



Freezing kinetic parameters influence allergenic and infective potential of *Anisakis simplex* L3 present in fish muscle

Isabel Sánchez-Alonso^a, Noelia Carballada-Sangiao^a, Miguel González-Muñoz^b, Alfonso Navas^c, Susana C. Arcos^c, Angel Mendizábal^d, Francisco Cuesta^a, Mercedes Careche^{a,*}

^a Institute of Food Science, Technology and Nutrition, Consejo Superior de Investigaciones Científicas (ICTAN-CSIC), C/ José Antonio Novais 10, 28040, Madrid, Spain

^b Department of Immunology, University Hospital La Paz, Paseo de la Castellana 261, 28046, Madrid, Spain

^c Department of Biodiversity and Evolutionary Biology, Museo Nacional de Ciencias Naturales, CSIC, C/ José Gutiérrez Abascal, 2, 28006, Madrid, Spain

^d Department of Sanitary, Quality and Consumption Services, Junta Municipal de Arganzuela, Ayuntamiento de Madrid, Paseo de la Chopera 10, 28045, Madrid, Spain

ARTICLE INFO

Keywords:

Anisakis
Freezing
Allergen
Agar penetrative ability
Survival in gastric juice
Respiratory analysis
Viability
Fish muscle

ABSTRACT

The effect of freezing rate, temperature and storage time of *Anisakis* L3 infecting fish mince in controlled conditions were studied in terms of larval viability and parasite antigen release. In order to determine the potential infectivity of larvae that might accidentally survive a freezing process, their agar penetration ability, survival in artificial gastric juice, and respiratory control were analysed in infected hake muscle in conditions that produced various survival rates. For that a series of experiments was conducted with 10–30 L3 per 75 g of mince, frozen and stored to provide a variety of freezing kinetic conditions and storage times. The results highlight the importance of a number of kinetic parameters in the survival of *Anisakis* larvae, not only final temperatures or storage times. High freezing rates, preferred in order to maintain optimum fish eating quality, are able to kill *Anisakis* larvae faster, but may cause a higher release of antigens to the surrounding medium. The characteristics of the surviving L3 were impaired as compared to the controls. No relationship between the latter and the percentage of viability due to different freezing conditions was found, which suggest that any larva which shows any sign of movement after freezing should be treated as equally infective.

1. Introduction

Anisakis spp. can cause infections in humans, through consumption of fish parasitized with live larvae in the third stage (L3) when the fish is consumed raw or when the processing, storage or cooking practices are insufficient to kill the larvae (Audicana & Kennedy, 2008; Mattiucci, Cipriani, Levens, Paoletti, & Nascetti, 2018; Moneo, Carballada-Sangiao, & González-Muñoz, 2017; Nieuwenhuizen & Lopata, 2014). Exposure to the parasite is mainly characterized by gastrointestinal manifestations of epigastralgia, nausea, abdominal pain and diarrhoea (Shimamura, Muwanwella, Chandran, Kandel, & Marcon, 2016), and also by a characteristic allergic reaction such as IgE-mediated hypersensitivity, angioedema, urticaria or anaphylaxis (Mattiucci et al., 2017; Moneo et al., 2017; Ventura, Napolitano, Menga, Cecere, & Asero, 2013).

EU Regulation No 1276/2011 on freezing of fish (at least -20°C in all parts of the product for not less than 24 h, or -35°C for not less

than 15 h) is compulsory for fish business operators and covers a wide range of freezing conditions that would have various effects on the characteristics of fish muscle after freezing, and therefore on quality. Although freezing is one of the methods most commonly used to preserve food, the process itself may bring about some sensory and technofunctional modifications, especially at slow freezing rates or high storage temperatures.

Experiments with isolated L3 suggest that fast freezing rates would be more efficient for a given final temperature (Sánchez-Alonso et al., 2018) in terms of inactivation. Although there is some evidence from experiments using infected fish muscle (Deardorff & Throm, 1988), the effect of freezing rate on viability of *Anisakis* L3 is often masked by other confounding factors as final temperature or holding time in the freezer, the geometry of the sample or the sample size. Moreover, there is no systematic information on how other freezing kinetic parameters such as initial temperatures, the prefreezing, phase change, or tempering times, would affect viability of *Anisakis* L3.

* Corresponding author.

E-mail addresses: isabel.sanchez@csic.es (I. Sánchez-Alonso), noeliacarba@hotmail.com (N. Carballada-Sangiao), miguel.gonzalez.munoz@salud.madrid.org (M. González-Muñoz), alfonsos@mncn.csic.es (A. Navas), scobacho@mncn.csic.es (S.C. Arcos), mendizabalaa@madrid.es (A. Mendizábal), mcareche@ictan.csic.es, m.careche@csic.es (M. Careche).

<https://doi.org/10.1016/j.foodcont.2020.107373>

Received 5 February 2020; Received in revised form 19 May 2020; Accepted 20 May 2020

Available online 14 June 2020

0956-7135/ © 2020 Elsevier Ltd. All rights reserved.

Safety authorities recommend preventive measures relating to freezing of fish that may contain anisakids, to be applied in households. However, these measures may not be applied correctly (Sánchez-Alonso et al., 2018), partly owing to the operating conditions of some domestic freezers (e.g. Evans, Foster, & Brown, 2014). This may lead to survival of *Anisakis* L3 under certain conditions, since they are moderately freezing-tolerant (Wharton & Aalders, 2002). Despite the fact that using the mobility as the only indicator for *Anisakis* L3 viability is not sufficient to assess its infectivity (EFSA, 2010), there are insufficient data concerning the physiological characteristics of freeze-surviving larvae (Sánchez-Alonso et al., 2018, 2019), and there is no information about the possible relation of the latter with the percentage of viability in a given batch (i.e. to what extent very low percentages of mobility would be associated with higher impairment of the infectivity potential of those surviving larvae).

The two principal clinical allergic responses due to *Anisakis* (EFSA, 2010) include gastroallergic anisakiasis, where the allergic symptoms are concurrent with acute gastric parasitism, and thus derive from live larvae, and allergy to *Anisakis*, which results from contamination of fishery products with allergens, with no need for the presence of live parasites, and allergic reactions can occur after ingestion of 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; Vidaček et al., 2009, 2010, 2011). It is widely accepted that a previous infection with live larvae is needed to elicit an allergic reaction. Thus any treatment that inactivates *Anisakis* L3 is also crucial as a preventive measure to avoid sensitization (EFSA, 2010), but there is no information about the effects of different freezing conditions on allergen release to the medium.

Overall, it is important to study the pathogenic potential of *Anisakis* L3 from a broader perspective embracing a wider number of factors as well as indicators. This would help to define more precisely the point at which the parasites are no longer viable/infective without compromising the quality of the fish products. It would also aid in gathering information on the interaction of freezing conditions and allergenic potential.

In this work, the effect of freezing rate, temperature and storage time of *Anisakis* L3 infecting fish muscle in controlled conditions were studied in terms of larval viability and parasite antigen release. *Anisakis* L3 species variability was also considered as an aleatory variable. In order to determine the potential infectivity of larvae that might accidentally survive a freezing process, their agar penetration ability, survival in artificial gastric juice or saline solution, and respiratory control were analysed in infected hake muscle in conditions that provided various survival rates.

2. Materials and methods

2.1. Raw material

2.1.1. Fish muscle

Hake (*Merluccius merluccius*), Atlantic horse mackerel (*Trachurus trachurus*) and mackerel (*Scomber scombrus*) were caught in the Atlantic Northeast fishing area (FAO area 27, subareas 27.7, 27.8 and 27.6.a, respectively); yellow horse mackerel (*Caranx rhonchus*) was caught in the Mediterranean Sea (FAO area 37, subarea 37.1.1). The gutted fish was purchased and filleted from a local fishmonger, transported to the laboratory refrigerated and then kept at 4.6 ± 0.3 °C until further processing (within 1 h). On arrival, the fish fillets were visually inspected and any larvae detected were removed from the muscle. Protein, moisture and ash content were determined for each batch according to AOAC (1995), and fat content was determined according to the method of Bligh & Dyer (1959).

2.1.2. Larvae

Anisakis L3 from heavily infected ovaries and viscera of hake caught in the Northeast Atlantic fishing area and obtained from the central fish market in Madrid (Mercamadrid) were used. After receiving the larvae, they were immediately separated from the tissue with forceps, rinsed with 0.85% NaCl, placed in groups of 50 individuals in tubes containing 10 mL of 0.85% NaCl and stored at 4.6 ± 0.3 °C until needed.

In order to establish the percentage of representation of species or their heterozygote genotypes (Mattiucci et al., 2016), at least 40 clean, frozen stored (-22.0 ± 0.3 °C) L3 per batch were individualized and subjected to identification. We have used as diagnostic marker the ITS1 region of the nuclear ribosomal DNA (rDNA). DNA was extracted and purified from the caudal region of individual L3s using the Speedtools Tissue DNA Extraction Kit (Biotools) and following the manufacturer's instructions. Molecular identification was carried out using PCR-RFLP. The forward primer A 5'-GTCTGAATTCGTAGGTGAACCTGCGGAAGGATCA-3' and reverse primer B 5'-GCCGGATCCGAATCCTGGTTAGTTCTTTTCCT-3' (D'Amelio et al., 2000) were used in reactions containing 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, 200 mM each of dATP, dCTP, dGTP and dTTP and 1 unit of DNA polymerase (Biotools B&M Labs, S.A., Madrid, Spain). Initial denaturalization (2 min at 94 °C) was followed by 35 cycles of 94 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min and by a final 7 min extension at 72 °C. Amplified DNA fragments were digested with the restriction enzymes HhaI and HinfI (New England Biolabs, Massachusetts, MA, USA) following the manufacturer's instructions. Restriction fragments were separated by electrophoresis in Tris-Borate-EDTA (TBE)-buffered 2.5% agarose gel, stained with SYBERsafe and visualized with UV illumination.

2.2. Experimental infection of muscle

Minced fish were spiked with *Anisakis* L3. For this purpose, fillets were minced in a meat mincer machine with a 3 mm diameter holes. Each Petri dish (88 mm Ø and 11 mm h) was approximately half-filled with minced fish, live larvae were put on top of the mince, and then the dish was filled with additional mince (final weight 74.7 ± 2.5 g); then the Petri dish was covered with a lid and immediately used for freezing. In some experiments the height of the Petri dishes was doubled or tripled (22 mm and 33 mm, respectively). The preparation of the samples was similar to that of the previous ones but in these cases the dishes contained 139 ± 1 and 206 ± 2 g mince, respectively.

