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Anisakis simplex Larva Killed by High-Hydrostatic-Pressure Processing

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ABSTRACT

Anisakis simplex is a common nematode parasite present in many marine fish, including finfish and squid. It can pose a public health problem if it is not destroyed during food processing. *Anisakis* larvae were isolated from fish tissue, and their survival of high-pressure treatments in distilled water and physiological isotonic solution was assayed. Treatment at a pressure of 200 MPa for 10 min at a temperature between 0 and 15°C kills all *Anisakis* larvae, with a lack of motility being used as an indicator of larval death. Lower pressures can be successfully employed down to 140 MPa, but with lower pressures, the treatment time must be increased by up to 1 h to kill all larvae. Meanwhile, most larvae treated for >10 min at pressures of >120 MPa were dead, with the autofluorescence method being used to determine death. Cycles of compression and decompression increase the destruction of larvae compared with a single pressure treatment for a similar treatment time. Our results indicate that high-pressure treatment is an alternative nonthermal method for killing this nematode. The possible mechanism of death and damage by pressure is discussed, and uses for this treatment in food processing are suggested.

Anisakiasis, also known as larva migrans visceral or eosinophilic granuloma, is a widespread disorder resulting from the consumption of contaminated fish. Its incidence in Spain and many other countries has risen dramatically over the last decade, and it constitutes a public health threat to humans and causes economic losses for fish-processing industries *(18, 21).* Transmitted through ingestion of fish and squid containing stage III larvae (despite the possible inadequacy of the term ''larva'' in referring to nematode juvenile stages, its use is widespread and common in most articles on the topic of foodborne parasites) of parasites from the *Anisakidae* family, the recent increase in the incidence of anisakiasis has been attributed to changes in sea resources, food processing, and cooking.

The genus *Anisakis* comprises three known species, *A. simplex, A. typica,* and *A. physeteris,* with *A. simplex* being the most frequent and widely distributed. Other species of water nematodes belonging to *Anisakidae* are also causative agents of zoonosis, but anisakiasis seems to prevail among fishborne parasitoses. Species in *Anisakidae* can be identified by several external characteristics of the larvae, such as length and color (e.g., a maximum of 30 mm and white to pale pink for *Anisakis*), which allows easy differentiation from the common *Pseudoterranova.*

This parasitosis has been described in a number of countries *(21),* with its frequency being especially high in Japan *(27).* In spite of the high public concern about this zoonosis in Spain, it has been described only recently *(3).* A special risk factor is the recent popularity of fish preparation methods involving little or no cooking, both traditional (sushi and sashimi, ceviche, etc.) and industrially introduced (e.g., carppacios and cold smoked preparations), together with a trend toward minimally processed foods.

Because of the massive presence of parasites and the impossibility of infection control for wild fish, measures can be taken only at the processing and consuming stages. *Anisakis* is sensitive to high temperatures, so normal cooking or thermal treatments for the sterilization of preserves guarantee product safety. Hot smoking is also safe, but there are some doubts about cold smoking, a process in which the temperature does not rise above 40°C. These larvae are also sensitive to freezing temperatures *(12, 16).* The conditions called for by different safety regulations range from 24 h at temperatures lower than −20°C to 7 days at temperatures lower than −23°C or 14 h at temperatures lower than −35°C *(15, 21).* Fish conserved in a high-salt concentration (20 to 30%) require a longer period (4 weeks) for larva destruction *(11).* In marinated fish, larvae can survive for as long as 10 weeks *(17).* New methods include the use of ionizing radiation, which, although useful for the destruction of *Anisakis,* is limited by both legislation and consumer acceptability.

High-pressure processing is currently the subject of many studies regarding its application to different aspects of food processing *(19).* Its more general uses include selective enzyme inactivation *(5, 14),* texture modification *(24),* and sterilization or reduction of microbial load *(7).* High pressure is able to modify large macromolecular assemblies, while it has very little or no effect on small molecules. The preservation of organoleptic and nutritional properties is the main advantage of high-pressure processing for modern food manufacturing.

