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Title: Visceral larvae as a predictive index of the overall level of fish batch infection in European anchovies (Engraulis encrasicolus): a rapid procedure for Food Business Operators to assess marketability

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Keywords: Visible nematode larvae, digestion procedure, marketability, self-monitoring plan, larvae per gram.

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Abstract: The European anchovy (Engraulis encrasicolus), one of the most important pelagic fish resources in the Mediterranean Sea, is frequently infected by anisakid larvae. Food Business Operators (FBOs) should use appropriate sampling plans and analytical methods to avoid commercialization of massively infected batches and reduce the risk of transmission of viable zoonotic larvae. In this study, performed at FishLab (Department of Veterinary Sciences of the University of Pisa) during 2016, an official sampling plan was associated with a digestion protocol for the inspection of anchovies. Considering that anisakid larvae are usually located in the fish visceral cavity and in the adjacent muscles (VM), this part was analyzed. In particular, we assessed the reliability of the digestion of a subsample of 150 g (±30 g) of VM, randomly collected from 29 specimens, in estimating the marketability of the anchovies' batch. Fifty-seven samples of 29 anchovies were collected. Each anchovy was sectioned to separate VM. All the subsamples were digested, and visible larvae counted. A high correlation between the number of larvae in VM regions and in the total batch was observed, indicating a very significant contribution of the VM region on total number of parasites. The Mean Abundance (MA) was used to assess the batch marketability according to a threshold calculated on the basis of the maximum number of nematodes tolerated per sample. Considering that the MA can be calculated only when the number of examined specimens is known, the number of visible Larvae per gram of tissue (LpG) was calculated on 150 g (±30 g) of VM subsamples. A LpG marketability threshold was calculated dividing the maximum number of tolerated nematodes by the average weight of a sample of 29 anchovies calculated considering data available in literature. To evaluate the diagnostic performance of the LpG threshold, the marketability of 57 batches assessed on the basis of the MA threshold was assumed as the gold standard. The proposed LpG showed very high Specificity and Sensitivity. These findings suggest that the analysis of VM is representative of the overall infestation of the batch, both when considering the absolute number of parasites and the

LpG, and may represent a valid alternative to the whole anchovy digestion. In particular, the use of an automated digestive method, coupled with the aforesaid sampling plan, could allow the procedure to be used by FBOs in operational conditions.

Pisa, 21st December 2016

Dear Editor,

Please find enclosed the manuscript entitled "**Visceral larvae as a predictive index of the overall level of fish batch infection in European anchovies (*Engraulis encrasicolus*): a rapid procedure for Food Business Operators to assess marketability"** to be considered for publication in International Journal of Food Microbiology.

The European anchovy (*Engraulis encrasicolus*), one of the most important pelagic fish resource in the Mediterranean Sea, is frequently infected by anisakid larvae. European anchovies are often sold ungutted and consumed raw in traditional recipes. Although fishery products to be consumed raw or almost raw must be frozen (not more than - 20 °C or - 35°C in all parts of the product for not less than 24 or 15 hours), FBOs must ensure that fishery products that are obviously contaminated with visible parasites are not placed on the market for human consumption. In fact, visible parasites alter the commercial quality of fishery products making them unfit for human consumption. Moreover, some species may be responsible for zoonotic infections, making the products injurious to health. It is up to FBOs to carry out their own checks at all stages in the production of fishery products (Commission Regulation EC 2074/2005). Thus, it is necessary that Food Business Operators (FBOs) use appropriate sampling plans and analytical methods to avoid that massively infected fish reach the market.

The aim of this study was to set up a standardized protocol, based on digestion, for the inspection of anchovies using Trichineasy®(CTSV srl, Brescia), a complete grinding, digestion and filtration instrument recently validated by the Italian National Reference Centre for Anisakiasis. In fact, the use of Trichineasy® coupled with the official sampling plan proposed by the Lombardy Region (Italy) Circular (Circular Letter VS8/C790/94), could allow the procedure to be used by FBOs in their operational conditions for the assessment of fish batch marketability. Considering that the majority of anisakid larvae are located in the fish visceral cavity and/or embedded in the visceral organs and in the adjacent muscles (VM), the analysis was focused on this body portions. The reliability of the digestion of 150gr (±30gr) of VM randomly collected from 29 specimens in estimating the overall infection and marketability of the fish batch was assessed. First, the marketability was assessed on the basis of the Mean Abundance (MA). Then, considering that the MA can be calculated only when the number of examined specimens is known, the number of Larvae per Gram (LpG) of tissue of anchovies was used for the statistical analysis concerning the 150gr (±30) VM subsample. To evaluate the diagnostic performance of the LpG threshold, the

marketability of 57 batches assessed on the basis of the MA threshold was assumed as the gold standard.

The proposed LpG showed very high Specificity and Sensitivity. The results of the present research highlight that a 0.03 LpG threshold and the related corrections can be a useful tool for taking decisions on the marketability of anchovies. The proposed method is aimed not only to prevent commercialization of repugnant products, but also to reduce the parasitological risk for those products intended to be used for the preparation of uncooked products.

The manuscript has not been published elsewhere nor is it being considered for publication elsewhere. All authors have approved this manuscript, agree to the order in which their names are listed, declare that no conflict of interests exists and disclose any commercial affiliation.

Yours sincerely,

Andrea Armani

Dear Editor,

We are sending you back the revised version of the manuscript entitled "*Visceral larvae as a predictive index of the overall level of fish batch infection in European anchovies (Engraulis encrasicolus): a rapid procedure for Food Business Operators to assess marketability*". Thank you for considering the manuscript for publication after revision. The manuscript has been implemented according the suggestions of the reviewer.