2.3. Freezing

Various freezing experiments were performed in home freezers with working temperatures set at temperatures ranging from -10 to -30 °C (models CHS-441-30, GS-310, GS-454, Radiber S.A., Barcelona, Spain; GGPV 5520, Liebherr GmbH Korneuburg, Austria) and -80 °C (NU-9668E, NuAire, Inc., USA), chosen to reach the target temperature and freezing rate combinations. The freezing finished when each experimental unit (i.e. Petri dish) reached the target temperature (i.e. -10 , -15 , -20 or -28 °C). In some experiments, samples were frozen and stored for 12 and 24 h at the corresponding temperatures. After freezing and/or frozen storage, samples were allowed to thaw overnight at -5 °C. Temperatures (freezing and thawing) were measured both in the thermal centre of the samples and inside the freezer/fridge, using T-type thermocouples and recording (scanning time of 10 s) with a DaqPRO model 5300 data logger (Fourier Systems Ltd.). From the time-temperature data the following parameters were obtained: initial (T_{initial}) and final (T) temperature (°C), time (min) to reach -1 °C (t_1), time to decrease from -1 to -7 °C (t_c), and from -7 °C to the final temperature (t_3); the time for the sample to reach the lowest temperature (t_c , total freezing time) is the sum of t_1 , t_c and t_3 . Total freezing and thawing time ($t_{\text{freeze/thaw}}$) was also calculated. A minimum of 2 and a maximum of 9 experimental units per experiment (i.e. Petri dishes

with minced hake spiked with 10–30 L3) were used, with an average of ~3 per experiment.

2.4. Modelling of freezing time

Thermophysical parameters were deduced from the equations of Choi and Okos (Choi & Okos, 1986; ASHRAE Handbook—Refrigeration SI Edition, 2010a, chap. 19) using the chemical compositions of hake and horse mackerel. Time-temperature experimental data from hake with 11 mm thickness geometries obtained from the previous experiments performed with minced fish were used to calculate the surface heat transfer coefficient following the method of Cuesta, Lamúa, & Alique (2012). The total freezing time was calculated by estimating the prefreezing, freezing and subfreezing times separately. The prefreezing and subfreezing times were calculated according to Cuesta, Lamúa, & Moreno (1990) and Cuesta & Lamúa (1995). Following the method of Cleland (Cleland, Cleland, & Earle, 1987a; Cleland, Cleland, & Earle, 1987b; ASHRAE Handbook—Refrigeration SI Edition, 2010b, chap. 20), freezing times were calculated. Thus freezing curves for -10°C and -20°C and 11 and 22 mm thickness were modelled.

2.5. Recovery of larvae

Larvae were recovered from the mince after visual inspection, and in some cases with the aid of UV light (366 nm) (Karl & Leinemann, 1993; Vidaček et al., 2010), and were then placed in 0.85% NaCl for further analysis. The remaining mince was stored at -30°C until use for the extraction of parasite antigens.

2.6. Viability

Movement of the larvae spontaneously or in response to stimulation with tweezers was observed according to EFSA (2010). Mobility of frozen and thawed larvae was assessed just after thawing. For those that did not show any movement just after thawing, we checked whether they moved after incubation with 0.85% NaCl for 10 min at 37°C or after 4 h and 24 h at room temperature. The larvae without mobility were then considered non-viable. Viability was expressed as percentage of mobile L3.

2.7. Agar penetration test

This test was performed according to Arizono, Yamada, Tegoshi, & Yoshikawa (2012), as modified by Sánchez-Alonso et al. (2018). Mobile larvae were placed in 100 mL jars 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_2 , and the number of larvae that penetrated into the solid agar was monitored after 24 h. Data were expressed as the percentage of L3 able to penetrate agar.

2.8. Survival in artificial gastric juice

Survival of *Anisakis* L3 in artificial gastric juice (pH 1.8) during time was determined as described by Sánchez-Alonso et al. (2018). 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 adjusted to 1.8 with 1 M HCl. Mobile larvae were incubated in this medium at 37°C in a 5% CO_2 atmosphere and checked periodically for mobility for up to 260 h.

2.9. Respiratory control analysis in *Anisakis* L3

Assessment of the oxygen consumption rate (OCR) of *Anisakis* larvae during various mitochondrial respiration states was performed

according to Sánchez-Alonso et al. (2019) using a single-chamber Clark electrode (model DW1/AD, Hansatech Instruments Ltd., Norfolk, England). A total of 25 cycles (loops, 8 min each) of OCR measurements was performed. Metabolic modulators – FCCP (carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone, Sigma-Aldrich, Inc.) and sodium azide (Sigma-Aldrich, Inc.) – were used to obtain data for initial, maximal and azide-insensitive respiration. Larvae were first allowed to warm at 37°C and were then carefully placed in the chamber containing 1000 μL of M9 buffer (22 mM KH_2PO_4 , 42 mM Na_2HPO_4 , 86 mM NaCl and 1 mM MgSO_4) at 37°C . Typically, the initial respiration was measured for the first 56 min (7 loops). After addition of 125 μL of 160 mM FCCP, OCR was measured for about 11 loops. Then 125 μL of 500 mM azide was added and OCR was measured for 7 loops. The OCR values were expressed as $\text{nmol O}_2/\text{min}/\text{worm}$.

2.10. Antigenicity and allergenicity

2.10.1. Quantification of *A. simplex* antigens in fish muscle

Ten g of thawed mince was homogenized (24,000 rpm, 30 s, room temperature) in 30 mM NaCl, 10 mM Tris-HCl, pH 6.8, 1:3 (w:v) using an Ultra-Turrax T25 (Janke & Kunkel IKA-Labortechnik, Staufen, Germany). The resulting mixture was sonicated (17 W, 30 s) in a Microson ultrasonic cell disruptor (Misonix XL-2000 Series, Farmingdale, NY, USA), maintaining the temperature of the mixture at $\leq 10^{\circ}\text{C}$. It was then incubated with orbital agitation (Tube Rotator Model SB3, Stuart, Barloworld Scientific Ltd., UK) at 40 rpm for 15 min at room temperature and centrifuged (Heraeus Multifuge 3L-R centrifuge) at $5000 \times g$ for 30 min. The supernatant was stored and parasite antigens were further extracted from the pellet by repeating the homogenization, stirring and centrifugation steps twice in the same conditions as above. The resulting pellet was discarded, and the supernatants were freeze-dried (Christ Beta 2–8 LDplus, Germany) and stored at -80°C . When needed, the lyophilized samples were resuspended in 1 mL of distilled water.

The antigenicity of the *Anisakis* L3 was studied by dot blot using rabbit anti-*A. simplex* crude extract polyclonal antibody and rabbit anti-recombinant (r)Ani s 4 polyclonal antisera (Carballeda-Sangiao et al., 2016; Rodríguez-Mahillo et al., 2007). Three microlitres of the extracts was dotted in duplicate onto a nitrocellulose membrane and air dried. The membrane was rehydrated with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 , pH 7.4). After blocking with 3% Nonidet P-40 (NP-40) in PBS for 1 h, the membrane was incubated with rabbit anti-*Anisakis simplex* antigens, anti-Ani s 4, or pre-immune anti-sera (1/10000 dilution in 128 mM NaCl, 9 mM Tris HCl, 1% BSA, 1% Tween 20) for 1 h at room temperature, washed with TTBS (25 mM Tris-HCl, 137 mM NaCl, 2.7 mM KCl, 0.05% Tween 20), then incubated with alkaline-phosphatase-labelled goat anti-rabbit IgG (Sigma-Aldrich, St. Louis, MO, USA), (1/10000), developed for 30 min with 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) alkaline phosphatase substrate solution (Bio-Rad Inc., USA) and stopped with PBS. A reference curve of an *A. simplex* crude extract was made in duplicate as a measurement of parasite antigens, or of rAni s 4. Membranes were scanned and Quantity One® software (Bio-Rad Laboratories, Hercules, CA) was used to define the boundaries of protein dots, to assess pixel number and density. In order to obtain final μg parasite antigens/g or Ani s 4/100 g of muscle, the non-specific pre-immune signal was first subtracted from each dot.

2.10.2. Anti Ani s 4 and IgE western blotting

Western blots were performed as previously described (Rodríguez-Mahillo et al., 2007). Briefly, samples were separated by SDS-PAGE on 4–20% Tris-glycine gel (Novex, Life Technologies, Grand Island, NY, USA). Proteins were transferred to nitrocellulose membranes by passive diffusion at room temperature overnight in TBS buffer (25 mM Tris-HCl, 137 mM NaCl, 2.7 mM KCl). Membranes were blocked with PBS, 3% NP-40, for 1 h at room temperature.

Table 1

Freezing parameters obtained by freezing minced hake infected with *Anisakis* L3 at -10 , -15 , -20 , -28 °C. T, final temperature (°C), t_1 , precooling time (min) or time to reach -1 °C, t_c , characteristic freezing time (min) or time to traverse the temperature range from -1 to -7 °C, t_3 , tempering time (min) or time from -7 °C to reach T, and t_t , the total freezing time (min) to reach T. Values are mean $\pm$ standard deviation. At least two replicates were performed per case (a total of 83 cases), with either 10 or 20 L3 per experimental unit, yielding a total of 1088 *Anisakis* L3 from 8 different batches.

Set T (°C)	Freezing parameters				
	Real T (°C)	t_1 (min)	t_c (min)	t_3 (min)	t_t (min)
-10	-10.0 ± 0.00	35.2 ± 2.82	173 ± 9.58	18.0 ± 2.68	226 ± 13.25
	-10.2 ± 0.13	44.7 ± 1.00	151 ± 5.47	10.0 ± 0.25	205 ± 6.54
	-10.0 ± 0.03	35.8 ± 2.09	165 ± 9.09	8.53 ± 3.19	209 ± 12.86
	-10.1 ± 0.05	33.4 ± 2.00	158 ± 8.05	10.5 ± 2.35	202 ± 7.27
	-10.1 ± 0.02	29.8 ± 1.61	160 ± 8.38	10.6 ± 1.99	200 ± 9.80
	-10.1 ± 0.04	32.2 ± 1.62	158 ± 10.32	14.5 ± 3.45	204 ± 14.70
	-10.1 ± 0.03	26.3 ± 0.00	113 ± 2.12	5.67 ± 0.24	145 ± 2.36
	-10.0 ± 0.01	22.8 ± 0.25	112 ± 1.55	5.67 ± 0.17	141 ± 1.45
	-10.0 ± 0.05	24.7 ± 1.64	111 ± 3.51	5.34 ± 0.58	140 ± 3.23
	-10.5 ± 0.12	27.6 ± 0.51	106 ± 3.72	6.78 ± 1.64	140 ± 2.89
	-10.3 ± 0.75	25.2 ± 1.00	97.0 ± 2.80	4.50 ± 1.30	127 ± 2.89
	-10.8 ± 0.18	6.44 ± 0.96	31.4 ± 3.31	2.11 ± 0.26	40.0 ± 3.46
	-15.1 ± 0.02	45.6 ± 1.06	154 ± 5.86	30.7 ± 1.54	231 ± 6.49
	-15.0 ± 0.00	25.0 ± 1.88	117 ± 2.24	19.5 ± 0.47	162 ± 3.66
-15	-15.0 ± 0.00	25.7 ± 0.23	111 ± 1.18	18.8 ± 0.23	156 ± 1.18
	-15.0 ± 0.00	28.7 ± 0.00	113 ± 4.00	19.7 ± 1.18	162 ± 5.18
	-15.2 ± 0.08	28.3 ± 0.17	105 ± 2.03	17.8 ± 0.93	152 ± 2.89
	-15.5 ± 0.13	3.83 ± 0.44	20.0 ± 7.08	9.11 ± 2.30	33.0 ± 4.36
	-20.1 ± 0.07	45.1 ± 0.92	150 ± 4.77	67.2 ± 3.71	262 ± 1.83
	-19.8 ± 0.34	26.0 ± 1.00	114 ± 0.98	43.6 ± 2.30	183 ± 2.52
-20	-20.0 ± 0.03	23.4 ± 1.83	111 ± 0.93	42.0 ± 1.89	176 ± 1.77
	-20.0 ± 0.00	24.3 ± 0.84	108 ± 3.33	45.4 ± 2.44	178 ± 2.31
	-20.1 ± 0.07	27.9 ± 0.84	104 ± 2.36	34.6 ± 1.67	166 ± 3.21
	-27.2 ± 0.35	42.3 ± 0.73	142 ± 3.91	138 ± 3.75	322 ± 0.00
-28	-29.0 ± 0.20	26.7 ± 1.77	101 ± 2.30	188 ± 3.54	316 ± 0.00

For anti Ani s 4 blotting they were subsequently incubated with rabbit anti-rAni s 4 for 1 h at room temperature, washed with TTBS, then incubated with horseradish peroxidase (HRP)-labelled goat anti-rabbit IgG (GeneTex, GTX213110-01L) (1/10000 in TTBS and 0.5% STF) and developed with horseradish peroxidase substrate according to the instructions of the manufacturer (Bio-Rad, Immun-Star™WesternC™Chemiluminescent Kit, no. 170–5070).

