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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.

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 and 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)

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the overall prevalence values in this fish species 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 <24 or 15 h, 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 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 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 >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 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 19,274 anchovies, analyzed in previous studies (Table 1). 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

VM and HT subsamples belonging to 5 samples were used for the optimization of the digestion procedure using the Trichineasy® (Cammilleri et al., 2016). The loading of the samples was conducted after the "mixer" step in order to avoid tissue homogenization (http://www.ctsv.biz/image-ctsv/PDF/TrichinEasy-anisakis.pdf). In order to test the recovery of parasites after the digestion, 5 frozen *Anisakis* spp. larvae were added to each subsample analyzed. All the larvae were collected from naturally infected *E. encrasicolus* and stored at −20 °C for a maximum of one month. Increasing weights (100–150–200 g with a tolerance of 10%) and different digestion times (15–20–30 min) were tested during the trials. The temperature was set at 37 °C, the blades were maintained at the minimum speed and all the digestions were performed adding 1 L of water, with 50 mL of 10% HCl and 10 g of pepsin