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Survival of *Anisakis simplex* in Arrowtooth Flounder (*Atheresthes stomia*) during Frozen Storage†

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ABSTRACT

Survival of naturally occurring larvae of *Anisakis simplex* in fresh arrowtooth flounder (*Atheresthes stomia*) was determined after storage for specified periods at four freezing temperatures. All larvae were killed by 96, 60, 12, and 9 h at temperatures of −15, −20, −30, and −40°C, respectively. The average percentages of live larvae per fillet at the next shortest holding time were as follows: 72 h at −15°C, 0 to 3%; 48 h at −20°C, 11 to 30%; 9 h at −30°C, 5%; and 6 h at −40°C, 0 to 3%. Larval survival was directly related to fillet thickness or weight ($P \leq 0.05$). Larval death was directly correlated to freezing temperatures. Holding time necessary to kill larval nematodes decreased as storage temperature decreased.

Individuals who consume raw or undercooked marine fish are at risk of ingesting larval nematodes of the family Anisakidae, which can cause anisakiasis. Three genera of anisakid larvae are reported to cause human diseases, *Anisakis*, *Pseudoterranova*, and *Contracaecum*, although *Anisakis* is most often implicated. These anisakids are present as adults in the gastrointestinal tract of marine mammals. Eggs are passed in the feces of infected marine mammals and subsequently embryonate in the ocean waters. After hatching, the nematode larvae are eaten by small invertebrates, such as small crustacea. If infected crustacea are consumed by fishes or squid, the third-stage larvae migrate from the intestinal tracts into the mesenteries, viscera, or muscles. When the fishes or squid are eaten by marine mammals, the larvae develop into adults in the stomach or intestine of the new host, thus completing the life cycle of the parasite. If infected fish are consumed by humans, larvae may penetrate into the mucosa of the gastrointestinal tract and cause severe abdominal pain, nausea, vomiting, diarrhea, and urticaria *(2)*.

Symptoms can occur 1 to 12 h after consumption of raw or undercooked seafood *(10)*. Usually, treatment requires removal of the larval nematodes with forceps guided by fiberoptic endoscopy *(2)*. In acute cases without appropriate medical intervention, patients may have intermittent abdominal pain, nausea, and vomiting persisting from weeks to several years *(10)*.

Cold smoking and brining are inadequate treatments to kill the nematodes in seafood. Thermal processing is the most effective way to kill the third-stage larvae of *Anisakis.* Heating fish to 65°C by conventional cooking for 10 min or by microwaving to 170°C *(1)* killed all anisakid worms present in fish fillets. Freezing is also an effective processing method to kill the worms. Blast freezing salmon to −35°C or colder for 15 h can effectively kill anisakid worms *(5)*, with more than 99% of the *Anisakis* larvae killed after 1 h at this temperature. Freezing herring in 100-lb (ca. 45-kg) blocks at −30°C for 16 h and then storing at −12°C can kill almost all of the anisakid worms within the first week *(7)*. Other studies demonstrated that storing rockfish for 5 days at −20°C or lower can kill anisakids. According to the U.S. Food and Drug Administration (FDA) Food Code *(14)*, fish products that are raw or uncooked throughout should be frozen at or below −20°C (−4°F) for 7 days or at −35°C (−31°F) for 15 h in a blast freezer.

Since, currently, existing data for freezing are somewhat limited, the purpose of this study was to further define freezing times and temperatures to kill anisakid nematodes in fish fillets. Arrowtooth flounder fillets were stored for specific periods at −15, −20, −30, and −40°C to determine the minimum time required to kill the parasites.

MATERIALS AND METHODS

Fish samples. Arrowtooth flounder (*Atheresthes stomias*), harvested in Alaska or the Canadian Pacific, was selected as the species to study because of its tendency to be naturally infected with nematodes and because of its local availability (Seattle, Wash.). Fish in the round were donated by a local seafood company and sent by overnight mail on wet ice to the University of Washington. On arrival, the fish were manually filleted (skin on), and measurements of weight and thickness of the fillets were recorded before freezing. Fillets were individually placed into 1-qt

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TABLE 1. *Survival of* Anisakis simplex *in arrowtooth flounder after frozen storage*