For IgE immunoblotting, membranes were subsequently incubated with the sera of *A. simplex*-allergic patients ($n = 5$, *Anisakis*-specific IgE > 16 kU/L) (1/20 in TTBS and 0.5% STF), mouse anti-human IgE monoclonal antibody (1 mg/mL; Ingenasa, Madrid, Spain; 1/1000 in TTBS and 0.5% STF) and HRP-labelled goat anti-mouse IgG (Dako, Denmark, P0447).

2.11. Experiments conducted

2.11.1. Study of the effect of freezing kinetics on the pathogenic potential of *Anisakis* L3 in experimentally infected minced hake at a fixed geometry

A set of eleven independent trials was performed with minced hake infected with *Anisakis* L3 under controlled conditions. For this purpose, eleven batches of minced hake were parasitized with a total of 1088 *Anisakis* L3 from 8 different batches, making a total of 25 cases studied (Table 1). In one of the batches, the effect of frozen storage time and final freezing temperature was also analysed, using an additional 240 L3 and 8 additional cases. Viability in all the cases (25 plus 8 additional from frozen stored) and parasite antigen release in a subset of 14 cases (8 from Tables 1 and 6 from frozen stored) were measured.

In order to confirm the effect of freezing rate on parasite antigen release, an additional trial was performed with hake mince infected with 30 L3/10 g muscle at two freezing rates, using 120 additional larvae.

2.11.2. Survival of *Anisakis* L3 from other species and specimen thicknesses

Four different trials were performed with minced hake, horse mackerel and mackerel experimentally infected with 20 L3 per Petri

dish, with three Petri dish thicknesses (11, 22 and 33 mm), and a total of 480 treated and 120 control L3 was used.

2.11.3. Study of the characteristics of surviving *Anisakis* L3

The characteristics studied were: a) *Anisakis* penetration into an agar layer, b) survival in artificial gastric juice, c) oxygen consumption rate (OCR), and d) ability to survive and OCR upon refrigeration after thawing.

The characteristics of surviving *Anisakis* L3 after freezing, with a survival percentage of 50% ($\sim S_{50}$), were studied in terms of penetration into agar in three independent experiments. Petri dishes (10 or 20 L3/mince) were parasitized with a total of 410 *Anisakis* L3 and subjected to freezing. The agar penetration ability of the surviving larvae (i.e. 196 L3) was compared to that of untreated controls (3 experiments, 160 L3). An agar penetration test was also performed in the trials studied in section 2.11.2.

Survival of *Anisakis* L3 in artificial gastric juice after freezing ($\sim S_{50}$) was studied in two experiments (158 L3) and compared to that of untreated larvae (102 L3).

Respiration after freezing and thawing and survival at refrigerated temperatures after thawing were studied in four additional experiments, using one batch of *Anisakis* L3 and four batches of hake (3.0 kg/batch). The same freezing conditions as before were used, but in this set of experiments 20 L3 were placed in each Petri dish instead of 10. Six Petri dishes were used per trial, thus making 480 treated *Anisakis*. Four measurements were performed on separate days with the control L3 (i.e. 40 L3 unfrozen and stored in the refrigerator at 4 °C).

2.12. Statistical analysis

The survival of L3 (i.e. % viability) was averaged over experiments (i.e. average of at least two Petri dishes). Percentage viability a) upon freezing or b) upon storage was analysed by one-way analysis of variance (ANOVA) as affected by either freezing temperature (a) or frozen storage time (b) as the main factor, with freezing times (t_1 , t_c , t_3 , t_t) or

percentage of *Anisakis* larva (*A. simplex* s.s., *A. pegreffii*, heterozygote genotypes) or T (final temperature achieved) as covariates. The same test was used for the study of the modification of parasite antigen or allergen release to fish muscle, with T as the factor and t_1 , t_c , t_3 , t_t and storage time as covariates. The homogeneity of variances was checked by the Levene test and pairwise comparison was conducted by the Bonferroni test.

One-way ANOVA was used for the study of % survival as a function of t_c range (i.e. fast, medium, slow), for the effect of frozen storage time on parasite antigens, and for the effect of storage time on the oxygen consumption rate parameters after thawing (initial, FCCP, azide-insensitive). Significant differences among means were determined by a Bonferroni multiple range test in those cases in which the prerequisite of homogeneity of variances was fulfilled. Otherwise, Tamhane's post hoc test was employed.

Factor analysis was performed in order to study the relation between temperature and freezing time parameters, on the one hand, and % viability, on the other. Stepwise regression analysis was performed with freezing (i.e. T_{initial} , T, t_1 , t_c , t_3 , t_t) or storage ($t_{\text{freeze/thaw}}$) parameters as independent variables and % viability as the dependent variable. The Durbin-Watson test was used to check for independence of residuals.

The comparison of the control and frozen-thawed larvae in terms of agar penetration test or oxygen consumption rate was analysed by a *t*-test of independent samples, using the Levene test to analyse the equality of variances. The differences with regard to survival in gastric juice were analysed by the Kaplan-Meier test, using the mortality per period. The significance level was established at 5%. IBM SPSS Statistics software V24.0.0.1 for Windows (IBM Corp., Armonk, NY, USA) was used for statistical analysis.

2.13. UNE-EN ISO 9001 certification

The Institute of Food Science, Technology and Nutrition (ICTAN-CSIC) has been certified under UNE-EN ISO 9001 for "Management and execution of research projects and contracts in the area of Food Science and Technology and Nutrition" (certificate number ER-0366/2015).

2.14. Ethics statement

Ethics approval is not applicable according to EU Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes, since the hakes used in this study were caught for commercial purposes, more specifically for human consumption, and not for research purposes, so the capture of the animals was not part of the experimental activity proposed in this work. The experimental design did not involve the manipulation of the live animals but of tissues (e.g. muscle fillets and viscera parasitized with *Anisakis* L3) extracted from already non-living specimens acquired from a local fishmonger and a central fish market.

The study with human samples was approved by the Ethics Committee of University Hospital La Paz-Hospital Carlos III (Madrid, Spain), and the participants were asked to sign an informed consent form.

3. Results

3.1. Effect of freezing rate, temperature and time on the viability and antigen release of *Anisakis* larvae in infected hake

3.1.1. Freezing kinetics

Fig. 1 shows representative curves describing the time and temperature profiles of minced hake subjected to three freezing rates, obtained in freezers with different working powers. The first decrease in temperature involves the removal of sensible heat from the samples, or prefreezing, with a given time (t_1). When the freezing point is reached,

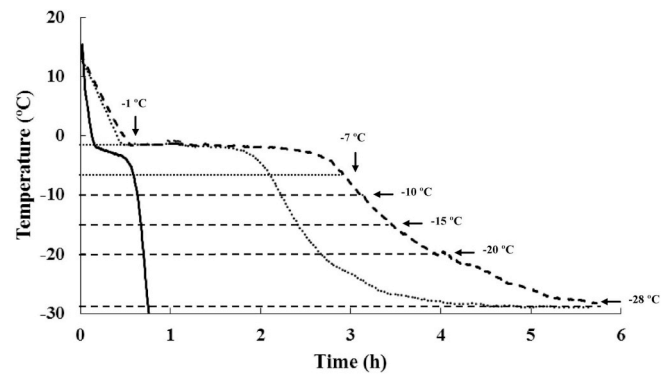


Fig. 1. Representative time and temperature profiles of minced hake subjected to three freezing regimes. Each profile exhibits a different characteristic freezing time (t_c), (~30 min solid line, ~100 min dotted line, and ~150 min dashed line), measured as the minutes taken to traverse the temperature range from -1 to -7 °C (vertical arrows). The horizontal arrows represent the final freezing temperatures set for each of the experiments (-10 , -15 , -20 , -28 °C).

the crystallization process begins and there is a freezing plateau until most of the water has been converted to ice (phase change). The characteristic freezing time of this phase change, i.e. from -1 to -7 °C, is defined as t_c . Further removal of heat leads to a decrease in temperature until the final freezing temperature (T) is reached, with time t_3 (tempering time), so total freezing time (t_t) is the sum of t_1 , t_c and t_3 .

The kinetic data for the experiments with minced hake, i.e. the final temperatures achieved, the prefreezing time, phase change interval, tempering, and total freezing time (T, t_1 , t_c , t_3 and t_t , respectively) are shown in Table 1. The average final temperatures reached in the thermal centre of the minced fish samples (i.e. -10.15 ± 0.27 °C, -15.17 ± 0.21 °C, -20.02 ± 0.16 °C, and -28.11 ± 0.98 °C), were well within the target values. Freezing time parameters t_1 , t_c and t_3 were, respectively, in the ranges of ~4–46, 20–173, and 2–188 min, as expected for the conditions set for each experiment, such as the freezer equipment and the ambient temperature of the freezer. Depending on the above factors, the total freezing times were between 33 and 322 min. The proximate composition of the various batches of hake ranged from 79.0 to 81.3% moisture, 17.5–20.0% protein, 0.8–1.2% ash and 0.9–1.0% fat content.

3.1.2. Effect of freezing kinetics on viability

One-way ANOVA of viability as a function of T with either t_1 , t_c , t_3 or t_t as covariate (Table 2) showed that *Anisakis* L3 survival was significantly affected not only by T but also by freezing time parameters t_1 , t_c and t_t ($P < 0.001$). Factor analysis, including the thermal variables which showed a significant effect in ANOVA (T, t_1 , t_c , t_t), together with L3 survival percentage, yielded two principal components (PCs), which explained 94.6% of the total variance (Table 3). The time components t_1 , t_c and t_t were grouped into the first PC, T was extracted into PC2, and the survival percentage was extracted in PC1 and PC2, thus suggesting that viability loss could be dependent on both the cooling and freezing times (represented by PC1) and the final temperature (represented by

Table 2

Anisakis viability as a function of set final freezing temperature (°C) with freezing time parameters (min) as covariates. Abbreviations as in Table 1.

