

THE FREEZING RESISTANCE OF ANTARCTIC FISH: III. AN EXPERIMENTAL STUDY OF THE DEATH OF SUPERCOOLED FISH RESULTING FROM CONTACT WITH ICE

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ABSTRACT. Survival times of several species of supercooled Antarctic fish in contact with ice were compared. Cloacal and muscle temperatures were measured, post-mortem ice formation was studied and differences in behaviour were observed. Variations were found in the freezing resistance of the different species, and the experimental observations were partly explained by the effect of blood solutes on crystal growth, the presence of structurally caused depressions of tissue freezing points and the behaviour of the fish. The distribution of the various species studied at Signy Island and South Georgia was apparently well correlated with the variations in freezing resistance. When applied to biological materials and particularly in studies of freezing resistance, the normal definition of freezing point proved inadequate, since factors affecting the initiation and propagation of freezing must be considered.

POLAR fish experience low environmental temperatures and may be supercooled for all or part of the year. Spontaneous freezing of supercooled fish does not appear to occur, thus the ability to supercool enables polar fish to survive low temperatures provided they either avoid contact with ice or else have some means of avoiding freezing of their tissues and body fluids if contact with ice occurs. This paper describes the effects of contact with ice on four species of supercooled Antarctic fish.

The freezing resistance of various species of fish has been studied by various workers. Scholander and others (1953) found that the Alaskan blackfish (*Dallia pectoralis*) could, in common with many other animals, survive partial but not total freezing. Scholander and others (1957) and Gordon and others (1962) studied various species of Arctic fish. They found that deep-water fish were supercooled all year round and were easily killed by contact with ice, but under natural conditions these fish did not encounter ice and so were in no danger of freezing. *Myoxocephalus scorpius* and *Gadus ogac* were also studied, both in summer and winter. The serum freezing points of these two species were lower in winter than in summer, although not low enough to prevent slight supercooling of the fish in winter. In summer, supercooling of the fish did not occur in nature, but under experimental conditions supercooled *M. scorpius* could not survive contact with ice. In winter, both *M. scorpius* and *G. ogac* were able to resist freezing for periods of hours when supercooled in the presence of ice. A similar partial resistance to freezing was found in *Cottus scorpius* by Eliassen and others (1960). It appeared that acclimated specimens of *C. scorpius* could tolerate a certain degree of supercooling in the presence of ice better than unacclimated specimens. DeVries (1970) has studied various species of Antarctic fish at McMurdo Sound. Two species of deep-water fish, *Rhizophila dearborni* and *Liparis* sp., were found to be slightly supercooled and easily killed by contact with ice, whereas various *Trematomus* species living in the ice-laden surface waters were found to be extremely resistant to freezing. It was concluded that the *Trematomus* species owed their freezing resistance to a serum glycoprotein which appeared to act as a freezing-point depressant. It is, however, questionable whether the glycoprotein acts as a true freezing-point depressant (Smith, 1972b).

The data given here were obtained from three *Notothenia* and one *Trematomus* species at Signy Island in the South Orkney Islands. Previous workers (I. Everson and R. Ralph, unpublished results) found that one of the *Notothenia* species, *N. neglecta*, could, in contrast to the other *Notothenia* species, be supercooled in the presence of ice and survive for several hours before death occurred. This observation was confirmed in the present study and a possible explanation is given. In addition, the body temperatures of the fish were measured, sites of ice formation in the bodies of the fish were investigated and factors thought to affect the survival of the fish under potentially lethal conditions were studied.

MATERIALS AND METHODS

Four species of Signy Island fish were studied. These were *Notothenia neglecta*, *N. rossii*, *N. gibberifrons* and *Trematomus newnesi*. Details of fishing methods have been given previously

(Smith, 1972a). The experiments described here were carried out on the *Notothenia* species only in summer and on *T. newnesi* only in winter.

