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

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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 to 30 L3 per 10 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.

Keywords

Anisakis, freezing, allergen, agar penetrative ability, survival in gastric juice, respiratory analysis, viability, fish muscle

Highlights

- Other kinetic parameters besides T or storage time affect *Anisakis* L3 survival
- Fast freezing rates are able to kill *Anisakis* larvae even at –10 °C
- Fast freezing rates may cause a higher release of antigens
- Biological or physiological parameters are impaired in freeze-surviving L3

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; Nieuwenhuizen & Lopata, 2014; Moneo et al., 2017; Mattiucci et al., 2018). Exposure to the parasite is mainly characterized by gastrointestinal manifestations of epigastralgia, nausea, abdominal pain and diarrhoea (Shimamura et al., 2016), and also by a characteristic allergic reaction such as IgE-mediated hypersensitivity, angioedema, urticaria or anaphylaxis (Ventura et al., 2013; Moneo et al., 2017; Mattiucci et al., 2017).

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.

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 et al., 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 (Moneo et al., 2005; Audicana & Kennedy, 2008; Vidacek et al., 2009, 2010, 2011; Carballeda-Sangiao et al., 2014; Nieuwenhuizen & Lopata, 2014). It is widely accepted that a previous infection with live larvae is needed to elicit an allergic

105 reaction. Thus any treatment that inactivates *Anisakis* L3 is also crucial as a preventive
106 measure to avoid sensitization (EFSA, 2010), but there is no information about the
107 effects of different freezing conditions on allergen release to the medium.
108
109 Overall, it is important to study the pathogenic potential of *Anisakis* L3 from a broader
110 perspective embracing a wider number of factors as well as indicators. This would help
111 to define more precisely the point at which the parasites are no longer viable/infective
112 without compromising the quality of the fish products. It would also aid in gathering
113 information on the interaction of freezing conditions and allergenic potential.
114
115 In this work, the effect of freezing rate, temperature and storage time of *Anisakis* L3
116 infecting fish muscle in controlled conditions were studied in terms of larval viability
117 and parasite antigen release. *Anisakis* L3 species variability was also considered as an
118 aleatory variable. In order to determine the potential infectivity of larvae that might
119 accidentally survive a freezing process, their agar penetration ability, survival in
120 artificial gastric juice or saline solution, and respiratory control were analysed in
121 infected hake muscle in conditions that provided various survival rates.
122

123 2. Materials and Methods

124 2.1. Raw material

125 *2.1.1. Fish muscle*

126 Hake (*Merluccius merluccius*), Atlantic horse mackerel (*Trachurus trachurus*) and
127 mackerel (*Scomber scombrus*) were caught in the Atlantic Northeast fishing area (FAO
128 area 27, subareas 27.7, 27.8 and 27.6.a, respectively); yellow horse mackerel (*Caranx*
129 *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 and 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′-GTCGAATTCGTAGGTGAACCTGCGGAAG GATCA-3′ and reverse primer B 5′-GCCGGATCC GAATCCTGGTTAGTTTCTTTTCCT-3′ (D'Amelio et al., 2000) were used in reactions containing 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 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 hours at the corresponding temperatures. After freezing and/or frozen storage, samples were allowed to thaw overnight at 4–5 °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_t, total freezing time) is the sum of t_1, t_c and t_3. Total freezing and thawing time ($t_{freeze/thaw}$) 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, 2010a) 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 et al. (2012). The total freezing time was calculated by estimating the prefreezing and freezing times separately. The time required to reach the starting freezing point was calculated according to Cuesta et al., (1990) and Cuesta & Lamúa, (1995). Finally, following the method of Cleland (Cleland et al., 1987a,b; ASHRAE 2010b), freezing

205 times for –10 °C and –20 °C were calculated for the case of thicker samples (i.e. 22 mm
206 thickness).
207
208 **2.5. Recovery of larvae**
209 Larvae were recovered from the mince after visual inspection, and in some cases with
210 the aid of UV light (366 nm) (Karl & Leinemann (1993); Vidaček et al., 2010), and
211 were then placed in 0.85% NaCl for further analysis. The remaining mince was stored at
212 –30 °C until use for the extraction of parasite antigens.
213
214 **2.6. Viability**
215 Movement of the larvae spontaneously or in response to stimulation with tweezers was
216 observed according to EFSA (2010). Mobility of frozen and thawed larvae was assessed
217 just after thawing. For those that did not show any movement just after thawing, we
218 checked whether they moved after incubation with 0.85% NaCl for 10 min at 37 °C or
219 after 4 h and 24 h at room temperature. The larvae without mobility were then
220 considered non-viable. Viability was expressed as percentage of mobile L3.
221
222 **2.7. Agar penetration test**
223 This test was performed according to Arizono et al. (2012), as modified by Sanchez-
224 Alonso et al. (2018). Mobile larvae were placed in 100 mL jars with 10 mL of artificial
225 gastric juice (pH 1.8) on top of ~57 mL of solid agar (0.75% agar and 0.9% NaCl). Each
226 jar was incubated at 37 °C with 5% of CO_2, and the number of larvae that penetrated
227 into the solid agar was monitored after 24 h. Data were expressed as the percentage of
228 L3 able to penetrate agar.
229

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 2,000 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 hours.

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 µM 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 nmol O_2/min/worm.

255 **2.10. Antigenicity and allergenicity**

256 *2.10.1. Quantification of* A. simplex *antigens in fish muscle*

257 Ten g of thawed mince was homogenized (24,000 rpm, 30 s, room temperature) in 30
258 mM NaCl, 10 mM Tris-HCl, pH 6.8, 1:3 (w:v) using an Ultra-Turrax T25 (Janke &
259 Kunkel IKA-Labortechnik, Staufen, Germany). The resulting mixture was sonicated (17
260 W, 30 s) in a Microson ultrasonic cell disruptor (Misonix XL-2000 Series, Farmingdale,
261 NY, USA), maintaining the temperature of the mixture at ≤10 °C. It was then incubated
262 with orbital agitation (Tube Rotator Model SB3, Stuart, Barloworld Scientific Ltd., UK)
263 at 40 rpm for 15 min at room temperature and centrifuged (Heraeus Multifuge 3L-R
264 centrifuge) at 5,000 × *g* for 30 min. The supernatant was stored and parasite antigens
265 were further extracted from the pellet by repeating the homogenization, stirring and
266 centrifugation steps twice in the same conditions as above. The resulting pellet was
267 discarded, and the supernatants were freeze-dried (Christ Beta 2-8 LDplus, Germany)
268 and stored at –80 °C. When needed, the lyophilized samples were resuspended in 1 mL
269 of distilled water.

270

271 The antigenicity of the *Anisakis* L3 was studied by dot blot using rabbit anti-*A. simplex*
272 crude extract polyclonal antibody and rabbit anti-recombinant (r)Ani s 4 polyclonal
273 antisera (Rodríguez-Mahillo et al., 2007; Carballeda-Sangiao et al., 2016). Three
274 microlitres of the extracts was dotted in duplicate onto a nitrocellulose membrane and
275 air dried. The membrane was rehydrated with PBS (137 mM NaCl, 2.7 mM KCl, 10
276 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4). After blocking with 3% Nonidet P-40 (NP-
277 40) in PBS for 1 hour, the membrane was incubated with rabbit anti-*Anisakis simplex*
278 antigens, anti-Ani s 4, or pre-immune antisera (1/10000 dilution in 128 mM NaCl, 9
279 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 (Rodriguez-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.

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 Table 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.