Freezer temperature (°C)	Time (h)[a]	No. of fillets	Mean ± SD thickness of fillet (mm)	Mean ± SD weight of fillet (g)	Average initial no. of live larvae per fillet	Average final no. of live larvae per fillet	Mean survival (%)
−15	60	10	17.2 ± 3.8	286.0 ± 130.4	26	1.2	3.0
	72	10	14.0 ± 2.3	151.6 ± 61.9	12	0.0	0.0
	72	10	15.3 ± 2.9	210.6 ± 62.1	21	0.3	2.7
	96	4	20.8 ± 1.3	360.5 ± 69.3	33	0.0	0.0
	96	10	15.5 ± 2.2	184.1 ± 50.1	23	0.0	0.0
−20	24	10	19.0 ± 3.1	378.9 ± 104.2	7	2.5	39.8
	36	10	12.8 ± 1.8	159.5 ± 32.4	10	5.2	51.4
	48	4	14.3 ± 0.5	197.3 ± 32.6	10	3.5	29.6
	48	10	15.6 ± 1.4	222.8 ± 40.4	71	7.1	11.2
	60	10	16.0 ± 1.6	247.5 ± 44.7	26	0.0	0.0
	60	10	17.6 ± 3.1	388.3 ± 149.8	14	0.0	0.0
	72	10	14.8 ± 2.7	210.3 ± 68.6	64	0.0	0.0
	72	10	16.4 ± 2.8	282.5 ± 66.9	29	0.0	0.0
−30	1	5	19.2 ± 3.4	360.2 ± 127.8	14	13.6	100.0
	2	5	15.4 ± 1.9	219.0 ± 28.7	34	5.0	27.5
	4	9	15.3 ± 1.4	214.4 ± 29.0	28	0.0	0.0
	4	10	20.0 ± 4.1	430.9 ± 164.8	35	14.0	38.0
	6	10	14.4 ± 2.3	176.8 ± 59.5	17	0.0	0.0
	6	10	15.7 ± 4.1	278.9 ± 147.1	32	2.0	4.7
	9	10	18.6 ± 3.2	282.6 ± 114.0	22	1.0	4.7
	12	10	16.7 ± 1.1	304.2 ± 57.1	31	0.0	0.0
	12	10	15.5 ± 1.5	155.4 ± 45.3	193	0.0	0.0
	24	10	16.2 ± 2.2	290.4 ± 86.7	60	0.0	0.0
−40	1	4	16.0 ± 2.6	248.5 ± 90.2	27	6.0	20.0
	2	5	16.8 ± 1.5	277.0 ± 66.4	17	0.0	0.0
	2	10	13.7 ± 1.9	176.1 ± 45.4	28	3.0	12.6
	3	10	20.0 ± 3.2	353.1 ± 124.5	41	8.0	20.3
	6	9	16.0 ± 2.7	364.6 ± 185.8	13	0.0	0.0
	6	10	15.3 ± 1.4	208.1 ± 49.8	10	0.4	2.9
	9	10	15.3 ± 2.5	176.0 ± 69.7	29	0.0	0.0
	9	10	18.2 ± 3.5	264.3 ± 112.2	44	0.0	0.0
	9	10	16.0 ± 2.2	161.0 ± 46.3	199	0.0	0.0
	12	10	15.0 ± 1.2	203.8 ± 49.6	9	0.0	0.0
	24	10	15.2 ± 2.0	219.4 ± 44.6	36	0.0	0.0
	24	10	15.3 ± 2.5	212.1 ± 75.0	15	0.0	0.0

[a] Listing of duplicate times indicates different runs.

Ziploc plastic bags, closed, held in the 0 to 5°C walk-in refrigerator for 1 to 3 days, and placed in single layers in a −15°C front-loading freezer compartment of a refrigerator, a −20°C chest freezer, or a −30°C or −40°C walk-in, nonblast freezer. Batches, usually consisting of 10 fillets, were removed from the freezers at specific times. Storage time (Table 1) was defined as the time from when batches of fillets were placed in the freezer until they were removed. Immediately after removal from the freezers, the fillets were thawed for 1 to 2 h in cold running water (18°C) while still in the plastic bags. After thawing, the fillets were promptly analyzed. Occasionally, fillets were stored in the 0 to 5°C refrigerator for 1 to 2 days after thawing and before analysis. Maintaining fillets in the refrigerator for this period did not appear to affect the viability of the nematodes. Gibson *(6)* provided a review of previous work and stated that after 20 days of storage at 4°C, 75% of *Anisakis* larvae were still motile, although they were no longer infectious in guinea pigs.

