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# Pathogenic potential of Anisakis L3 after freezing in domestic freezers



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#### ABSTRACT

Anisakis L3 were subjected to freezing in domestic freezers and the risk posed by those larvae that might possibly survive freezing was analysed by determining their agar penetration ability, survival in gastric juice, allergenic potential and oxygen consumption rate. Anisakis L3 gradually became non-viable as the temperature fell from -1 °C to -28 °C. The faster the freezing rate, the greater the decrease in survival, but viability was not found to be affected by the proportions of Anisakis species per batch (A. simplex s.s. 83–100%, A. pegreffii 0–17%, and their heterozygote genotypes at the ITS region of rDNA 0–10%). Surviving larvae after freezing presented a drastically reduced ability to penetrate into a layer of solid agar, as compared to controls, but still 5% of them penetrated into this medium. About 80% of surviving larvae died within 24 h under conditions simulating those of gastric fluid, whereas up to 85% of the controls were able to resist for 24 h. Allergen release during freezing and after freezing was higher than in controls, and this release was compatible with a contribution of both passive (by rupture of the cuticle) and active liberation of excretion/secretion allergens during and after freezing, suggesting that the pathogenic potential of these frozen and thawed larvae cannot be discarded. These results could be of use for more precise risk assessment and providing guidelines for consumers.

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#### 1. Introduction

The importance of *Anisakis* sp. infection of fish is well recognized by the fisheries sector and food safety authorities since humans can get infected by *Anisakis* larvae in the third stage (L3) through consumption of fish parasitized with live L3. This can occur when the fish is being consumed raw or when the technological processes, cooking or storing practices are insufficient to kill the larvae (Audicana & Kennedy, 2008; Moneo, Carballeda-Sangiao, & González-Muñoz, 2017; Nieuwenhuizen & Lopata, 2014).

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Anisakiasis is mainly characterized by gastrointestinal manifestations of epigastralgia, nausea, abdominal pain and diarrhoea (Shimamura, Muwanwella, Chandran, Kandel, & Marcon, 2016). Other symptoms associated with exposure to the parasite include a characteristic allergic reaction such as IgE-mediated hypersensitivity, angioedema, urticaria or anaphylaxis (Moneo et al., 2017; Ventura, Napolitano, Menga, Cecere, & Asero, 2013). It is generally accepted that an initial contact with live L3 is needed to develop allergic symptoms, but once sensitized they can be observed after ingestion of fish containing allergens even in products that have been correctly treated to inactivate the larvae since there are some allergens with high thermal stability (Audicana & Kennedy, 2008; Carballeda-Sangiao et al., 2014; Moneo et al., 2005; Nieuwenhuizen & Lopata, 2014; Vidacek et al., 2010, 2011, 2009).

One of the points in prevention of the risk of *Anisakis* infection is the application of technological treatments to ensure the mortality of any larvae present in the fish (EFSA, 2010). Besides freezing

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(Lanfranchi & Sardella, 2010; Oh et al., 2014), several physical and chemical treatments are currently under study (Anastasio et al., 2016; Brutti et al., 2010; Oh et al., 2014; Pascual, Antonio, Cabo, & Pineiro, 2010), but freezing of fish muscle and subsequent frozen storage is at present the most suitable technological treatment to control the risk of infection through consuming raw or undercooked infected fish (EFSA, 2010). EU Regulation No 1276/2011 on freezing of fish stipulates that at least  $-20\,^{\circ}\mathrm{C}$  must be reached in all parts of the product for not less than 24 h, or  $-35\,^{\circ}\mathrm{C}$  for not less than 15 h. Observance of this regulation is compulsory for food business operators, and food safety authorities give general freezing recommendations for households to avoid infection.

Quantitative risk assessment models show that anisakiasis is an underdiagnosed disease (http://cordis.europa.eu/result/rcn/189395 accessed February 2017; Bao et al., 2017). This might be due in part to improper application of the regulation by consumers or small business operators since *Anisakis* L3 are moderately freezing-tolerant (Wharton & Aalders, 2002).

There are at least 12 accepted species within the genus Anisakis [A. simplex sensu stricto (s.s.), A. berlandi (former A. simplex C), A. pegreffii, A. typica, A. ziphidarum, A. physeteris, A. brevispiculata, A. paggiae, A. nascettii and A. schupakovi plus two as yet undescribed species, Anisakis sp 1 and Anisakis sp 2, according to larvae genotypes] (Mattiucci and Nascetti 2006; Mattiucci et al., 2014). Factors such as metabolic status of L3 known to affect other anisakids (Stormo, Praebel, & Elvevoll, 2009) or different species of Anisakis simplex can potentially have an influence on freezing survival, but less attention has been paid to them.