The objective of this study was to identify the conditions applicable to high-pressure treatments for killing *An-*

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isakis larvae, both isolated and in fish tissues. The effects of these treatments on fish tissues and the practical viability of the treatment must also be taken into consideration.

MATERIALS AND METHODS

Stage III *A. simplex* larvae were obtained by the Laboratorio Municipal de Higiene, Ayuntamiento de Madrid, Spain, from heavily parasitized fish and kept at 4°C for a few hours before transport in ice to the laboratory. Larvae were kept at 4°C in contact with the original fish tissues until they were used, for up to 5 days. *A. simplex* was identified by its general morphological characteristics *(22, 33)*.

High-pressure processing was performed with a GEC ALSTHOM ACB (Nantes, France) high-pressure instrument. The pumping time necessary to reach the pressures used in this study was 1 to 2 min, and the decompression time was 10 to 30 s. Temperatures were recorded with a steel-coated thermocouple, and a data capture system (Fluke Helios I) connected to a computer was used to register data. In a large pressure vessel (2.35 liters), the temperature cannot be kept perfectly constant during a pressurization treatment because of the associated adiabatic heat, which increases the internal temperature during a pressure increase and reduces it during a pressure release *(25)*. Consequently, the temperature data in this work are always the averages over the whole pressurization process. The working temperature ranged from 0 to 15°C.

Experiments were performed either with deionized Milli-ρ water (Millipore, Bedford, Mass.) or with sterile physiological isotonic solution (0.9% NaCl water solution). The nematodes were placed in the selected medium in a plastic container, with any contact between the larvae and the external pressurizing fluid (75% ethylene glycol/water, employed to avoid freezing damage to the equipment) being avoided.

Several methods can be used to differentiate between dead and live larvae. The observation of motility is one of the more widely used methods *(1)*. Other methods include the coloring of dead larvae with a variety of dyes, such as methylene blue, and the observation of the emission of fluorescence by dead nematodes when excited by UV radiation *(11, 32)*.

With plastic forceps, batches of 10 normally motile larvae were placed in a plastic cuvette (2 by 2 cm) with 4 to 5 ml of medium, and their motility was determined at ×6.5 magnification with the help of video equipment (Hitachi Color Camera KP-C501E, Hitachi Denshii, Ltd., Tokyo, Japan). Then the larvae were transferred into a 1.5-cm^3 Eppendorf container full of medium for pressurization. Pressure-treated larvae were transferred to the cuvette, and their motility was observed after a few minutes to allow temperature equilibration and then again after 2 to 4 h. The number of motile individuals as well as their type of motility was recorded. According to their movement, larvae could be classified into three categories: normal motility, total lack of movement, and spiral-spring residual agitation. The highest degree of motility detected during 1 min of continuous observation was reported.

In deionized water or saline solution, *Anisakis* larvae showed vigorous spontaneous swimming movements at room temperature, but when the temperature was lowered to 0 to 5°C, movement stopped; it resumed after the temperature was again raised to room temperature. Movement intensity varied with batches of parasites, but in control experiments the movement was constant for several hours.

After the motility tests had been conducted, larvae were transferred to a sheet of unbleached filter paper, and fluorescence was observed after excitation with a 6-W UV lamp (Uvtev, Model LF106M, Cambridge, UK) emitting at 312 nm. The color uptake of the larvae in a 0.1% solution of methylene blue was assayed for selected samples. Larvae were placed in a cuvette with this solution for several hours at room temperature. The larvae were removed and superficially dried on filter paper prior to observation. Control samples were taken to minimize the variation associated with larvae of different origins. These samples were kept at room temperature in the medium under observation for the same time as the treated samples, and then their motility, fluorescence, and dye uptake were determined in the same way.

The error was calculated as the standard deviation of dead larvae in the set of experiments (comprising 10 individuals each) performed under the same conditions. The number of experiments constituting these sets varied, as the regions of the border between what were considered innocuous conditions and what were considered deadly conditions for *A. simplex* were repeatedly explored.