Reviewer #3: This manuscript is improved over the original version and all of my comments have been adequately addressed. There are still a few grammatical corrections to be made. I have attached a number of minor revisions for the authors to consider.

Introduction
Pg. 5, line 100, revise to "…are aimed at avoiding…"
Addressed: see line 89
Pg. 6, line 109, revise to "Although the visual inspection of fish through direct observation without candling has been…"
Addressed: see line 97-98
Pg. 6, line 128, revise to "…take advantage of the application..."
Addressed: see line 114

Materials and Methods
Pg. 8, line 158, revise to "…the average weight (411.99 g, SD ± 165.41) of a sample of…"
Addressed: see line 141
Pg. 8, line 178, should this be a "minimum" of one month or a "maximum"?
Addressed: see line 161
Pg. 9, line 190, revise to "through observation on a microscope…"
Addressed: see line 173
Pg. 10, line 212, need to close the parentheses which started on line 210; ie. "(…species examined, (Bush et al., 1997)) of each sample..."
Addressed: see line 194
Pg. 10, line 213, add the word "larvae" after "0.30"
Addressed: see line 195

Results and Discussion
Pg. 16, line 361, revise to something like "The corrected LpG is appropriate for samples weighing from 200 to 470 g…"
Addressed: see line 335
Pg. 17, line 396, revise to "…may be responsible for human infection."
Addressed: see line 369
Pg. 18, line 409, replace "alive" with "live"
Addressed: see line 382

Conclusion
Pg. 18, line 419, correct to "issued"
Addressed: see line 392

Highlights

- Visible parasites in Viscera/adjacent muscle (VM) were used as a predictor of infection
- Anchovies' marketability was assessed according to Larvae per gram (LpG) of VM tissue
- LpG showed good diagnostic performances when compared to the gold standard
- The method is an easy tool for inspecting anchovies timely as they reach the market
- The method will prevent the commercialization of massively infected fish

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Visceral larvae as a predictive index of the overall level of fish batch infection in European anchovies (*Engraulis encrasicolus*): a rapid procedure for Food Business Operators to assess marketability

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Abstract

The European anchovy (*Engraulis encrasicolus*), one of the most important pelagic fish resources in the Mediterranean Sea, is frequently infected by anisakid larvae. Food Business Operators (FBOs) should use appropriate sampling plans and analytical methods to avoid commercialization of massively infected batches and reduce the risk of transmission of viable zoonotic larvae. In this study, performed at FishLab (Department of Veterinary Sciences of the University of Pisa) during 2016, an official sampling plan was associated with a digestion protocol for the inspection of anchovies. Considering that anisakid larvae are usually located in the fish visceral cavity and in the adjacent muscles (VM), this part was analyzed. In particular, we assessed the reliability of the digestion of a subsample of 150 g (±30 g) of VM, randomly collected from 29 specimens, in estimating the marketability of the anchovies' batch. Fifty-seven samples of 29 anchovies were collected. Each anchovy was sectioned to separate VM. All the subsamples were digested, and visible larvae counted. A high correlation between the number of larvae in VM regions and in the total batch was observed, indicating a very significant contribution of the VM region on total number of parasites. The Mean Abundance (MA) was used to assess the batch marketability according to a threshold calculated on the basis of the maximum number of nematodes tolerated per sample. Considering that the MA can be calculated only when the number of examined specimens is known, the number of visible Larvae per gram of tissue (LpG) was calculated on 150 g (±30 g) of VM subsamples. A LpG marketability threshold was calculated dividing the maximum number of tolerated nematodes by the average weight of a sample of 29 anchovies calculated considering data available in literature. To evaluate the diagnostic performance of the LpG threshold, the marketability of 57 batches assessed on the basis of the MA threshold was assumed as the gold standard. The proposed LpG showed very high Specificity and Sensitivity. These findings suggest that the analysis of VM is representative of the overall infestation of the batch, both when considering the absolute number of parasites and the LpG, and

may represent a valid alternative to the whole anchovy digestion. In particular, the use of an automated digestive method, coupled with the aforesaid sampling plan, could allow the procedure to be used by FBOs in operational conditions.

Keywords

Visible nematode larvae, digestion procedure, marketability, self-monitoring plan, larvae per gram.

1. Introduction

The European anchovy (*Engraulis encrasicolus*) has a high commercial value (http://www.iucnredlist.org/details/summary/198568/1) and represents the most important pelagic fish resource in the Mediterranean Sea (Lleonart and Maynou, 2003). In Italy, *E. encrasicolus* is the main fished species by weight, corresponding to 25-35% of the total catches of marine fishes between 2010 and 2014 (http://www.fao.org/fishery/topic/16140/en).

Although there is a growing tendency in producing prepared and preserved products, fresh anchovies are still largely requested from the markets. Italy is among the main importers of this product (Eurofish, 2012).

Among the most important biohazards related to the consumption of raw anchovies, there is the presence of viable nematode larvae belonging to the Anisakidae family (Alonso-Gómez *et al*., 2004; Cipriani *et al*., 2016; Daschner *et al*., 1998; Mattiucci *et al*., 2013). In fact, even though according to a recent systematic review (Colombo et al., 2016) the overall prevalence values are quite low, particularly high values have been reported in some areas, such as in Sardinia (Piras *et al*., 2014) and Croatia (Mladineo and Poljak, 2014). The zoonotic infection, known as anisakidosis, is acquired through the consumption of raw or undercooked marine fish or cephalopods infected by third stage larvae of Anisakidae nematodes, most frequently belonging to *Anisakis* and *Pseudoterranova* genera (Lymbery and Cheah, 2007). In the Mediterranean Region the zoonotic risk is mainly associated with the presence of *A. pegreffi* (Bernardi *et al*., 2011; Mattiucci *et al*., 2008; Mladineo *et al*., 2012).