Factor & Covariate	Model	Factor	Covariate	R ² adj
Temperature & Precooling time (t_1)	***	***	***	0.81
Temperature & Characteristic freezing time (t_c)	***	***	***	0.79
Temperature & Tempering time (t_3)	**	**	NS	0.46
Temperature & Total freezing time (t_t)	***	***	***	0.81

Table 3

Factor loading patterns of the freezing parameters and % survival. Abbreviations as in Table 1.

Variables	Components	
	1	2
Prefreezing time (t_1)	0.95	-0.12
Characteristic freezing time (t_c)	0.94	0.11
Total freezing time (t_t)	0.82	-0.53
Final freezing temperature (T)	-0.02	0.99
% Survival	0.70	0.67

Two components extracted which explained 94.6 of total variance.

PC2). Since the significant effects ($P < 0.001$) of the time parameters on the mortality of *Anisakis* L3 could be partly a consequence of a correlation among them, stepwise regression analysis was performed with % viability as the dependent variable and T and the time parameters t_1 , t_c and t_t (which together covered the total freezing time) as independent variables. This showed that the best model (Table 4, row a) included T, and only t_c among the time parameters ($R^2_{adj} = 0.767$, $P < 0.001$). However, when t_t was included, the best model was achieved with T and that variable ($R^2_{adj} = 0.836$, $P < 0.001$) (Table 4, rows a and b). This may be because t_t carries part of the information related to the final freezing temperature.

According to the known effect of lowering T on the % survival of *Anisakis* L3, Fig. 2a reflects that the higher the final temperature, the higher the variance. In order to display the effect of t_c on the area of maximum variance, viability data at -10°C were assigned to three ranges of t_c [slow (125–177 min), intermediate (90–120 min), and fast (10–40 min)] (Fig. 2b). It can be seen that the percentage of viable larvae with fast freezing rates can be 0 even at freezing temperatures as high as -10°C . The same trend was found when viability was plotted against t_t (not shown).

3.1.3. Effect of storage time and temperature on viability

The percentage of mobile *Anisakis* L3 at a fixed freezing rate (i.e. t_c 149 $\pm$ 5 min) as a function of storage time and T (Fig. 3) decreased significantly both with lowering the final temperature and with prolonging the storage time. After 12 h at -10°C , -15°C , -20°C the percentage of viable larvae decreased to values close to half of the initial ones, and after 24 h no viable larvae were recovered except in the case of the samples stored at -15°C , where one larva out of 19 showed signs of mobility. Stepwise regression analysis showed that both T and storage time were significant in the model (Table 4, row c).

3.2. Survival of *Anisakis* L3 in fish muscle from other fish species and thicknesses

One of the aspects that may affect the freezing kinetics is the

Table 4

Stepwise regression analyses with the freezing or frozen storage parameters as independent variables and % viability as the dependent variable. Initial temperature ($T_{initial}$), final freezing temperature (T), characteristic freezing time (t_c), total freezing time (t_t), storage time at a given freezing temperature ($t_{storage}$), total freezing and storage time until thawing ($t_{freeze/thaw}$).

Model	Species	Thickness (mm)	Storage time	Other	Significance & R^2_{adj}	Temperature factors	Time factors
a	hake	11	no		*** 0.77	T ***	t_c ***
b	hake	11	no		*** 0.84	T ***	t_t ***
c	hake	11	yes	fixed t_c	*** 0.81	T **	$t_{storage}$ ***
d	hake, mackerel, horse mackerel	11, 22, 33	no		*** 0.62	T ***	t_t **
e	hake, mackerel, horse mackerel	11, 22, 33	yes		*** 0.64	T ***	t_t *** $t_{freeze/thaw}$ ***

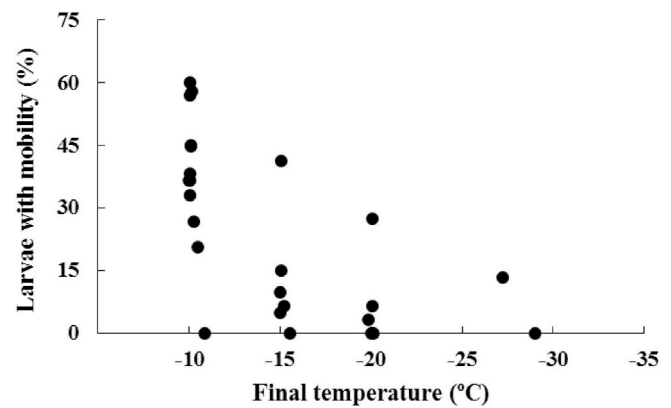


Fig. 2a. Viability (% L3 with mobility) of *Anisakis* L3 infecting minced hake as a function of final freezing temperature. Data corresponding to the experiments represented in Table 1.

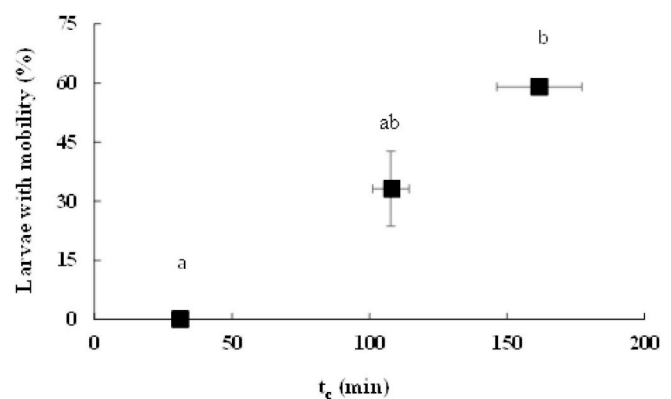


Fig. 2b. Viability (% L3 with mobility) of *Anisakis* L3 infecting minced hake as a function of the characteristic freezing time (t_c) at -10°C . Experimentally obtained t_c values (see Table 1) were assigned to three ranges corresponding to fast (10–40 min), intermediate (90–120 min) and slow (125–177 min) freezing rates. Data are average $\pm$ standard deviation. Different letters (a, b) indicate significant differences in viability.

composition of the food to be frozen (i.e. water, lipids, proteins, ash) which would affect their thermophysical properties. Results from previous sections were obtained by experimentally infecting minced fish from a lean species such as hake in cylinders with 9 cm ϕ and 11 mm h. In this section, freezing time was calculated as an example for hake (0.7% fat) and also for two horse mackerel minces (i.e. semi-fatty species) which may have varying fat content depending of a series of factors, including seasonality (in these particular cases, 2.0 and 4.3% fat), and for two thicknesses (11, 22 mm), with a starting temperature of 15.5°C , final temperature of -10 or -20°C in the thermal centre,

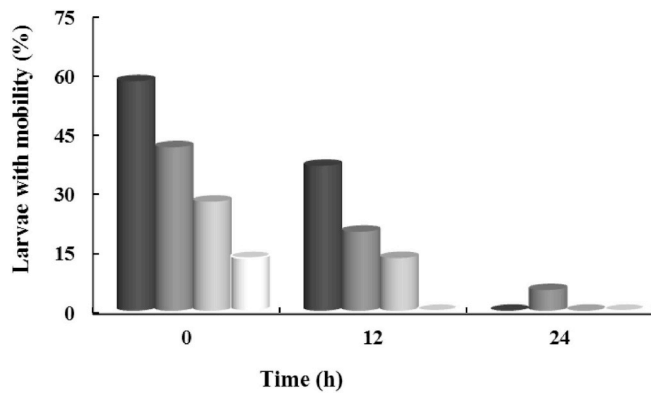


Fig. 3. Viability (% L3 with mobility) of *Anisakis* L3 infecting minced hake as a function of final temperature (-10 °C black, -15 °C dark grey, -20 °C light grey, -28 °C white) and frozen storage time at a characteristic freezing time, $t_c = 149.29 \pm 6.75$ min.

Table 5

Simulation of total freezing times as a function of thickness and composition of the sample for final temperatures of -10 °C and -20 °C, with initial temperature of 15.5 °C.

Species	Diameter (mm)	Height (mm)	External T (°C)	Incipient freezing T (°C)	Final T (°C)	Time (min)
hake (0.7% fat)	9	11	-30	-0.81	-10	118
	9	11	-30	-0.81	-20	145
	9	22	-30	-0.81	-10	203
	9	22	-30	-0.81	-20	250
horse mackerel (4.3% fat)	9	11	-30	-0.90	-10	111
	9	11	-30	-0.90	-20	143
	9	22	-30	-0.90	-10	194
	9	22	-30	-0.90	-20	250
horse mackerel (2.0% fat)	9	11	-30	-0.87	-10	114
	9	11	-30	-0.87	-20	144
	9	22	-30	-0.87	-10	198
	9	22	-30	-0.87	-20	251

and ambient temperature in the freezer of -30 °C. The theoretical freezing curves were very close to the experimental ones (not shown), and simulation of the freezing times (Table 5) indicated, for example, that doubling the thickness of the sample involved an increase of nearly 72–74% in freezing time, whereas increasing the fat content had less influence on total freezing time.

In order to establish if fish species (and their composition) have an influence in *Anisakis* L3 survival during freezing this parameter was additionally studied for horse mackerel and mackerel (semi-fatty species, four batches), with proximate compositions that ranged from 73.2 to 79.0% moisture, 19.9–22.9% protein, 1.2–1.5% ash and 2.0–4.8% fat content.

Data from these assays were added to the database with all the values, and a stepwise regression analysis that related the kinetic freezing parameters with percentage viability was performed. In these conditions the final temperature and total freezing time also entered as factors (Table 4, row d), as in the previous model. However, it was observed that the initial temperature before the cooling and freezing process entered as an additional factor in the model, and there was a trend towards a higher % survival of *Anisakis* L3 at higher T_{initial} values ($T_{\text{initial}} 14.6 \pm 3.6$ °C; range 4.9–20.9 °C).

When samples that had been frozen and stored for up to 24 h were included, total freezing/thawing time ($t_{\text{freeze/thaw}}$) was also significant in the model (Table 4e). No effect of fish species was found, but the small sample size of the mince with fatty species does not allow being conclusive in this respect.

3.3. Parasite antigen release

No parasite antigens or allergens (i.e. Ani s 4) were detected by dot blot in the extracts from the minced fish after removal of all *Anisakis* larvae from the muscle. Western blotting revealed with anti rAni s 4 and chemiluminescence showed that this allergen was only detected in the samples that had been frozen at the fastest freezing time (i.e. 31 min) (Fig. 4a). In a separate experiment, hake was parasitized with a higher amount of L3 (30 L3/10 g) and two freezing rate conditions were assayed ($t_c = 139 \pm 11.6$ and 16 ± 1.2 min, respectively). Fig. 4b shows parasite antigen release to muscle after removal of *Anisakis* L3, and significant differences between the two freezing rates were found by dot blot. In addition, western blotting revealed with anti rAni s 4 and chemiluminescence also showed striking differences in the recognition of this allergen between these two groups of samples, thus suggesting an effect of freezing rate on parasite antigen release.

