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High occurrence of Anisakidae at retail level in cod (*Gadus morhua*) belly flaps and the impact of extensive candling

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ABSTRACT

The presence of Anisakidae at retail level, after the routine screening via candling, was investigated in cod, the most commonly consumed fish species in Belgium. A total of 780 pre-packed belly flap samples destined for one branch of retail shops were collected from a Belgian wholesale company. To recover all larvae, each sample was first candled and thereafter enzymatically digested. Larvae were morphologically identified to the genus level and a subset was additionally molecularly confirmed by amplification of the ITS fragment and *Hin*fI/*Hha*I enzyme restriction. The PCR/RFLP profiles of *Contracaecum* spp. were determined and confirmed with sequencing by the European Reference Laboratory for Parasites (Istituto Superiore di Sanità). The positivity rate of Anisakidae in the individual cod samples was 18% [95%-CI: 15–21%], with a mean intensity of one larva [range: 1–6]. Belly flaps were sold packed primarily by two, with a one-in-three chance of buying an infected package. *Pseudoterranova* spp. infections (single infections) were most frequently detected (positivity rate 9% [95%-CI: 7–11]), closely followed by *Anisakis* spp. (7% [95%-CI: 6–9]). Co-infections of *Pseudoterranova* spp. and *Anisakis* spp. comprised 8% of the infections, with a positivity rate of 1% [95%-CI: 1–3%]. All belly flaps reportedly were candled prior to our sampling, nonetheless our results indicated that an additional candling screening before packaging would identify an extra third of the infections and larvae. In 19 of the 139 infected samples, all larvae were recovered by the additional candling, thereby removing the infection risk for consumers. In conclusion, this study shows that cod belly flaps infected with zoonotic parasites reach the Belgian consumer. Although a second candling step at retail level could be helpful in reducing the consumer risk, additional measures are needed since 66% of infections would still remain undetected.

1. Introduction

The third larval stage (L3) of the marine nematodes Anisakidae occurs in a wide range of fish hosts. Zoonotically important species are, among others, *Anisakis* spp., *Pseudoterranova* spp., and, to a lesser extent, *Contracaecum* spp., causing gastro-intestinal complaints in humans after the consumption of raw or undercooked fish, cephalopods, and derived products containing live L3 larvae (Buchmann

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and Mehrdana 2016). In addition, allergic symptoms against the (heat-resistant) antigens can occur, even without exposure to a viable larva (Audicana and Kennedy 2008; Moneo et al. 2017; Nieuwenhuizen and Lopata 2014; Pozio 2013).

In fish, larvae commonly infect the viscera, although both ante- as post-mortem migration to the muscles can occur (Cipriani et al. 2016; Mattiucci and Nascetti 2008; Quiazon et al. 2011; Smith and Wootten 1975). From the muscles (fillets), the belly flaps are the most frequently infected part, containing most of the larvae, probably due to their close proximity to the abdominal cavity (Mercken et al. 2020a; Mercken et al. 2020b).

The EU Regulations (EC) No 853/2004 and No 1276/2011 detail control measures to be implemented in the industry, focusing on the freezing of fish destined for raw consumption and on visual inspection to avoid commercialization of obviously contaminated products. A routinely used detection method of anisakid nematodes in fish products is candling, whereby fish fillets are placed on a light table, allowing a better visualization of the larvae. While this technique has the major advantage of not affecting the fish quality and thus allowing consumption afterwards, it is labour intensive and as such highly costly. Moreover, not all larvae can be detected and removed, depending on the fish (colour, thickness, skin), the larvae (size, colour) and the skill of the inspector (Karl and Leinemann 1993; Levsen et al. 2005; Petrie et al. 2007). Due to a lack of alternatives, candling is currently the most widely implemented industrial screening method. In laboratory settings, highly accurate methods, such as enzymatic digestion and the UV press method, are commonly used, but the total destruction of the fish fillets renders these methods unfit for routine, large scale use in the food industry (Gómez-Morales et al. 2018).