The presence of anisakid larvae in fish is a natural condition throughout the supply chain and the complete elimination of the parasitological hazard from fishery products is not feasible. Although fishery products to be consumed raw or almost raw must be frozen (not warmer than - 20°C or - 35°C in all parts of the product for not less than 24 or 15 hours, respectively), FBOs must ensure that fishery products obviously contaminated with visible parasites (all parasites longer than 10 mm

according to Codex Alimentarius Commission, 1971) are not placed on the market for human consumption (Commission Regulation (EU) No. 1276/2011). Visible parasites alter the commercial quality of fishery products making them unfit for human consumption (Council Regulation (EC) No 2406/1996; Reg. (EC) No 178/2002). Moreover, some species may be responsible for zoonotic infections, making the products injurious to health (Reg. (EC) No 178/2002). Thus, the implementation of preventive measures by FBOs is compulsory at all stages of the fishery chain: from the primary production to the administration (Commission Regulation EC 2074/2005). Preventive measures, such as good manufacturing practices and HACCP programs, are aimed ~~to~~ at avoiding commercialization of unsafe products, reducing the parasitological risk to acceptable levels. While the preventive measures that FBOs can apply before harvest are limited, those applied after the capture can have an impact on consumers' health (D'Amico *et al*., 2014).

Preventive measures are particularly important in fish like anchovies that are sold ungutted, since larval migration from the viscera to the muscle generally occurs after the capture (Cipriani *et al*., 2016; Šimat *et al*., 2015). FBOs should use appropriate sampling plans and analytical methods provided by European or national laws to assure food safety. In the absence of such methods, scientifically validated alternatives can be used (Regulation (EC) No 852/2004). Although the visual inspection of fish through direct observation without candling has been ~~Although the visual inspection by direct observation of fish (not candling) has been~~ established as the official method for anisakid larvae detection in the European Union (Commission Regulation (EC) No 2074/2005), a unique official sampling protocol is still lacking. In the Lombardy Region (Italy), for example, Circular Letter VS8/C790/94 authorized official controls of visible nematode larvae on anchovies based on the visual inspection of a sample of 29 whole specimens from batches of more than 600 anchovies. The sampling of 29 anchovies was chosen in order to detect a prevalence not lower than 10% from an infinite population (Cannon and Roe, 1982). In a previous work (Guardone *et al*., 2016) the sampling plan proposed by the Lombardy Region (Italy) was compared to the UV press

method (Karl and Leinemann, 1993) and to a digestion procedure. The visual inspection was statistically comparable to the digestion procedure in detecting visible parasites and assessing the batch marketability (Guardone *et al*., 2016). However, the digestion procedure is still widely considered the most sensitive method for larval detection (Bernardi *et al*., 2011; Fraulo *et al*., 2014; Llarena-Reino *et al*., 2013; Rossi *et al*., 2015). Standard operating procedures applied to fish species have been published by the European Union Reference Laboratory for Parasites (http://www.iss.it/binary/crlp/cont/SOP_Artificial_digestion_of_fish_fillet.pdf).

Seafood inspection for the detection of parasites can take advantage of the application ~~take advantage from the application~~ of new laboratory methods (Bao *et al*., 2017). The use of Trichineasy® (CTSV srl, Brescia), a complete grinding, digestion and filtration instrument recently validated for the digestion of fish tissue by the Italian National Reference Centre for Anisakiasis (Cammilleri *et al*., 2016), can speed up the digestion procedure reducing the overall time of analyses and allowing the procedure to be used also by FBOs in their operational conditions to assess the marketability of fish.

The aim of this study was to set up a standardized sampling protocol, based on digestion, for the inspection of anchovies. Considering that most anisakid larvae are located in the fish visceral cavity and/or embedded in the visceral organs (Bernardi *et al*., 2011; Cipriani *et al*., 2016; Mladineo *et al*., 2012; Šimat *et al*., 2015) and in the adjacent muscles (belly flap) (Adams *et al*., 1997; EFSA, 2010) the analysis was focused on this body portion. In particular, the reliability of the digestion of 150 g (±30 g) of viscera and adjacent muscles (VM) randomly collected from 29 specimens in estimating the overall infection and the marketability of the fish batch was assessed.

2. Material and Methods

2.1 Sampling

Fifty-seven samples of 29 anchovies (*E. encrasicolus*), for a total of 1652 specimens, were randomly sampled at the wholesale market of Viareggio (Lucca, Italy) from different batches of anchovies caught in the Western Mediterranean Sea (FAO area 37.1.3) and in the Central Mediterranean Sea (FAO area 37.2.1). Anchovies were sampled at landing and, to preclude the possibility of postmortem larval migration from viscera to muscle and to maintain as much as possible the original localization of the larvae (Cipriani *et al.*, 2016), they were kept on ice for a maximum of 24 h and then frozen. All the samples were then transferred to the FishLab (Department of Veterinary Sciences of the University of Pisa), and maintained at - 20°C until the analysis. Five additional samples, not included in the statistical analysis, were used for the optimization of the digestion procedure (see section 2.3).