Nevertheless, when the extracts from mince that had contained the lowest numbers of *Anisakis* (i.e. 10 L3/75 g) were revealed with patients' sera, all the western blots showed IgE reacting proteins with molecular weights ranging from 10 to 80 kDa (Fig. 4c).

3.4. Effect of *Anisakis* L3 species on viability

The *Anisakis* L3 used were found to be mainly *A. simplex* s.s. (71–100%), coexisting with *A. pegreffii* (0–21%) and with heterozygote genotypes (0–17%) (Table S1). In order to study viability as a function

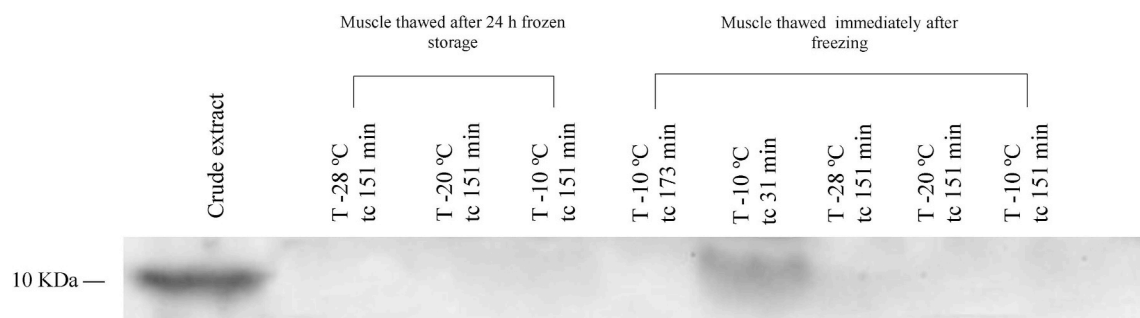


Fig. 4a. Immunoblots, revealed with anti rAni s 4 antiserum, of the fish mince that had been infected with 10 L3 per 75 g of muscle, frozen at -10 , -20 or -28 °C at different t_c values (173, 151 or 31 min), and thawed (immediately after reaching the target temperature or after frozen storage for 24 h). The antigen extraction was performed after complete removal of the larvae from the muscle.

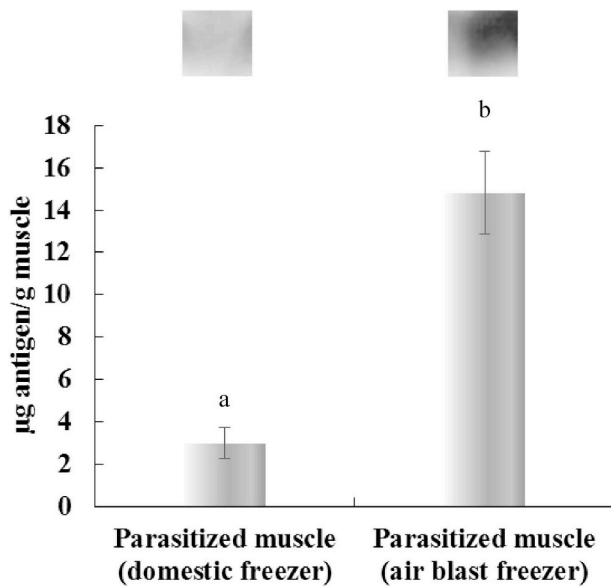


Fig. 4b. *Anisakis* L3 antigen release to the fish mince ($\mu\text{g g}^{-1}$) upon freezing at -20°C and subsequent frozen storage for up to 5 days. Data corresponding to domestic and air blast freezing (average $t_c 139 \pm 11.6$ and 16 ± 1.2 respectively). Different letters (a, b) indicate significant differences. Inserts show Ani s 4 in each band detected by immunoblotting and revealed with anti rAni s 4 antiserum.

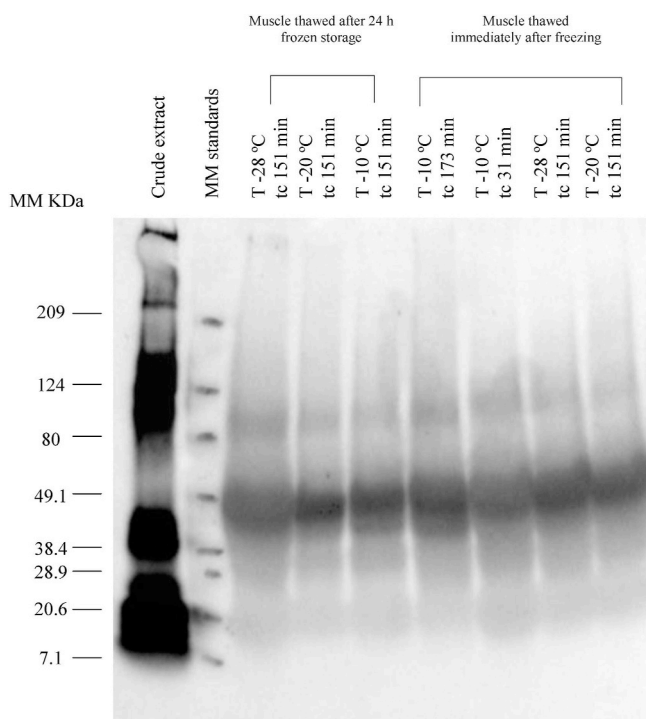


Fig. 4c. IgE immunoblots, revealed with a pool of sensitized patients' sera, of the fish mince that had been infected with 10 L3 per 75 g of muscle, frozen at -10 , -20 or -28°C at different t_c values (173, 151 or 31 min), and thawed (immediately after reaching the target temperature or after frozen storage for 24 h). The antigen extraction was performed after complete removal of the larvae from the muscle.

of *Anisakis* species, a subset of data corresponding to the experiments where the final temperature was set at -10°C (i.e. hake in Petri dishes with 11 mm thickness) was used, in order to avoid redundancy among taxonomic units (*A. simplex*, *A. pegreffii*, heterozygote genotypes). One-way ANOVA as a function of temperature and either *Anisakis simplex*

Table 6

Anisakis viability as a function of set final freezing temperature ($^\circ\text{C}$) and % of *Anisakis* species as covariates.

Factor & Covariate	Model	Factor	Covariate	R ² adj
Temperature & <i>Anisakis simplex</i> s.s.	**	**	NS	0.42
Temperature & <i>Anisakis pegreffii</i>	**	**	NS	0.42
Temperature & heterozygote genotypes	***	***	*	0.57

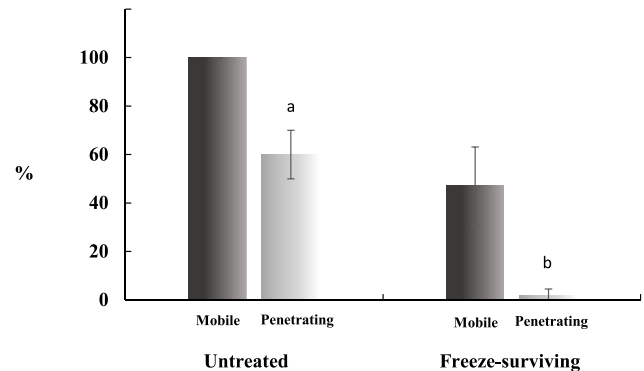


Fig. 5a. Percentage of mobile (dark grey) and agar-penetrating (light grey) *Anisakis* L3. Data from untreated (i.e. stored at $4-5^\circ\text{C}$) and freeze-surviving ($\sim S_{50}$) larvae. Error bars are standard deviation ($n = 3$ independent experiments). Different letters (a, b) show significant differences in the agar test between the untreated and freeze-surviving L3.

s.s., *A. pegreffii* or the heterozygote genotypes as cofactor (Table 6) showed a small correlation between the proportion of the heterozygote genotypes and viability after freezing ($P < 0.05$).

3.5. Characteristics of surviving *Anisakis* L3

3.5.1. Penetrative ability

Samples were treated to obtain a minimum of 40% viable L3 per trial (-10°C , at t_c higher than 140 min). The agar penetration ability of L3 that had been frozen in conditions that yielded 47% survival ($n = 408$) (Fig. 5a) was drastically impaired, with values close to 1.7% with respect to surviving larvae, in contrast to the penetrative ability of untreated L3, which was close to 60%. As a result, the t -test showed significant differences between means as regards the penetrative ability in the two groups of larvae (i.e. untreated and frozen/thawed).

Fig. 5b shows survival percentages ranging from 10 to 61% for a series of experiments with different species and geometries. No relation was observed between survival percentage and penetrative ability. The pooled data (885 L3, 322 surviving) showed that about 1% of them ($n = 8$) were able to penetrate into solid agar, suggesting that potentially any surviving larva may be equally infective regardless of the survival percentage in the trial.

3.5.2. Behaviour of surviving *Anisakis* L3 in artificial gastric juice

Survival in gastric juice was also impaired in frozen and thawed L3 as compared to the controls (Fig. 6), and the Kaplan-Meier test showed that significant differences were found between untreated L3 and those that survived freezing. However, after 68 h 11% of treated L3 were still surviving in this medium. These results confirmed the ones obtained in isolated larvae frozen in saline solution (Sánchez-Alonso et al., 2019).

3.5.3. Oxygen consumption rate of surviving *Anisakis* L3 after thawing and during storage at 4°C

Since alterations in oxygen consumption rate are considered an indicator of mitochondrial dysfunction, another characteristic studied was the respiration of *Anisakis* L3 subjected to freezing. We previously

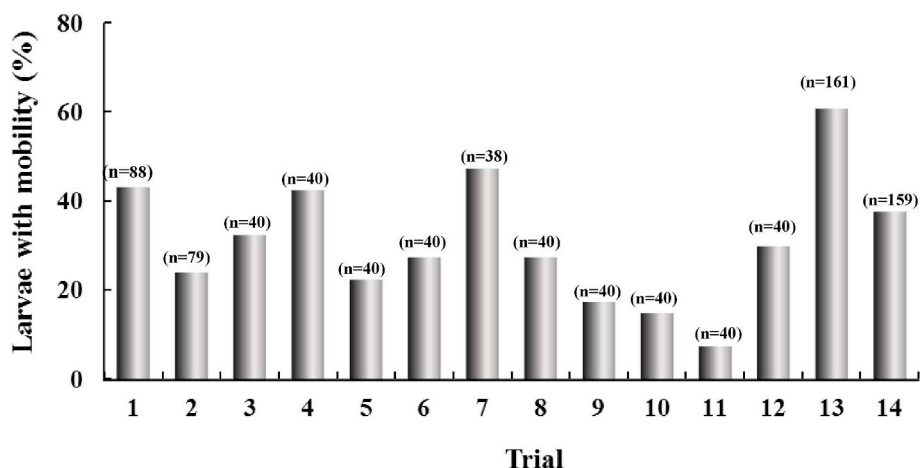


Fig. 5b. Range of viability (% L3 with mobility) of *Anisakis* L3 infecting minced fish in the different trials, regardless of species (i.e. hake, mackerel or horse mackerel), *Anisakis* batch (6 batches) or thickness of experimental units (i.e. 11, 22 or 33 mm).

