

Scanning Electron Microscopy of *Anisakis* Larvae following Different Treatments

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ABSTRACT

Ingestion of fish parasitized with *Anisakis* larvae can produce infestation and/or allergy in consumers. Technological and food processing treatments have been applied to parasitized fish in order to kill the larvae and avoid the infestation; however, their influence on allergenicity has not been studied. Four lots of hake (*Merluccius merluccius*) steaks artificially parasitized with *Anisakis* larvae were subjected to two storage chilling ($5^{\circ}\text{C} \pm 1^{\circ}\text{C}$) and freezing ($-20^{\circ}\text{C} \pm 1^{\circ}\text{C}$) treatments and two food processing treatments of heat (final temperature 86.3°C) and microwave (final temperature 66.9°C) and studied by scanning electron microscopy, environmental scanning electron microscopy (ESEM) (acid [pH = 2] and water preparations), and emission of fluorescence. *Anisakis* larvae were resistant to acid conditions, remaining alive after treatment. Larvae in the heat- and microwave-treated lots presented coagulated and disrupted zones in the cuticle with release of fluids. The cylindrical shape changed to a dehydrated appearance mainly observed by ESEM. Fluorescence was only noticeable in the frozen larvae. Larvae without apparent changes, together with dehydrated ones, were observed by ESEM in the frozen lot; nevertheless, no disruptions in the cuticle were perceptible. Further studies are needed in order to elucidate if the changes observed in the cuticle reduce the resistance of the parasites to the action of gastric enzymes in the gastrointestinal tract and to determine the release of allergens to the flesh by the live larvae during chilled storage of the fish.

The consumption of fish parasitized with Anisakidae larvae is a problem in countries where fish is traditionally eaten raw or undercooked. Anisakiasis (anisakidosis) is a human disease that includes infections (infestations) by nematode parasites having larval stages in aquatic hosts. The main nematodes to cause disease in humans are *Anisakis simplex* and *Pseudoterranova decipiens*; nevertheless, other *Anisakis* sp. and *Pseudoterranova* sp. have been detected (33–36). The final hosts of the parasites are marine mammals (pinnipeds and cetaceans), and the parasites reach sexual maturity in the intestinal tracts of these mammals. The parasite eggs are expelled with the feces in the water. There, the eggs embryonate and undergo at least one moult. These larvae may infect small crustaceans, mainly euphausiids, where they molt to the third stage. Fish and cephalopods are parasitized with third-stage larvae (L3) when they eat euphausiids, parasitized fish, or cephalopods and become paratenic hosts. When an infected fish is consumed by another fish, the larvae may penetrate the viscera and infect the new fish. In the infected fish, the majority of larvae are associated with the gut, mesenteries, and peritoneum. It is accepted that infection of the fish flesh is due to migration of the larvae once the fish is dead. Migration to the muscle is accelerated at higher temperatures and longer times of storage. Nevertheless, migration of the larvae

differs with the fish species and with fish found in the North or South Hemisphere (58, 69). The taxonomy, cycle of the Anisakidae larvae, parasitized fish species, organs infected, and postmortem migration of the larvae in the fish and transmission to man have been studied for decades (18, 28, 36, 40, 44, 46, 57, 58, 69). The prevalence of the Anisakidae larvae and the intensity of the infection in wild fish vary with the fish species, fishing area, season and individual characteristics of the fish (48, 65). When the fish parasitized with *Anisakis* larvae is consumed by humans, it can cause two types of problems: infestation with the parasite (anisakiasis) and allergy. Anisakiasis occurs when the live larva is eaten. The live larvae migrate from the gastrointestinal tract and are embedded or penetrate the gastrointestinal mucosa causing tissue reaction. The clinical signs depend on the zone of the gastrointestinal tract where the larva is located and also if the response is to an acute or chronic infection (3, 12, 22, 27, 32, 38, 45, 49, 51, 67, 68). Clinical symptoms in humans due to the consumption of parasitized fish with live Anisakidae larvae have been described for decades, mainly in countries where raw, undercooked fish or fish subjected to mild treatments where the larvae remain alive is consumed (67, 70). However, the first recognized case relating human allergic reaction to consumption of fish parasitized with *Anisakis* sp. is from 1990 (25). After that, many other cases with differing degrees of intensity have been diagnosed (6, 13, 23, 26, 29). Allergy

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to the parasite occurs in individuals who have been previously sensitized to the live parasite. The prevalence, geographical distribution, frequency, and extent of sensitization among the general population are unknown in spite of the large amount of clinical work done in these areas at present.

