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The response of *Anisakis* larvae to freezing

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Abstract

Anisakis third stage larvae utilize a variety of fish as intermediate hosts. Uncooked fish are rendered safe for human consumption by freezing. Larvae freeze by inoculative freezing from the surrounding medium but can survive freezing at temperatures down to −10°C. This ability may be aided by the production of trehalose, which can act as a cryoprotectant, but does not involve recrystallization inhibition. Monitoring of fish freezing in commercial blast freezers and under conditions which simulate those of a domestic freezer, indicate that it can take a long time for all parts of the fish to reach a temperature that will kill the larvae. This, and the moderate freezing tolerance of larvae, emphasizes the need for fish to be frozen at a low enough temperature and for a sufficient time to ensure that fish are safe for consumption.

Introduction

Marine mammals (cetaceans and pinnipeds) are the definitive hosts of *Anisakis* but this nematode uses a wide variety of fish as its second intermediate host. Larvae encyst in the visceral cavity and musculature of fish and humans can be infected if they consume uncooked or poorly-cooked fish and fish products containing live larvae (Smith & Wootten, 1978). The increasing popularity of raw fish dishes, such as sushi and sashimi, has led to increasing concerns regarding the potential for human infections with *Anisakis*, particularly where such dishes have not been professionally prepared (Oshima, 1987; Takabe *et al.*, 1998). Freezing at a low enough temperature and for a time sufficient to kill *Anisakis* larvae has been an important procedure that renders raw fish products safe for human consumption (Gustafson, 1953; Deardorff *et al.*, 1984; Deardorff & Throm, 1988; Karl & Priebe, 1991). However, we know little of the response of *Anisakis* larvae to freezing, other than they are killed if subjected to a sufficient degree of freezing stress. In this paper we determine the response of *Anisakis* larvae to freezing, define the freezing conditions that will kill larvae in some New Zealand commercial fish species and investigate some of the factors that may confer a moderate ability to survive freezing in this nematode.

Cold tolerant nematodes survive subzero temperatures either by surviving freezing or they avoid freezing by having an eggshell or a sheath that prevents inoculative freezing from ice in the surrounding medium (Wharton, 1995). *Anisakis* larvae are in contact with the water that is contained within the tissues of their fish hosts. When the fish freezes the nematodes may freeze by inoculative freezing. The capsule of host connective tissue, which usually surrounds the larvae (Smith & Wootten, 1978) may, however, prevent inoculative freezing and allow the nematode to avoid freezing and the larvae to supercool in the presence of external ice. In the absence of a structure that prevents inoculative freezing the nematode will freeze. Some nematodes, however, can survive ice forming within their bodies (Wharton, 1995). The free-living Antarctic nematode *Panagrolaimus davidi*, for example, will survive extensive intracellular ice formation and temperatures as low as −80°C (Wharton & Brown, 1991; Wharton & Ferns, 1995; Wharton & Block, 1997). Here we determine to what extent *Anisakis* third stage larvae (L3) display cold tolerance (can survive subzero temperatures) and, if they do so, whether they use freeze avoidance or freeze tolerance.

Materials and methods

Anisakis L3 were dissected from the viscera of barracouta (*Thyristes atun*), obtained from commercial inshore trawlers fishing off the coast of Otago, South Island, New Zealand. Fish were stored on ice after capture and were dissected fresh, soon after landing.

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Barracouta are heavily infected with *Anisakis* L3 (Wharton *et al.*, 1999), which were kept refrigerated at 4°C and used within five days.

Freezing of L3 on a microscope cold stage

Individual L3 were sandwiched between two coverslips in a drop of 0.9% NaCl on a thermoelectric cold microscope stage with a computer control system. This allows the control of a cycle of cooling and warming, and the nematode to be observed during freezing and thawing. The design and operation of the cold stage has previously been described (Wharton & Rowland, 1984; Wharton & Allan, 1989; Wharton & McCormick, 1993). Briefly it uses thermoelectric cooling modules, the hot faces of which are cooled by fluid from a refrigerated circulator (Haake F3Q). The specimen temperature is monitored via a NiCr/NiAl thermocouple and a Comark electronic thermometer. A thermocouple is also interfaced to a computer which compares the temperature to that determined by a programmed temperature cycle and pulses power to the thermoelectric cooling modules to match the two.