328 *2.11.2. Survival of* Anisakis *L3 from other species and specimen thicknesses*

329 Four different trials were performed with minced hake, horse mackerel and mackerel
330 experimentally infected with 20 L3 per Petri dish, with three Petri dish thicknesses (11,
331 22 and 33 mm), and a total of 480 treated and 120 control L3 was used.

332

333 *2.11.3. Study of the characteristics of surviving* Anisakis *L3*

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

337

338 The characteristics of surviving *Anisakis* L3 after freezing, with a survival percentage of
339 50% (~S_{50}), were studied in terms of penetration into agar in three independent
340 experiments. Petri dishes (10 or 20 L3/mince) were parasitized with a total of 410
341 *Anisakis* L3 and subjected to freezing. The agar penetration ability of the surviving
342 larvae (i.e. 196 L3) was compared to that of untreated controls (3 experiments, 160 L3).
343 An agar penetration test was also performed in the trials studied in section *2.11.2*.

344

345 Survival of *Anisakis* L3 in artificial gastric juice after freezing (~S_{50}) was studied in two
346 experiments (158 L3) and compared to that of untreated larvae (102 L3).
347 Respiration after freezing and thawing and survival at refrigerated temperatures after
348 thawing were studied in four additional experiments, using one batch of *Anisakis* L3 and
349 four batches of hake (3.0 Kg/batch). The same freezing conditions as before were used,
350 but in this set of experiments 20 L3 were placed in each Petri dish instead of 10. Six
351 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

377 regression analysis was performed with freezing (i.e. $T_{initial}$, T, t_1, t_c, t_3, t_t) or storage
378 ($t_{freeze/thaw}$) parameters as independent variables and % viability as the dependent
379 variable. The Durbin-Watson test was used to check for independence of residuals.
380
381 The comparison of the control and frozen-thawed larvae in terms of agar penetration
382 test or oxygen consumption rate was analysed by a t-test of independent samples, using
383 the Levene test to analyse the equality of variances. The differences with regard to
384 survival in gastric juice were analysed by the Kaplan-Meier test, using the mortality per
385 period as a weight. The significance level was established at 5%. IBM SPSS Statistics
386 software V24.0.0.1 for Windows (IBM Corp., Armonk, NY, USA) was used for
387 statistical analysis.
388
389 **2.13. UNE-EN ISO 9001 certification**
390 The Institute of Food Science, Technology and Nutrition (ICTAN-CSIC) has been
391 certified under UNE-EN ISO 9001 for "Management and execution of research projects
392 and contracts in the area of Food Science and Technology and Nutrition" (certificate
393 number ER-0366/2015).
394
395 **2.14. Ethics statement**
396 Ethics approval is not applicable according to EU Directive 2010/63/EU of the
397 European Parliament and of the Council of 22 September 2010 on the protection of
398 animals used for scientific purposes, since the hakes used in this study were caught for
399 commercial purposes, more specifically for human consumption, and not for research
400 purposes, so the capture of the animals was not part of the experimental activity
401 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

Figure 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, 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 minutes. The proximate composition of the various batches of hake ranged from 79.0 to 81.3% moisture, 17.5 to 20.0% protein, 0.8 to 1.2% ash and 0.9 to 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 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_3 (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^2adj=0.767, $P<0.001$). However, when t_t was

452 included, the best model was achieved with T and that variable (R^2adj=0.836, P<0.001)
453 (**Table 4, rows a and b**). This may be because t_t carries part of the information related
454 to the final freezing temperature.
455
456 According to the known effect of lowering T on the % survival of *Anisakis* L3, **figure**
457 **2a** reflects that the higher the final temperature, the higher the variance. In order to
458 display the effect of t_c on the area of maximum variance, viability data at –10 °C were
459 assigned to three ranges of t_c [slow (125–177 min), intermediate (90–120 min), and fast
460 (10–40 min)] (**Figure 2b**). It can be seen that the percentage of viable larvae with fast
461 freezing rates can be 0 even at freezing temperatures as high as –10 °C. The same trend
462 was found when viability was plotted against t_t (not shown).
463
464 *3.1.3. Effect of storage time and temperature on viability*
465 The percentage of mobile *Anisakis* L3 at a fixed freezing rate (i.e. t_c 149±5 min) as a
466 function of storage time and T (**Figure 3)** decreased significantly both with lowering the
467 final temperature and with prolonging the storage time. After 12 hours at –10 °C, –15
468 °C, –20 °C the percentage of viable larvae decreased to values close to half of the initial
469 ones, and after 24 hours no viable larvae were recovered except in the case of the
470 samples stored at –15 °C, where one larva out of 19 showed signs of mobility. Stepwise
471 regression analysis showed that both T and storage time were significant in the model
472 (**Table 4, row c**).
473
474 **3.2. Survival of *Anisakis* L3 in fish muscle from other fish species and thicknesses**
475 One of the aspects that may affect the freezing kinetics is the composition of the food to
476 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 Ø 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, 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 to 22.9% protein, 1.2 to 1.5% ash and 2.0 to 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

501 additional factor in the model, and there was a trend towards a higher % survival of
502 *Anisakis* L3 at higher $T_{initial}$ values ($T_{initial}$ 14.6±3.6 °C; range 4.9–20.9 °C).
503
504 When samples that had been frozen and stored for up to 24 h were included, total
505 freezing/thawing time ($t_{freeze/thaw}$) was also significant in the model (**Table 4e**). No effect
506 of fish species was found, but the small sample size of the mince with fatty species does
507 not allow being conclusive in this respect.
508
509 **3.3. Parasite antigen release**
510 No parasite antigens or allergens (i.e. Ani s 4) were detected by dot blot in the extracts
511 from the minced fish after removal of all *Anisakis* larvae from the muscle. Western
512 blotting revealed with anti rAni s 4 and chemiluminiscence showed that this allergen
513 was only detected in the samples that had been frozen at the fastest freezing time (i.e. 31
514 min) (**Figure 4a**). In a separate experiment, hake was parasitized with a higher amount
515 of L3 (30 L3/10 g) and two freezing rate conditions were assayed (t_c=139±11.6 and
516 16±1.2 min, respectively). **Figure 4b** shows parasite antigen release to muscle after
517 removal of *Anisakis* L3, and significant differences between the two freezing rates were
518 found by dot blot. In addition, western blotting revealed with anti rAni s 4 and
519 chemiluminiscence also showed striking differences in the recognition of this allergen
520 between these two groups of samples, thus suggesting an effect of freezing rate on
521 parasite antigen release.
522
523 Nevertheless, when the extracts from mince that had contained the lowest numbers of
524 *Anisakis* (i.e. 10 L3/75 g) were revealed with patients' sera, all the western blots showed
525 IgE reacting proteins with molecular weights ranging from 10 to 80 kDa (**Figure 4c**).

526

527 **3.4. Effect of *Anisakis* L3 species on viability**

528 The *Anisakis* L3 used were found to be mainly *A. simplex s.s.* (71–100%), coexisting
529 with *A. pegreffii* (0–21%) and with heterozygote genotypes (0–17%) (**Table S1**). In
530 order to study viability as a function of *Anisakis* species, a subset of data corresponding
531 to the experiments where the final temperature was set at –10 °C (i.e. hake in Petri
532 dishes with 11 mm thickness) was used, in order to avoid redundancy among taxonomic
533 units (*A. simplex*, *A. pegreffii*, heterozygote genotypes). One-way ANOVA as a function
534 of temperature and either *Anisakis simplex s.s.*, *A. pegreffii* or the heterozygote
535 genotypes as cofactor (**Table 6**) showed a small correlation between the proportion of
536 the heterozygote genotypes and viability after freezing ($P<0.05$).