Anisakidosis can be prevented by subjecting the fish to treatments in which the larva is killed, although there is disagreement about the intensity of the treatments to kill the larvae, mainly due to differences in the size, composition of the samples, and even variations of the Anisakidae in their ability to survive at low temperatures, because different species or genetically different populations may have different responses to the applied treatment (1, 8, 17, 20, 24, 50, 59, 63). Most of the published works concerning the survival of the Anisakidae larvae are related to freezing and frozen storage. Nevertheless, papers related to the effect of the application of other technologies on the mortality of the Anisakidae larvae have also been published (high temperature or prolonged cooking, microwave processing, high pressure, pH, salt concentration, irradiation, etc.) (1, 15, 39, 53, 54, 63, 66).

Three groups of allergens from *Anisakis simplex* have been described: somatic, the most abundant; excretory-secretory, considered histolytic enzymes that help the parasite to penetrate the gastric mucous and inhibit certain enzymatic systems present in the gastric tract (37, 43); and surface allergens, expressed in the cuticle of the parasite with a role in the development of chronic stimulus (65).

There is controversy among immunologists about allergy caused by the dead parasite (4, 5, 7, 9, 64). This disagreement may be because differences in species or genetically diverse populations, habitat, antemortem conditions, and killing methods of the parasite (freezing, high temperature, etc.) may cause changes in the allergens or in the release of allergens from the parasite to the gastrointestinal tract of the consumer. Proteins have a different response to denaturation and aggregation by storage (freezing and frozen time and temperature) or processing conditions (high temperature, microwave processing, etc.), as some species evolved with adaptive mechanisms that allow them to survive in harsh environments at the extremes of temperature (11, 14, 16, 19, 55, 56). It is important to know how the treatments given to the fish affect the parasite.

The objective of this preliminary study is to detect the effect of different storing and processing treatments on the structure of *A. simplex*.

MATERIALS AND METHODS

Sample preparation. To standardize the conditions of each treatment, we prepared sandwiches of hake steaks as follows: approximately 40 to 60 live *Anisakis simplex* larvae obtained from parasitized hake (*Merluccius merluccius*) ovaries were placed on the dorsal muscle of a steak (1 cm thick) of thawed hake and covered with other steak. Each sandwich was vacuum sealed in heat-resistant bags (WIPAK/GRYSPEERT model PAE 110 K FP; permeability to oxygen 30 cc/m²/24 h measured at 23°C/75% relative humidity; ILPRA Systems España, S.L., Mataró, Spain) and stored in chilled conditions (5°C ± 1°C) or immediately frozen and stored at -20°C. After 20 h of chilled storage, five sandwiches were individually heated for 8 min at 95°C ± 1°C in a

water bath. Another five sandwiches were taken out of the sealed bags and processed individually in a microwave-resistant container covered with microwave-resistant film in a commercial 900-W microwave oven (Fagor Elegance, model MW3-245 B, Fagor, Spain) operating at maximum (100%) power for 3 min. Temperature in the thermal center of the treated sandwiches was recorded immediately after treatment. The times for cooking the fish sandwiches (water bath or microwave treatments) were selected in order to cook fish sandwiches of this size. After treatments, the sandwiches were vacuum packed again and stored at 5°C for 24 h. Afterwards, one sandwich subjected to each treatment was opened to measure emission of fluorescence. The remaining sandwiches were used for electron microscopy studies.

Emission of fluorescence. Emission of fluorescence of the nematodes in the treated and control lots was examined by exposing the open sandwiches to UV light (366 nm) and observing the fluorescence emission in a darkened room, holding the UV light approximately 15 cm above the surface of the fish steak.

Composition of the *Anisakis* cuticles. Composition of the *Anisakis* cuticles was analyzed from samples prepared as for environmental scanning electron microscopy (ESEM). An integrated system analysis with an energy dispersive spectrometer and wavelength dispersive spectrometer of X ray coupled with the FEI-Quanta 200 microscopy (Oxford Instruments, Oxford, UK) was used. Identification of elements was made according to a database of internal standards provided by Oxford Instruments with INCA platform software. No element was omitted in the spectral analyses. Analysis of cuticle was performed semiquantitatively in ≥15 larvae per lot.

Electron microscopy. The microstructure of the larvae was analyzed by ESEM, which permits the imaging of fresh material with no prior specimen preparation (metallization) and by scanning electron microscopy (SEM).