Larval nematodes were cooled rapidly to 2°C, held at this temperature for 2 min and then cooled at 0.5°C min^{-1} to various subzero temperatures. The larvae were held at the test temperature for 2 min and then warmed to 2°C at 2°C min^{-1}. The temperature at which the medium and the larvae froze was recorded and their appearance during freezing and melting noted.

Freezing and survival

Individual L3 were transferred to the cold stage and subjected to the cooling/warming cycle described above. Whether or not the L3 had frozen during cooling was noted. After rewarming, L3 were removed from the cold stage, transferred to 0.9% NaCl and incubated at 4°C for 24 h. The L3 were then allowed to warm to room temperature and their survival assessed by whether they moved in response to stimulation with a dissection needle. The L3 were exposed to minimum temperatures in the range −2°C to −15°C with a total of 5–8 larvae being exposed to each test temperature. The %survival and the %frozen was calculated for each test temperature. The temperatures at which 50% of the L3 froze (F_{50}) and were killed (S_{50}) were calculated using the methods of probit analysis (Finney, 1952).

The cold stage would only accommodate one *Anisakis* L3 at a time and so a multi-specimen cooling block, which holds six 0.5 ml Eppendorf tubes and thus allows a large number of larval nematodes to be exposed to a cooling/warming cycle (Wharton & McCormick, 1993), was used to study the effect of the duration of exposure to subzero temperatures on survival. The L3 in 0.9% NaCl were transferred to Eppendorf tubes in the specimen holders of the cooling block and rapidly cooled to −1.5°C. Freezing of samples was initiated by adding a small ice crystal. Samples were held at −1.5°C for 1 h, to ensure that they froze completely, and then were cooled further at 0.5°C min^{-1} to −5°C, −10°C or −15°C. They were held at these temperatures for various times up to 5 h, the cooling was then terminated and the samples allowed to warm to room temperature. Survival was assessed after 24 h and the S_{50} calculated, as before.

Freezing patterns of fish

To determine the pattern of freezing of fish under conditions similar to those in a domestic freezer, the fish were placed in an insulated chamber lined with coils of tubing, through which fluid from the refrigerated circulator was circulated. A small fan within the chamber ensured an even temperature. The temperature was monitored using a platinum-resistance temperature probe connected to the refrigerated circulator, which thus controlled the temperature within the chamber. Thermocouples were placed inside the viscera, fillets (flesh) and just beneath the skin of whole (ungutted) red gurnard (*Chelidonichthys kumu*). Outputs from the thermocouples were monitored via a Macintosh computer and a Maclab A/D interface (Analog Digital Instruments Ltd, London). Temperature records were analysed using a computer programme (Chart v3.2.7, Analog Digital Instruments Ltd). Although the temperature of the chamber decreased steadily, freezing of the fish elevated the temperature of fish tissues to their melting point as a result of the release of latent heat of fusion. The temperature at which fish tissues froze and the duration of the freezing event could thus be determined from the temperature record. Three fish were frozen to −20°C and one fish was frozen to −10°C.

To determine the freezing of fish under commercial conditions, dataloggers (Stowaway Xti, Energy Engineering, Auckland, New Zealand) with attached temperature probes were placed in boxes of processed fish. These were placed in commercial blast freezers (−35°C to −38°C) at the premises of Otakou Fisheries, Dunedin, New Zealand. Three 10 kg boxes of filleted blue cod (*Parapercis colias*), four of whole New Zealand sole (*Peltorhamphus novaezeelandiae*) and two 20 kg boxes of headed and gutted monkfish (*Kathetostoma giganteum*) were used. The dataloggers recorded temperatures every 30 s and produced a temperature trace which allowed the temperature and duration of the freezing of the fish to be determined.

Potential adaptations for freezing tolerance in Anisakis *L3*

Concentrations of sugars and sugar alcohols, which could be acting as cryoprotectants, were determined using gas chromatography, as described by Wharton *et al.* (2000). The mean dry weights of individual *Anisakis* L3 were determined after drying at 60°C for 24 h. Each sample consisted of 5, 10 or 20 L3.