2.2 Samples preparation

In order to obtain a representative estimate, the average weight (411.99 g, SD ± 165.41) of a sample of 29 anchovies has been calculated on 19274 anchovies, analyzed in previous studies (Table 1)~~: it resulted to be 411.99 g (SD ±165.41)~~. Considering that the manufacturer set the maximum amount of tissue at 200 g (http://www.ctsv.biz/image-ctsv/PDF/TrichinEasy-anisakis.pdf), it was not possible to digest the whole amount at once using Trichineasy®. Therefore, the samples were divided in subsamples of lower weight and, assuming that most larvae are located in the visceral cavity and/or embedded in the visceral organs and in adjacent tissue (VM portion) (Cipriani *et al.*, 2016), the fish body was divided in 2 parts. Each anchovy was sectioned in order to separate the central part of the body, containing the viscera surrounded by the belly flaps and the dorsal muscles (VM), from the head and the tail (HT). The sections were obtained by performing two cuts perpendicular to the anchovy's body, the first one in correspondence of the gills' operculum, and the second in correspondence of the anus (Fig. 1). Then, the pools of VM and HT were weighed separately.

2.3 Optimization of the digestion procedure and final protocol

155 VM and HT subsamples belonging to 5 samples were used for the optimization of the digestion
156 procedure using the Trichineasy® (Cammilleri *et al.*, 2016). The loading of the samples was
157 conducted after the "mixer" step in order to avoid tissue homogenization
158 (http://www.ctsv.biz/image-ctsv/PDF/TrichinEasy-anisakis.pdf). In order to test the recovery of
159 parasites after the digestion, 5 frozen *Anisakis* spp. larvae were added to each subsample analyzed.
160 All the larvae were collected from naturally infected *E. encrasicolus* and stored at - 20°C for a
161 ~~minimum~~ maximum of one month. Increasing weights (100-150-200 g with a tolerance of 10%) and
162 different digestion times (15-20-30 min) were tested during the trials. The temperature was set at
163 37°C, the blades were maintained at the minimum speed and all the digestions were performed
164 adding 1 L of water, with 50 mL of 10% HCl and 10 g of pepsin from PLYtricons® (CTSV srl,
165 Brescia). At the end of the digestion procedure, the digested material was filtered through the
166 filtering part of the Trichineasy (mesh 180 µm). The retained material was washed from the filter
167 and divided in Petri dishes in order to create a thin layer of a few mm. Then, the Petri dishes were
168 observed under natural and UV light (UltraBright UV Transilluminator, 302/365 nm, Maestrogen,
169 Las Vegas, USA) for the detection of anisakid larvae.
170 In consideration of the provisions of the Regulation (EC) No 853/2004 and of the definition
171 given by the Codex Alimentarius Commission (1971) only the visible larvae detected by visual
172 inspection were counted and collected. Then, they were identified to genus level following Sakanari
173 and McKerrow (1989) and Berland (1989), through observation on a microscope ~~through observation~~
174 ~~at optical microscope~~ (Nikon Eclipse E200) and then stored in 70% alcohol.
175 On the basis of the amount of undigested tissue (<5% of the initial weight according to
176 Commission Regulation EC 2075/2005) the final digestion protocol was set at 20 min for maximum
177 150 g (±30 g) of tissue. The degree of variability in the subsamples' weight was voluntarily
178 introduced to simulate the real conditions of utilization in the field.

All the VM and HT subsamples belonging to the 57 samples were digested separately according to the optimized protocol above described. If the VM and HT subsamples' weight exceeded 150 g (±30 g) 2 digestions were performed: the first one of 150 g (±30 g) randomly sampled from the pool, the second one of the remaining tissue (Fig. 2 shows the whole procedure).

2.4 Statistical analysis

For each sample of 29 anchovies, the number of visible larvae present in the total tissue sample was correlated with the number of visible larvae present only in the abdominal regions (VM); in addition, the number of parasites present in 150 g (±30 g) of the abdominal regions (VM) was correlated with the total number of parasites present per gram of the total sample, calculating the Larvae per gram (LpG). Pearson's r correlation coefficient was calculated, by means of SPSS ® vs. 11 for windows. Results were considered significant when *p* values were lower than 0.05. In order to quantify the contribution of the number of parasites in the selected body portions to the total number of parasites, the R^2 coefficient was then calculated.

2.4.1 Mean abundance index. The mean abundance (MA) (total number of individuals of a particular parasite species in a sample of a particular host species divided by the total number of hosts of that species examined (Bush *et al*., 1997)) of each sample was calculated after its complete digestion and used to assess the batch marketability according to a threshold of 0.30 larvae as proposed in a previous work (Guardone *et al*., 2016) on the basis of the maximum number of parasites allowed by the Liguria Region in Circular n. 1 of 1997 per batch of anchovies. In fact, regulation states that acceptable batches are those harboring a maximum number of three larvae in 10% of the collected fish. Batches are usually composed by 29 anchovies, therefore a cut-off of MA equal to 0.30 was set. On the basis of the calculated MAs, batches were divided in acceptable (MA<0.30) and unacceptable (MA>0.30) (Guardone *et al*., 2016).