Monitoring of time to reach target temperature. To monitor the time for the fillets to reach the target temperature in the freezers, a temperature probe was connected to a strip chart recorder (Flatbed Recorder, single channel, model Bd111, Kipp and Zonen, Delft BV, Holland). Standardization of the strip chart recorder was achieved by inserting the probe into a thermos of wet ice water (0°C) or a wet ice water solution saturated with NaCl (−21.1°C). The NaCl-saturated ice water was prepared from 750 g of ice and 500 g of NaCl.

After the flounder were filleted, weighed, and measured, the temperature probe was inserted into the center of the thickest part of the fillet. Each fillet was placed into a 1-qt Ziploc plastic bag then placed in the −15, −20, −30, or −40°C freezers. Time for the fillet to reach the lowest temperature was calculated from the output recorded on the strip chart.

The method for calculating the freezing periods A and B is shown in Figure 1. Freezing period A is composed of the time from the attainment of 0°C until the time corresponding to the intersection of a straight line tangent to the steepest section of the freezing curve below 0°C and a line at 0°C. Freezing period B consists of the time at the end of freezing period A until the time

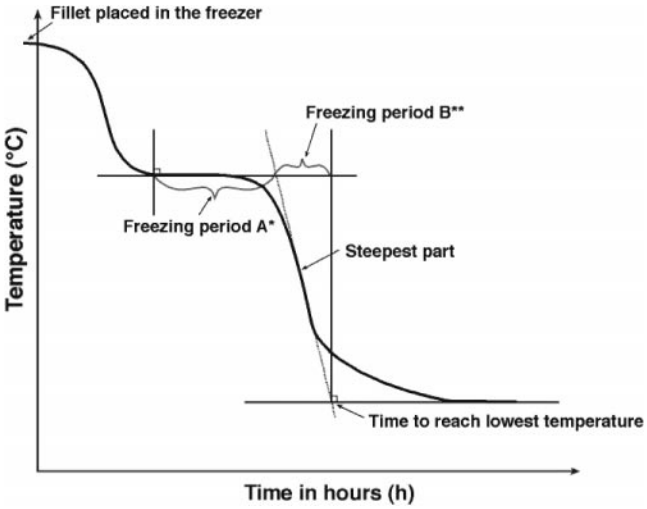

FIGURE 1. *General representation of a freezing curve. During freezing period A (*), the temperature of the fillet does not change and remains essentially at 0°C. Freezing period B (**) consists of the projected time for the temperature of the fillet to decrease from the end of period A (essentially 0°C) to the lowest temperature in the freezer (usually the target temperature).*

where the line tangent to the steepest section of the freezing curve intersects a line that indicates the lowest temperature attained. The freezing rate equaled the difference in temperature between the beginning and the end of freezing period B divided by freezing period B (in hours).

Digestion procedure. Nematodes were isolated by the pepsin digestion method described originally by Van Velzen *(15)* and later by Adams et al. *(1)*, except that cold tap water rather than distilled water was used to rinse away hydrolyzed protein. After thawing, fillets were placed in the digesting solution until the flesh was completely hydrolyzed, leaving the nematodes intact. All larvae were removed with forceps from a pan and placed in petri dishes that contained cold tap water. Identification of the nematodes was performed according to the taxonomic parameters given by Nagasawa and Moravec *(9)*, Larizza and Vovlas *(8)*, and Smith and Wootten *(12)*.

Nematodes were counted and assessed for viability. After being placed under a dissecting microscope, nematodes were gently probed with forceps. All larvae that showed movement spontaneously were considered to be alive and were reported as ''live.'' If the larvae did not show spontaneous movement but moved after being stimulated with forceps, the nematode was regarded as moribund and was reported as ''live.'' If there was no reaction after being stimulated three times, the nematode was reported as ''dead.''

Statistical analysis. Linear regression analyses of freezing time, fillet weight, and fillet thickness as the independent variables and percentage of survival as the dependent variable were performed using the least squares method of Microsoft Excel 97 (Microsoft Corp, Redmond, Wash.); the correlation was considered statistically significant when $P \leq 0.05$. Because the number of worms in each fillet had no significant correlation to percentage of survival, data from all fillets that contained worms were used in the analyses.