The most consumed fish species in Belgium is Atlantic cod (*Gadus morhua*) (VLIZ 2018). In the Northeast Atlantic Ocean, cod is intensively fished for the European market, with 20% being fished by Iceland (VLIZ 2018). Previous studies on the prevalence of ascaridoids in cod muscle fillets on the Belgian market showed a prevalence of 35% in 1999 (Piccolo et al. 1999) and 47% in 2019 (Mercken et al. 2020b). Although the study of Mercken et al. presents recent findings, the sample size of cod was limited, and the samples were collected at the arrival point of fish at wholesale level. Data regarding Anisakidae infections in cod at retail level (i.e. after industrial candling) are required to assess the risk for consumers, but are currently lacking.

The objectives of this study were (1) To investigate the positivity rate, intensity, abundance, and larvae species distribution of Anisakidae in pre-packed cod fillets at retail level; and (2) To evaluate whether an additional screening with the candling technique at retail level would contribute to a reduction of the number of Anisakidae infections/larvae.

2. Material and methods

2.1. Sample collection & processing

Fish samples ($n = 780$) were collected from a Belgian wholesale company during a time span of one year (September 2018–September 2019). Weekly, cod samples were provided by the company in a modified atmosphere package (MAP) destined for one branch of retail shops. One package contained either two or six belly flaps of 125 g each and all belly flaps were analysed individually (one single belly flap = one sample). The belly flaps were derived from fish caught in the Icelandic fishing grounds (FAO 27.5.a) and were reported to be candled at processing in Iceland. Samples were stored refrigerated (2 °C) and were processed within two working days.

First, candling was performed under similar conditions as in an industrial setup and visible larvae were counted and collected. Thereafter, samples were digested in a pepsin/HCl solution consisting of 8 ml HCl 25% (PanReac AppliChem) and 5 g pepsine 1:10000 NF (Chem-Lab) for 1 l solution, with a ratio of 1 l for 50 g of sample (Jackson et al. 1981; Karl and Leinemann 1993). Samples were stirred for 15 min at 44 °C and all remaining larvae were counted and collected after sieving.

2.2. Larvae identification

Identification to the genus level was achieved through light microscopy, using the identification keys of (Berland 1961; Petter and Maillard 1988). All un-identified larvae (48) and a random selection of larvae from each morphological identified genus group (24 *Pseudoterranova* spp. and 42 *Anisakis* spp.) were further identified with PCR-RFLP. Amplification of the ITS fragment with the primers NC5 (5′-GTA GGT GAA CCT GCG GAA GGA TCA T-3′) and NC2 (5′-TTA GTT TCT TTT CCT CCG CT-3′) (Zhu et al. 1998) was performed following the protocol of the European Reference Laboratory for Parasites (ISS 2018). Restriction by the *Hin*fI and *Hha*I enzymes allowed identification of *Anisakis simplex s.s.*, *Anisakis pegreffii*, *Anisakis simplex*/*pegreffii hybrid* genotype, *Pseudoterranova* spp. (*P. decipiens s.s.*), and *Hysterothylacium* spp. (*H. aduncum*), in accordance with the protocol of the European Reference Laboratory for Parasites (ISS 2018). In case of unclear molecular results, sequencing of the ITS fragment was performed.

The PCR/RFLP profile of *Contracaecum osculatum* was determined by ISS through gel electrophoresis and sequencing of two L3 larvae of *Contracaecum osculatum* isolated from Atlantic cod (*Gadus morhua*) caught in the Baltic Sea. According to the available *Contracaecum osculatum* gene sequences encompassing the 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, (GenBank acc. Number AB277825.1) in silico amplification with the primer NC2 and NC5 will result in a 964 bp amplicon (see Supplementary Fig. A1). In silico digestion with the restriction enzyme *Hin*fI will result in two fragments of 501pb and 463 bp, whereas digestion with *Hha*I will occur in three fragments of 377 bp, 375 bp and 212 bp.