To look for recrystallization inhibition, ten L3 were homogenized in 1 ml of buffer (Tris-HCl, 100 mmol, pH 7.8), centrifuged at 10,000 *g* for 10 min and the supernatant taken. Droplets (15 μl) were frozen using the 'splat-freezing' technique (Knight *et al.*, 1988) and transferred to a microscope cold stage at −20°C, mounted on a Zeiss Axiophot Photomicroscope. The specimen was allowed to warm to −8°C, annealed at −8°C for 30 min and ice crystals photographed under polarized light (Ramløv *et al.*, 1996). The maximum diameter of the ten largest ice

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crystals were measured. Samples of buffer were also analysed as a control.

Results

Freezing of L3 on a microscope cold stage

Nematodes ceased movement and became coiled as soon as cooling commenced. The L3 froze almost immediately after the freezing of the surrounding medium. Both larvae enclosed in a capsule of the host tissue and larvae free of host tissue froze in this fashion. The freezing temperature of the medium was -4.01 ± 0.15°C (mean ± 1 SEM). The larval nematodes supercooled by only 0.44 ± 0.07°C (the difference between the freezing temperature of the medium and that of the nematode). Ice formation within the larval nematodes could be seen as a darkening, which commenced at the head or the tail and travelled through the body until the whole of the nematode darkened. This darkened appearance lightened and disappeared during warming, indicating the melting of the ice within the nematode.

Freezing and survival

The proportion of frozen larval nematodes increased with decreasing temperature (fig. 1), the freezing of the nematodes coincided with that of the surrounding medium. Nematode survival also declined with exposure to decreasing temperatures. The decline in survival did not, however, coincide with an increase in the proportion of frozen nematodes. The S_{50} was -9.12 ± 0.25°C, whilst the F_{50} was -4.07 ± 0.56°C. The difference between the S_{50} and the F_{50} was significant (*t* test: $t = 8.23$, $P < 0.001$). Survival was higher than that predicted if each nematode that froze died (fig. 2). The difference between observed and predicted survival was significant ($X^2 = 140.94$, $P = 0.001$).

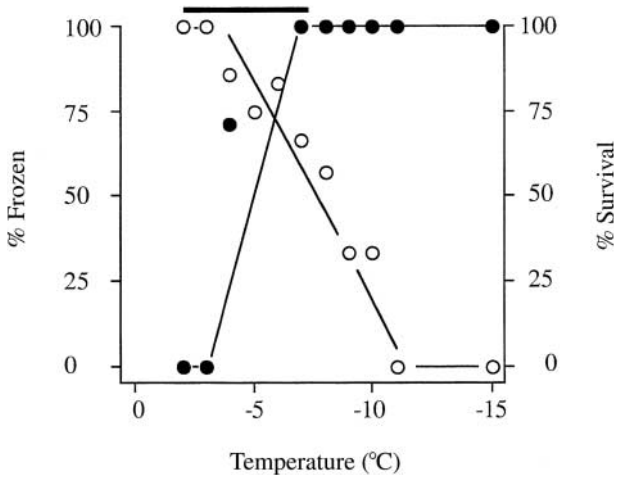

Fig. 1. Survival (○) and freezing (●) of *Anisakis* L3 cooled to various subzero temperatures at 0.5°C min^{-1}. Five to eight L3 were frozen to each temperature. The line at the top of the graph represents the range of temperatures at which the medium froze.

No L3 survived exposure to −15°C, even after spending only 10 min at that temperature. Frozen L3 did, however, survive exposure to −5°C and −10°C. Survival declined with time spent at both temperatures (fig. 3). The S_{50}s were 2.71 ± 0.31 h at −5°C and 0.57 ± 0.17 h at −10°C. Larval nematodes were killed significantly faster at −10°C than at −5°C (*t*-test comparing S_{50} values: $t = 4.03$, $P < 0.001$).