537

538 **3.5. Characteristics of surviving *Anisakis* L3**

539 *3.5.1. Penetrative ability*

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

547

548 **Figure 5b** shows survival percentages ranging from 10 to 61% for a series of
549 experiments with different species and geometries. No relation was observed between
550 survival percentage and penetrative ability. The pooled data (885 L3, 322 surviving)

551 showed that about 1% of them (n=8) were able to penetrate into solid agar, suggesting
552 that potentially any surviving larva may be equally infective regardless of the survival
553 percentage in the trial.
554
555 *3.5.2. Behaviour of surviving* Anisakis *L3 in artificial gastric juice*
556 Survival in gastric juice was also impaired in frozen and thawed L3 as compared to the
557 controls (**Figure 6**), and the Kaplan-Meier test showed that significant differences were
558 found between untreated L3 and those that survived freezing. However, after 68 hours
559 11% of treated L3 were still surviving in this medium. These results confirmed the ones
560 obtained in isolated larvae frozen in saline solution (Sánchez-Alonso et al., 2019).
561
562 *3.5.3. Oxygen consumption rate of surviving* Anisakis *L3 after thawing and during*
563 *storage at 4 °C*
564 Since alterations in oxygen consumption rate are considered an indicator of
565 mitochondrial dysfunction, another characteristic studied was the respiration of *Anisakis*
566 L3 subjected to freezing. We previously showed that *Anisakis* L3 OCR responds to the
567 modulators FCCP and azide, so it can be incorporated as an additional measurement for
568 *in vivo* assessment of mitochondrial function (Sánchez-Alonso et al., 2019).
569
570 **Figure 7a** shows a representative profile of the oxygen consumption rate of untreated
571 *Anisakis* L3 (n=3 runs). The first part of the curve, i.e. prior to addition of the metabolic
572 modulator FCCP, was constant. The addition of FCCP progressively increased the
573 oxygen consumption rate, and the addition of sodium azide led to a drastic decrease of
574 this value. From these values, the basal (i.e. initial minus azide), maximum (FCCP

575 minus azide) and spare (i.e. FCCP minus initial) respiration were obtained, as described
576 elsewhere (Sánchez-Alonso et al., 2019).
577
578 Larvae that survived freezing gradually lost mobility upon storage in 0.85% NaCl at 4
579 °C (**Figure 7b**), but after 168 h some larvae were still mobile. Initial respiration rates of
580 larvae that survived freezing measured just after thawing were not significantly different
581 from those of untreated L3 (**Figure 7c**) ($P>0.05$). FCCP respiration decreased abruptly
582 and significantly upon freezing ($P<0.001$), and then no significant differences were
583 observed during storage at 4 °C. These results suggest that the infective potential of
584 these freeze-surviving larvae cannot be discounted, but they displayed impaired
585 metabolic health as compared to the untreated controls. It was previously shown
586 (Sánchez-Alonso et al., 2019) that larvae that did not survive freezing had significantly
587 lower basal metabolism than the controls, and addition of FCCP had no effect on the
588 respiration rate.
589
590 **4. Discussion**
591 **4.1. Effect of freezing kinetics on *Anisakis* L3 viability**
592 Two aspects of freezing treatment were evaluated in conditions of fixed geometry of the
593 samples: the freezing process itself, which includes the kinetic parameters and final
594 temperatures reached, and the effect of frozen storage, or holding time, up to 24 hours
595 after reaching T. The results concerning the effect of T (**Figures 2a**, **3**, **Tables 2**, **3**, **4**)
596 and holding time (**Figure 3**, **Table 4**) on infected fish muscle were in line with
597 previously published work, which found that lowering the temperature and increasing
598 the holding time increased the % mortality of *Anisakis* L3 in fish (e.g. Gustafson, 1953;

599 Deardorff et al., 1984; Deardorff & Throm, 1988; Karl & Leinemann, 1989; Adams et
600 al., 2005; Lanfranchi & Sardella, 2010; Oh et al., 2014; Podolska et al., 2019).
601
602 It has been suggested that the freezing rate affects *Anisakis* mortality (Deardorff &
603 Throm, 1988; Adams et al., 2005; Sánchez-Alonso et al., 2018), and it may be one of
604 the underlying factors in the differences in mortality found in the literature at a given T.
605 For example, some authors point out that freezing at –20 °C and then storing the fish for
606 up to 24 h may not be sufficient to inactivate anisakids (Bier, 1976; Deardorff et al.,
607 1984; Adams et al., 2005), whereas others report that below –20 °C larvae may die
608 within 2 minutes (Gustafson, 1953). We observed that a decrease in the time that
609 elapsed for maximum crystal growth, t_c, significantly ($P<0.001$) affected *Anisakis*
610 mortality during freezing (Tables 2, 4, **Figure 2b**), confirming our previous results for
611 larvae frozen in saline solution (Sánchez-Alonso et al., 2018). Thus, at sufficiently fast
612 freezing rates (i.e. short t_c or t_t) (**Figure 2b**) *Anisakis* larvae become non-viable at high
613 temperatures (i.e. –10 °C), which would mean approximately 0.2 °C/min for the
614 transition from –1 to –7 °C (t_c) or 0.3 °C/min if the total time in the freezer is taken into
615 account. Taken together, these results contrast with the suggestion that there seems to be
616 a critical temperature for the mortality of *Anisakis* which is close to –18 °C (EFSA,
617 2010).
618
619 Adams et al. (2005) stated the importance of reporting the freezing rate and type of
620 freezer used in studies on *Anisakis* mortality, and we additionally suggest that the
621 freezing rate could be taken into account as a factor in recommendations. Including
622 freezing rate as a parameter in the design of conditions for *Anisakis* mortality would
623 have an additional advantage, since freeze-induced alterations are accompanied by

624 modifications in the characteristics of fish muscle. With slow freezing, formation of
625 extracellular ice crystals occurs, fibres are separated into groups and myofibrils are
626 compressed, deformed and fragmented in some places (e.g. Grujić et al., 1993). With
627 fast freezing, the effect on fish muscle is minimized, so the freezing rate positively
628 affects the quality of the product.
629
630 In industrial processing, freezing times can range from a few minutes to more than a
631 day, depending on the product, geometry and freezing system (Johnston et al., 1994). In
632 order to take into account all the freezing kinetics variables, freezing simulations could
633 be useful for giving more precise recommendations to consumers and industry. We
634 performed a simulation in Table 5, and the FAO (Johnston et al.,1994) also provides
635 examples of freezing times for fish products in a large number of set-ups. Nowadays,
636 several software packages are available that could help in the adaptation of
637 recommendations to obtain the final time/temperature/freezing rate/holding time
638 combinations. Thus, in specific scenarios, conditions to meet both safety in terms of
639 larvae mortality and fish quality at short holding times may be achieved.
640
641 We also found that the initial temperature before freezing significantly affected the %
642 viability of *Anisakis* L3 (Table 4d and e), and the higher the initial temperature, the
643 higher the survival of these nematodes. Initial temperature entered as an aleatory
644 variable, and it would have to be studied separately as a design factor, since in practice,
645 especially in households, the variability in starting conditions can be very great. The
646 possible protective role of relatively high starting temperatures should be studied in the
647 light of modifications in expression patterns such as heat shock proteins (Chen et al.,
648 2015), excretory-secretory proteins (Palomba et al., 2019) or the accumulation of