2.4.2 Larvae per gram index (LpG). Considering that the MA index can be applied only when the number of specimens composing the sample is known, in the present work we calculated the

visible LpG of tissue of anchovies, obtaining a measure that does not imply that the assessor needs to know the exact number of fish tested before the analysis is performed (as it may happen for prepared products that have lost their anatomical integrity). For this reason, LpG was used for the statistical analysis concerning the VM subsample of 150g (±30). A LpG marketability threshold was calculated dividing the maximum number of tolerated nematodes (9) per each sample of 29 anchovies (Circular n. 1 of 1997 of the Liguria Region) by the average weight of a sample of 29 anchovies. As mentioned in section 2.2. the average weight of a sample of 29 anchovies has been calculated on 19274 anchovies, analyzed in previous studies (Table 1) and the standard deviation (SD) and the Relative Standard Deviation (RSD) were calculated in order to estimate weight dispersion around the mean. The LpG threshold value calculated was 0.025, with a 95% Confidence Interval of 0.019 to 0.030 parasite/g. Therefore, LpG equal or higher than 0.030 were considered not suitable for human consumption, being out of the range of values expected in the population (with 95% of probability).

Then, we proceeded to calculate the LpG for the 57 batches herein analyzed (average weight=362.17 g, SD ±81.57), and, as previously mentioned, batches with LpG equal or higher than 0.030 were considered not suitable for human consumption. For all the 57 samples of this study, the LpG was calculated first on the total batch (dividing the number of total parasites of a sample by the total sample weight); in addition, the same index was calculated on the 150 g (±30 g) VM subsample (dividing the number of parasites found in 150 g (±30 g) of VM by the weight of the corresponding VM subsample). Correlation between the two methods was estimated by means of *Pearson's r* and, considering that the digestion of low amounts of tissues could be more suited for practitioners in the field, the LpG calculated on 150 g (±30 g) of VM tissues was used also for evaluating the diagnostic performance of the LpG index.

In order to evaluate the diagnostic performance of the LpG threshold, the marketability assessed on the basis of the MA threshold was assumed as the gold standard.

229 Sensitivity (Sn), Specificity (Sp), Positive and Negative Predictive values (PPV and NPV,
230 respectively) and all the related 95% Confidence Intervals (95% C.I.) were calculated by means of
231 EPINFO®.

232 **3. Results and Discussions**

233 ***3.1 Digestion procedure***

234 Even though the artificial digestion method represents the gold standard for the detection of
235 parasites in fish tissues, it needs a long time of execution to analyze the whole fish sample
236 (Cavallero *et al*., 2015; Fraulo *et al*., 2014; Guardone *et al*., 2016; Llarena-Reino *et al*., 2013). In
237 fact, according to the Circular of the Lombardy Region, which is the most frequently adopted by
238 Italian wholesale fish markets for the detection of visible nematode larvae (D'Amico *et al*., 2014),
239 at least 29 anchovies must be analyzed per batch. Thus, the fish sample must be generally divided in
240 subsamples (the weight of which depends on the equipment used) which need to be processed
241 separately. In this study, a new digestion protocol for the detection of anisakid larvae in anchovies
242 using Trichineasy® was developed and coupled with the sampling plan proposed by the Lombardy
243 Region Circular (Circular Letter VS8/C790/94) in order to make it applicable by FBOs.

244 A protocol for the digestion of 100 g of fish using Trichineasy® has been recently validated by
245 Cammilleri *et al*., (2016). However, in the case of anchovies 100 g represents approximately ¼ of
246 the total weight of a sample of 29 anchovies (section 2.2). Thus, in the present study some
247 preliminary trials were conducted to assess the digestibility of increasing weights of subsamples of
248 VM and HT (100-150-200 g with a tolerance of 10%). As regards the SOP for the artificial
249 digestion in beakers proposed by the European Union Reference Laboratory for Parasites
250 (http://www.iss.it/binary/crlp/cont/SOP_Artificial_digestion_of_fish_fillet.pdf), it uses 2 L per 100
251 g of fish fillet. It follows that this procedure requires a high amount of reagents and equipment for
252 the analysis of a sample of 29 anchovies. Moreover, the procedure has been developed for fish

253 muscle. These limits make available digestion procedures less suitable to be routinely applied on
254 field by FBOs, where the marketability of fish must be assessed rapidly.

255 In a previous study (Guardone *et al*., 2016) a temperature of 44°C during the digestion did not
256 affect the recovery of *Anisakis* larvae. However, in this study, considering the presence of blades
257 and the use of a precast kit, that does not allow optimization in reagents concentration, the
258 temperature was set at 37°C and the blades were kept at minimum speed. In fact, all the reaction
259 parameters (pH value, pepsin concentration, temperature and stirring blades) could affect the
260 recovery of the larvae (Bernardi *et al*., 2011; Guardone *et al*., 2016; Llarena-Reino *et al*., 2013).
261 Moreover, the optimization of the digestion parameters could allow the recovery of viable larvae
262 when fresh anchovies are analyzed (Cammilleri *et al*., 2016). Loading the samples after the "mixer"
263 step (see Section 2.3) reduced the fragmentation of the parasites. A high number of fragments were
264 found only in 3 massively infected samples which presented a MA (5.7; 3.0; 2.9) much higher than
265 the threshold (see section 3.2). However, the judgment on batch marketability was not affected by
266 the number of fragments, which were not included in the statistical analysis.

267 Once the final protocol has been set all the HT subsamples were analyzed in a single digestion
268 since their average weight was 143.08 g (SD ±31.34 g). In the case of VM subsamples, with an
269 average weight of 218.79 g (SD ±58.31 g), 2 digestions were needed in most of the cases.
270 Considering that 2 digestions increase to more than 40 min the duration of the procedure, the
271 possibility to digest a single aliquot of 150 g (±30 g) of VM randomly collected from a VM
272 subsample belonging to 29 specimens was verified. Then, the visible larvae found in 150 g (±30 g)
273 of VM was used as a predictive index of the overall level of fish batch infestation and marketability.