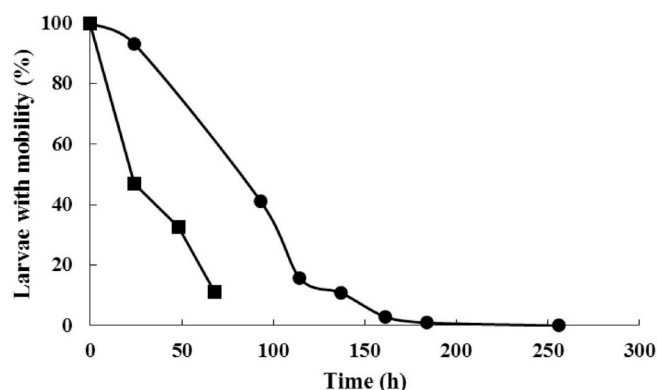


Fig. 6. Viability (% L3 with mobility) of *Anisakis* L3 upon incubation in artificial gastric juice at 37 °C. Untreated (circles) ($n = 1$) and freeze-surviving L3 (squares) ($\sim S_{50}$) ($n = 2$).

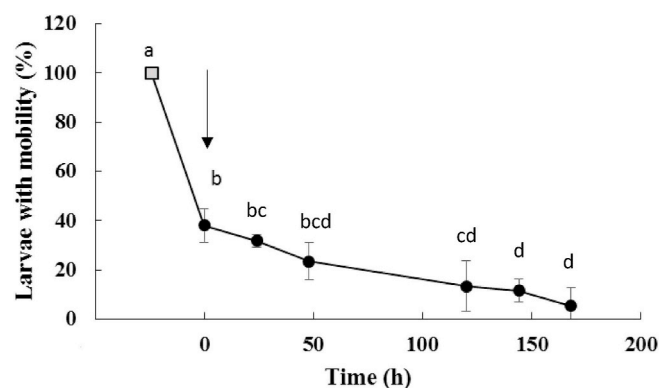


Fig. 7b. Viability (% L3 with mobility) of *Anisakis* L3 upon freezing at -10 °C (before the arrow) and upon storage at 4–5 °C for up to 168 h after thawing (after the arrow) ($n = 4$). Error bars show the standard deviation. Different letters (a, b, c, d) indicate significant differences.

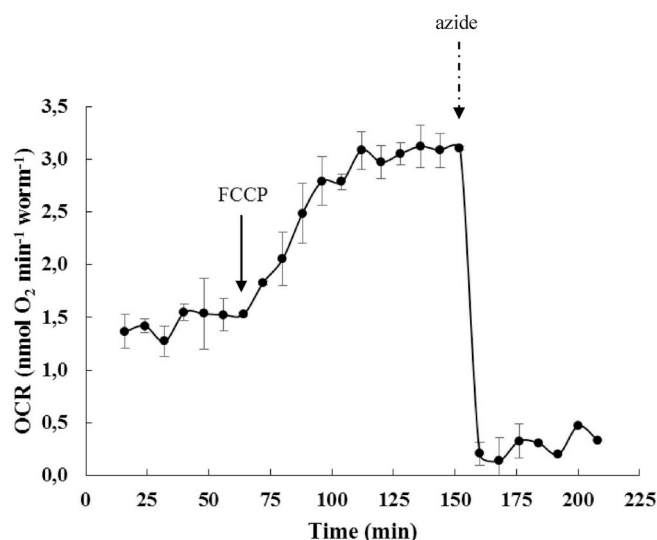


Fig. 7a. Representative data of oxygen consumption rate (OCR, $\text{nmol O}_2 \text{ min}^{-1} \text{ worm}^{-1}$) profile of untreated *Anisakis* L3, showing the initial (i.e. without metabolic modulators), maximum (i.e. after FCCP addition) and azide-insensitive respiration. Data represent the average of 3 experiments. The arrows show the point of FCCP (solid) and sodium azide (dotted) additions.

showed that *Anisakis* L3 OCR responds to the modulators FCCP and azide, so it can be incorporated as an additional measurement for *in vivo* assessment of mitochondrial function (Sánchez-Alonso et al., 2019).

Fig. 7a shows a representative profile of the oxygen consumption rate of untreated *Anisakis* L3 ($n = 3$ runs). The first part of the curve, i.e. prior to addition of the metabolic modulator FCCP, was constant. The addition of FCCP progressively increased the oxygen consumption rate, and the addition of sodium azide led to a drastic decrease of this value. From these values, the basal (i.e. initial minus azide), maximum (FCCP minus azide) and spare (i.e. FCCP minus initial) respiration were obtained, as described elsewhere (Sánchez-Alonso et al., 2019).

Larvae that survived freezing gradually lost mobility upon storage in 0.85% NaCl at 4 °C (Fig. 7b), but after 168 h some larvae were still mobile. Initial respiration rates of larvae that survived freezing measured just after thawing were not significantly different from those of untreated L3 (Fig. 7c) ($P > 0.05$). FCCP respiration decreased abruptly and significantly upon freezing ($P < 0.001$), and then no significant differences were observed during storage at 4 °C. These results suggest that the infective potential of these freeze-surviving larvae cannot be discounted, but they displayed impaired metabolic health as compared to the untreated controls. It was previously shown (Sánchez-Alonso et al., 2019) that larvae that did not survive freezing had significantly lower basal metabolism than the controls, and addition of FCCP had no effect on the respiration rate.

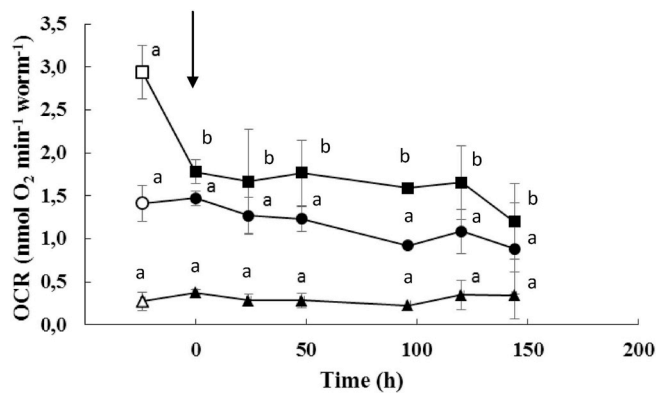


Fig. 7c. OCR ($\text{nmol O}_2 \text{ min}^{-1} \text{ worm}^{-1}$) of surviving *Anisakis* L3 upon freezing at -10°C (before the arrow) and upon storage at $4-5^\circ\text{C}$ for up to 144 h after thawing (after the arrow). Symbols ($\square, \blacksquare, \circ, \bullet, \triangle, \blacktriangle$) represent maximum, initial and azide-insensitive respiration, respectively, before (open) and after (closed) freezing. Error bars show the standard deviation. Different letters (a, b) indicate significant differences during incubation time for each respiration phase of the OCR profile ($n = 4$).

4. Discussion

4.1. Effect of freezing kinetics on *Anisakis* L3 viability

Two aspects of freezing treatment were evaluated in conditions of fixed geometry of the samples: the freezing process itself, which includes the kinetic parameters and final temperatures reached, and the effect of frozen storage, or holding time, up to 24 h after reaching T. The results concerning the effect of T (Figs. 2a and 3, Tables 2–4) and holding time (Fig. 3, Table 4) on infected fish muscle were in line with previously published work, which found that lowering the temperature and increasing the holding time increased the % mortality of *Anisakis* L3 in fish (e.g. Adams, Ton, Wekell, MacKenzie, & Dong, 2005; Deardorff, Raybourne, & Desowitz, 1984; Deardorff & Throm, 1988; Gustafson, 1953; Karl & Leinemann, 1989; Lanfranchi & Sardella, 2010; Oh et al., 2014; Podolska et al., 2019).

It has been suggested that the freezing rate affects *Anisakis* mortality (Deardorff and Throm, 1988; Adams et al., 2005; Sánchez-Alonso et al., 2018), and it may be one of the underlying factors in the differences in mortality found in the literature at a given T. For example, some authors point out that freezing at -20°C and then storing the fish for up to 24 h may not be sufficient to inactivate anisakids (Bier, 1976; Deardorff et al., 1984; Adams et al., 2005), whereas others report that below -20°C larvae may die within 2 min (Gustafson, 1953). We observed that a decrease in the time that elapsed for maximum crystal growth, t_c , significantly ($P < 0.001$) affected *Anisakis* mortality during freezing (Tables 2 and 4, Fig. 2b), confirming our previous results for larvae frozen in saline solution (Sánchez-Alonso et al., 2018). Thus, at sufficiently fast freezing rates (i.e. short t_c or t_f) (Fig. 2b) *Anisakis* larvae become non-viable at high temperatures (i.e. -10°C), which would mean approximately 0.2°C/min for the transition from -1 to -7°C (t_c) or 0.3°C/min if the total time in the freezer is taken into account. Taken together, these results contrast with the suggestion that there seems to be a critical temperature for the mortality of *Anisakis* which is close to -18°C (EFSA, 2010).

Adams et al. (2005) stated the importance of reporting the freezing rate and type of freezer used in studies on *Anisakis* mortality, and we additionally suggest that the freezing rate could be taken into account as a factor in recommendations. Including freezing rate as a parameter in the design of conditions for *Anisakis* mortality would have an additional advantage, since freeze-induced alterations are accompanied by modifications in the characteristics of fish muscle. With slow freezing, formation of extracellular ice crystals occurs, fibres are separated into

groups and myofibrils are compressed, deformed and fragmented in some places (e.g. Grujić, Petrović, Pikula, & Amidžić, 1993). With fast freezing, the effect on fish muscle is minimized, so the freezing rate positively affects the quality of the product.

In industrial processing, freezing times can range from a few minutes to more than a day, depending on the product, geometry and freezing system (Johnston, Nicholson, Roger, & Stroud, 1994). In order to take into account all the freezing kinetics variables, freezing simulations could be useful for giving more precise recommendations to consumers and industry. We performed a simulation in Table 5, and the FAO (Johnston et al., 1994) also provides examples of freezing times for fish products in a large number of set-ups. Nowadays, several software packages are available that could help in the adaptation of recommendations to obtain the final time/temperature/freezing rate/holding time combinations. Thus, in specific scenarios, conditions to meet both safety in terms of larvae mortality and fish quality at short holding times may be achieved.

We also found that the initial temperature before freezing significantly affected the % viability of *Anisakis* L3 (Table 4d and e), and the higher the initial temperature, the higher the survival of these nematodes. Initial temperature entered as an aleatory variable, and it would have to be studied separately as a design factor, since in practice, especially in households, the variability in starting conditions can be very great. The possible protective role of relatively high starting temperatures should be studied in the light of modifications in expression patterns such as heat shock proteins (Chen, Cheng, & Shih, 2015), excretory-secretory proteins (Palomba et al., 2019) or the accumulation of metabolites (i.e. cryoprotectants such as trehalose) (Łopieńska-Biernat et al., 2019) in response to thermal stress, among other factors.

