.58	2.81 ± 7.61	2.81429	1.00	0.00	57.893	7.048	54.818
SSE48	70	5.7 ± 2.7	1 ± 0	0.06 ± 0.23	0.05714	0.00	0.00	0.055	3.899	13.597
SSH48	70	34.3 ± 5.54	2.25 ± 3.23	0.77 ± 2.08	0.72857	0.00	0.00	4.346	4.869	26.491
SST48	70	38.57 ± 5.7	2.25 ± 3.04	0.83 ± 2.08	0.78571	0.00	0.00	4.345	4.791	25.942

Table 3 Demographic infection values and descriptive statistics for anisakids counts in defined variables.

Pair of variables	**N**	**Spearman (*r*)**	**t (N-2)**	***p*-level**
MPE12 - MPT12	163	0.188116	2.43031	0.016185
MPH12 - MPT12	163	0.956773	41.74187	0.000000
MPG48 - MPE48	166	0.211691	2.77384	0.006182
MPG48 - MPH48	166	0.527729	7.95636	0.000000
MPG48 - MPT48	166	0.512729	7.64793	0.000000
MPE48 - MPH48	166	0.292033	3.91030	0.000135
MPE48 - MPT48	166	0.380792	5.27385	0.000000
MPH48 - MPT48	166	0.988358	83.18953	0.000000
SSE12 - SSH12	166	0.261501	3.46958	0.000666
SSE12 - SST12	166	0.263223	3.49412	0.000612
SSH12 - SSG12	166	0.343702	4.68707	0.000006
SSH12 - SST12	166	0.982838	68.23008	0.000000
SSG12 - SST12	166	0.349530	4.77751	0.000004
SSE48 - SST48	70	0.312904	2.71669	0.008355
SSH48 - SST48	70	0.926179	20.25402	0.000000
SSG48 - SST48	70	0.258036	2.20241	0.031030

Table 4 Spearman Rank Order Correlations between variables. Spearman correlation coefficient (*r*) and *p*-level (value of the statistical significance at 0.05) are given for pairs of variables which present correlation. Pairs without some intensity of correlation have not been taken into consideration.

	Micromesistius poutassou						***Scomber scombrus***					
	Epaxial		**Hypaxial**		**Total**		**Epaxial**		**Hypaxial**		**Total**	
	12h	**48h**	**12h**	**48h**	**12h**	**48h**	**12h**	**48h**	**12h**	**48h**	**12h**	**48h**
F	0.074	0.292	0.029	0.580	1.096	0.491	0.000	1.099	0.028	0.009	0.028	5.778
***p*-level**	0.785	0.600	0.865	0.447	0.297	0.484	0.992	0.298	0.867	0.924	0.866	0.019
R^2	-	-	-	-	-	-	-	-	-	-	-	0.079

Table 5 Statistics of simple linear regression of gut vs. muscular (epaxial, hypaxial and total) parasites using log-transformed data. F (test for statistical significance of the regression equation), *p*-level (value of the statistical significance at 0.05) and the coefficient of determination R^2, are represented for *Micromesistius poutassou* and *Scomber scombrus.*

Merluccius merluccius

	Gonads	Liver
N	25	25
Prevalence (% ± CI)	0.64 ± 0.13	0.84 ± 0.10
Mean Intensity	9.2	21.23
Mean Abundance	6.1	17.84

Table 6 Infection values for *Anisakis* spp. in the gonads and livers of European hake *Merluccius merluccius*.

FIGURE LEGENDS:

Fig. 1 Box-whisker graph of anisakid counts in fish gut and musculature (epaxial, hypaxial and total). The number of *Anisakis* spp. larvae (vertical axis) is represented for each variable defined and studied (horizontal axis).

Fig. 2A-C Gonads (A and B) and liver (C) heavily infected with *Anisakis* spp. larvae. The parasites are located encysted inside both organs as well as covering them

Fig. 2D-F Histological sections of liver and gonads infected with *Anisakis* spp. larvae, stained with hematoxylin and eosin, 40X. **D** Cross-section of an embedded larva inside the female reproductive tract. Black arrow: Rests of an old capsule (melanin granules) surrounding the parasite. **E** Longitudinal section of an embedded larval inside the male reproductive tract. **F** Cross-section of four embedded larvae inside the liver.