649 metabolites (i.e. cryoprotectants such as trehalose) (Łopieńska-Biernat et al., 2019) in
650 response to thermal stress, among other factors.
651
652 **4.2. Antigen and allergen recognition**
653 Part of the allergic symptoms that have been related to consumption of frozen fish may
654 actually derive from larvae that remained alive even after the recommended measures
655 had been followed (Alonso et al., 2016), or, alternatively, to low adherence to
656 recommendations, resulting either from poor interpretation of the recommendations or
657 from the variability of freezing conditions in domestic freezers (Sánchez-Alonso et al.,
658 2018). Furthermore, exposure to *Anisakis* L3 allergens can occur as a result of
659 consumption of dead larvae or pieces of fish that have been in contact with larvae even
660 though the larvae are not present at the time of consumption (Audicana & Kennedy,
661 2008; Solas et al., 2008). Allergen detection in a subset of samples in which the
662 parasites had been removed from the mince before extraction (**Figure 4c**), supports the
663 results of those authors and suggests that allergenic potential, in terms of the possibility
664 of triggering an allergic reaction based on the fact that the allergenic epitopes are
665 conserved as observed by IgE immunoblotting, albeit with low concentrations of
666 allergens, cannot be ruled out.
667
668 Allergenic potential can also be measured by quantifying the presence of parasite
669 individual allergens of known clinical and technological relevance. They include the
670 minor excretion/secretion allergen Ani s 4, which is a pepsin- and heat-resistant
671 excretion/secretion allergen whose clinical importance derives from its association with
672 anaphylaxis (Moneo et al., 2005). In order to analyse whether there was a difference in
673 allergen release as a function of any of the kinetic parameters when the larvae were

674 removed from the muscle, parasite antigens and Ani s 4 were analysed in all the samples
675 in Table 1. Rodriguez-Mahillo et al. (2010) were able to detect Ani s 4 concentrations as
676 low as 2.5 ng/g muscle in frozen, stored, naturally parasitized belly flaps from hake.
677 Assuming ~2 µg Ani s 4/L3 (Rodriguez-Mahillo, 2006), the maximum detection of this
678 allergen for 10 L3/75 g muscle, if all the Ani s 4 had been released to the media, should
679 be 0.27 µg/g. Since all the larvae had been removed, for a positive signal in the dot blot
680 analysis (i.e. >2.5 ng/g) at least 1% of Ani s 4 would have to be released to the muscle.
681 The fact that no signal was observed with 10 L3/75 g, except for the samples frozen at
682 the highest freezing rate (**Figure 4a**), suggests that the antigen release, either by an
683 active or by a passive mechanism, was below this level. Nevertheless, to demonstrate
684 that these allergens retain their biological activity, functional assays such as the basophil
685 activation test would be necessary.
686
687 The effect of freezing rate on antigen release observed in **Figures 4a** and **b** could be a
688 consequence of damage or modification of the permeability of the cuticle at faster
689 freezing conditions (manuscript in preparation). According to EFSA (2010), further
690 studies are needed to elucidate whether the changes observed in the cuticle reduce the
691 resistance of the parasites to the action of the gastric enzymes in the gastrointestinal
692 tract. We propose, additionally, to explore whether this resistance might, in turn, be
693 different if the technological conditions, prior to the action of the enzymes, cause a
694 different allergen release, depending on the intensity or the conditions of the treatments
695 applied.
696

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), and its use has recently been adopted to differentiate between *Anisakis* L3 species (Suzuki et al., 2010; Arizono et al., 2012) 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 (**Figures 5a–b**) and survival in gastric juice (**Figure 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 (**Figure 7c**) was impaired in the treated larvae, although their basal respiration was not significantly different from that of the controls, also in agreement

722 with previous results (Sánchez-Alonso et al., 2019). The lack of relationship between
723 the % viability after freezing (**Figure 5b**) and the penetrative ability (i.e. 1% on
724 average,) suggests that any larva which shows any signs of movement or respiration
725 ability should be treated as equally infective even if the thermal freezing conditions
726 were considered harsh enough.
727
728 **5. Conclusions**
729 This set of experiments with *Anisakis* L3 infecting hake muscle highlights the
730 importance of a number of kinetic parameters in the survival of *Anisakis* larvae, not
731 only final temperatures or storage times. The fact that high freezing rates are able to kill
732 *Anisakis* larvae faster, and at the same time are preferred to maintain the best eating
733 quality of fish, suggests that technological conditions can be designed to meet both
734 safety in terms of larva mortality and fish quality.
735
736 However, faster freezing rates may cause a higher release of antigens to the surrounding
737 medium. The relative importance of this in terms of recommendations for sensitized
738 patients needs to be examined in the light of further studies where the effect of damage
739 to the cuticle on resistance to gastric enzymes is addressed.
740
741 The characteristics of the surviving larvae after freezing (i.e. penetrative ability in solid
742 agar, survival in gastric juices and saline solutions, basal and FCCP respiration ability)
743 were impaired as compared to the controls, but the lack of relationship between the %
744 survival due to freezing conditions and the other indicators suggests that any larva
745 which shows any sign of movement after freezing should be treated as equally infective.
746

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752

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935 **Figure captions**

936

937 **Figure 1.** Representative time and temperature profiles of minced hake subjected to
938 three freezing regimes. Each profile exhibits a different characteristic freezing time (t_c),
939 (~30 min solid line, ~100 min dotted line, and ~150 min dashed line), measured as the
940 minutes taken to traverse the temperature range from –1 to –7 °C (vertical arrows). The
941 horizontal arrows represent the final freezing temperatures set for each of the
942 experiments (–10, –15, –20, –28 °C).

943

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

947

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

953

954 **Figure 3.** Viability (% L3 with mobility) of *Anisakis* L3 infecting minced hake as a
955 function of final temperature (–10 °C black, –15 °C dark grey, –20 °C light grey, –28
956 °C white) and frozen storage time at a characteristic freezing time, t_c=149.29 ± 6.75
957 min.

958

959 **Figure 4a.** Immunoblots, revealed with anti rAni s 4 antiserum, of the fish mince that
960 had been infected with 10 L3 per 75 g of muscle, frozen at –10, –20 or –28 °C at
961 different t_c values (173, 151 or 31 min), and thawed (immediately after reaching the
962 target temperature or after frozen storage for 24 h). The antigen extraction was
963 performed after complete removal of the larvae from the muscle.
964
965 **Figure 4b.** *Anisakis* L3 antigen release to the fish mince (μg g^{-1}) upon freezing at –20
966 °C and subsequent frozen storage for up to 5 days. Data corresponding to domestic and
967 air blast freezing (average t_c, t_c=139±11.6 and 16±1.2 respectively). Different letters (a,
968 b) indicate significant differences. Inserts show Ani s 4 in each band detected by
969 immunoblotting and revealed with anti rAni s 4 antiserum.
970
971 **Figure 4c.** IgE immunoblots, revealed with a pool of sensitized patients' sera, of the
972 fish mince that had been infected with 10 L3 per 75 g of muscle, frozen at –10, –20 or –
973 28 °C at different t_c values (173, 151 or 31 min), and thawed (immediately after
974 reaching the target temperature or after frozen storage for 24 h). The antigen extraction
975 was performed after complete removal of the larvae from the muscle.
976
977 **Figure 5a.** Percentage of mobile (dark grey) and agar-penetrating (light grey) *Anisakis*
978 L3. Data from untreated (i.e. stored at 4–5 °C) and freeze-surviving (~S_{50}) larvae. Error
979 bars are standard deviation (n=3 independent experiments). Different letters show
980 significant differences in the agar test (a, b) between the untreated and freeze-surviving
981 L3.
982