To perform the ESEM studies, pieces of parasitized tissue (muscle and ovaries) were immersed in water for a period of approximately 30 min, after which the *Anisakis* larvae were removed from the muscle with tweezers. Live *Anisakis* separated from the chilled stored lot and ovaries were immersed in glacial acetic acid solution (pH 2) (acid treatment) or in tap water (water treatment) for 7 days, after which selected specimens were examined. Larvae removed from heated, frozen, and microwave-treated lots were maintained in water until examined (maximum time 60 min) with an ESEM (FEI-Quanta 200) operating up to 30 kV in low-vacuum mode (environmental mode) in which the column is under a high-pressure range of 13.3 to 5.320 pascals using water vapor from a built-in water reservoir for preserving the integrity of the hydrated samples.

For SEM observation, individual *Anisakis* larvae and small blocks of parasitized tissue (1.5-cm-sided cubes) were cut from the sandwiches in all lots. The samples were fixed with a mixture (1:1, vol/vol) of paraformaldehyde (4%) and glutaraldehyde (0.2%) in 0.1 M phosphate buffer (pH 7.2), postfixed with OsO₄, washed, dehydrated in increasing concentrations of acetone, critical point dried, sputter coated with gold-palladium in a metallizer (Balzer SCD 040, Liechtenstein) and scanned by SEM (Jeol JSM 6400, Akishima, Tokyo, Japan) at 20 kV.

A large number of micrographs were taken in ESEM and SEM in order to select the most representative ones.

RESULTS

Migration of the larvae from the inner surface of the sandwiches to the interior of the flesh after 19 h of chilled

TABLE 1. Main components of the cuticle of *Anisakis* larvae analyzed by energy dispersive spectrometry in semiquantitative mode^a

Component	Control ^b	Acid treatment ^c	Heated	Microwaved	Frozen ^d
Cl	— ^e	4.71	—	—	—
Na	8.67	—	2.47	4.22	4.40
Mg	2.45	—	2.18	1.50	1.26
P	5.42	8.61	3.92	—	—
S	8.31	17.13	19.63	5.69	3.76
K	7.82	16.51	—	3.97	3.17
Ca	13.84	14.37	45.52	41.34	40.76

^a Data expressed as percent of the element in weight present in cuticle according to each treatment. Number of larvae per lot ≥15.
^b Live *Anisakis* larvae after 7 days in water.
^c Live *Anisakis* larvae after 7 days at pH 2.
^d Frozen *Anisakis* larvae after 44 h frozen at −20°C.
^e —, not detected.

storage at 5°C was observed. Some larvae were detected outside the sandwiches, free in the internal surface of the bag. Movement and migration of the larvae were evident in the ovaries and in the chilled lot after 5 days storage; the longer the storage period, the deeper the penetration in the muscle. Movement was not observed in the frozen, heated, or microwave-treated lots. The temperature in the thermal center of the fish sandwiches in the heated lot reached >60°C after 2 min of immersion in the water bath fixed at 95°C ± 1°C. Maximum temperature recorded was 86.3°C (±1°C) after 8 min treatment. In the microwave-treated sandwiches, the temperature recorded in the thermal center of four sandwiches immediately after the treatment was 66.9°C (±0.5°C). No movement of the larvae was observed in the frozen stored lots (−20°C, 48 h) after thawing the sandwiches at 5°C overnight.

Emission of fluorescence. Emission of fluorescence by the larvae in the open sandwiches when examined under UV light was clearly observed in the frozen lots. Nevertheless, the fluorescence decreased or even disappeared after the thawed sandwiches were heated in a water bath (95°C ± 1°C for 8 min). No clear or very little fluorescence was emitted by the *Anisakis* larvae in the chilled, heated, and microwave-treated lots, irrespective of the treatment.

Composition of the *Anisakis* cuticle. The *Anisakis* cuticle showed differences among treatments (Table 1). There was a significant increase in Ca in the heat, microwave, and freezing treatments compared to the control (water treatment; 7 days in tap water) and acid treatments (pH 2, 7 days). *Anisakis* in the control and acid treatment was obtained alive from the ovaries; however, in the heated, microwave, and frozen treatments the larvae received the treatment inside the fish muscle, which may modify the results if any interaction between muscle and larvae occurs. No element was omitted in the spectral analysis, so absence of elements such as Na and Mg (acid treatment), K (heat treatment), and P (microwave and freezing) in addition to the presence of Cl in the acid treatment should be stressed. Unexpectedly, microwave and freezing treatments gave similar results on the composition of the cuticle of the examined larvae.