Refrigerated aquarium

Freezing experiments were carried out in a refrigerated aquarium of approximately 90 l. capacity. Ethylene glycol at -20°C was circulated through a cooling coil immersed in the aquarium. The water in the aquarium was continually stirred and could be maintained at -1.8° to -2.0°C . A shelf of wire mesh could be raised or lowered to regulate the depth of water available to the fish. An inner container of fine nylon mesh on a rigid frame was used to supercool fish in the absence of ice. The mesh was fine enough to obstruct the passage of most ice crystals in the aquarium, but occasionally very small ice crystals either passed through the mesh or formed on "motes" in the inner container. When this happened the stirrers were switched off and the small ice crystals were skimmed off the water surface.

"Seeding" of supercooled fish

Fish which were supercooled in the absence of ice were "seeded" by touching their gills or skin with ice. In most cases, ice from a deep-freeze at -20°C was used so that visible freezing of the tissue occurred immediately.

Temperature measurement

Cloacal temperatures of the fish were measured with a thermistor or thermometer inserted several centimetres into the cloaca. Muscle temperatures were measured by inserting a thermistor 1–2 cm. into an oblique incision in the dorsolateral musculature so that it lay vertically above the base of the pectoral fin. Thermistors were held in place by a single stitch through the skin. Differences between body temperatures of the fish and water temperatures were measured after the fish had been equilibrated for at least 1 hr. in water of constant temperature. For experiments of several hours duration, the thermistors were calibrated before and after each experiment, and the results were corrected to allow for the small "drift" in calibration. The corrected results were probably within 0.01°C of the true values.

Post-mortem examination

Serum from some fish was analysed by methods described previously (Smith, 1972a, b). Some skin and gill samples were preserved in Bouin's fluid.

The extent of ice formation in the fish was investigated by opening the skull, body cavity and pericardium, and probing for ice.

The colours of the liver and gills of each fish were observed to determine whether restriction of the peripheral blood circulation occurred. In addition, the heart was touched with a probe to see if a heart beat could be elicited.

The lengths of the fish were measured to 0.5 cm. and skin thicknesses were measured to 0.005 cm. using skin-fold callipers. Skin for measurement was removed from approximately the same place on each fish.

RESULTS

Body temperatures of fish in equilibrium with surroundings

Cloacal and muscle temperatures of the three *Notothenia* species compared with water temperatures are shown in Table I. The specimens of *Trematomus newnesi* were too small for cloacal or muscle temperatures to be measured. The results were obtained with quiescent fish in equilibrium with their surroundings, but it was noticed that activity had very little effect on body temperature. Precise water temperatures were not measured during the freezing experiments (following section) but in general it was found that fish could be cooled to within 0.1° to 0.3°C of the aquarium temperature (-1.8° to -2.0°C). Metabolic heat production did not, therefore, enable the fish to survive low temperatures by raising their body temperatures above the freezing point of their body fluids.

TABLE I. TEMPERATURE DIFFERENCES BETWEEN ANTARCTIC FISH AND SEA-WATER

 $\Delta(\text{cloaca})$ = difference in temperature ($^{\circ}\text{C}$) between cloaca and sea-water. $\Delta(\text{muscle})$ = difference in temperature ($^{\circ}\text{C}$) between dorsal muscles and sea-water.

Species	Water temperature ($^{\circ}\text{C}$)	$\Delta(\text{cloaca})$	$\Delta(\text{muscle})$
<i>Notothenia neglecta</i>	1.06	0.09	—
	1.02	0.04	—
	0.98	0.18	—
	0.97	0.14	—
	0.93	0.10	—
	0.90	0.10	0.13
	0.88	0.26	—
	0.75	0.04	—
	-0.62	0.09	0.04
	-0.70	0.13	0.04
	Mean values	0.12	0.07
<i>N. rossii</i>	1.93	0.12	0.07
	1.80	0.20	0.09
	1.20	0.12	0.12
	1.02	0.25	—
	0.92	0.22	0.18
	0.75	0.16	0.07
	0.71	0.07	—
	0.58	0.10	—
	0.50	0.27	0.10
	0.50	0.21	0.14
	0.10	0.16	0.07
	0.10	0.11	0.07
	0.02	0.21	0.21
	-1.09	0.07	0.04
	Mean values	0.16	0.11
<i>N. gibberifrons</i>	0.91	0.30	—

Freezing experiments

Freezing experiments were carried out on all four species of fish. Each species will be considered separately.