983 **Figure 5b.** Range of viability (% L3 with mobility) of *Anisakis* L3 infecting minced
984 fish in the different trials, regardless of species (i.e. hake, mackerel or horse mackerel),
985 *Anisakis* batch (6 batches) or thickness of experimental units (i.e. 11, 22 or 33 mm).
986
987 **Figure 6.** Viability (% L3 with mobility) of *Anisakis* L3 upon incubation in artificial
988 gastric juice at 37 °C. Untreated (circles) (n=1) and freeze-surviving L3 (squares) (~S_{50})
989 (n=2).
990
991 **Figure 7a.** Representative data of oxygen consumption rate (OCR, nmol O_2 min^{-1}
992 $worm^{-1}$) profile of untreated *Anisakis* L3, showing the initial (i.e. without metabolic
993 modulators), maximum (i.e. after FCCP addition) and azide-insensitive respiration. Data
994 represent the average of 3 experiments. The arrows show the point of FCCP (solid) and
995 sodium azide (dotted) additions.
996
997 **Figure 7b.** Viability (% L3 with mobility) of *Anisakis* L3 upon freezing at –10 °C
998 (before the arrow) and upon storage at 4–5 °C for up to 168 hours after thawing (after
999 the arrow) (n=4). Error bars show the standard deviation. Different letters (a, b, c)
1000 indicate significant differences.
1001
1002 **Figure 7c.** OCR (nmol O_2 min^{-1} $worm^{-1}$) of surviving *Anisakis* L3 upon freezing at –10
1003 °C (before the arrow) and upon storage at 4–5 °C for up to 144 hours after thawing
1004 (after the arrow). Symbols (□,■,○,●,△, ▲) represent maximum, initial and azide-
1005 insensitive respiration, respectively, before (open) and after (closed) freezing. Error bars
1006 show the standard deviation. Different letters (a, b, c) indicate significant differences
1007 during incubation time for each respiration phase of the OCR profile (n=4).

Table

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 ± 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 (ºC)	Freezing parameters				
	Real T (ºC)	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	-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
	-15.0±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	-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
	-20.0±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
-28	-27.2±0.35	42.3±0.73	142±3.91	138±3.75	322±0.00
	-29.0±0.20	26.7±1.77	101±2.30	188±3.54	316±0.00

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**
Precooling 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

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 ***	$T_{initial}$ **	t_t **	
e	hake, mackerel, horse mackerel	11, 22, 33	yes		*** 0.64	T ***	$T_{initial}$ **	t_t ***	$t_{freeze/thaw}$ ***

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

Table 6. *Anisakis* viability as a function of set final freezing temperature (°C) and % of *Anisakis* species as covariates.

Factor & Covariate	Model	Factor	Covariate	R^2 adj
Temperature & *Anisakis simplex s.s.*	**	**	NS	0.42
Temperature & *Anisakis pegreffii*	**	**	NS	0.42
Temperature & heterozygote genotypes	***	***	*	0.57

Figure 1.

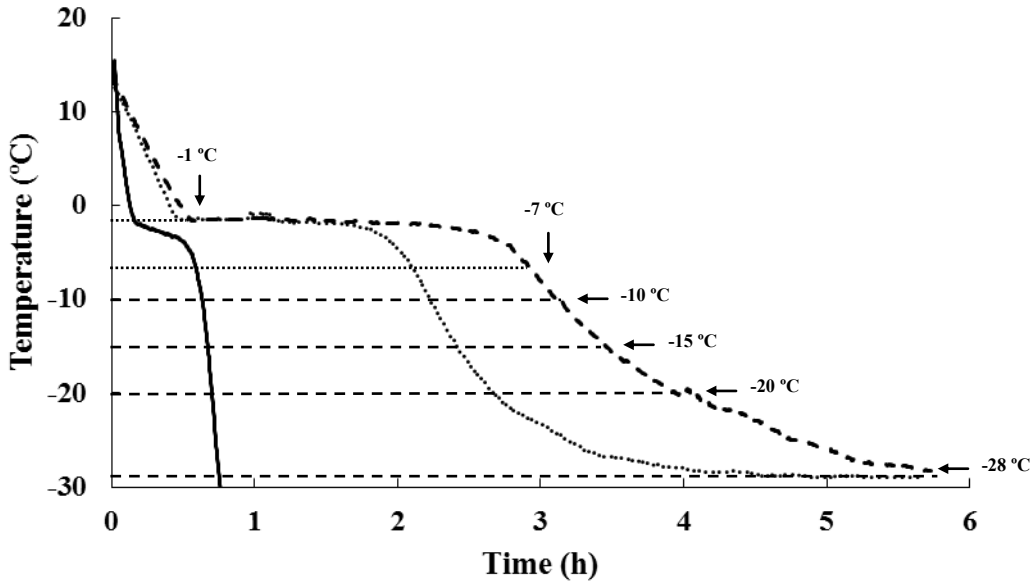

20
10
0
-10
-20
-30
Temperature (°C)
0
1
2
3
4
5
6
Time (h)
-1 °C
-7 °C
-10 °C
-15 °C
-20 °C
-28 °C

Figure 2a.

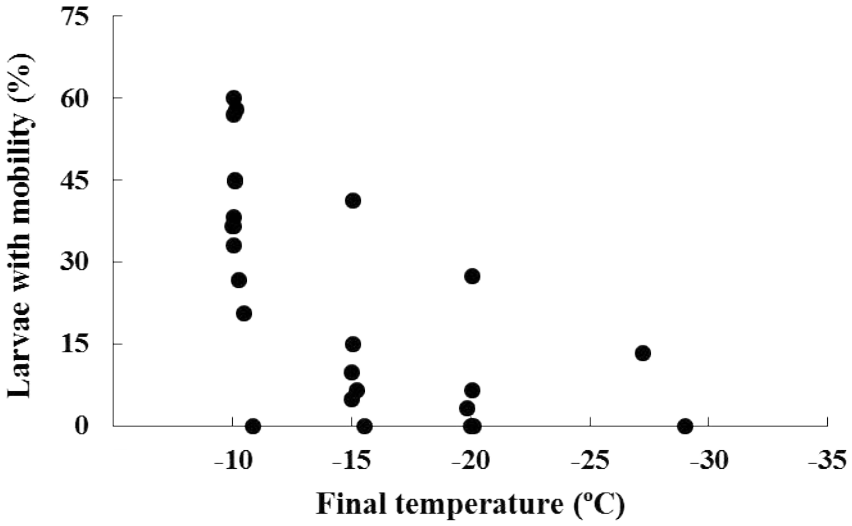

75
60
45
30
15
0
Larvae with mobility (%)
-10
-15
-20
-25
-30
-35
Final temperature (ºC)

Figure 2b.

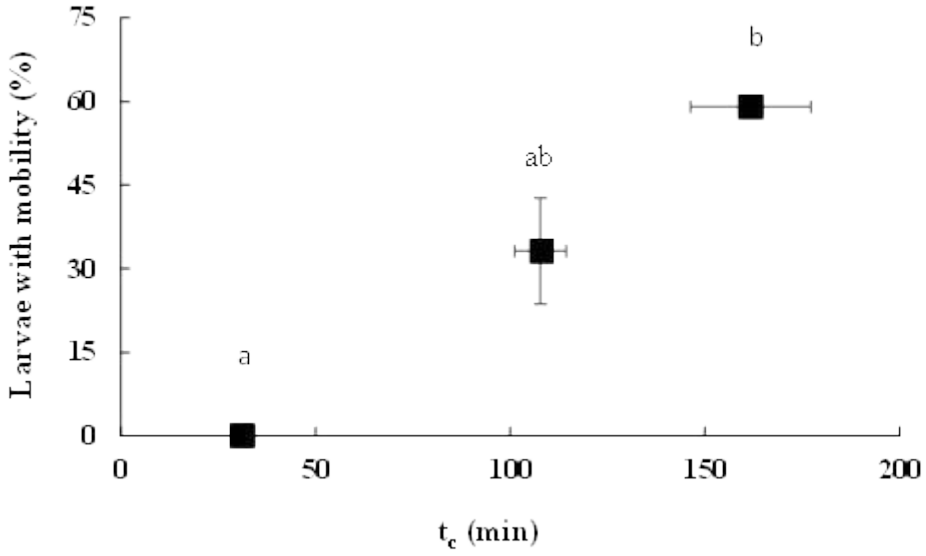

75
60
45
30
15
0
Larvae with mobility (%)
a
ab
b
0
50
100
150
200
t_c (min)

Figure 3.