274 The same number of larvae was found when Petri dishes were observed under natural and UV
275 light. The utilization of the UV light can speed up the procedure making the parasite detection
276 quicker. However, considering that UV light instruments are not always available among FBOs, the

277 Petri dishes were observed also under natural light. In this latter case the parasite detection is
278 favored if the digested material is examined against a dark surface.

279 ***3.2 Parasites number and localization***

280 A total of 640 visible larvae were found after the digestion of the 57 batches of anchovies (total
281 number of anchovies=1652). All the visible larvae were morphologically identified as *Anisakis* spp.
282 Of these larvae, 603 were found in VM and 37 in HT. Three hundred eighty-four larvae were found
283 in all the subsamples of 150 g (±30 g) of VM. Statistical analyses evidenced very strong correlation
284 between the total number of parasite in 150 g (±30 g) of VM and the total number of parasite in the
285 sample of 29 anchovies ($r=0.976$ $p<0.05$). Similarly, when the LpG was calculated, the correlation
286 between the Number of parasite/gram of fish tissue of 150 g (±30 g) of VM and the Number of
287 parasite/gram calculated on the total batch weight was also highly significant ($r=0.98$ $p<0.05$). The
288 calculation of R^2 between the number of larvae in the sample and those present only in the 150 g
289 (±30 g) VM region, evidenced a value of 0.953, implying that 95% of the variability in the total
290 number of parasites in the batch, is related to the number of larvae in VM. Similar results were
291 obtained when LpGs of VM regions were related to those measured on the whole batch weights
292 ($R^2=0.95$). However, even if the R^2 are quite relevant, the purpose of this study was not to establish
293 a regression equation to quantify the number of parasite to be expected in a portion of fish given the
294 total number of parasites, considering that the level of contamination is quite heterogeneous in
295 nature and the collected sample may be biased. In this study, 94.2% of the recovered larvae were
296 found in the VM portion confirming this as the elective site of localization of *Anisakis* spp. In
297 particular, a very similar percentage (96%) was found by Cipriani *et al*., (2016) although in the
298 latter survey the ratio was calculated only considering viscera and not the adjacent muscles.

299 These results suggest that the analysis of the selected body part is representative of the overall
300 infestation of the batch, both when considering the absolute number of parasites and the LpG.

Therefore, when samples of VM to be analyzed exceed 180 g, practitioners could extract a 150 g (±30 g) portion to perform parasite detection and LpG calculation.

3.3. Mean Abundance and Larvae per Gram.

The MA is among the most important descriptors to quantify parasite numbers in a host sample or population. MA carries the same information of mean intensity, but it correlates with prevalence (Rózsa *et al*., 2000) and, especially in the case of small fish, sold in batches, it could be used to estimate the degree of infestation (Guardone *et al*., 2016).

When it is impossible to calculate the number of fish specimens of a sample, such as in the case of 150 g (±30 g) of VM analyze in this study or in RTE products, the LpG, expressing the number of parasite per gram of sample, can be used. Also in this case, it is essential to define a threshold value to discriminate between marketable and not marketable products. To date, variable thresholds have been set for particular kind of products: 20 nematodes per 1000 g (0.02 parasite/gram) was established in the case of salmon fillets (Karl *et al*., 2014) and 2 or more visible parasites per 1000 g (0. 002 parasite/gram) in the case of frozen blocks of fish fillets, minced fish flesh and mixtures of fillets and minced fish flesh (Codex Alimentarius Commission, 1989). To the best of our knowledge, no threshold has been proposed for anchovies.

The diagnostic performance of the 0.030 LpG as a rapid screening tool to adequately identify anchovies' batches not suitable for human consumption, evidenced very high values of Sensitivity (Sn=1; 95% C.I.: 0.75-1) and Specificity (Sp=1; 95% C.I.: 0.90-1). All samples showing MA greater than 0.3 were also characterized by LpG greater than the proposed threshold (see Table 2). Moreover, negative predictive value of 1 (95% C.I.: 0.90-1) indicates that the probability of samples testing negative for LpG to be true negative (MA<0.3) is really high, therefore the decision can be considered "sure". Similarly, a high positive predictive value was recorded (PPV=1 95% C.I.: 0.75-1), indicating that also the probability of a sample testing positive with the LpG threshold to be

positive also with the MA method is extremely high, therefore indicating the method as a valid alternative to the more commonly used MA.

In order to verify the applicability of the proposed threshold, and considering the high values of RSD calculated, Microsoft Excel for Windows was used for simulating the distribution of LpG values by varying anchovies' weights and the number of larvae present. The results showed that when 18 or more larvae are present in the sample analyzed (no matter its weight) the LpG is always greater that the threshold. When the number is lower, the LpG proposed is able to discriminate samples with more than 9-10 larvae only when samples weight ranges from 250 to 330 g. For this reason, a corrected measure was proposed for a better fit of the variation in weights: LpG±delta (Δ). In this formula Δ represents the difference between the sample weight and 330 g (maximum level tolerated by the proposed threshold) divided by 10,000. The corrected LpG is appropriate for samples weighing ~~The corrected LpG correctly performs on samples weigh~~ from 200 to 470 g, range in which the majority of the reported mean weight of a sample of 29 anchovies (ranging from 249.34 to 567.22) is included(Table 1). The average weight calculated on the collected 57 batches in this study was 362.17 g (SD ±81.57 g), also falling within the specified range. However, another simulation was performed on samples outside the proposed range (which may be possible given the RSD values) and the screening of unacceptable batches needed threshold modification (see Table 3). This simulation was made in order to take into account that the LpG measure could be influenced by the selection of smaller/larger fish, for example depending on harvesting season.