Notothenia neglecta

Freezing experiments were carried out on 41 fish. Cloacal and/or muscle temperatures were monitored continuously in 12 experiments and cloacal temperatures were measured at intervals in most other experiments.

The freezing experiments were of two types: those in which fish were supercooled in the presence of ice, and those in which fish were supercooled in the absence of ice and then "seeded" by contact with ice.

Supercooling of Notothenia neglecta in the presence of ice. 28 fish were put, one at a time, in the refrigerated aquarium at -1.8° to -2.0°C . The wire-mesh shelf was raised to restrict the fish to the upper level where ice crystals were most abundant and stirring ensured continual contact of the fish with ice. The times taken for the fish to die were noted.

The death of a fish was preceded by occasional gasps and increased activity. Violent convulsions followed, after which the fish lost the ability to remain upright, respiration ceased and the head of the fish bent back with the gill covers open. When the convulsions ceased, the only movement of the fish was an occasional slight quivering of the body. Death was assumed to have occurred when the fish lay on its back with the gill covers dilated. This usually occurred within 5 min. of the first convulsion.

Survival times of the fish ranged from 15 to 490 min. Fish which died quickly showed the

first signs of distress when their cloacal temperature fell to the approximate freezing point of their blood serum. Well vascularized portions of the fish must have been significantly supercooled at this stage, since blood passing through the gills is cooled to the temperature of the surrounding water (Lyman, 1968). Ice formed in the eyes of the fish during the experiments, but the time at which this occurred was unrelated to the survival time. The lengths and skin thicknesses of the fish were measured after death and the sera of 10 fish were analysed.

Survival times were plotted against the following variables: initial temperature of the fish, length, skin thickness, and serum protein, total carbohydrate, reducing sugar and freezing point. The data are summarized in Table II. No correlation was found between survival times and any of the variables.

The only factor which appeared to affect the survival times was the behaviour of the fish. It was found that quiescent fish survived for much longer periods than more active fish. Quiescent fish lay practically motionless in the aquarium and their respiratory movements were minimal, whereas active fish opened their mouths on respiration and also "coughed" at intervals. These observations suggested that the fish could not be "seeded" by ice through their exposed body surface but instead could be "seeded", possibly at the gills, by ice crystals entering their mouths. This hypothesis was tested in the following experiments.

"Seeding" of supercooled *Notothenia neglecta*. 10 fish were supercooled in the absence of ice and a small portion of the gills or body of each fish was frozen by contact with ice at -20°C . One "gill-seeded" and one "body-seeded" fish survived more than 24 hr. Survival times of the other fish are summarized in Table II. The average survival time of "gill-seeded" fish was approximately equal to the average survival time of fish supercooled in the presence of ice, which suggests that "gill-seeding" rather than "body-seeding" occurred in the latter case. In addition, the maximum survival time recorded for a quiescent fish in continual bodily contact with ice (490 min.) was nearly twice the maximum recorded for a "body-seeded" fish (255 min.). It is unlikely that a fish could survive contact with ice for so long if "body-seeding" by ice at -1.8°C were possible. It is reasonable to conclude, therefore, that "body-seeding" of supercooled *N. neglecta* is unlikely to occur under natural conditions and that survival in the presence of ice is possible as long as ice crystals are excluded from the mouth and gills.

The lengths and skin thicknesses of the "seeded" fish were measured and serum samples were analysed. The results are summarized in Table II. They resemble those obtained in the first series of freezing experiments in that no correlation was found between survival times and the other variables. The average concentrations of serum constituents in the fish which died in the freezing experiments differed slightly from those of summer or winter fish (Smith, 1972a) and may have been the result of slight osmoregulatory disturbance or freezing injury. Two important features of the analytical results were the demonstration that no "antifreeze" was released into the blood in response to the experimental conditions, and the indication that freezing of the blood (resulting in significantly increased concentrations of serum constituents) did not occur.