Freezing patterns of fish

A typical freezing pattern for a red gurnard frozen at −20°C is shown in fig. 4 and the freezing parameters are summarized in table 1. The skin froze first, followed by the fillet and the viscera. The freezing of skin was rapid, there being little flattening of the trace which indicates that the tissue is being held at its freezing (melting) point. The fillets and viscera took longer to freeze and all tissues took over 5 h to reach the temperature of the chamber. A similar pattern was observed in fish frozen at −10°C, but freezing took even longer with the fillets and viscera taking 10 h to reach the temperature of the chamber.

The freezing patterns of boxes of fish in a commercial blast freezer are summarized in table 2. The time taken for the fish to freeze and to reach the temperature of the freezer depended upon the mass of fish. Ten-kilogram boxes of blue cod and New Zealand sole took over 5 h to freeze and over 15 h to reach freezer temperature. Twenty-kilogram boxes of monkfish took more than three times as long to freeze and had still not reached freezer temperature after nearly 28 h. The minimum temperature reached was -22.9 ± 1.7°C.

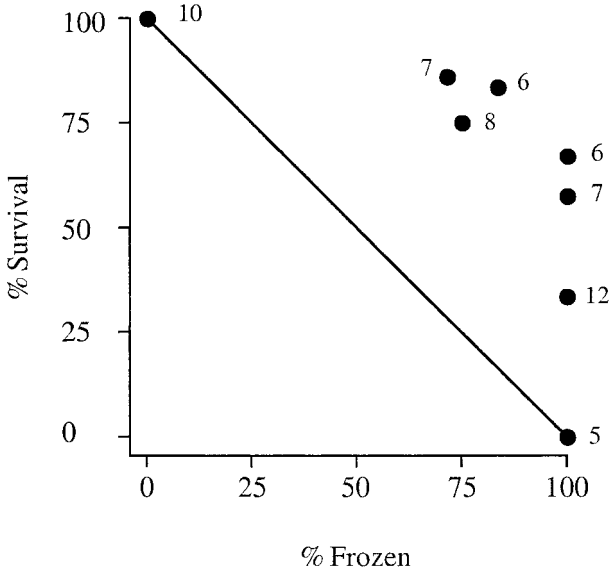

Fig. 2. The effect of freezing on the survival of *Anisakis* L3 cooled to various subzero temperatures at 0.5°C min^{-1}. The line is the survival predicted if each larval nematode that froze died. Many of the points lie above the line, indicating freezing tolerance. The number of larval nematodes associated with each data point is indicated.

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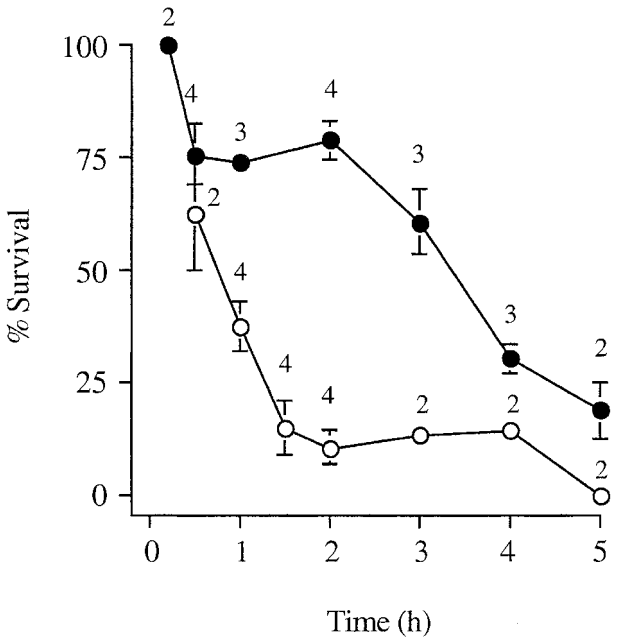

Fig. 3. The effect of the duration of freezing on the survival of *Anisakis* L3 after freezing at −5°C (●) or −10°C (○). Vertical lines are standard errors ($N = 2–4$).