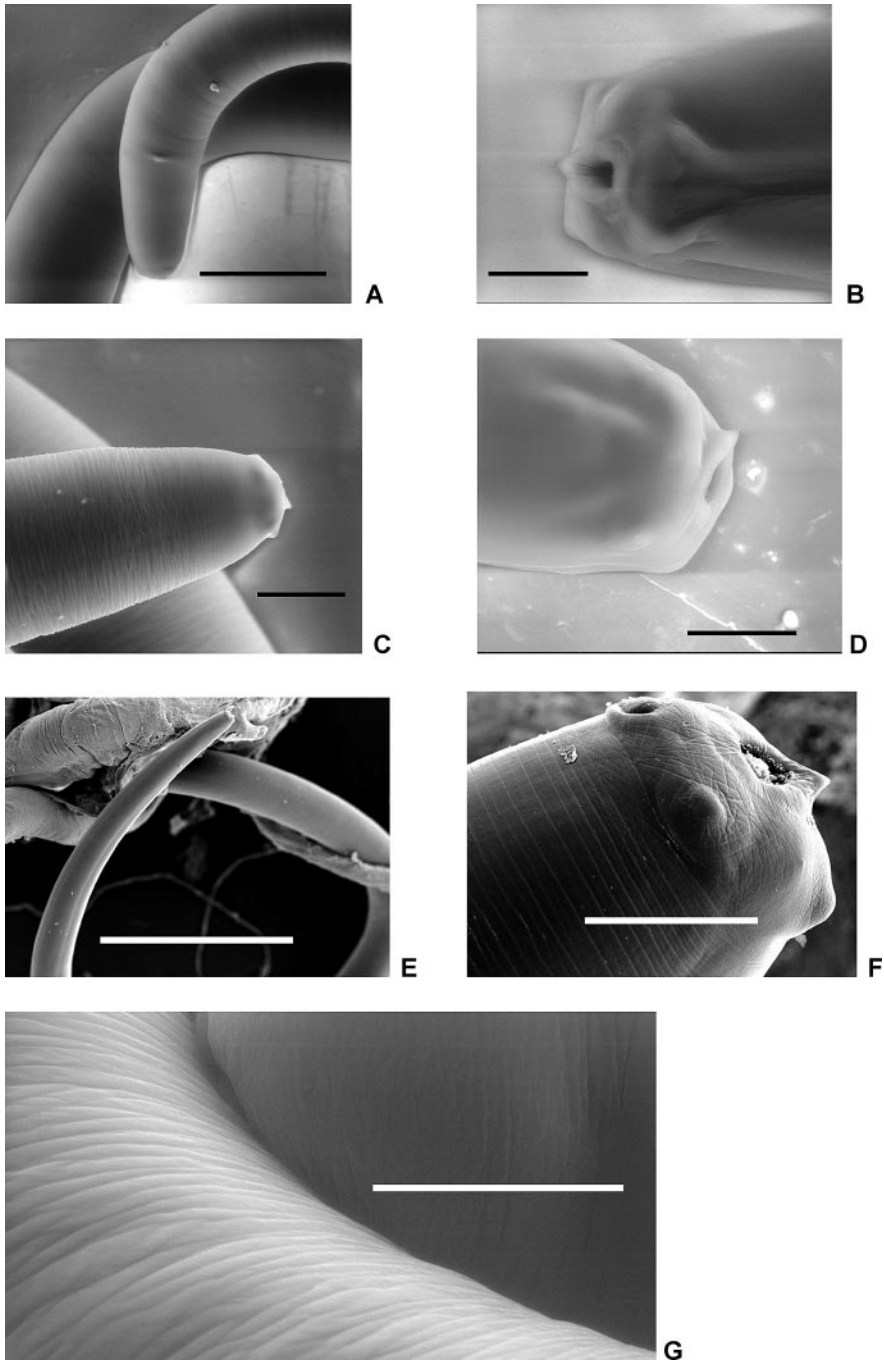
Electron microscopy. No differences were found by ESEM in *Anisakis* larvae obtained from the ovaries (live *Anisakis*), irrespective of the treatment of the sample prior to be examined by ESEM (Fig. 1). The nematodes remained alive after 7 days in the acid treatment (pH 2). In both cases, no changes were detected in the cuticle of the parasite, neither in the excretory pore nor the boring tooth. The shape of the body of the *Anisakis* remained cylindrical, with no apparent changes observed. Figure 1E and 1F shows *Anisakis* larvae obtained from the chilled sandwiches examined by SEM. As with the parasites examined by ESEM, the larvae examined by SEM did not show any apparent damage in the cuticle or in the shape.