Living and pressure-dead larvae were observed by low-temperature scanning electron microscopy (cryo-SEM). Observations were carried out with an electron scanning microscope (Zeiss DSN-960, Jena, Germany) equipped with a low-temperature stage (Oxford CT-1500, UK). Sample larvae were introduced in nitrogen slush (vaporizing liquid nitrogen) and were then transferred into the low-temperature stage in the prechamber of the microscope (held at −180°C), where the sample was fractured. After fracture, the sample was placed onto the microscope stage for etching at −90°C for 2 min. It was then reintroduced into the prechamber, where it was shadowed with gold and subsequently introduced into the microscope, where observation was carried out at a temperature of −150 to −160°C. Samples were observed with both secondary and retrodispersed electrons, and the best images were selected in each case. Treated and untreated larvae were also observed by optical microscopy with a Nikon Optiphot Microscope (Nikon, Tokyo, Japan) with an objective of ×10 and the dark-field method. Micrographs were taken with a Nikon 35-mm camera optically coupled to the microscope.

The effect of high-pressure treatment on nematodes was determined by the "fish fillet sandwich" technique *(1)*. Fresh fillets (ca. 1 cm thick, 7 cm long, and 5 cm wide) of Nile perch (*Lates nilotica*) were employed a few hours after they were purchased. The motility of 20 nematodes was tested in distilled water, and then these nematodes were placed on top of the internal face of one fish fillet. A similar fillet was placed on top of them, closing the sandwich. The sandwich was vacuum-packed and either submitted to pressure treatments as described (at a temperature between 10 and 15°C) or left sealed and untreated for an equivalent period as a control. Finally, sandwiches were opened, and all nematodes were recovered. The nematodes were transferred to distilled-water containers, and their motility was tested as described. In addition, their fluorescence on UV illumination was tested.

RESULTS AND DISCUSSION

Table 1 presents data on the viability of *A. simplex* larvae treated by high pressure in distilled water. Nematode death is reported on the bases of both lack of movement for a long period and fluorescence. Standard errors are quoted. We observe that a long treatment time at low pressure can have the same effect as a short treatment time at high pressure. The two regions in which at least 90% nonmotile nematodes and >70% fluorescent larvae can be expected are indicated in Table 1.

The fluorescence emitted by pressure-treated *A. simplex* larvae was visually evaluated. Untreated individuals

TABLE 1. *Death of* A. simplex *larvae subjected to different treatments*[a]

Pressure (MPa)	Treatment time (min)													
	1	3	5	7	10	15	20	25	30	40	50	60	70	240
50														40 70 1
100			0 10 1		20 ± 0 20 ± 14 2				*60 ± 27* *78 ± 32* *3*					
120				*50 ± 15* *90 ± 0* *6*										
140												**100** **100** **1**		
150					*60 ± 60* *70 ± 60* *2*			*50* *100* *1*	**100** **100** **1**					
160					*80* *100* *1*	**100** **100** **1**								
170					*88 ± 15* *100 ± 0* *4*	**100 ± 0** **100 ± 0** **2**					**100** **100** **1**	**100** **100** **1**	**100** **100** **1**	
180			*82 ± 15* *100 ± 0* *5*	**90** **100** **1**	**91 ± 17** **100 ± 0** **11**	**90 ± 14** **90 ± 14** **2**		**93 ± 12** **100 ± 0** **3**	**100** **100** **1**	**100** **100** **1**				
190	*70* *90* *1*		**90 ± 17** **97 ± 6** **3**		**91 ± 16** **100 ± 0** **7**	**100 ± 0** **100 ± 0** **3**								
200		*80* *100* *1*	**90** **100** **1**		**100** **100** **1**									
300	*70* *100* *1*													

[a] Each entry shows the percentage of nonmotile individuals, followed by the percentage of fluorescent individuals, followed by the number of experiments, with each experiment involving 10 individuals. Standard deviations are shown where more than one experiment was carried out. Boldface entries represent the conditions under which at least 90% nonmotile (dead) larvae can be expected. Italic entries represent the conditions under which at least 70% of the larvae were fluorescent.