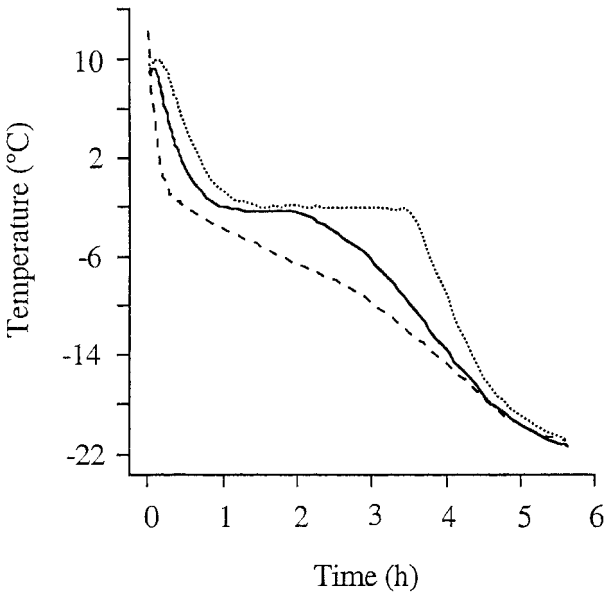

Fig. 4. Cooling profiles produced by red gurnard tissues exposed to −20°C. Thermocouples were placed beneath the skin (---), in the flesh (—) and in the viscera (. . .).

Potential adaptations for freezing tolerance

The principal sugars and sugar alcohol detected by gas chromatography ($N = 5$) were glycerol (9.22 ± 1.86 $\mu g\,mg^{-1}$ dry weight), trehalose (44.75 ± 9.34 $\mu g\,mg^{-1}$ dry weight) and glucose (3.61 ± 1.44 $\mu g\,mg^{-1}$ dry weight).

Ice crystal size increased markedly during annealing at −8°C in both the nematode supernatant and the buffer control. The ice crystal size after 30 min at −8°C was significantly smaller in the nematode supernatant than in the buffer control (fig. 5, ANOVA: $F_{1,6} = 9.52$, $P = 0.02$).

Discussion

Anisakis L3 froze when the medium surrounding them froze. The capsule of host tissue surrounding them did not provide any barrier to ice nucleation. This nematode thus shows no potential for freezing avoidance by supercooling. The L3 do, however, have a moderate ability to survive freezing. A proportion of L3 observed to have frozen on the microscope cold stage recovered, indicating that they are freezing tolerant. Some L3 survived freezing at −10°C for up to 4 h and at −5°C for 5 h. None, however, survived even a brief exposure to −15°C. Other authors have reported similar responses to freezing, with some survival of *Anisakis* L3 at high subzero temperatures but no survival once the internal temperatures of the fish that contained them reached temperatures lower than −15°C (Gustafson, 1953; Deardorff & Throm, 1988). These studies did not demonstrate that the nematodes froze under the conditions to which they were exposed, but the present results indicate that *Anisakis* L3 would be expected to do so.

This moderate freezing tolerance of *Anisakis* L3 is perhaps surprising since they are unlikely to be naturally exposed to freezing in the fish species that we tested. Dead or dying fish washed up on the shores of Arctic waters may be exposed to freezing conditions before ingestion by a carnivore. However, it seems unlikely that marine mammals would have the opportunity to ingest such fish with any frequency. The moderate freezing tolerance of *Anisakis* L3 is thus unlikely to have any adaptive significance and is perhaps a consequence of some other aspect of their biology.

The ability to inhibit recrystallization could be

Table 1. Freezing parameters for red gurnard frozen at −20°C.

Parameter	Skin	Fillet	Viscera
Start of freezing			
Time (h)	0.27 ± 0.04*	1.09 ± 0.11	1.36 ± 0.17
Temperature (°C)	−0.9 ± 0.3	−1.1 ± 0.1	−1.2 ± 0.2
Duration of freezing (h)	0.25 ± 0.07	1.58 ± 0.21	2.06 ± 0.04
Time to reach −20 °C (h)	6.05 ± 0.45	5.35 ± 0.53	5.6 ± 0.65

*Mean ± SE, $N = 3$.

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Table 2. Freezing parameters for various fish species frozen in a commercial blast freezer (−35°C to −38°C).