Changes in the surface of the *Anisakis* larvae were detected by ESEM in the samples subjected to heat treatment (Fig. 2). Coagulated and disrupted zones in the surface of the parasite are clearly observed in Figure 2A. Figure 2B shows alterations in the shape and in the surface of the parasite. The cylindrical shape was lost, giving a dehydrated image of the body. Disrupted zones in the cuticle with emission of internal material out of the body cavity are also observed. A halo of emission apparently adhered to the cuticle of the parasite is clearly shown in Figure 2C. Penetration of the *Anisakis* larvae in the fish muscle and changes in the cylindrical appearance of the parasite in the heated lot are observed by SEM in Figure 2D and 2E, respectively. Nevertheless, the dehydrated appearance is more marked when observed by ESEM.

Microwave treatment caused alterations in the parasites quite similar to the ones observed by heat, even though the treatment was shorter and the final temperature lower than in the heat-treated samples (Fig. 3). A clear break in the cuticle is observed in Figure 3C (ESEM) and 3E (SEM). An emission of material from the interior of the parasite is clearly appreciated in Figure 3C. As in the heated lot, the dehydrated appearance is more marked when the larvae are observed by ESEM.

Figure 4 shows the *Anisakis* larvae after frozen storage at −20°C. Parasites with no apparent changes were found as shown in Figure 4A (ESEM) and 4C and 4D (SEM). In Figure 4D, the mucron is clearly visible. Nevertheless, parasites with changes in the shape (Fig. 4B) similar to the

FIGURE 1. *Anisakis* larvae extracted from hake ovaries observed by ESEM: A and B, acid treatment (pH2, 7 days) and C and D, water treatment (chilled stored, 7 days). *Anisakis* larvae extracted from hake muscle chilled stored for 44 h, observed by SEM: E and F; G, striations on lateral side (ESEM, water treatment). The excretory pore, boring tooth, and incipient lips are clearly observed in B, D, and F. Bars: A = 300 μ m, B = 50 μ m, C = 100 μ m, D = 50 μ m, E = 3 mm; F = 50 μ m, and G = 50 μ m.



ones observed in treatments where the temperature is raised (Figs. 2B and 3A) were also observed in the frozen lot by ESEM. Surface protuberances with no apparent rupture of the cuticle were evident (Fig. 4B).

DISCUSSION

Fluorescence has been used to detect nematodes in fish muscle. A blue or bluish-white color emitted by *Anisakis* when examined with UV light has been described (1, 2). Detection of the parasites through exposure to UV light is improved by a preliminary freeze and thawing of the sample, but the procedure does not distinguish between live and dead parasites (2). However, other authors have associated the emission of fluorescent white color with the death of the *Anisakis* larvae (24). In our case, a fluorescent bluish

white emission by the *Anisakis* larvae was clearly observed only in the frozen lot. Work is in progress to measure intensity of the fluorescent emission and to elucidate changes due to the treatment applied.

The *Anisakis* larvae were very resistant to the strong acidic conditions used in the preparation of the live parasites to be observed by ESEM. It is known that the adult parasite and the larvae are very resistant to the acidic conditions of the gastric juice of the host. Moreover, the *A. simplex* L3 larvae secrete allergens when incubated in conditions that mimic the gastric juice (41), which can explain the acute allergic symptoms that appear in some patients.

Evident changes were found in the larvae in the heat- and microwave-treated sandwiches where the temperature measured in the thermal center of the sandwiches was