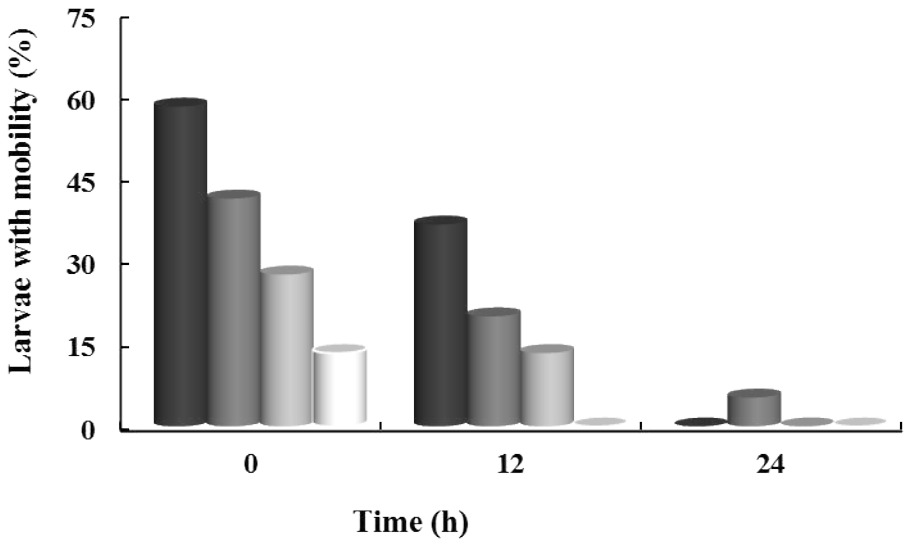


Figure 4a.

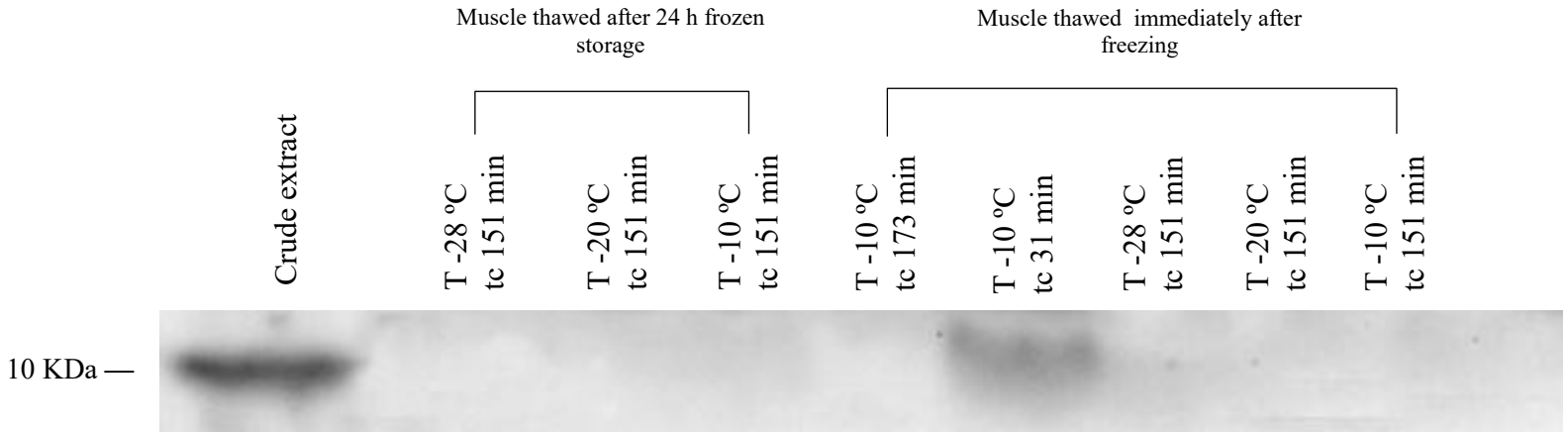

Muscle thawed after 24 h frozen storage
Muscle thawed immediately after freezing
Crude extract
T -28 ºC tc 151 min
T -20 ºC tc 151 min
T -10 ºC tc 151 min
T -10 ºC tc 173 min
T -10 ºC tc 31 min
T -28 ºC tc 151 min
T -20 ºC tc 151 min
T -10 ºC tc 151 min
10 KDa —

Figure 4b.

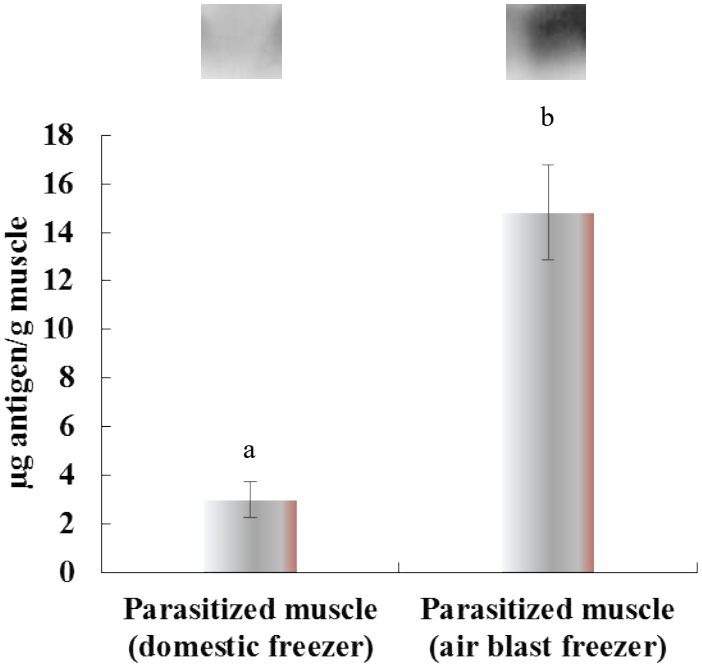

18
16
14
12
10
8
6
4
2
0
µg antigen/g muscle
a
b
Parasitized muscle (domestic freezer)
Parasitized muscle (air blast freezer)

Figure 4c.