Temperature changes in Notothenia neglecta. Cloacal and/or muscle temperatures were recorded for 22 of the fish used in the freezing experiments. A typical example is shown in Fig. 1. Individual variations occurred depending on the experimental conditions and the sizes of the fish but several general features were evident. Cooling of the fish obeyed an approximately Newtonian law and, as expected, small fish cooled more rapidly than large fish. Small irregularities occurred in the temperature-time curves, indicating that metabolic heat was produced or lost in a series of "pulses" which were possibly related to heart beat and/or respiration. An abrupt temperature rise of 0.1° to 0.3°C occurred on the death of the fish. This was probably due to release of latent heat as ice formed in the fish rather than to the effect of the convulsions which preceded death, since, under ordinary conditions, activity had little effect on body temperature. Cloacal and muscle temperatures in the same fish were found to be similar and to undergo comparable changes.

The cloacal temperatures of 33 fish were measured at varying times before and after death. The temperatures at which death occurred varied and, since only a small temperature rise occurred on death, the temperatures immediately after death (range -0.61° to -1.90°C ,

TABLE II. DEATH BY FREEZING OF *Notothenia neglecta*—SUMMARY OF DATA

(Results are given as means. Figures in parentheses are the number of specimens in the sample followed by the ranges of results.)

<i>Physical parameters</i>	<i>Fish supercooled with ice present</i>	<i>"Gill-seeded" fish</i>	<i>"Body-seeded" fish</i>	<i>"Gill-seeded" fish plus "body-seeded" fish</i>	<i>Total</i>
Survival time (min.)	71 (28, 14-490)	77 (6, 5-150)	188 (2, 120-255)	105 (8, 5-255)	79 (36, 5-490)
Initial temperature (°C)	-0.03 (28, +0.90 to -1.50)	—	—	—	—
Length (cm.)	39.0 (28, 25-51.5)	38.0 (6, 35-39.5)	40.5 (2, 37.5-43.5)	38.5 (8, 35-43.5)	39.0 (36, 25-51.5)
Skin thickness (cm.)	0.085 (23, 0.060-0.110)	0.085 (6, 0.065-0.100)	0.085 (2, 0.080-0.090)	0.085 (8, 0.065-0.100)	0.085 (31, 0.060-0.110)
Serum analysis					
Protein (mg./ml.)	64.7 (10, 59.1-75.9)	64.8 (6, 56.6-72.7)	69.5 (2, 62.2-76.7)	65.9 (8, 56.6-76.7)	65.2 (18, 56.6-76.7)
Total carbohydrate (mg./ml.)	15.1 (10, 13.6-17.9)	13.2 (6, 11.0-14.6)	14.4 (2, 12.4-16.3)	13.8 (8, 11.0-16.3)	14.4 (18, 11.0-17.9)
Reducing sugar (mg./ml.)	4.8 (10, 3.5-6.3)	5.8 (6, 4.6-7.1)	5.9 (2, 4.3-7.4)	5.8 (8, 4.3-7.4)	5.2 (18, 3.5-7.4)
Freezing point (°C)	-1.10 (10, -1.01 to -1.25)	—	—	—	—