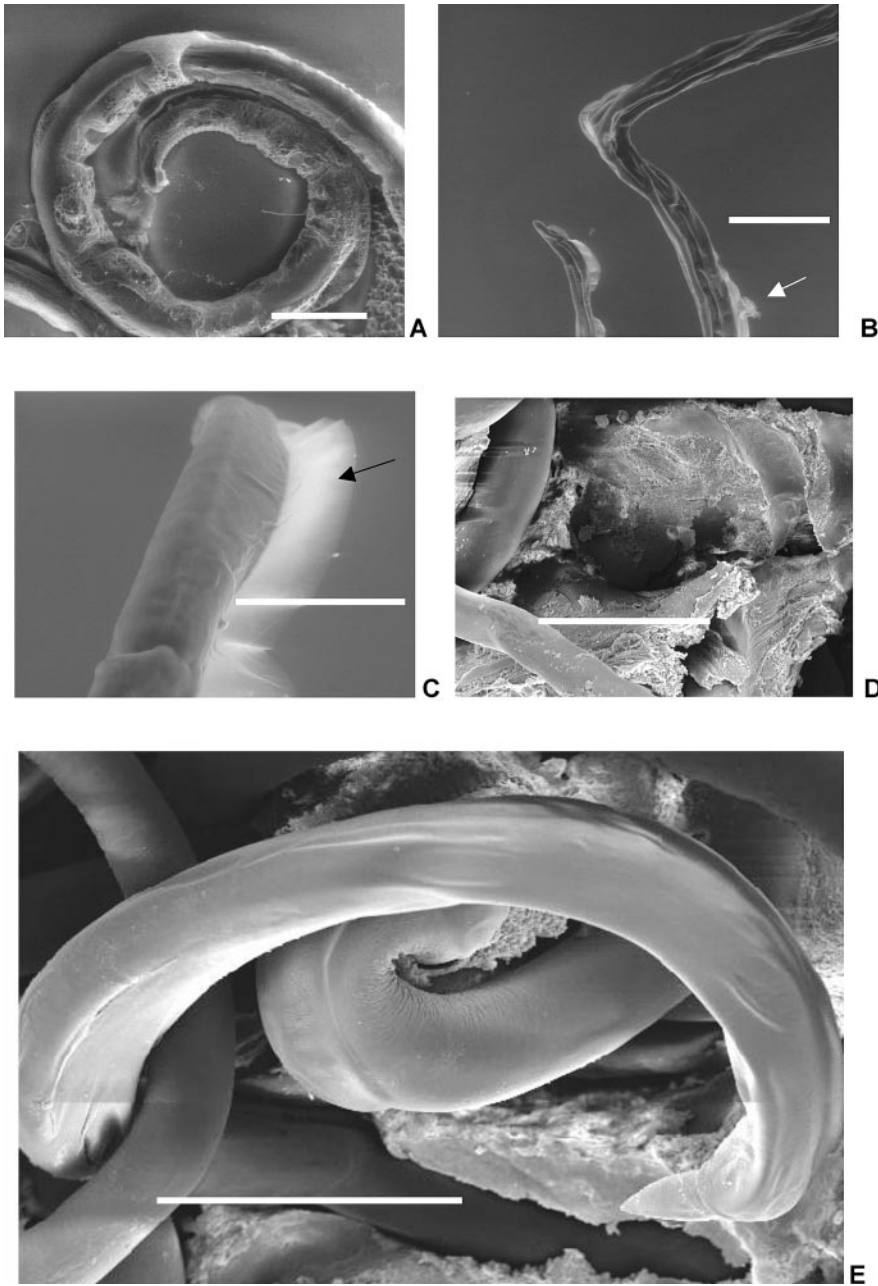


FIGURE 2. *Anisakis* larvae extracted from hake muscle after heating. ESEM: A through C; SEM: D and E. Bars: A and B = 1 mm; C = 200 μ m; D and E = 1 mm. Arrow in B indicates where the cuticle is broken and matter from the interior is expelled. Arrow in C indicates where a halo is observed.

above 66°C. This temperature has been described as sufficient to kill the parasites. No survival of *A. simplex* has been detected in arrowtooth flounder after microwave processing when the samples were heated at temperatures $\geq 65^\circ\text{C}$ (1). The U.S. Food and Drug Administration (63) recommends an internal temperature of 74°C to microwave process fish. The inactivation of parasites in microwave-processed seafood is mainly due to the temperature reached in the sample. Microwave heating generates heat within the material and heats the entire volume, although the final temperature necessary to kill the parasites differs with the species. Nevertheless, any other intrinsic properties of the electromagnetic waves may contribute to the changes observed in the parasites. According to our results, in the treatments that generate heat, the cuticle of *Anisakis* appears disrupted and coagulated, and the body shape changes from a cylindrical form full of fluids to a dehydrated appearance. This

may be due to the combined action of the increase of the internal pressure and changes in the permeability of the cuticle (breakages and changes in size of the pores), which accelerate the release of the internal fluids from the body cavity of the parasites in the conditions of ESEM observation.

Freezing and frozen storage for short time (48 to 72 h) of the parasitized fish caused different response in the *Anisakis* larvae. Parasites with no apparent changes either in the cuticle or in the shape, together with larvae showing a dehydrated shape, were observed by ESEM. Nevertheless, no breakage of the cuticle and no emission of internal matter were evident in the dehydrated individuals.