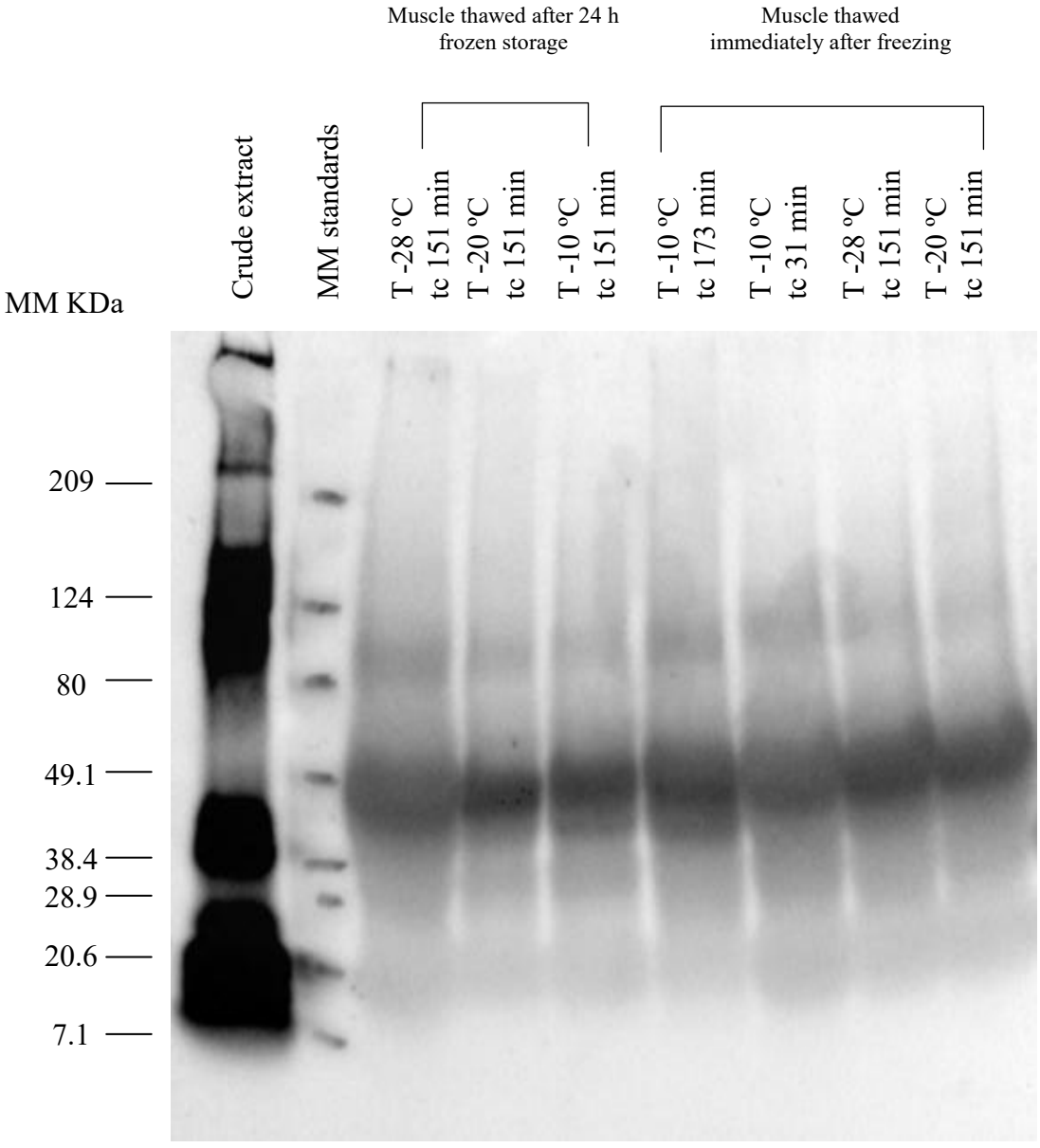

Muscle thawed after 24 h frozen storage
Muscle thawed immediately after freezing
Crude extract
MM standards
T -28 ºC tc 151 min
T -20 ºC tc 151 min
T -10 ºC tc 151 min
T -10 ºC tc 173 min
T -10 ºC tc 31 min
T -28 ºC tc 151 min
T -20 ºC tc 151 min
MM KDa
209
124
80
49.1
38.4
28.9
20.6
7.1

Figure 5a.

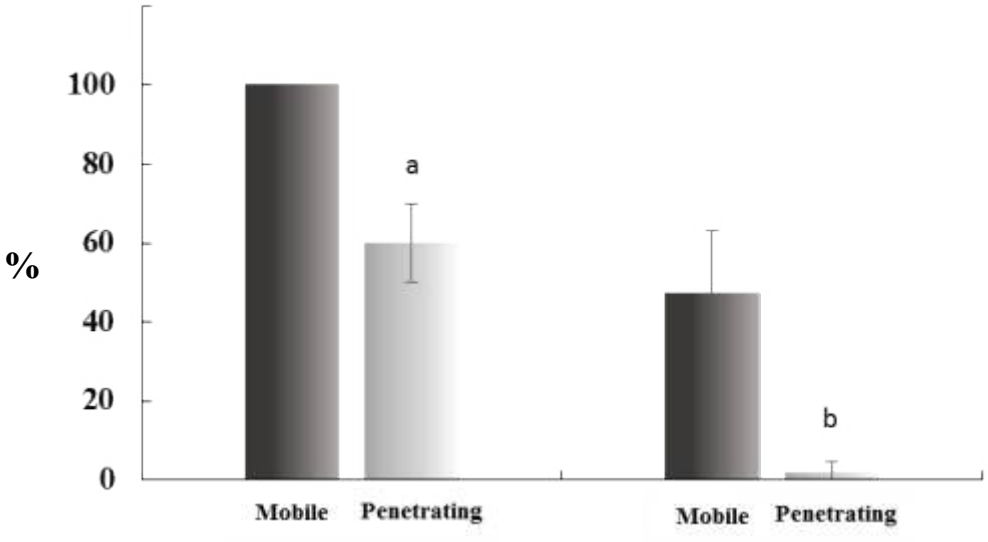


Figure 5b.

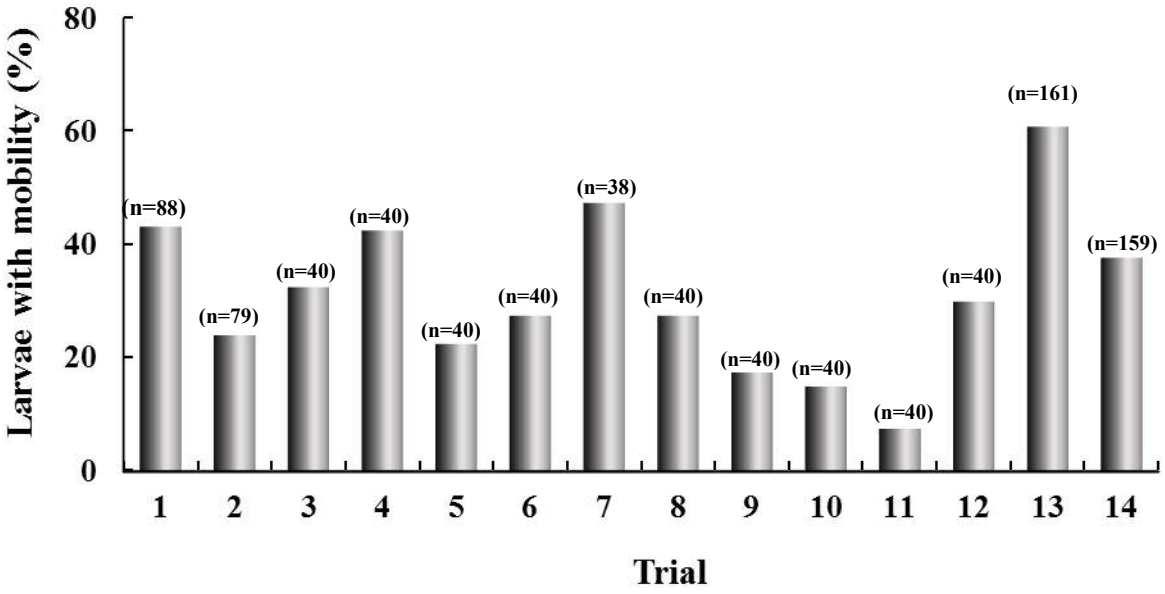

Larvae with mobility (%)
80
60
40
20
0
(n=88)
(n=79)
(n=40)
(n=40)
(n=40)
(n=40)
(n=38)
(n=40)
(n=40)
(n=40)
(n=40)
(n=161)
(n=159)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Trial

Figure 6.