RESULTS

Holding times required to kill *Anisakis simplex* larvae and the temperatures required were inversely related. The higher (warmer) the freezer temperature, the longer the time required to kill the larvae. Holding times effective in killing all larvae were confirmed with at least two separate batches of fish. In the −15°C freezer, storage for 96 h resulted in no survival of larvae (Table 1), whereas 60- and 72-h storage periods resulted in approximately 3% survival of larvae. At −20°C, a holding time of 60 h was required to kill all larvae, whereas survival ranged from 11 to 51% when the fillets were stored for 24 to 48 h. In the −30°C walk-in, nonblast freezer, only 12 h were required to kill all larvae; survival was 0 to 38% after 2 to 9 h, whereas 100% of the larvae survived after 1 h. When fillets were stored in a −40°C walk-in, nonblast freezer, a period of 9 h was required to kill all of the larvae, whereas 0 to 20% of the larvae survived after 1 to 6 h.

The time required for the internal section of the fillet to reach the lowest temperature, the lowest temperature attained, the two freezing periods A and B, and the freezing rate were calculated from data obtained with the temperature probe and strip chart recorder (Table 2). The times required to reach the lowest temperatures in the four different freezers ranged from 7.4 to 9.5 h, with no significant correlation with freezer temperature. The lowest temperatures achieved inside the fillets averaged 1 to 2°C lower than the respective ambient freezer temperatures, except for the −40°C freezer, where the fillets averaged 10°C lower. Frequent use of the −40°C freezer caused parts of the room to be cooled to temperatures as low as −50°C (Table 2) but

TABLE 2. *Time in the freezer for arrowtooth flounder fillets to reach the lowest temperature*

Freezer temperature (°C)	No. of fillets	Mean ± SD thickness (mm)	Mean ± SD weight (g)	Mean ± SD time to reach lowest temperature (h)	Mean ± SD lowest temperature (°C)	Mean ± SD freezing period A (h)[a]	Mean ± SD freezing period B (h)[a]	Mean ± SD freezing rate (°C/h)
−15	4	14.9 ± 2.6	200.5 ± 56.9	7.9 ± 0.4	−16.8 ± 0.7	2.6 ± 0.2	1.7 ± 1.0	11.2 ± 4.8
−20	16	17.1 ± 1.7	313.1 ± 95.4	9.5 ± 1.8	−21.4 ± 1.6	3.4 ± 0.9	2.1 ± 0.9	11.4 ± 4.9
−30	7	15.9 ± 5.5	200.3 ± 157.7	7.4 ± 3.4	−31.6 ± 1.3	2.3 ± 1.1	1.1 ± 3.3	31.1 ± 12.1
−40	6	16.3 ± 3.2	205.2 ± 84.6	8.8 ± 1.9	−50.2 ± 2.1	1.1 ± 0.2	1.0 ± 0.2	48.9 ± 7.7

[a] Freezing period A: the temperature of the fillet does not change and remains essentially at 0°C. Freezing period B: the time for the temperature of the fillet to decrease from the end of period A (essentially 0°C) to the lowest temperature in the freezer (the target temperature).

did not allow the temperature to be higher than −40°C. The average of freezing period A of the fillets in the four freezers ranged from 1.1 to 3.4 h, and freezing period B ranged from 1.0 to 2.1 h, with no correlation to freezer temperature. The freezing rate was −11.6 (−15°C freezer), −11.4 (−20°C freezer), −31.1 (−30°C freezer), and −48.9°C/h (−40°C freezer). In the −30 and −40°C freezers, the fillets reached the lowest temperatures in 1 h. Based on the difference between the total time required to kill all larvae (Table 1) and the time required to reach the lowest temperature (Table 2), the time that fillets remained at the lowest internal temperature can be calculated as follows: at −15°C, 88.1 h; at −20°C, 50.5 h; at −30°C, 4.6 h; and at −40°C, 0.2 h.

Regression analyses were performed to determine if storage time, thickness of the fillet, or weight of the fillet was significantly correlated to percentage of survival of larvae at each freezer temperature. For regression analyses of storage time, the storage time and the percentage of survival (average per batch of fillets) data (Table 1) were analyzed for each freezer temperature. Data from the shortest period up to and including the batches that confirmed the minimum lethal freezing time (2 batches at −20 and −30°C; 3 batches at −40°C) were entered into the regression analysis. For regression analyses of fillet thickness and weight versus percentage of survival, if the average percentage of survival for the batch was between 10 and 90%, then the data for the individual fillets for that batch were entered into the analysis. For regressions of storage time and percentage of survival, significant negative correlations ($P \leq 0.05$) were found at −20 (Fig. 2a), −30 (Fig. 2b), and −40°C (Fig. 2c). For regressions of thickness or weight as the independent variables and percentage of survival as the dependent variable, there was a trend of a positive correlation at all temperatures. Significant correlations were found at −40°C for thickness ($P = 0.05$) and weight ($P = 0.03$) (Fig. 3a and 3b, respectively).