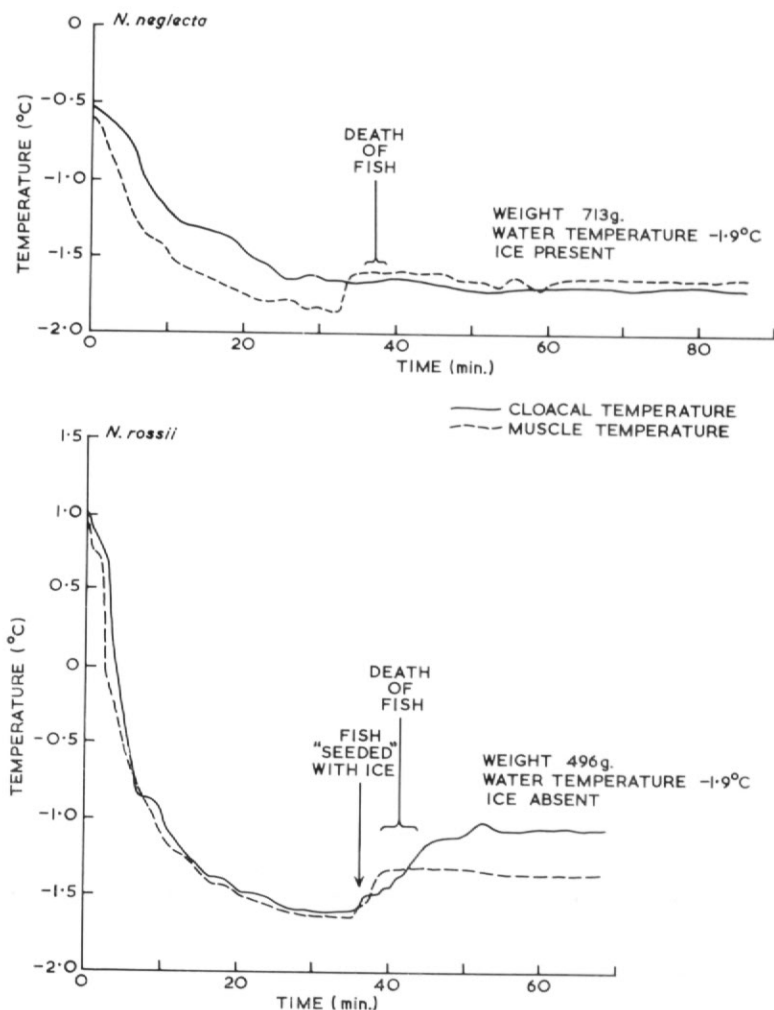


Fig. 1. Graphs of temperature vs time for *Notothenia neglecta* and *N. rossii*.

mean -1.48°C) differed only slightly from those prior to death. Between 30 and 360 min. after death, however, the cloacal temperatures ranged from -1.33° to -1.70°C with a mean of -1.54°C . These values were lower than the freezing points of various *N. neglecta* body fluids which ranged from -0.854° to -1.032°C (Smith, 1972b) and can be accounted for by slow post-mortem propagation of ice in the fish. The resulting slow release of latent heat would lead to an equilibrium between fish and water at a lower temperature than the freezing point of the fishes' body fluids. Slow propagation of ice in the fish could be caused either by the effect of body-fluid solutes on the rate of crystallization of ice (Lusena, 1955) or, more probably, by the effect of tissue structure on the growth of ice crystals resulting in a structurally caused depression of tissue freezing point (Bloch and others, 1963).

Preliminary temperature measurements (Smith, 1970) suggested that seasonal variation possibly accounted for differences in post-mortem temperatures of the fish, but the more detailed results reported here indicate that this is not the case.

Ice formation in Notothenia neglecta after death by freezing. 12 fish were examined at varying times up to 120 min. after death by freezing. Variation between individual specimens occurred but several general features were apparent.

Before death occurred, ice formed in the eyes of all specimens but, as previously mentioned, this bore no apparent relation to the survival times of the fish.

Immediately after death, no ice could be found in the bodies of the fish, although approximately 2 g. of ice must have formed in order to cause the small rise in body temperature that occurred on death. 30 min. after death, ice was found in the urine, endolymph and cerebrospinal fluid of most specimens. The urine and endolymph were almost completely frozen at this stage but only small amounts of ice were found in the cerebrospinal fluid. Ice formation in the head appeared to start in the endolymph and then to spread to the cerebrospinal fluid in which substantial amounts of ice had formed by 2 hr. after death. Ice was also found to encase the otoliths of specimens in which freezing of the cerebrospinal fluid had begun.

No ice was found in the brain, muscular tissue or viscera either at death or up to 2 hr. afterwards; and, in particular, no ice was found in the blood, heart or pericardium of any specimen. This latter observation demonstrated the freezing resistance of *N. neglecta* blood which appears to be related primarily to its high blood-sugar concentration (Smith, 1972a).