The survival of *Anisakis* L3 during freezing is usually measured in terms of their viability, i.e. their ability to move spontaneously or in response to stimulation with tweezers (EFSA, 2010). But viable larvae may not be infective. Since most of the studies found in the literature do not take into account the infective characteristics of larvae that have survived technological treatments, a higher margin of prevention is given by authorities by assuming that any larva with mobility is able to infect a human (EFSA, 2010). It is considered that the penetrative ability of the larvae may play an important role in the pathogenicity through invasion of the gastrointestinal mucosa of patients, and one of the ways of addressing this issue is by studying the penetration of the larvae into an agar layer together with the rate of mortality in an artificial gastric fluid (Arizono, Yamada, Tegoshi, & Yoshikawa, 2012; Oishi & Hiraoki, 1973; Ruitenberg, 1970, pp. 1–138). In addition, little information is available about differences in allergenicity as a function of factors such as species (Arcos et al., 2014; Quiazon, Zenke, & Yoshinaga, 2013) or technological treatments aimed at inactivating the larvae. Such information would give more insight into the overall pathogenic potential of Anisakis and assist the design of optimal technological applications.

The aim of this work was to study the risk posed by *Anisakis* L3 when frozen in domestic freezers in terms of viability, penetration into agar as an indication of potential infectivity, and allergenic potential. For this purpose, factors affecting the survival of larvae during a freezing process were studied, including inhomogeneity of temperature in domestic freezers, freezing rate and *Anisakis* species. The risk posed by those larvae that might possibly survive freezing was analysed by determining their ability to penetrate into agar gels, survival in artificial gastric juice, allergenic potential and metabolic status in terms of oxygen consumption rate.

#### 2. Materials and methods

#### 2.1. Sample preparation

Anisakis L3 from heavily infected ovaries and viscera of hake

caught in the North-East Atlantic fishing grounds and obtained from the central fish market in Madrid (Mercamadrid) were used at regular intervals. Infected tissue was sent to the laboratory and larvae were removed from the tissue with forceps, cleaned by successive washings with water or 0.85% NaCl, analysed for viability (see 2.3), placed in groups of 50 in test tubes containing 0.85% NaCl, and stored at 4 °C. Before using the larvae, they were again checked for viability.

#### 2.2. Taxonomic identification

Larvae from the various batches were used and stored at  $-20\,^{\circ}\mathrm{C}$  until needed. At least 40 individuals were used in the experiments in each batch. Larvae were individualized and each specimen was subjected to taxonomic identification following D'Amelio et al. (2000) and Abollo, Paggi, Pascual, and D'Amelio (2003) in order to establish the percentage of representation of each species or their heterozygote genotypes at the ITS region of rDNA (Mattiucci et al., 2016) (referred to as heterozygote genotypes thereafter) in the experiments.

#### 2.3. Viability

Movement of the larvae was observed according to EFSA (2010). Those larvae that moved spontaneously or in response to stimulation with tweezers were considered alive. The assessment of viability of frozen and thawed larvae was done just after thawing.

For those that did not show any movement just after thawing, we confirmed that they did not move after incubation for 4 h and 24 h with 0.85% NaCl or acetic acid solution, and they were then considered dead.

#### 2.4. Survival in artificial gastric juice

Survival of *Anisakis* L3 in artificial gastric juice (pH 1.8) during time was determined as described by Arizono et al. (2012). The medium consisted of 0.1% pepsin (proteolytic activity 1:10,000 NF [U.S. National Formulary], equivalent to 2000 International Pharmaceutical Federation [FIP]-U/g; Panreac, Castellar del Vallés, Spain), 0.1% porcine stomach mucin (Sigma-Aldrich, St. Louis, MO), 0.12% NaCl, 0.02% KCl and 2% HCl. The pH was further adjusted to 1.8 with 1 M HCl. Mobile larvae (10 tubes, 10 L3/tube) were incubated in 10 mL of this medium at 37 °C in a 5% CO<sub>2</sub> atmosphere. The medium was changed every 24 h and dead larvae were removed. Repeated observations of the mobility of larvae were made until all the larvae were considered non-viable.

#### 2.5. Agar penetration test

The test was performed according to Arizono et al. (2012). Mobile larvae were placed in 100 mL jars (10 jars, 10 L3 per jar) with 10 mL of artificial gastric juice (pH 1.8) on top of ~57 mL of solid agar (0.75% agar and 0.9% NaCl). Each jar was incubated at 37 °C with 5% of CO<sub>2</sub> and the number of larvae that penetrated into the solid agar was recorded for up to 24 h.

#### 2.6. Oxygen consumption rate

Oxygen consumption was measured in untreated and treated larvae as described by Navas et al. (2007) with a Clark cell-type oxygen electrode (model DW1/AD, Hansatech Instruments Ltd., Norfolk, England) using a 2.5 mL chamber. Each measurement was made with 10 larvae in 1 mL 0.85% NaCl.

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