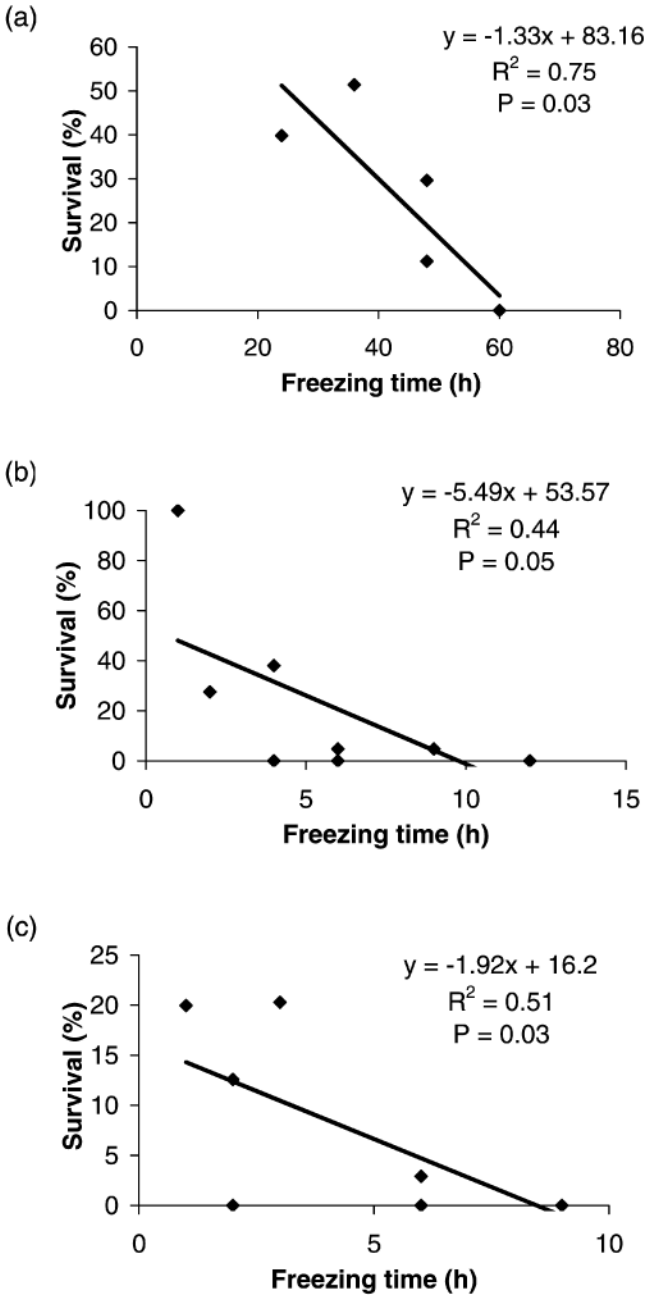

FIGURE 2. *Effects of freezing time on the survival of* Anisakis simplex *larvae in arrowtooth flounder fillets. Time and average percentage of survival plotted from Table 1 up to and including the shortest time effective in killing all larvae (60 h at −20°C, 12 h at −30°C, 9 h at −40°C). (a) −20°C* (n = 6); *(b) −30°C* (n = 9); *(c) −40°C* (n = 9).

DISCUSSION

This investigation was prompted by several needs. The FDA Food Code for retailers *(14)* and the FDA Center for Food Safety and Applied Nutrition's *Fish and Fishery Products Hazards and Controls Guide (13)* recommend freezing at −20°C (−4°F) or below (internal or external) for 7 days or −35°C (−31°F) or below (internal) for 15 h to kill parasites in fish intended for consumption in a raw or undercooked state. Even though this information is widely available, processors in the industry often hold fish at temperatures other than −20 or −35°C and question the effective processing times required at these alternate freezer temperatures. From a regulatory and consumer safety standpoint, the minimum effective processing times need to be known so that a safety factor can be added before making recommendations to the public. Alternatively, if agency recommendations are assumed to have extra processing time added to the minimum needed, then the minimum should be known so that fish are not processed for less than that amount of time.