The cuticle of parasitic nematodes is highly resistant to strong acids and digestive enzymes, regardless of whether the nematodes are alive or have been killed by freezing or conventional heating (1). The cuticle of nematodes is a

FIGURE 3. *Anisakis* larvae extracted from hake muscle after microwave treatment. ESEM: A through C; SEM: D and E. Bars: A = 1 mm; B and C = 200 μ m; D = 3 mm; E = 700 μ m. Arrows in C and E indicate where the cuticle is broken.

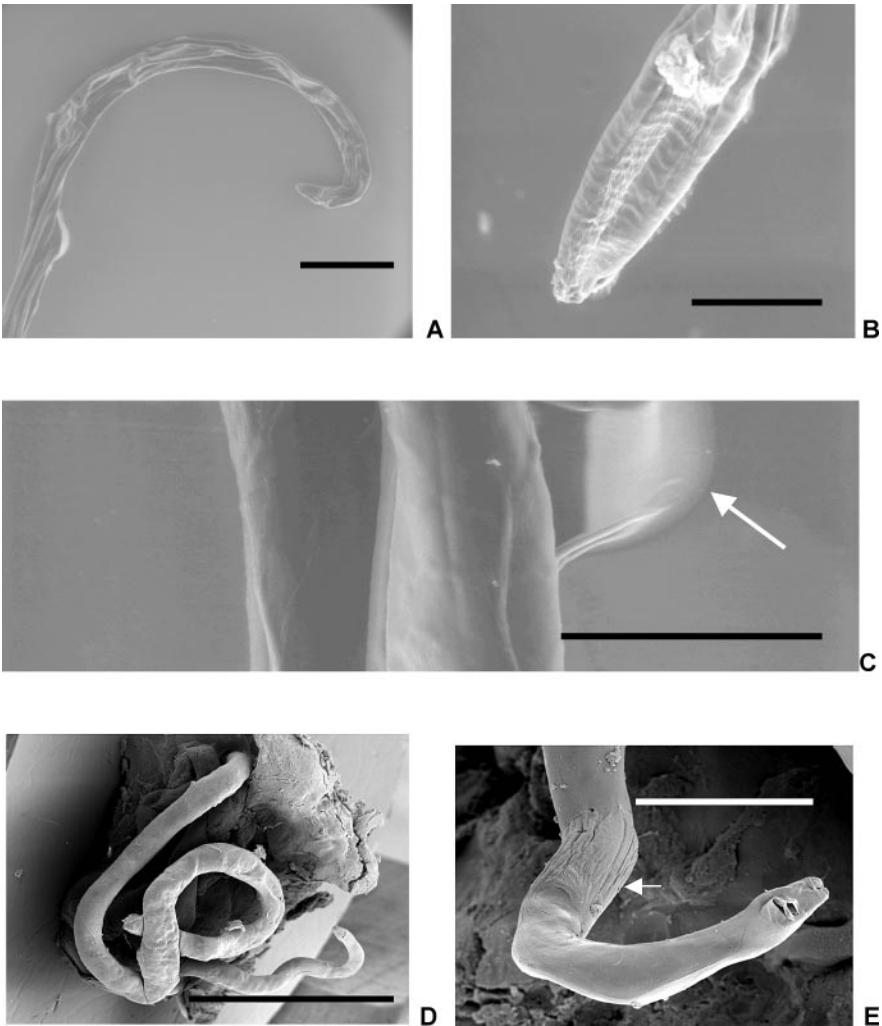
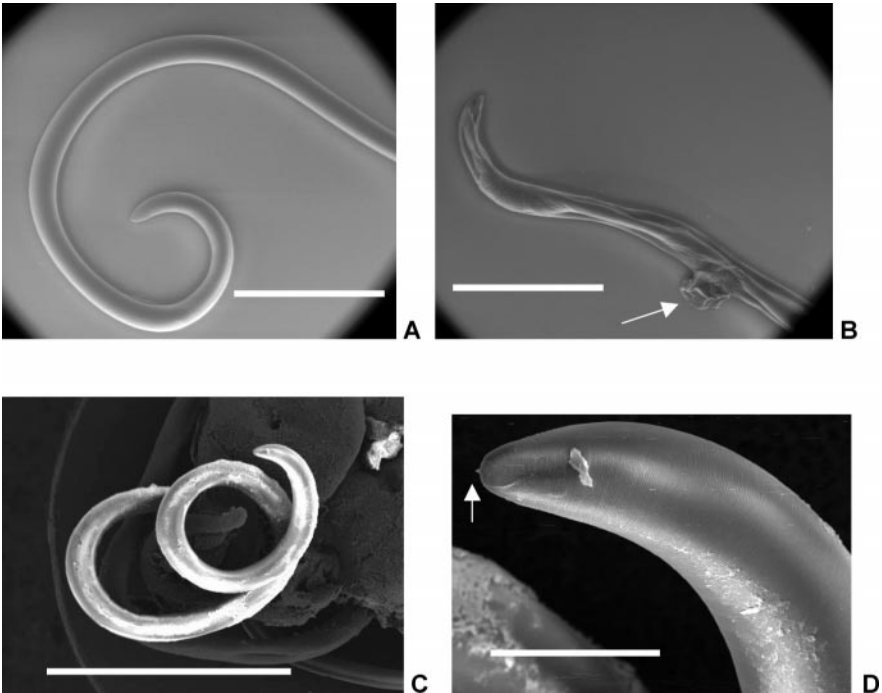