were invariably not fluorescent, and occurrence of fluorescence was considered proof of death, or at least damage to the larvae *(28, 30, 32)*. All fluorescent nematodes were nonmotile or showed altered motility (Tables 1 and 2), as observed at pressures as low as 120 MPa and for times as short as 7 min. It seems unlikely that these damaged nematodes were able to implant themselves into the host tissues. The definition of the border between lethal and nonlethal conditions was reduced by *A. simplex* lot-to-lot variability. The general health of the nematodes before the experiment could have influenced their resistance to pressure treatments. In control experiments, all larvae were motile and nonfluorescent for the entire observation period (4 to 6 h).

Data presented in Table 1 correspond to experiments carried out at temperatures of 0 to 5°C. Higher temperatures (up to 15°C) were tested, and no appreciable difference was found (data not shown). It must be noted that the temperature range was constrained by the intent not to freeze the sample. Freezing was avoided to differentiate between the damaging effects of freezing and those of high pressure on *A. simplex* and also to check the effect of high pressures under conditions where fish tissues were not damaged by freezing. The low temperature limit had to take into consideration the reduction of temperature associated with depressurization (ca. 3 to 4°C). A high temperature limit was set at 15°C, because the quality of fish can decline above this temperature. Selected experiments confirmed that results were not altered when pressure treatments were carried out with saline isotonic solution (data not shown).

Treatment with 200 MPa for 10 min can be recommended as safe, although a longer treatment at a lower pressure may be advisable (the lowest effective pressure found was 120 MPa), depending on the possible deleterious effect of high pressure on the food product itself. (Often, a pressurization time longer than 5 to 10 min is considered not economically viable. This figure can be increased if this

TABLE 2. *Death of* A. simplex *larvae subjected to cyclic pressure treatments*[a]

Pressure (MPa)	Total treatment time[b] 4 (2 × 2)	6 (3 × 2)	12 (2 × 6)	15 (3 × 5)	20 (2 × 10)	30 (6 × 5)
100				20		*60*
				30		*90*
				1		*1*
120			*40 ± 0*			
			90 ± 0			
			2			
150	10				*80*	
	20				*100*	
	1				*1*	
160	*90*					
	100					
	1					
170	*75 ± 29*	**100 ± 0**				
	100 ± 0	**100 ± 0**				
	4	**2**				
180	**100 ± 0**					
	100 ± 0					
	2					
320				**100**		
				100		
				1		

[a] Each entry shows the percentage of nonmotile individuals, followed by the percentage of fluorescent individuals, followed by the number of experiments, with each experiment involving 10 individuals. Standard deviations are shown where more than one experiment was carried out. Boldface entries represent the conditions under which at least 90% nonmotile (dead) larvae can be expected. Italic entries represent the conditions under which at least 70% of the larvae were fluorescent.

[b] The number of cycles × cycle duration (min) is shown in parentheses. Ramping time was not considered.

process is not considered an additional treatment but is integrated into the production line.) Color, texture, and lipid oxidation are the main effects reported for high-pressure-treated fish foods. Color alterations, possibly due to oxidative changes in myoglobin, have been observed in tuna even at 50 MPa *(23)* and in bluefish at >100 MPa *(10).* The texture of fish muscle can be altered by high-pressure treatments, as has been reported for cod at 400 MPa *(2)*; for turbot *(9),* for which myosin becomes denatured between 100 and 200 MPa; and for bluefish at >100 MPa *(4).* Fish lipids exhibit a variety of behaviors, depending on the species: turbot lipids are oxidized at 180 MPa *(9),* while those of cod are not affected at 400 MPa *(2).* Therefore, 140 MPa seems to be a pressure level that does not produce important alterations in fish *(9).*

In Table 2, the results of experiments with cycled pressurization-depressurization treatments are summarized. Data correspond to experiments at a temperature of 0 to 5°C. For control experiments, all larvae were motile and nonfluorescent. Cycling treatment has been reported to be useful for the sterilization of microorganisms by hydrostatic pressure. Here it produced a stronger nematode inactivation (after both the nonmotility and the fluorescence methods) than did single treatments for the same treatment time under pressure (Tables 1 and 2). This result may be related to the pressure increase or decrease or to actions on different physiological stages of the organism (i.e., as has been observed for bacterial spores, for which a pressure treatment induces a less resistant stage). Alternatively, the additional time under pressure resulting from pressure ramping times (1 to 2 min) may account for the increased lethality. The additional cost, the additional technical complication, and the increased equipment use time quite likely outweigh the possible advantages of cycling.