In this study, fish fillets were held in the freezer for a total of 96 h or for 88.1 h at the internal temperature of −15°C to kill all anisakid nematodes. This type of nonblast freezer at this temperature is similar to those used by consumers, showing that a minimum storage time of 4 full days is required to kill all worms; holding at −15°C for 3 days resulted in some survival (0 to 3%) of worms.

Using a −20°C chest freezer, a storage time of 60 h in the freezer or 50.5 h at an internal temperature of −20°C were required to kill all *Anisakis* nematodes. The Food Code recommends 7 days or 168 h at a temperature of −20°C, which represents a 2.8-fold safety factor. In our

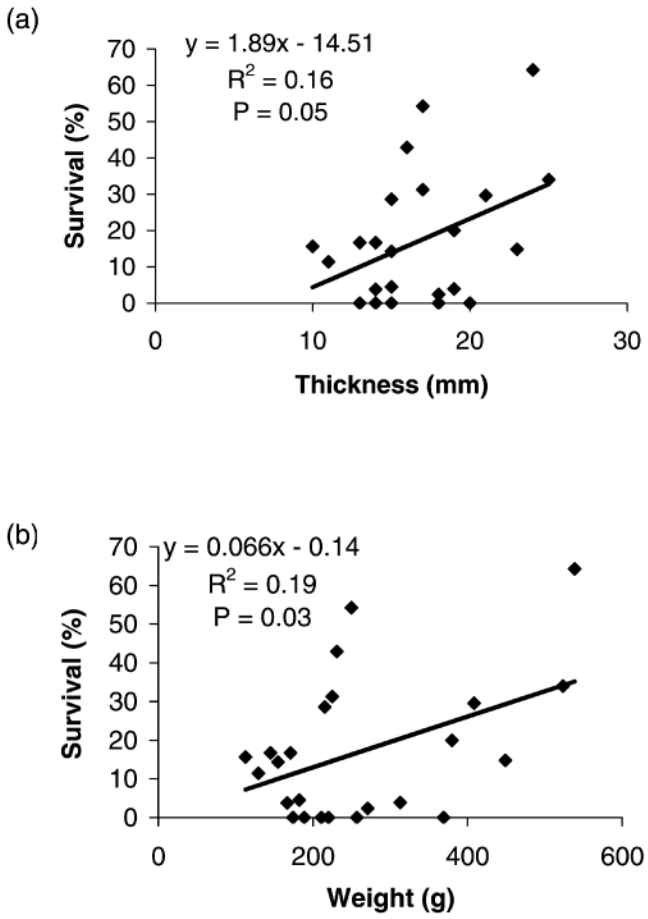

FIGURE 3. *Percentage of survival of* Anisakis simplex *larvae in fillets of arrowtooth flounder at −40°C. (a) Effect of thickness of the fillet. (b) Effect of weight of the fillet. Fillets were stored at −40°C for 1, 2, or 3 h. Data from 24 fillets are plotted individually.*

study, a storage time of 48 h resulted in survival of 11 to 30% of the worms in the fillets, emphasizing the importance of a severalfold safety factor when making recommendations. Our data are in direct contrast to the Netherlands' Green Herring Laws, where a temperature of −20°C or colder is recommended for only 24 h *(11)*; however, factors such as fish size or thickness (discussed below) may affect recommendations. Deardorff et al. *(4)* recommended holding Hawaiian snappers (four species) in the round for 1 day at −20°C and imported rockfish (*Sebastes* sp.) in the round for 5 days at this temperature. Their work demonstrated differences in required freezing times for different species of fish.

For fish held in a −30°C walk-in, nonblast freezer, a total holding time in the freezer of 12 h or for 4.6 h at an internal temperature of −30°C was required to kill all *Anisakis* nematodes. If the internal temperature data at −30 and −40°C are interpolated, a processing time at −35°C of approximately 2.4 h (average of 4.6 + 0.2 h) is obtained. Deardorff and Throm *(5)* and the Food Code recommend 15 h in a −35°C blast freezer. Since blast freezers can lower the temperature of fish from −15 to −40°C in less than 1 h, it can be concluded that for arrowtooth flounder, the Food Code recommendation represents an approximate three-fold safety factor. Deardorff and Throm *(5)* performed their research on sockeye salmon (*Oncorhynchus nerka*) and canary rockfish (*Sebastes pinniger*), with the fish presented in the round or dressed (headed and gutted). Their work illustrates the differences in required freezing time or temperature that can be attributed to species of fish and/or thickness of the seafood product.