Parameter	Blue cod[1]	Sole[2]	Monkfish[3]
Start of freezing			
Time (h)	1.78 ± 0.43*	4.53 ± 1.01	5.02 ± 0.17
Temperature (°C)	−1.0 ± 0.52	−1.2 ± 0.12	−1.1 ± 0.1
Duration of freezing (h)	5.07 ± 0.67	5.03 ± 1.16	16.7 ± 0.26
Time to reach freezer temperature (h)	15.87 ± 1.75	17.82 ± 1.29	>27.9**

[1]10-kg boxes, $N = 3$; [2]10-kg boxes, $N = 4$; [3]20-kg boxes, $N = 2$.
*Mean ± SE; **Dataloggers were removed before freezer temperature was reached.

important for the survival of freezing-tolerant nematodes (Ramløv *et al.*, 1996). Although ice crystal size was significantly smaller in the *Anisakis* supernatant than that in buffer controls, the amount of recrystallization inhibition was small (about 9% inhibition of ice crystal growth). In the supernatant taken from homogenates of the freezing-tolerant Antarctic nematode *Panagrolaimus davidi*, the recrystallization inhibition is of the order of 64% (calculated from data in Ramløv *et al.*, 1996). The suppression of ice crystal growth exhibited in *Anisakis* extracts is less than that taken to indicate the presence of recrystallization inhibition (D.A. Wharton, unpublished results). *Anisakis* L3, however, contained trehalose at concentrations similar to that found in *P. davidi* (Wharton *et al.*, 2000). Trehalose is known to act as a cryoprotectant in a variety of invertebrates (Lee, 1991). The main functions of trehalose in parasitic stages of ascarid nematodes appear to be as a store of carbohydrates and as a blood sugar which is transported to provide the tissues with carbohydrates (Barrett, 1981; Behm, 1997). The moderate freezing tolerance of *Anisakis* may be a consequence of the presence of trehalose in its tissues but is unlikely to be of adaptive significance.

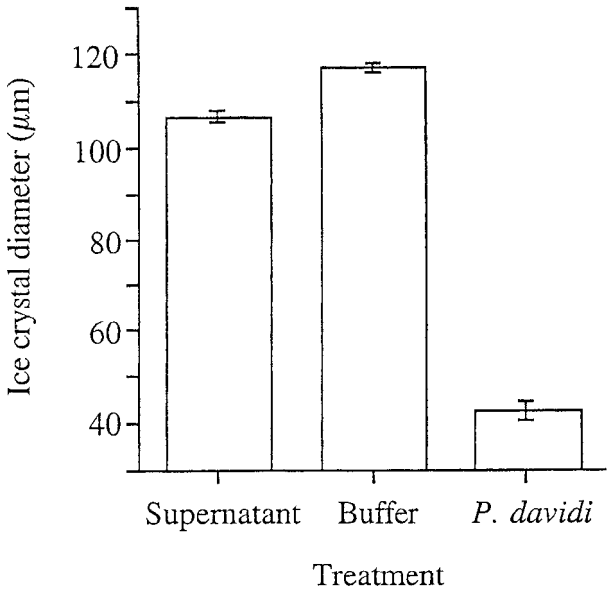

Fig. 5. Ice crystal size (mean ± 1 SEM, $N = 4$) of *Anisakis* supernatant and Tris/HCl buffer after annealing for 30 min at −8°C. Data for the freezing-tolerant Antarctic nematode *Panagrolaimus davidi* is shown for comparison (from Ramløv *et al.*, 1996).

Fish take a long time to freeze and to cool to the temperature of the freezer in which they are placed. This, and the observation that *Anisakis* L3 have a moderate ability to survive freezing, underlines the fact that fish must be frozen for a sufficient time and at a sufficiently low temperature to ensure that larvae are killed. Whether such a temperature is reached will depend on the temperature of the freezer, the mass of the fish in the container and the duration of exposure. We found that 20-kg containers of fish did not reach ambient temperatures (−35°C) even after 28 h exposure. The US FDA recommendation is for −35°C for 15 h or −20°C for 7 days (Sakanari & McKerrow, 1989), whilst the EU regulations on the freezing of fish to kill nematode parasites specify 'a temperature of not more than −20°C in all parts of the product for not less than 24 hours' (European Commission, 1991). The important part of this statement is 'in all parts' of the product (Howgate, 1998). Considerably longer than 24 h in a freezer may be necessary to achieve the temperature necessary to render the product safe for human consumption.

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