After the proposed threshold variation, the corrected LpG was calculated on all the 57 samples herein analyzed. Three samples showed LpG<0.042 but the MA for the whole batch was 0.31 (higher than 0.30 set as threshold for marketability), corresponding to 9 larvae per 29 anchovies. However, according to the experience gained by the Official Authorities in decades of sampling performed at the Wholesale market of Milan (D'Amico et al., 2014) a tolerance of 10% in the number of parasite has been introduced. This means that the MA threshold value varies from 0.310

(9/29) to 0.344 (10/29), indicating that samples with 9 parasites could be still considered acceptable for consumption. In this case, the proposed LpG index did not produce a miss-classification on samples harboring more than 10 larvae, therefore not compromising consumer safety.

All in all, the proposed method, may be a useful tool to assure product suitability and protect consumers. In the other cases (sample weight <200 g or >470 g), we propose arrangements in order to decrease the frequency of false positive and negative results. However it should be noticed that these cases are not frequent, since they approach the tail of the weight distribution. Considering data from literature, where often massive infestation is reported, the diagnostic performance of LpG are not impaired, however, more studies, on a greater number of batches and with a different level of parasitic infestation need to be performed in order to verify LpG and the herein proposed corrections.

3.4 Consumers' safety in the spotlight

According to the Reg. (EC) No 178/2002, unsafe food should not be placed on the market. In particular, food injurious to health and unfit for human consumption is considered unsafe. More specifically, "*In determining whether any food is unfit for human consumption, regard shall be had to whether the food is unacceptable for human consumption according to its intended use, for reasons of contamination, whether by extraneous matter or otherwise, or through putrefaction, deterioration or decay"* (Reg. (EC) No 178/2002). Visible parasites, such as some nematode larvae, that can be immediately perceived by consumers, make the fish unfit for consumption for aesthetic reasons. In addition, some nematode species may be responsible ~~of~~ for human infection.

Anchovies may be responsible for the transmission of larval nematodes of the genus *Anisakis* (Mattiucci *et al.*, 2013). In the last two decades an increasing number of human cases of anisakiasis have been diagnosed in many parts of the world, as a consequence of a greater awareness of this parasitic disease and of improvement in its diagnosis (Mattiucci *et al*., 2013; Umehara *et al*., 2007). In particular, in European countries the increased occurrence of this infection has been related to an

increase in the popularity of raw and/or undercooked fish (Mattiucci *et al*., 2011). Cases of human anisakiasis have been reported in Italy since 1996 (Stallone *et al*., 1996).

Considering that the gutting of anchovies on board is not feasible, as it is extremely time consuming for FBOs, other procedures aiming at reducing the parasitological risk for the final consumer must be implemented. Since the parasites located in the viscera contributes to the overall level of infestation of the ungutted fish, procedures that prevent the commercialization on massively infected fish need to be applied. This is particular true in the case of fresh anchovies that reach the final consumers without undergoing a preventive freezing and can present ~~a~~live larvae emerging from their surface. Considering that FBOs are responsible for preventing the commercialization of such products, the development of standardized sampling protocols for the analysis of fish batches is needed. The implementation of preventive measures is aimed not only to prevent commercialization of repugnant products, but also to reduce the parasitological risk for those products intended to be used for the preparation of uncooked products. In fact, in Italy raw anchovies are often used for the preparation of typical products, traditionally prepared without thermal processing.

4. Conclusion

European Community regulations establish that fish heavily parasitized must be removed from the market (Commission Reg. EC 2074/2005). However, no official limits have been issued to clarify the maximum number of larvae that can be tolerated in a fish batch. The results of the present research highlight that a 0.030 LpG threshold and the related corrections can be a useful tool for taking decisions on the marketability of anchovies. The application of a corrected index will allow the possibility of calculating a correct parasitic load also for samples with weights far from the average, thus being a valid alternative to visual inspection. Moreover, the use of the LpG index discloses the possibility for an evaluation of larvae density in fish products where it is impossible to count the number of examined specimens, such as processed products that may be characterized by

a loss of anatomical integrity, for which the MA index cannot be applied for making a decision on marketability. However, considering that this is the first report on the use of the LpG threshold for anchovies, more studies on a larger number of samples are necessary in order to validate this method and better estimate its diagnostic potential.

Conflict of interest

The authors do not have any conflict of interest to declare.

Acknowledgements

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Figure captions

Fig 1. Preparation of anchovies.

The VM sections were obtained by performing two cuts perpendicular to the anchovy's body, the first one in correspondence of the gill operculum, and the second in correspondence of the anus

Fig 2. Diagram of the whole sampling procedure.

A sample of 29 anchovies was collected from each batch of anchovies at the Fish Market of Viareggio. VM and HT subsamples were prepared dividing each fish according to Fig. 1. Then the subsamples were digested separately following the illustrated protocol.