These observations confirmed the supposition arising from post-mortem temperature measurements that ice propagation in *N. neglecta* is slow. In addition, the lack of ice formation in the tissues of the fish is circumstantial evidence of a structurally caused freezing-point depression (Bloch and others, 1963) which could account for the fact that "seeding" of the fish with ice does not apparently occur through the body surface most likely to come into contact with ice.

The experimental technique was not sensitive enough to determine the site of initial ice formation in the fish, but, in view of the convulsions and loss of balance that preceded death, it is likely that some part of the central nervous system was involved. Evidence in favour of this was obtained by G. Holeton (personal communication), who monitored cardiac and respiratory movements during death by freezing of a single specimen of *N. neglecta*. The results indicated that heart beat ceased at the stage where violent convulsions occurred, respiration failed soon after, and the mode of death was consistent with freezing of part of the central nervous system. These observations complemented the experiments described here and showed that the criterion of death that was chosen (the time when the fish lay motionless on its back after the phase of violent convulsions) was in accord with the cessation of heart beat.

Appearance of Notothenia neglecta after death by freezing. The gills and viscera of *N. neglecta* were examined during the post-mortem search for ice in the bodies of the fish. No external signs of freezing were found. The digestive tract appeared normal and, in contrast to *Trematomus bernacchii* under cold stress (Potts and Morris, 1968), the gills and livers were the same colour as those of controls, showing that restriction of the peripheral circulation did not occur. The heart beat of frozen fish could be induced by touch for an hour or more after death.

Death by freezing of Notothenia neglecta—summary. Freezing of a supercooled fish by contact with ice could be delayed for a period of hours if the fish excluded ice crystals from its mouth and gills. The eyes of the fish were easily frozen but the time at which this occurred bore no relation to the survival time, nor did the initial temperature, length, skin thickness or serum composition of the fish affect survival time. No "antifreeze" was produced in response to potentially lethal conditions. Death was accompanied by violent convulsions and was probably due to freezing of part of the central nervous system. The small rise in body temperature that accompanied death by freezing was thought to be due to ice formation in the fish. The location of this small amount of ice was uncertain, although shortly after death ice was found in the urine, endolymph and cerebrospinal fluid. Ice did not form in the blood or tissues of the fish in the 2 hr. after death during which observations were carried out. The lack of ice formation in the blood was confirmed by serum analysis and was probably due largely to the high concentration of blood sugar. The lack of ice formation in the tissues implied that a structurally caused depression of tissue freezing point occurred in the fish. This could account for the

resistance to freezing of fish which excluded ice from their mouths and gills. The equilibrium cloacal temperatures attained after death were lower than the body-fluid freezing points of the fish; this was evidence in favour of a structurally caused freezing-point depression.

Notothenia rossii

Freezing experiments were carried out on 12 specimens of *N. rossii*. Cloacal and (with one exception) muscle temperatures were monitored in each case.

Supercooling of Notothenia rossii in the presence of ice. Five fish were put, one at a time, in the refrigerated aquarium at -1.9°C with ice present. All died very quickly and the first signs of distress became evident within a few minutes when the cloacal temperatures of the fish fell to about 0°C . The pattern of death resembled that of *N. neglecta* with the exception that respiratory failure occurred soon after the fish were put in the refrigerated aquarium. Suffocation may therefore have been partly responsible for the death of the fish, although freezing of part of the central nervous system was probably the main cause of death. The most obvious feature of the experiments was the low resistance to freezing of *N. rossii* compared with *N. neglecta*.

"Seeding" of supercooled Notothenia rossii. Seven fish were supercooled in the absence of ice. Two of these died when their cloacal temperatures reached about -1.5°C . The cause of death was not known but was probably due to contact with small ice crystals that either passed through the nylon mesh or else formed in the ice-free part of the aquarium. The other five fish were successfully supercooled and then seeded with ice at -20°C either on the body or gills as in the case of *N. neglecta*. All the fish died within 10 min. of being "seeded". There was no noticeable difference in survival time between "body-seeded" and "gill-seeded" fish. The low resistance to freezing of *N. rossii* was again obvious and the results indicated that "body-seeding" was as likely to be as lethal as "gill-seeding" under natural conditions.