2.3. Statistical analyses

The number of larvae recovered with candling refers to the larvae detected by candling in our laboratory. The total number of infected samples/larvae refers to the samples/larvae detected both with digestion and candling (the larvae from the latter would also have been identified with digestion as sole detection technique). For the individual samples, the following parameters were used as defined by Bush et al. 1997: the total number of samples (N); the number of infected samples ($N_{infected}$); the positivity rate with 95% Clopper-Pearson confidence interval [95%-CI] defined as the number of infected samples to the number of examined samples; the mean intensity (mI) with minimum-maximum range [min-max]; and the mean abundance (mA).

Above parameters were also defined for the candling method alone. The McNemar's chi-squared test was performed to evaluate the positivity rate with candling in comparison with the total number of infections detected. In addition, the sensitivity (Se) and negative predictive value (NPV) of candling in samples destined for the retail level were estimated, with the total number of infections as the gold standard. Lastly, species identification is presented as the number of larvae for each larvae species, given for the total number of larvae detected and for those detected with candling. Results were considered statistically significant when $P < 0.05$. All analyses were conducted in RStudio, using R version 3.6.1 (R Core Team, 2019).

3. Results

3.1. Positivity rate and intensity

Of the 780 examined belly flaps 139 were infected, resulting in an overall positivity rate of 18% [95%-CI: 15–21%] (Table 1). A total of 201 Anisakidae larvae were recovered, with a mI of one larva [range: 1–6] and a mA of 0.3 larva.

Morphological identification resulted in 77 *Pseudoterranova* spp., 76 *Anisakis* spp., and 48 unidentified larvae. Molecular confirmatory identification of 114 larvae (24 *Pseudoterranova* spp., 42 *Anisakis* spp. and 48 undefined larvae) resulted in 63 *P. decipiens*, 49 *A. simplex*, and two *C. osculatum* larvae. The morphologically undefined larvae were two *C. osculatum*, nine *A. simplex,* and 37 *P. decipiens* larvae. Molecular identification showed a 6% error rate of the morphological identification. Two *A. simplex* larvae were morphologically identified as *Pseudoterranova* spp. and four *P. decipiens* larvae were morphologically identified as *Anisakis* spp. The final identification (taken morphological and molecular results into account) can be found in Table 1.

The larvae of the genus *Pseudoterranova* were found most often, both in terms of number of infections (58% of the infected samples contained at least one *Pseudoterranova* spp. larva) and of number of larvae (58%). Furthermore, *Anisakis* spp. larvae were also frequently recovered, with a positivity rate of 9% [95%-CI: 7–11%].

In 45 out of 139 infected samples, more than one larva was recovered, including 11 samples with only *Anisakis* spp. larvae, 23 samples with only *Pseudoterranova* spp., and 11 samples with a coinfection of *Anisakis* spp. and *Pseudoterranova* spp. (data not shown).

3.2. Candling

The positivity rate using candling as the sole detection method was significantly lower (6% [95%-CI: 4–8%]) than the overall positivity rate ($p < 0.001$) (Table 1). The sensitivity of candling was 34% [95%-CI: 26–42%] and the NPV 87% [95%-CI: 85%–88%]. No false positive results were recorded. The performance of candling to detect infection was similar for samples only infected with *Pseudoterranova* spp. (Se = 33% [95%-CI: 22–46%]) or only infected with *Anisakis* spp. (Se = 30% [95%-CI: 18–43%]).

Supplementary Fig. A2 represents the sensitivity for different intensities of infection. When only one larva was present, the sensitivity was 24% [95%-CI: 16–34%]; $n = 94$). A sensitivity higher than 40% was observed in those samples with two larvae or more. Moreover, candling detected the infection in both samples containing five larvae and the sample with six larvae.

Candling led to the detection of 31% of all larvae (Table 1). A higher proportion of *Anisakis* spp. larvae (36%) was recovered compared to *Pseudoterranova* spp. larvae (28%). The two *Contracaecum* spp. larvae were not detected with candling.