FIGURE 4. *Anisakis* larvae extracted from hake muscle after freezing. ESEM: A and B; SEM: C and D. Bars: A and B = 2 mm; C = 1 mm; D = 400 μ m. Arrow in B indicates where the cuticle protrudes. Arrow in D indicates the mucron in the extreme posterior.



highly ordered, multilayered structure formed by the assembly of macromolecules secreted by the hypodermal cells. Four or five main layers, with much ultrastructural variation among the different species, genera, and developmental stages of nematodes have been described (30, 47, 71). The major structural elements of the nematode cuticle are small collagens, cuticulin, and glycoproteins (52). Several fish muscle proteins are denatured and aggregated at temperatures recommended for frozen storage of the fish. Changes in proteins depend on fish species and are more pronounced at higher temperatures and longer periods of storage (10, 21, 42, 60–62). We can assume that changes in the parasite cuticle during longer periods of frozen storage may also happen, modifying the structure of the cuticle and changing the resistance to heat and gastric juices and, consequently, the allergic response when the parasite is consumed. Research is needed to make clear the effect of different freezing and frozen storage (time-temperature) conditions in the *Anisakis* larvae.

Our results show differences in the parasites determined by the treatment applied. In the samples where the temperature rises (heat and microwaves), the surface of the parasites shows disrupted zones where the cuticle is damaged. Live larvae with a damaged cuticle may be susceptible to the action of enzymes and acid conditions of the gastric solutions when consumed, with the result of fragmentation and dissolution of the larvae and its death, before the attachment to and penetration of the mucosa (1). In this case, no anisakiasis occurs when the larva is ingested. However, the allergic response in sensitized individuals may increase due to the release of somatic allergens, some of them heat and pepsin resistant, which are not protected by the cuticle from the gastric juices. This would explain the cases of allergy in patients sensitized to *Anisakis* after the ingestion of well-cooked or canned fish that are described in the literature (9).

No changes in the L3 larvae stored at chill temperature were observed even in harsh acid conditions used in ESEM. However, highly dehydrated *Anisakis* has been detected by ESEM in lots treated by heat, microwaves, or freezing. Preparation of the samples for SEM and ESEM observation differs. In SEM, the samples were fixed and no further changes are expected, whereas in ESEM the L3 larvae extracted from the treated lots were maintained in water for up to 60 min before observation and up to 7 days in the water and acid treatment given to the live larvae. This suggests that dehydration occurs only when the *Anisakis* cuticle is damaged, even if no apparent breakage is observed as happen in the frozen lot.

It is important to keep in mind that, once the fish is dead and during the period in which the fish is chilled stored, the larvae migrate from the abdominal cavity of the fish to the flesh. In this process, the *Anisakis* larvae secrete and excrete enzymes to the fish muscle, which is accelerated according to the storage conditions (longer time, higher temperature, acid pH, etc.). These allergens may provoke an allergic response when the parasitized flesh is eaten by sensitized consumers, even when the larvae are killed before ingestion of the fish. This would explain the different

allergic responses observed when fish parasitized with dead larvae or only dead larvae are ingested. In the first case, an allergic response was obtained (4, 5, 9), whereas no allergic response occurred when dead larvae were orally supplied to sensitized patients (31, 64).

Additional research is needed to evaluate the effect of storage conditions prior to the death of *Anisakis* larvae and to detect the presence of *Anisakis* allergens in the tissues of parasitized fish, with the aim of elucidating the recognition of allergens when parasitized fish is consumed, even when the larvae are dead as a result of food processing or technological treatments.

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