Experiments under three sets of conditions yielding 100% mortality for isolated nematodes were carried out by the fish sandwich technique. The nematodes burrowed their way inside the fish tissue, even in the short period (5 to 10 min) before pressurization, and this finding was considered suitable proof of the proper host-parasite interaction and of the significance of these tests with regard to the behavior of *A. simplex* in its wild hosts. All fish sandwich experiments (10 min at 200 MPa, 15 min at 190 MPa, and 25 min at 170 MPa; 10 to 15°C in all cases) yielded 100% dead larvae, after both the motility (immediately after the test and 2, 6, and 12 h later) and the fluorescence tests. Meanwhile, for the control experiments, all larvae were motile and nonfluorescent.

The question of how pressure damages these organisms must be considered at this point. The effect of pressure on living matter has been approached in different ways as the subject of many studies *(8, 31).* On the one hand, the natural habitats of many organisms involve pressures on the order considered in this study. Pressures of about 109 MPa are attained in the deepest sea regions. At these pressures, fish and specially adapted microorganisms survive *(6, 31).* On the other hand, damage to physiological activity has been found at much lower pressures (e.g., in muscle at 30 to 50 MPa *(20)*). Other effects, such as red cell lysis, appear at 100 to 200 MPa *(34).* Experiments on food, often undertaken with the aim of attaining sterility, have demonstrated that pressures on the order of 700 MPa must be reached to obtain efficient vegetative microorganism inactivation *(8, 13, 26),* while deleterious effects on the characteristics of food products, especially on muscle tissues, have been reported at lower values.

The nematodes have some physiological characteristics that might make them sensitive to pressure changes. The maintenance of body turgidity requires that a relatively high hydrostatic pressure be maintained, implying a delicate balance of electrolytes *(29).* Damage caused by thermal treatments, especially those involving quick temperature increases, has been suggested to be due to the rapid increase and altered balance of internal pressure *(1).* High external pressure might affect the nematodes in several ways, e.g., by damaging the mechanisms for maintenance of hydroskeletal turgidity or by forcing water and solutes through the cuticle, altering the equilibrium. Alternatively, the rapid depressurization could have a similar effect on a very high transient inner pressure.