4. Discussion

Our results demonstrated that almost 1/5th of the cod belly flap fillets destined for the Belgian retail market were infected with Anisakidae larvae. Earlier findings already indicated the presence of infection in cod, as well as the presence of Anisakidae in the Icelandic fishing grounds in general (Gay et al. 2018; Klapper et al. 2018; Mehrdana et al. 2014; Mercken et al. 2020a; Mercken et al. 2020b; Mouritsen et al. 2010). In the northern part of the Northeast Atlantic Ocean, an increase of *Pseudoterranova* spp. and *Contracaecum* spp. in cod has been reported in the past decade, linked to an increase of their final hosts, i.e. seals (Mehrdana et al. 2014).

Gay et al. (2018) detected the presence of *Anisakis* spp. and *Pseudoterranova* spp. in cod caught in the Barents, Baltic and North Sea. The majority of larvae in the fillets were found in the ventral region, i.e. the belly flaps, with a positivity rate of 39% in those cod belly flaps. Likewise, Mehrdana et al. 2014 detected Anisakidae larvae in cod from the Baltic sea, with 58% of the larvae recovered from the belly flaps, the part of the muscles where higher levels of infection are expected due to their close proximity to the viscera, which is the primary infection site (Brooker et al. 2016; Mercken et al. 2020a; Mercken et al. 2020b; Pascual et al. 2018; Rodriguez et al. 2018).

Table 1
Positivity rate and intensity of Anisakidae species in individual cod samples ($n = 780$) and the evaluation of the candling method for the *detection of Anisakidae larvae.* With the number of infected samples ($N_{infected}$), the positivity rate with 95% confidence interval [95%-CI], the number of larvae (L), the mean intensity (mI) with minimum-maximum range [min-max] in the infected samples, the mean abundance (mA), the sensitivity (Se) with 95% confidence interval [95%-CI], the number of larvae recovered with candling ($L_{candling}$), and the proportion of larvae recovered with candling in comparison with the total number of larvae recovered as gold standard (% Candling).

Larvae species	$N_{infected}$	Positivity rate (%) [95%-CI]	L	mI [min-max]	mA	Se (%) [95%-CI]	$L_{candling}$	% Candling
Anisakis spp.*	57	7 [6–9]	71	1 [1–5]	0.1	30 [18–43]	24	**34%**
Pseudoterranova spp.*	69	9 [7–11]	100	1 [1–5]	0.1	33 [22–46]	28	**28%**
Contracaecum spp.*	2	0 [0–1]	2	1 [1–1]	0.003	0 [0–84]	0	**0%**
Co-infections**	11	1 [1–3]	28[a]	3 [2–6]	0.04	64 [31–89]	11	**39%**
Total	**139**	**18 [15–21]**	**201**	**1 [1–6]**	**0.3**	**34 [26–42]**	**63**	**31%**

The bold line represents the total.
* Infections only with this species (single infections).
** Co-infections of *Anisakis* spp. and *Pseudoterranova* spp. As these are co-infections, a minimum of two larvae are always present, which influences the mI, mA and Se (see also Supplementary Fig. A2).
[a] 16 *Pseudoterranova* spp. and 12 *Anisakis* spp.

In our previously published systematic review and meta-analyses, conducted to investigate the occurrence of Anisakidae in fish imported to the Belgian market, we collected (among others) data from 14 different studies reporting Anisakidae in cod in the North Atlantic Ocean (Mercken et al. 2020a). An overall prevalence of 33% was determined, comprising infections both in viscera and muscles. At the Belgian wholesale level, we demonstrated a prevalence of 40% [95%-CI: 32–64%] in cod fillets caught in the Northeast Atlantic Ocean (Mercken et al. 2020b). Those cod samples were a heterogeneous group of fillets (up to 3 kg), consisting of fillets with or without belly flaps, or only the loins, and were collected at the point of arrival in the wholesale company, before the industrial candling was performed. Of the five collected belly flap samples, four were infected with a range of 0.3–2 larvae per 125 g infected muscle. In the current study, we focused solely on belly flaps, ready for transport to retail stores. The removal of visible larvae during the industrial candling prior to our sampling could explain the lower positivity rate than previously reported. Still, a positivity rate of 18% [95%-CI: 15–21%] was found, with a mean intensity of one larva [range: 1–6] per 125 g sample. However, in reality consumers buy the cod fillets packed by either two or six, giving respectively a 31% or 74% chance of buying an infected package.