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573 **Table 1** References used for calculating the average weight of a sample of 29 anchovies.
574

References	Number of anchovies examined	Mean weight of one anchovy	Mean weight of 29 anchovies
Guardone *et al*., 2016	929	14.39	417.3
Pekmezci *et al.*, 2014[a]	250	8.88	257.52
Serracca *et al.*, 2014	1050	11.70	339.3
De Liberato *et al.*, 2013	1490	9.00	261
Mladineo & Poljak, 2014	120	17.73	514.17
Chaligiannis *et al*., 2012	77	13.20	382.8
Mladineo *et al*., 2012	4600	28.92	838.7
Ciccarelli *et al*., 2011[a]	5696	10.96	317.84
Rello *et al.*, 2009[a]	792	13.66[b]	396.14
		14.99[c]	434.71
Gutierrez-Galindo *et al*., 2010[a]	153	18.71	542.59
Anastasio *et al*., 2007[a]	4117	8.34	241.86
Total	**19274**		
Mean		**14.21**	**411.99**
SD		**5.70**	**165.41**
RSD		**40%**	**40%**

575
576 [a] In these studies the weight of the specimens has been calculated with FishBase length-weight
577 conversion system for anchovies
578 (http://www.fishbase.org/PopDyn/LWRelationshipList.php?ID=66&GenusName=Engraulis&Speci
579 esName=encrasicolus&fc=454); [b] data referred to 396 anchovies from the Gulf of Cadiz; [c] data
580 referred to 396 anchovies from Western Mediterranean Sea
581

582 **Table 2** Cross classification of anchovies batches considering MA index (gold standard) and the

583 herein proposed LpG: results show a complete agreement.

	MA		
LpG	**≥ 0.3**	**<0.3**	**total**
≥ 0.030	15	0	15
< 0.030	0	42	42
Total	15	42	57

584

585

586 **Table 3** LpG Threshold variation considering anchovies weights.

weight	**LPG**
125-199	0.042
200-470	0.030
471-550	0.036
551-600	0.041

587

588

Figure

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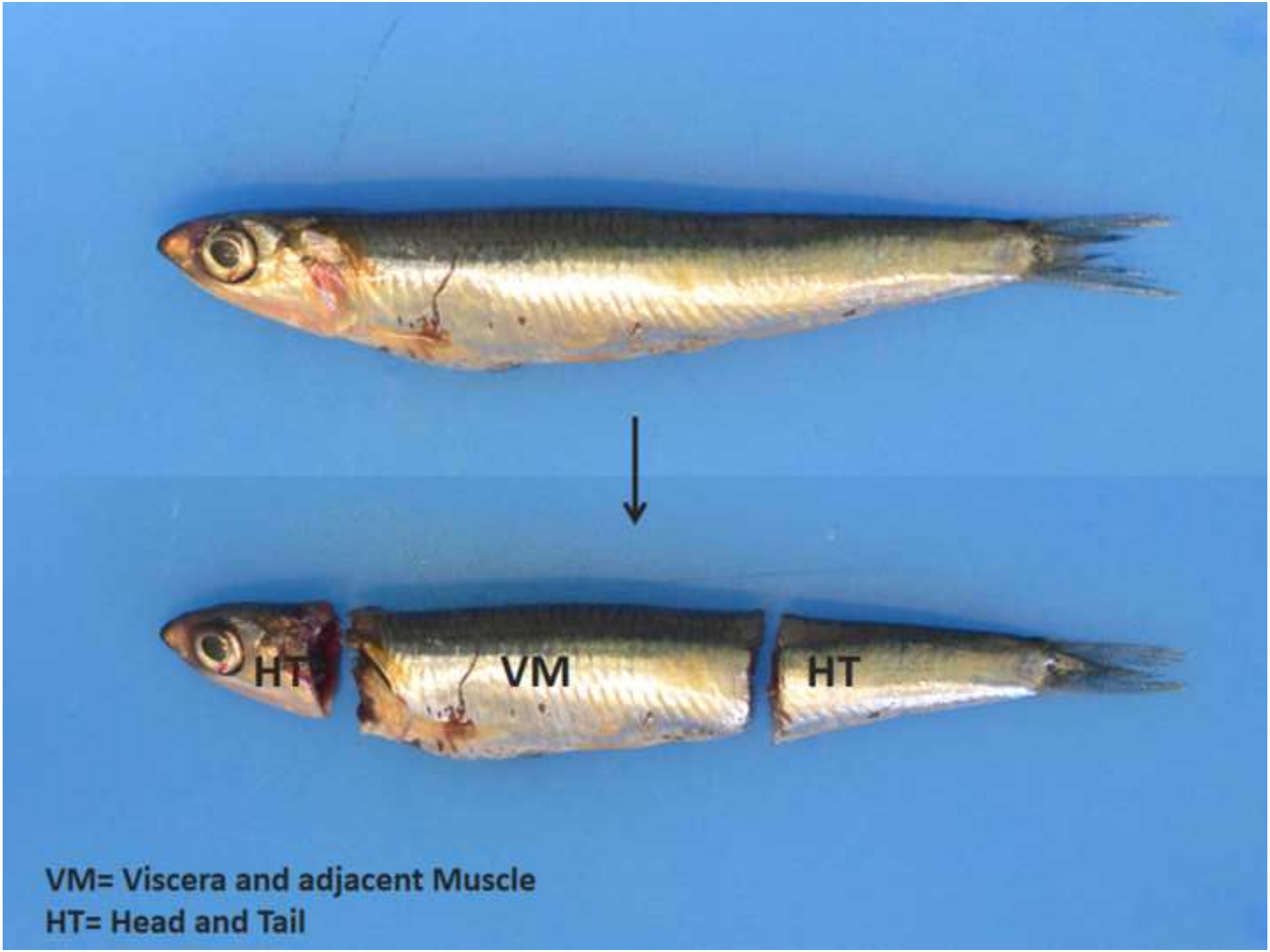

Figure
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