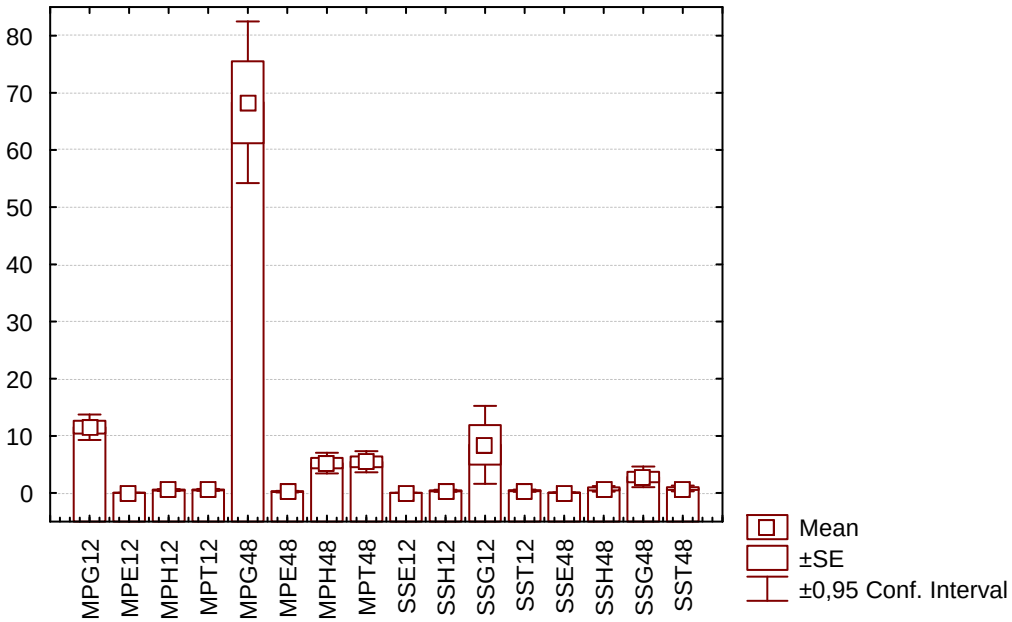

Fig. 1

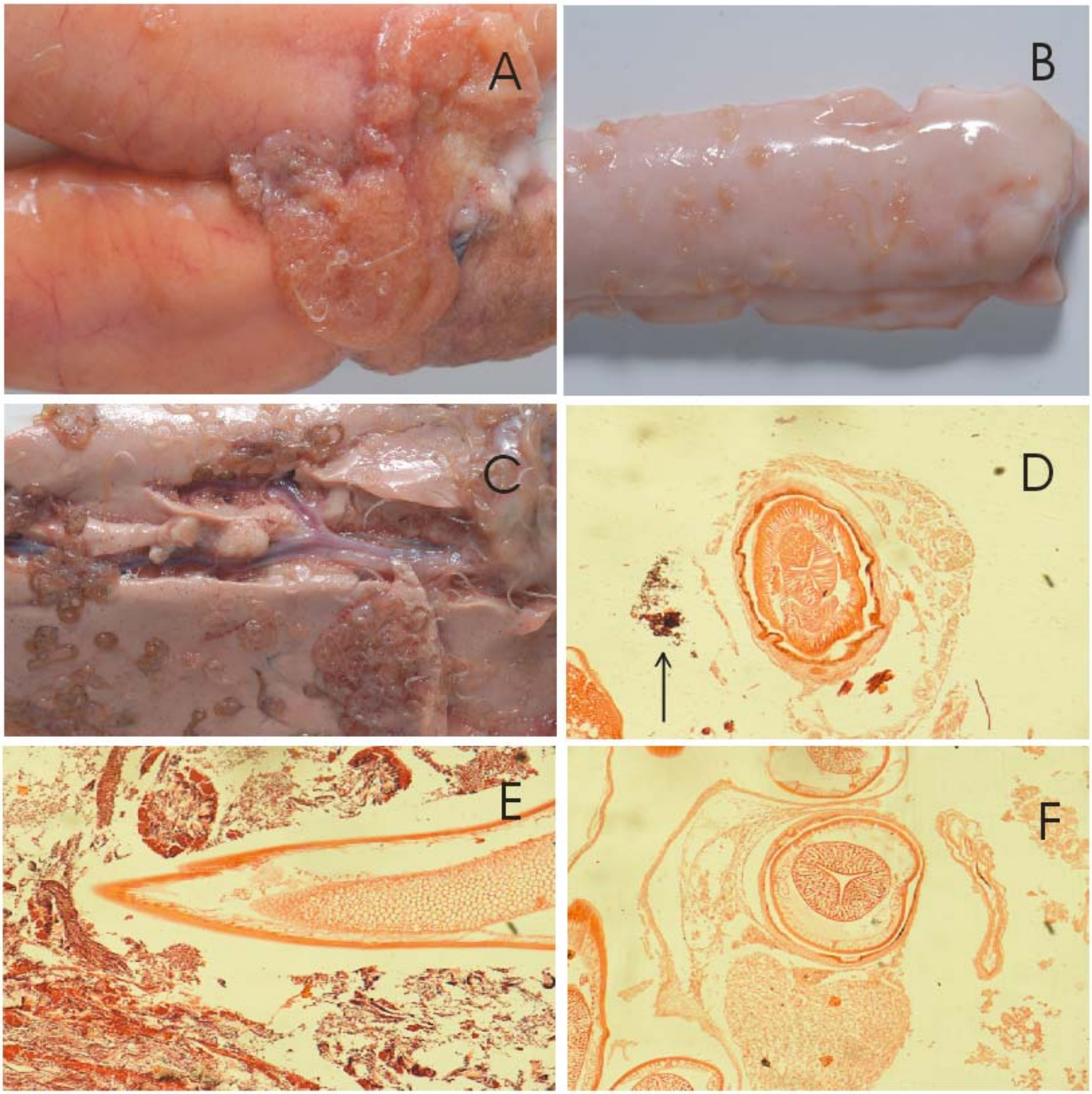

Fig. 2