The importance of reporting the rate of freezing and the type of freezer used (blast or nonblast) in these types of studies cannot be overemphasized. Blast freezers were not used in the current study; if they had been used, the lethal processing times would have been considerably shorter. Also, for household freezers, an observation must be stated to further support the additional time recommended in the FDA Food Code. In this study, the contents of the freezer and distribution of the fillets were easily controlled, whereas many freezers used by the consumer are subject to practices that raise the temperature. Household freezers are opened more frequently and nonfrozen products may be placed in the freezer.

The mechanism by which subfreezing temperatures kill nematodes has been hypothesized by Deardorff and Throm *(5)* and Wharton *(16)* to be the disruption of the body membranes by ice crystals, dehydration of the worm when the ice crystals are formed, and resulting increase in solute concentration. Besides the freezing rate and the type of freezer, other variables that can affect heat transfer and thereby survival of nematodes in fish are fish or fillet thickness and species of fish. For example, fish in the round (whole fish) may be more protective for the nematodes than dressed (headed and gutted) fish. Deardorff and Throm *(5)* proposed that the presence of viscera may slow the freezing process. At the least, whole fish are thicker than other processed forms, providing a buffer for the nematodes to the change in temperatures. Species of fish may simply provide some protection to freezing by a difference in size; however, those species that are cold tolerant may afford some protection to their respective parasites *(4)*. Additionally, some parasites are known to have developed their own tolerance to cold temperatures and freezing *(16)*. Differences in proximate composition and preparation of fillets (e.g., skin on or off) before freezing may also affect worm survival, especially in nonblast freezers. Dailey *(3)* stated that the size of the sample, temperature, and time of exposure to the freezing temperatures are the most important factors that affect the kill rate.

Our observations on the behavior of moribund worms agree with those reported by others. Deardorff and Throm *(5)* reported that of 3,545 third-stage larvae of *A. simplex*, only six survived blast freezing at −35°C for 15 h then held at −18°C for 1 h. These six worms showed no movement without tactile stimulation. The internal structures of the worms appeared to be severely damaged by dissociation of esophageal and intestinal tracts, which Deardorff and Throm *(5)* believed to have been due to the cooling and formation of intracellular ice crystals. Deardorff and Throm *(5)* proposed that moribund worms probably would not be able to penetrate tissues. Gibson *(6)* performed infection studies with *Anisakis* larvae and observed that those dam-

aged or broken had a shorter life span in saline at 5°C than undamaged larvae and that refrigeration could decrease the infectivity of the larvae. Although we agree with the hypotheses forwarded by these authors *(5, 6)*, no substantive data provide assurances that damaged larvae are not infective. Therefore, for regulatory purposes and public safety, we propose that moribund worms should be assumed to be alive and infectious when testing the effectiveness of different freeze-processing procedures.

The data in the current study suggest that the freezing time, weight of the fillet, or thickness of the fillet were sometimes significantly correlated with percentage of survival. In cases with significant correlations, an inverse relationship occurred between freezing time and percentage of survival, and positive correlations between either weight or thickness and percentage of survival were observed. The range and repetition of freezing times tested can affect the results of regression analysis. Deardorff and Throm *(5)* suggested that the rate of survival of nematodes may be attributed to thickness of the fish, which our data supported at −40°C.

For future studies that contribute to regulatory guidelines, we recommend that experiments include an internal probe in sample fillets to monitor the reduction in temperature so that the time to reach the lowest temperature can be recorded and the freezing rate calculated. The size and species of the fish should be reported, as well as how the fish are processed before freezing (e.g., in the round, dressed, or filleted). Thickness and weight should be recorded if fillets are being prepared. Moribund worms should be described and noted, but they should be counted as alive.

In conclusion, time and temperature recommendations within the FDA Food Code provide a general framework for seafood safety in regard to parasites. However, if more specific parameters are desired by industry or the consumer, then the variables mentioned herein must be considered and included within studies tailored specifically for the seafood product, including the species of fish and portion processed (e.g., fillets, steaks, dressed).

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