The larvae of the genus *Pseudoterranova* were found most often, both in terms of number of infections as number of larvae. *Anisakis* spp., which are the larvae species most linked to human pathogenicity (Buchmann and Mehrdana 2016), were also frequently detected. Our previous results also showed a higher number of *Pseudoterranova* spp. than *Anisakis* spp. in cod (Mercken et al. 2020b). In the study conducted by (Mehrdana et al. 2014), both prevalence and mean intensity of *Pseudoterranova* spp. in cod muscles (28.7%, 5.2) was higher compared to the prevalence of *Anisakis* spp. infections (8.1%, 2.0). The larvae of the genus *Contracaecum* are mainly associated with infection of the liver, infections in the muscles are rare (Haarder et al. 2014; Mehrdana et al. 2014), which could explain the much lower positivity rate of this genus in the present study.

The majority of larvae detected in the cod belly flaps were still viable (data not presented) and could therefore lead to anisakiasis after raw or undercooked consumption of the infected fish. The ingestion of a viable larvae can lead to gastric, intestinal, or ectopic anisakiasis (Ishikura et al. 1993). Symptoms range from mild epigastric pain, nausea, and vomiting to gastric ulceration and mucosa penetration (Shimamura et al. 2016). Ingestion of *Pseudoterranova* spp. larvae leads to infection of the gastrointestinal tract, mouth, and pharynx and is more associated with mild symptoms such as a tickling sensation, coughing and/or vomiting (Measures 2014; Torres et al. 2007).

In addition, allergic symptoms can occur in absence of an active infection. Fourteen allergens of *A. simplex* have been described so far (Moneo et al. 2017). Some allergens are freeze- and heat resistant, implying that allergy can manifest even after proper freezing or cooking of the fish. Besides the oral route, fish handlers are at risk for allergy through skin contact or respiratory tract inhalation when processing (candling) the fish (Moneo et al. 2017; Uña-Gorospe et al. 2018).

Results from the current study indicate a similar performance of candling to detect *Pseudoterranova* spp. or *Anisakis* spp. larvae. This is unexpected as *Anisakis* spp. larvae are smaller and often coiled-up, requiring a more attentive close-up inspection of the fish fillet. *Pseudoterranova* spp. larvae are spotted more easily since they are larger and darker (Buchmann and Mehrdana 2016; Hurst 1984). Therefore, in the industrial candling step, the latter might be more likely to be detected and removed, which could lead to an underestimation of the candling efficacy of the *Pseudoterranova* spp. larvae in our study.

Our results show the low efficacy of candling in general, which is currently the mostly used screening method in the fish industry (Levsen et al. 2005; Petrie et al. 2007). A second accurate candling step before packaging would detect another third of the infections and larvae. Nevertheless, candling underperformed in the detection and removal of all larvae. Only in six samples of the 45 with more than one larva present, all the larvae were found, resulting in a negative sample after candling.

5. Conclusion

We investigated the presence of Anisakidae larvae in cod belly flaps destined for the Belgian retail market and found 18% to be infected. In reality, consumers buy the cod fillets packed by either two or six, giving respectively a 31% or 74% chance of buying an infected package. The current industrial screening method lacks efficacy and the remaining Anisakidae larvae therefore present a

consumer risk, given that the dominant larvae species found, *A. simplex s.s.* and *P. decipiens s.s.*, are both zoonotic. While an additional candling of each sample could theoretically reduce the risk of exposure to an infected belly flap by a third, still two thirds of positive fillets would remain undetected after application of this costly and labour-intensive method. A detailed consumer risk assessment should thus be conducted to further assess the risk of exposure and to evaluate different interventions, such as the effect of an additional candling step on the reduction of the consumer's health risk.

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Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fawpar.2020.e00108.

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