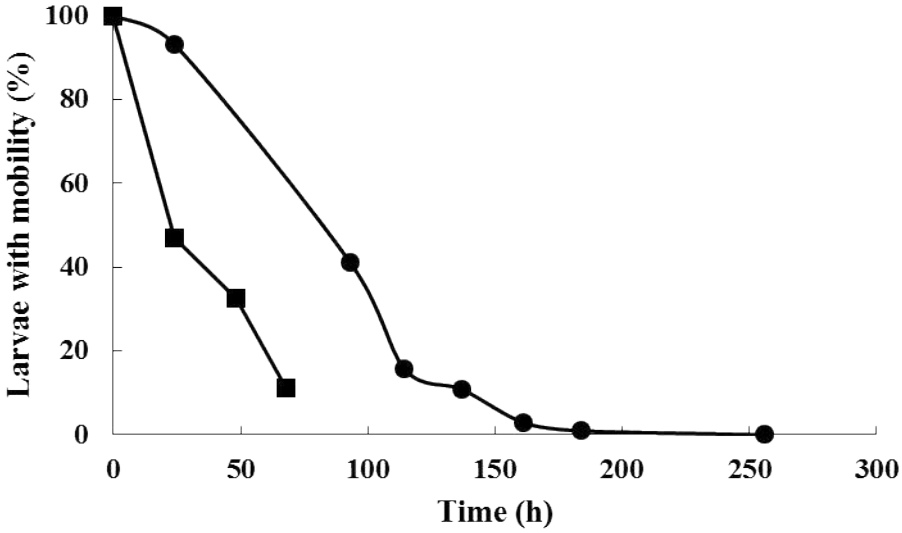

Larvae with mobility (%)
100
80
60
40
20
0
0
50
100
150
200
250
300
Time (h)

Figure 7a.

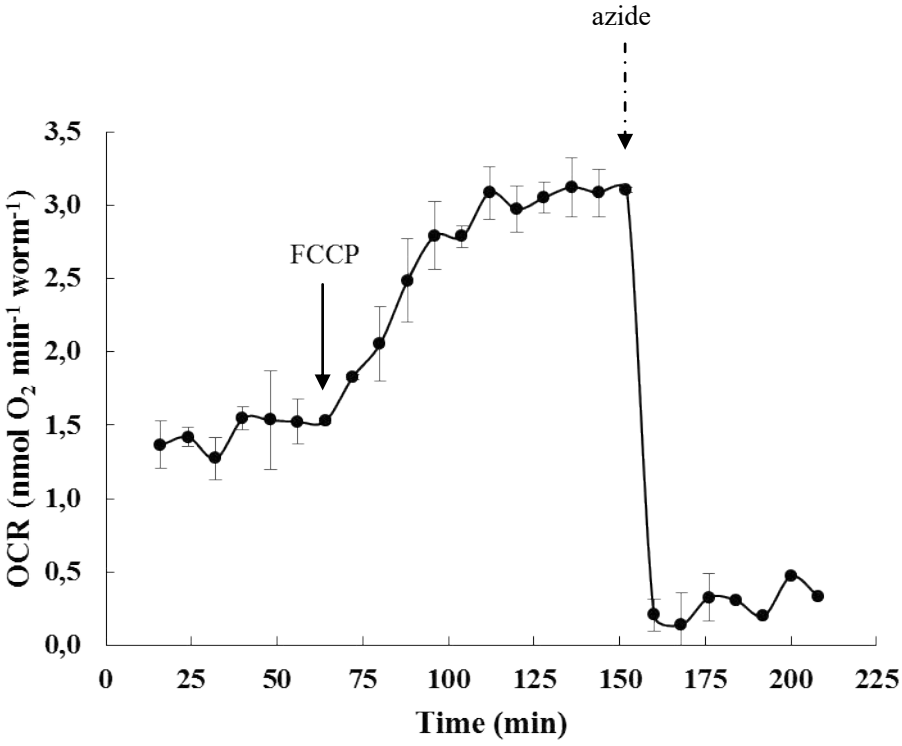

azide
FCCP
OCR (nmol O2 min-1 worm-1)
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
0
25
50
75
100
125
150
175
200
225
Time (min)

Figure 7b.

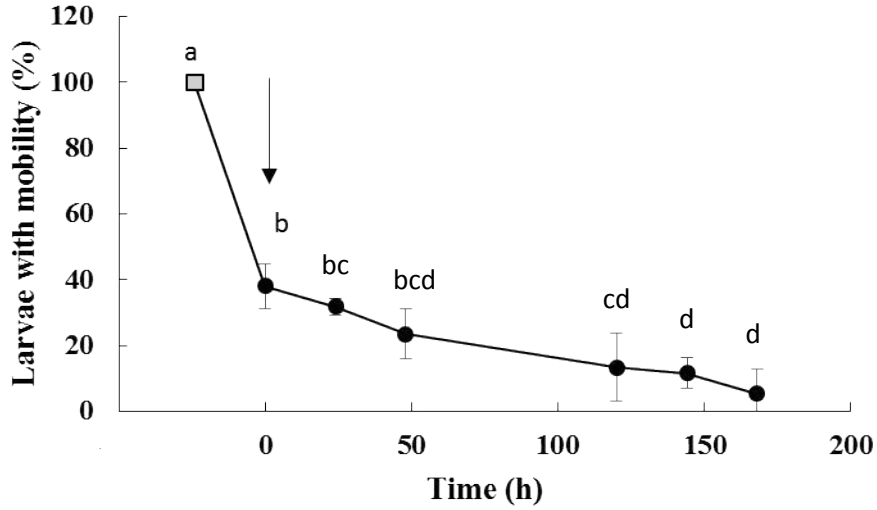
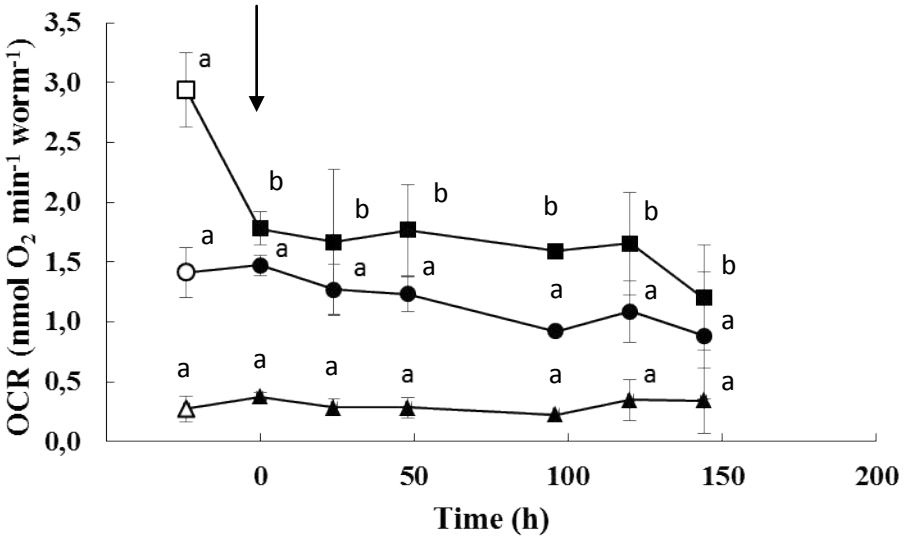

Larvae with mobility (%)
120
100
80
60
40
20
0
a
b
bc
bcd
cd
d
d
0
50
100
150
200
Time (h)

Figure 7c.


OCR (nmol O2 min-1 worm-1)
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
0
50
100
150
200
Time (h)
a
b
b
b
b
b
b
a
a
a
a
a
a
a
a
a
a
a
a
a
a
a
a

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

Batch	*A. simplex s.s.* %	*A. pegreffii* %	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