4.2. Antigen and allergen recognition

Part of the allergic symptoms that have been related to consumption of frozen fish may actually derive from larvae that remained alive even after the recommended measures had been followed (Alonso, Guix, Palop, Ros, & Santos, 2016), or, alternatively, to low adherence to recommendations, resulting either from poor interpretation of the recommendations or from the variability of freezing conditions in domestic freezers (Sánchez-Alonso et al., 2018). Furthermore, exposure to *Anisakis* L3 allergens can occur as a result of consumption of dead larvae or pieces of fish that have been in contact with larvae even though the larvae are not present at the time of consumption (Audicana & Kennedy, 2008; Solas et al., 2008). Allergen detection in a subset of samples in which the parasites had been removed from the mince before extraction (Fig. 4c), supports the results of those authors and suggests that allergenic potential, in terms of the possibility of triggering an allergic reaction based on the fact that the allergenic epitopes are conserved as observed by IgE immunoblotting, albeit with low concentrations of allergens, cannot be ruled out.

Allergenic potential can also be measured by quantifying the presence of parasite individual allergens of known clinical and technological relevance. They include the minor excretion/secretion allergen Ani s 4, which is a pepsin- and heat-resistant excretion/secretion allergen whose clinical importance derives from its association with anaphylaxis (Moneo et al., 2005). In order to analyse whether there was a difference in allergen release as a function of any of the kinetic parameters when the larvae were removed from the muscle, parasite antigens and Ani s 4 were analysed in all the samples in Table 1. Rodríguez-Mahillo, González-Muñoz, De las Heras, Tejada, & Moneo (2010) were able to detect Ani s 4 concentrations as low as 2.5 ng/g muscle in frozen, stored, naturally parasitized belly flaps from hake. Assuming $2 \mu\text{g}$ Ani s 4/L3 (Rodríguez-Mahillo, 2006), the maximum detection of this allergen for 10 L3/75 g muscle, if all the Ani s 4 had been released to the media, should be $0.27 \mu\text{g/g}$. Since all the larvae had been removed, for a positive signal in the dot blot analysis

(i.e. > 2.5 ng/g) at least 1% of *Anisakis* would have to be released to the muscle. The fact that no signal was observed with 10 L3/75 g, except for the samples frozen at the highest freezing rate (Fig. 4a), suggests that the antigen release, either by an active or by a passive mechanism, was below this level. Nevertheless, to demonstrate that these allergens retain their biological activity, functional assays such as the basophil activation test would be necessary.

The effect of freezing rate on antigen release observed in Fig. 4a and b could be a consequence of damage or modification of the permeability of the cuticle at faster freezing conditions (manuscript in preparation). According to EFSA (2010), further studies are needed to elucidate whether the changes observed in the cuticle reduce the resistance of the parasites to the action of the gastric enzymes in the gastrointestinal tract. We propose, additionally, to explore whether this resistance might, in turn, be different if the technological conditions, prior to the action of the enzymes, cause a different allergen release, depending on the intensity or the conditions of the treatments applied.

4.3. The effect of *Anisakis* species

Despite the differences in transcriptome expression patterns between *A. simplex* s.s. and *A. pegreffii* (Llorens et al., 2018), the results found in this work suggest that freezing kinetics may have a greater effect than the possible differences in *Anisakis* species within the range studied in this paper, in agreement with previous results (Sánchez-Alonso et al., 2018).

4.4. Characteristics of surviving *Anisakis* L3

Besides mobility, other indicators are being introduced to check the potential infectivity of surviving larvae, using various *in vitro* methods. Among them, the determination of the ability of *Anisakis* L3 to penetrate into agar was long considered an alternative way of studying infection in these nematodes (e.g. Ruitenberg, 1970, p. 138), and its use has recently been adopted to differentiate between *Anisakis* L3 species (Arizono et al., 2012; Suzuki, Murata, Hosaka, & Araki, 2010) or between unfrozen larvae and larvae frozen in water that have survived freezing (Sánchez-Alonso et al., 2018). Similarly, survival in artificial gastric juice (Arizono et al., 2012) and oxygen consumption rates as a measure of the metabolic status of L3 (Sánchez-Alonso et al., 2019) have also been adopted as indicators of potential infectivity.

The impairment of some of the characteristics of surviving *Anisakis* L3 as compared to the untreated controls in terms of penetrative ability (Fig. 5a and b) and survival in gastric juice (Fig. 6) are in agreement with results found previously in isolated larvae (Sánchez-Alonso et al., 2018). The respiration analysis also showed that maximum respiratory ability (Fig. 7c) was impaired in the treated larvae, although their basal respiration was not significantly different from that of the controls, also in agreement with previous results (Sánchez-Alonso et al., 2019). The lack of relationship between the % viability after freezing (Fig. 5b) and the penetrative ability (i.e. 1% on average) suggests that any larva which shows any signs of movement or respiration ability should be treated as equally infective even if the thermal freezing conditions were considered harsh enough.

5. Conclusions

This set of experiments with *Anisakis* L3 infecting hake muscle highlights the importance of a number of kinetic parameters in the survival of *Anisakis* larvae, not only final temperatures or storage times. The fact that high freezing rates are able to kill *Anisakis* larvae faster, and at the same time are preferred to maintain the best eating quality of fish, suggests that technological conditions can be designed to meet both safety in terms of larva mortality and fish quality.

However, faster freezing rates may cause a higher release of antigens to the surrounding medium. The relative importance of this in

terms of recommendations for sensitized patients needs to be examined in the light of further studies where the effect of damage to the cuticle on resistance to gastric enzymes is addressed.

The characteristics of the surviving larvae after freezing (i.e. penetrative ability in solid agar, survival in gastric juices and saline solutions, basal and FCCP respiration ability) were impaired as compared to the controls, but the lack of relationship between the % survival due to freezing conditions and the other indicators suggests that any larva which shows any sign of movement after freezing should be treated as equally infective.

Funding

This work was financed by the European Union's Seventh Framework Programme for Research, Technological Development and Demonstration under Grant Agreement 312068, EU PARASITE and the project ANIRISK (AGL2015-68248-C2) is cofunded by the Spanish Ministry of Economy and Competitiveness (MINECO) and FEDER funds from EU.

CRediT authorship contribution statement

Isabel Sánchez-Alonso: Conceptualization, Methodology, Validation, Investigation, Visualization, Writing - original draft. **Noelia Carballeda-Sangiao:** Methodology, Investigation. **Miguel González-Muñoz:** Conceptualization, Methodology, Resources, Writing - review & editing, Funding acquisition. **Alfonso Navas:** Conceptualization, Methodology, Writing - review & editing. **Susana C. Arcos:** Investigation, Methodology. **Angel Mendizábal:** Resources, Methodology. **Francisco Cuesta:** Methodology, Investigation, Formal analysis, Validation, Writing - review & editing. **Mercedes Careche:** Conceptualization, Methodology, Validation, Formal analysis, Writing - original draft, Visualization, Supervision, Resources, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodcont.2020.107373>.

References

- Adams, A. M., Ton, M. N., Wekell, M. M., MacKenzie, A. P., & Dong, F. M. (2005). Survival of *Anisakis simplex* in arrowtooth flounder (*Atheresthes stomia*) during frozen storage. *Journal of Food Protection*, 68(7), 1441–1446.
- Alonso, E., Guix, S., Palop, A., Ros, G., & Santos, J. A. (2016). Informe del Comité Científico de la Agencia Española de Consumo, Seguridad Alimentaria y Nutrición (AECOSAN) en relación a la alergia a *Anisakis*. *Revista del Comité Científico de la AECOSAN*, 24, 23–33.
- AOAC (1995). *Official methods of analysis of the association of official agricultural chemists*. Gaithersburg, Maryland, USA: AOAC International.
- Arizono, N., Yamada, M., Tegoshi, T., & Yoshikawa, M. (2012). *Anisakis simplex sensu stricto* and *Anisakis pegreffii*: Biological characteristics and pathogenetic potential in human anisakiasis. *Foodborne Pathogens & Disease*, 9(6), 517–521.
- ASHRAE Handbook—Refrigeration (SI Edition) (2010b). *Cooling and freezing times of foods*. Atlanta, GA: American Society of Heating, Refrigerating and Air-conditioning Engineers, Inc20.7–20.15 (chapter 20).
- ASHRAE Handbook—Refrigeration (SI Edition) (2010a). *Thermal properties of foods*. Atlanta, GA: American Society of Heating, Refrigerating and Air-conditioning Engineers, Inc19.1–19.2 (chapter 19).
- Audicana, M. T., & Kennedy, M. W. (2008). *Anisakis simplex*: From obscure infectious worm to inducer of immune hypersensitivity. *Clinical Microbiology Reviews*, 21(2), 360–379.
- Bier, J. W. (1976). Experimental anisakiasis - cultivation and temperature tolerance determinations. *Journal of Milk and Food Technology*, 39(2), 132–137.

- Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology*, 37(8), 911–917.
- Carballeda-Sangiao, N., Olivares, F., Rodríguez-Mahillo, A. I., Careche, M., Tejada, M., Moneo, I., et al. (2014). Identification of autoclave-resistant *Anisakis simplex* allergens. *Journal of Food Protection*, 77(4), 605–609.
- Carballeda-Sangiao, N., Rodríguez-Mahillo, A. I., Careche, M., Navas, A., Caballero, T., Dominguez-Ortega, J., et al. (2016). Ani s 11-Like protein is a pepsin- and heat-resistant major allergen of *Anisakis* spp and a valuable tool for *Anisakis* allergy component-resolved diagnosis. *International Archives of Allergy and Immunology*, 169(2), 108–112.
- Chen, H.-Y., Cheng, Y.-S., & Shih, H.-H. (2015). Expression patterns and structural modelling of Hsp70 and Hsp90 in a fish-borne zoonotic nematode *Anisakis pegreffii*. *Veterinary Parasitology*, 212(3–4), 281–291. <https://doi.org/10.1016/j.vetpar.2015.07.006>.
- Choi, Y., & Okos, M. R. (1986). Effects of temperature and composition on the thermal properties of foods. In L. Maguer, & P. Jelen (Vol. Eds.), *Food engineering and process applications: Transport phenomena: Vol. 1*, (pp. 93–101). New York: Elsevier, Applied Science Publishers 1986.
- Cleland, D. J., Cleland, A. C., & Earle, R. L. (Cleland, & Earle, 1987a). Prediction of freezing and thawing times for multi-dimensional shapes by simple formulae. I: Regular shapes. *International Journal of Refrigeration*, 10(3), 156–164.
- Cleland, D. J., Cleland, A. C., & Earle, R. L. (Cleland, & Earle, 1987b). Prediction of freezing and thawing times for multi-dimensional shapes by simple formulae. II: Irregular shapes. *International Journal of Refrigeration*, 10(4), 234–240.
- Cuesta, F. J., & Lamúa, M. (1995). Asymptotic modelling of the transient regime in heat conduction in solids with general geometry. *Journal of Food Engineering*, 24(3), 295–320.
- Cuesta, F. J., Lamúa, M., & Alique, R. (2012). A new exact numerical series for the determination of the Biot number: Application for the inverse estimation of the surface heat transfer coefficient in food processing. *International Journal of Heat and Mass Transfer*, 55(15–16), 4053–4062.
- Cuesta, F. J., Lamúa, M., & Moreno, J. (1990). Graphical calculation of half-cooling times. *International Journal of Refrigeration*, 13(5), 317–324.
- D'Amelio, S., Mathiopoulos, K. D., Santos, C. P., Pugachev, O. N., Webb, S. C., Picanco, M., et al. (2000). Genetic markers in ribosomal DNA for the identification of members of the genus *Anisakis* (Nematoda: Ascaridoidea) defined by polymerase chain reaction-based restriction fragment length polymorphism. *International Journal for Parasitology*, 30(2), 223–226.
- Deardorff, T. L., Raybourne, R. B., & Desowitz, R. S. (1984). Behavior and viability of third-stage larvae of *Terranova* sp. (type HA) and *Anisakis simplex* (type I) under coolant conditions. *Journal of Food Protection*, 47(1), 49–52.
- Deardorff, T. L., & Throm, R. (1988). Commercial blast-freezing of 3rd-stage *Anisakis simplex* larvae encapsulated in salmon and rockfish. *The Journal of Parasitology*, 74(4), 600–603.
- EFSA Panel on Biological Hazards (BIOHAZ) (2010). Scientific Opinion on risk assessment of parasites in fishery products. *EFSA Journal*, 8(4), 1543. [91 pp] www.efsa.europa.eu.
- EU Directive 2010/63/UE of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes. <https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2011:327:0039:0041:EN:PDF>.
- Evans, J. A., Foster, A. M., & Brown, T. (2014). Temperature control in domestic refrigerators and freezers. *3rd IIR international conference on sustainability and the cold chain, London. Refrigeration science and Technology* (pp. 142–149). 2014(1).
- Grujić, R., Petrović, L., Pikula, B., & Amidžić, L. (1993). Definition of the optimum freezing rate-1. Investigation of structure and ultrastructure of beef *M. longissimus dorsi* frozen at different freezing rates. *Meat Science*, 33(3), 301–318.
- Gustafson, P. V. (1953). The effect of freezing on encysted *Anisakis* larvae. *The Journal of Parasitology*, 39(6), 585–588.
- Johnston, W. A., Nicholson, F. J., Roger, A., & Stroud, G. D. (1994). *Freezing and refrigerated storage in fisheries. FAO fisheries technical paper – 340*. Rome: Food and Agriculture Organization of the United Nations. <http://www.fao.org/3/v3630e/V3630E06.htm#5.2%20Calculation%20of%20freezing%20times/> accessed 28 January 2020.
- Karl, H., & Leinemann, M. (1989). Viability of larval nematodes (*Anisakis* sp) in frozen herrings. *Archiv fuer Lebensmittelhygiene*, 40(1), 14–16.
- Karl, H., & Leinemann, M. (1993). A fast and quantitative detection method for nematodes in fish fillets and fishery products. *Archiv für Lebensmittelhygiene*, 44(5), 105–128.
- Lanfranchi, A. L., & Sardella, N. H. (2010). *Anisakis* survival after microwaving, freezing and salting fish from Argentina. *Food Science and Technology Research*, 16(5), 499–504.
- Llorens, C., Arcos, S. C., Robertson, L., Ramos, R., Futami, R., Soriano-Salvador, B., et al. (2018). Functional insights into the infective larval stage of *Anisakis simplex* s.s., *Anisakis pegreffii* and their hybrids based on gene expression patterns. *BMC Genomics*, 19, 592. <https://doi.org/10.1186/s12864-018-4970-9>.
- Łopieńska-Biernat, E., Stryński, R., Dmitryjuk, M., & Wasilewska, B. (2019). Infective larvae of *Anisakis simplex* (Nematoda) accumulate trehalose and glycogen in response to starvation and temperature stress. *Biology Open*, 8(3), <https://doi.org/10.1242/bio.040014>.
- Mattiucci, S., Accerra, V., Paoletti, M., Cipriani, P., Levsen, A., Webb, S. C., et al. (2016). No more time to stay 'single' in the detection of *Anisakis pegreffii*, *A. simplex* (s.s.) and hybridization events between them: A multi-marker nuclear genotyping approach. *Parasitology*, 143(8), 998–1011.
- Mattiucci, S., Cipriani, P., Levsen, A., Paoletti, M., & Nascetti, G. (2018). Molecular epidemiology of *Anisakis* and *anisakiasis*: An ecological and evolutionary road map. In D. Rollinson, & J. R. Stothard (Vol. Eds.), *Advances in parasitology: Vol. 99*, (pp. 93–263). <https://doi.org/10.1016/bs.apar.2017.12.001>.
- Mattiucci, S., Colantoni, A., Crisafi, B., Mori-Ubalchini, F., Caponi, L., Fazio, P., et al. (2017). IgE sensitization to *Anisakis pegreffii* in Italy: Comparison of two methods for the diagnosis of allergic anisakiasis. *Parasite Immunology*, 39(7), 1–10. <https://doi.org/10.1111/pim.12440>.
- Moneo, I., Caballero, M. L., Gonzalez-Muñoz, M., Rodríguez-Mahillo, A. I., Rodríguez-Perez, R., & Silva, A. (2005). Isolation of a heat-resistant allergen from the fish parasite *Anisakis simplex*. *Parasitology Research*, 96(5), 285–289.
- Moneo, I., Carballeda-Sangiao, N., & González-Muñoz, M. (2017). New perspectives on the diagnosis of allergy to *Anisakis* spp. *Current Allergy and Asthma Reports*, 17(5), 27. <https://doi.org/10.1007/s11882-017-0698-x>.
- Nieuwenhuizen, N. E., & Lopata, A. L. (2014). Allergic reactions to *Anisakis* found in fish. *Current Allergy and Asthma Reports*, 14(8), 455. <https://doi.org/10.1007/s11882-014-0455-3>.
- Oh, S. R., Kim, T. I., Hong, S. J., Ju, I. S., Lee, S. H., Kim, S. H., et al. (2014). Inactivation of *Anisakis* larvae in salt-fermented squid and pollock tripe by freezing, salting, and combined treatment with chlorine and ultrasound. *Food Control*, 40, 46–49.
- Palomba, M. L., Paoletti, M., Colantoni, A., Ruggeri, G., Nascetti, G., & Mattiucci, S. (2019). Gene expression profiles of antigenic proteins of third stage larvae of the zoonotic nematode *Anisakis pegreffii* in response to temperature conditions. *Parasite*, 26, 52. <https://doi.org/10.1051/parasite/2019055>.
- Podolska, M., Pawlikowski, B., Nadolna-Aityn, K., Pawlak, J., Komar-Szymczak, K., & Szostakowska, B. (2019). How effective is freezing at killing *Anisakis simplex*, *Pseudoterranova* *krabbei*, and *P. decipiens* larvae? An experimental evaluation of time-temperature conditions. *Parasitology Research*, 118(7), 2139–2147.
- Rodríguez-Mahillo, A. I. (2006). *Clonación y caracterización de alérgenos recombinantes de Anisakis simplex. Valoración de su utilidad en el diagnóstico de Anisakiasis* PhD Thesis. Universidad Autónoma de Madrid, Faculty of Sciences.
- Rodríguez-Mahillo, A. I., González-Muñoz, M., De las Heras, C., Tejada, M., & Moneo, I. (2010). Quantification of *Anisakis simplex* allergens in fresh, long-term frozen, and cooked fish muscle. *Foodborne Pathogens & Disease*, 7(8), 967–973.
- Rodríguez-Mahillo, A. I., Gonzalez-Muñoz, M., Gomez-Aguado, F., Rodríguez-Perez, R., Corcuera, M. T., Caballero, M. L., et al. (2007). Cloning and characterisation of the *Anisakis simplex* allergen Ani s 4 as a cysteine-protease inhibitor. *International Journal for Parasitology*, 37(8–9), 907–917.
- Ruiterberg, E. J. (1970). *Anisakiasis. Pathogenesis, serodiagnosis and prevention*. Vet. Fac. Univ. of Utrecht. Netherlands, Rijksuniversiteit Utrecht: Elinkwijk.
- Sánchez-Alonso, I., Carballeda-Sangiao, N., González-Muñoz, M., Navas, A., Arcos, S., Mendizábal, A., et al. (2018). Pathogenic potential of *Anisakis* L3 after freezing in domestic freezers. *Food Control*, 84, 61–69.
- Sánchez-Alonso, I., Navas, A., Arcos, S. C., González-Muñoz, M., Carballeda-Sangiao, N., & Careche, M. (2019). Respiratory analysis as a tool to detect physiological changes in *Anisakis* larvae subjected to stress. *Parasitology Research*, 118(4), 1127–1135.
- Shimamura, Y., Muwanwella, N., Chandran, S., Kandel, G., & Marcon, N. (2016). Common symptoms from an uncommon infection: Gastrointestinal anisakiasis. *Chinese Journal of Gastroenterology and Hepatology*. <https://doi.org/10.1155/2016/5176502> ID 5176502.
- Solas, M. T., García, M. L., Rodríguez-Mahillo, A. I., Gonzalez-Muñoz, M., De las Heras, C., & Tejada, M. (2008). *Anisakis* antigens detected in fish muscle infested with *Anisakis simplex* L3. *Journal of Food Protection*, 71(6), 1273–1276.
- Suzuki, J., Murata, R., Hosaka, M., & Araki, J. (2010). Risk factors for human *Anisakis* infection and association between the geographic origins of *Scomber japonicus* and *anisakid* nematodes. *International Journal of Food Microbiology*, 137(1), 88–93.
- Ventura, M. T., Napolitano, S., Menga, R., Cecere, R., & Asero, R. (2013). *Anisakis simplex* hypersensitivity is associated with chronic urticaria in endemic areas. *International Archives of Allergy and Immunology*, 160(3), 297–300.
- Vidacek, S., De las Heras, C., Solas, M. T., García, M., Mendizábal, A., & Tejada, M. (2011). Viability and antigenicity of *Anisakis simplex* after conventional and microwave heating at fixed temperatures. *Journal of Food Protection*, 74(12), 2119–2126.
- Vidacek, S., De las Heras, C., Solas, M. T., Mendizábal, A., Rodríguez-Mahillo, A. I., González-Muñoz, M., et al. (2009). *Anisakis simplex* allergens remain active after conventional or microwave heating and pepsin treatments of chilled and frozen L3 larvae. *Journal of the Science of Food and Agriculture*, 89(12), 1997–2002.
- Vidacek, S., De las Heras, C., Solas, M. T., Mendizábal, A., Rodríguez-Mahillo, A. I., & Tejada, M. (2010). Antigenicity and viability of *Anisakis* larvae infesting hake heated at different time-temperature conditions. *Journal of Food Protection*, 73(1), 62–68.
- Wharton, D. A., & Aalders, O. (2002). The response of *Anisakis* larvae to freezing. *Journal of Helminthology*, 76(4), 363–368.

Supplementary Table. *Anisakis* L3 species used in the various experiments.

Batch	<i>A. simplex</i> s.s. %	<i>A. pegreffii</i> %	Heterozygote genotypes %
A	73	10	17
B	71	21	8
C	91	9	0
D	100	0	0
E	100	0	0
F	83	17	0
G	82	6	11
H	93	7	0
I		n.a.	
J	100	0	0
K	98	0	2
L	94	2	4
M	96	0	4
N		n.a.	
O	98	2	0

n.a. Data not available