The damage caused to *A. simplex* by other physical

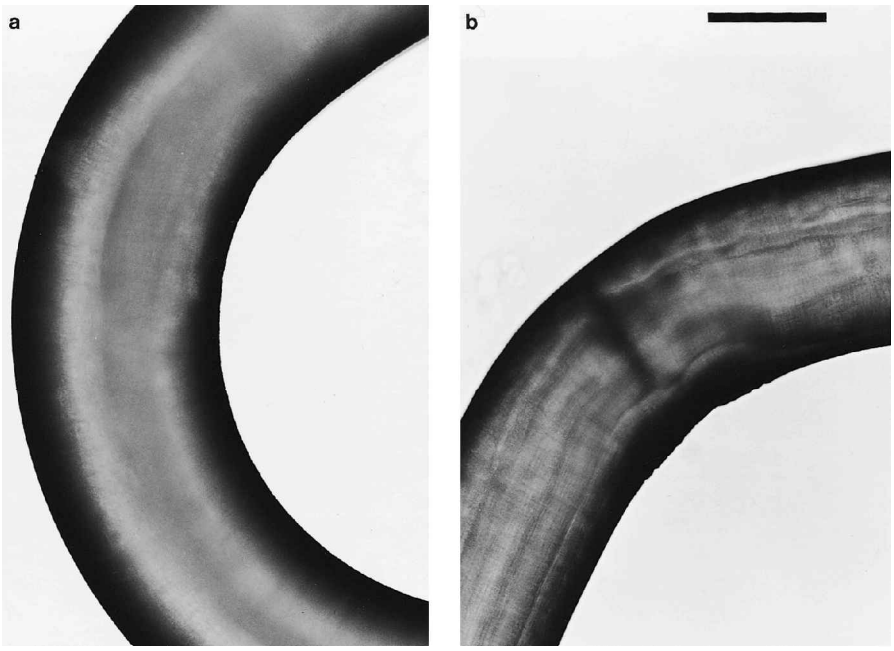

FIGURE 1. *Optical micrographs of (a) untreated and (b) pressure-dead* A. simplex *larvae. A ×10 objective and direct illumination were employed. The bar corresponds to 300 μm.*

treatments can be visualized by microscopy as ruptures in the cuticle and inner organs *(1).* For high-pressure treatments, external damage to the cuticles of larvae killed by high pressure was not observed by cryo-SEM for pressure-dead *A. simplex* larvae. Instead, a smooth, nonbroken surface indistinguishable for dead and living nematodes was found (data not shown). Figure 1 presents visible light micrographs of pressure-dead and living nematodes. As with cryo-SEM, no external damage was detected. The strong cuticles of parasitic nematodes may not reflect the extent of inner damage. For experiments at the higher pressures tested, when the other viability test indicated that larvae were dead, they did not dye with methylene blue, which could be another indication of the integrity of the cuticle of *A. simplex* in high-pressure treatments.

The fluorescence of dead larvae is likely related to internal alterations, such as protein coagulation *(30),* which may imply breakage of internal compartmentalization and mixture of inner fluids and would constitute a reasonable cause of death. Even in this state, residual movement similar to a watch spring very slowly unwinding can be seen. These nematodes did not revive later (i.e., they did not resume normal movement).

Several new avenues for research are suggested by these findings. The sufficiency of the fluorescence criterion or the confirmation that the "spring" state of the nematode corresponds to an inactive form would endorse the reliability of treatments at lower pressures. The possible variations in the effects of pressure when the larvae are present in a fish product must also be considered, as must the possible effects of these pressure levels or more elevated pressure levels on the allergenic capability of *A. simplex.* However, the preservation of cuticle integrity in high-pressure inactivation can have the beneficial effect of avoiding the dissemination of allergenic material in the fish tissues, reducing the effect of this material on sensitized consumers.

The pressure levels found here to make the consumption of treated fish safe are only slightly higher than those reported to maintain fish quality *(2, 4, 9, 23).* The pressure actually needed to kill parasites could be lower when combined with other treatments partially lethal to *A. simplex.* Pressure treatment could be appropriate for processed fish, such as fish in marinated and cold-smoked preparations. In these cases, pressure would not produce additional fish tissue structure alterations, because the tissues are already substantially modified.

Nematodes naturally implanted in fish tissues might behave differently (with a higher pressure resistance) than those that are isolated out of their natural medium. Considering the possible mechanisms of pressure killing, this possibility seems unlikely and has also been considered negligible in the study of other physical inactivation methods *(1).* Our data indicate that the inclusion of nematodes in fish tissues does not confer any protection against pressure damage. Hydrostatic pressure does not depend on sample thickness or any gradient. It "instantaneously" and homogeneously spreads to the whole of the sample. Meanwhile, for thermal or radiation methods, a thick fish covering can eliminate or delay the effect of cooking, freezing, or microwaving. Methods relying on chemical substances' diffusion through tissues, such as salting, marinating, or smoking the thick barrier of the fish, can also reduce its effective concentration and the effect on the parasite in the internal sample regions.

The success of *A. simplex* larvae destruction in fish samples predicts the utility of high-pressure processing for the destruction of these parasites in real fish and fish products. High-pressure treatment, a mild processing method, can constitute an alternative method for enforcing the safety of minimally processed fish foods when the destruction of *A. simplex* or other types of parasites is desired.

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