Temperature changes in Notothenia rossii. Cooling curves of the fish resembled those of *N. neglecta* in being approximately Newtonian in shape with small irregularities. A typical example is given in Fig. 1. Post-mortem cloacal and muscle temperatures were measured at intervals of up to 155 min. after death. Cloacal temperatures rose by 0.2° to 0.5°C on seeding the fish and approximate calculation showed that on average about 40 per cent more ice formed initially in *N. rossii* than in *N. neglecta* when death by freezing occurred. Equilibrium post-mortem cloacal temperatures averaged -1.2°C (range -1.06° to -1.34°C) and the corresponding muscle temperatures averaged -1.25°C (range -1.00° to -1.47°C). These temperatures were closer to the body-fluid freezing points than in *N. neglecta*, showing that post-mortem ice propagation occurred faster in *N. rossii* than in *N. neglecta*.

Ice formation in Notothenia rossii killed by freezing. Ice formed in the eyes of all but one fish during the course of the experiments. As in the case of *N. neglecta*, the initial site of ice formation could not be determined, although ice was found in the urine, endolymph and cerebrospinal fluid between 10 and 20 min. after death and in approximately the same quantities as were found in *N. neglecta* 30 min. after death. *N. rossii* differed from *N. neglecta* in that ice propagation continued until, by 155 min. after death, ice was found in the gall bladder, viscera and muscular tissue as well as in the urine, endolymph and cerebrospinal fluid. These observations confirmed the results of the body-temperature measurements by showing that ice forms faster in *N. rossii* than in *N. neglecta*. *N. rossii*, however, resembled *N. neglecta* in one important respect: ice did not appear to form in the blood.

Appearance of Notothenia rossii after death by freezing. As in the case of *N. neglecta*, there were no external signs of freezing and there was no indication of restriction of peripheral circulation. The heart beat of frozen fish could be induced by touching for an hour after death.

Death by freezing of Notothenia rossii—summary. *N. rossii* died quickly when supercooled and in contact with ice. "Body-seeding" and "gill-seeding" were both lethal under the experimental

conditions and appeared equally likely to occur in nature. Respiratory failure occurred before death and may have been partly responsible for the death of the fish, although freezing of part of the central nervous system was probably the main cause. Ice formed in the eyes of all specimens. The cloacal temperature rise that occurred on death showed that about 40 per cent more ice formed initially in *N. rossii* than in *N. neglecta*. Ice formed soon after death in the urine, endolymph and cerebrospinal fluid, and, by 2½ hr. after death, the bile, viscera and muscular tissue were partially frozen. No ice was found in the blood. The average equilibrium body temperature attained after death was slightly below the freezing point of the body fluids and restriction of the peripheral blood circulation did not occur.

Notothenia gibberifrons

Supercooling of Notothenia gibberifrons in the presence of ice. Five fish were put in the refrigerated aquarium at -1.9°C in the presence of ice. All died within 2–3 min. with ice in their eyes, their mouths open and their gills dilated. The cause of death was not established but was probably due to freezing of part of the central nervous system.

"Seeding" of supercooled Notothenia gibberifrons. "Seeding" experiments were not carried out on *N. gibberifrons* since attempts to supercool fish in the absence of ice were unsuccessful. Three fish died before reaching equilibrium with the aquarium temperature and a fourth fish died after supercooling to -1.8°C (cloacal temperature). Death was probably due to ice crystals in the aquarium that were too small to be retained by the nylon mesh surrounding the "ice-free" container.

Temperature changes in Notothenia gibberifrons. Post-mortem cloacal temperatures of the fish dropped steadily, reaching -1.7°C within 2 hr. of death. This indicated that little or no ice formed in the fish once the initial freezing of the various body fluids was complete.

Ice formation in Notothenia gibberifrons after death by freezing. The five fish which died after being supercooled in the presence of ice were removed from the aquarium at 30 min. intervals and examined for the presence of ice. Immediately after death, no ice was found except in the eyes. 30 min. after death, ice was found in the endolymph and cerebrospinal fluid, and by 90 min. after death the urine was frozen. No ice was found in the blood, brain, viscera or muscular tissue of any fish; this confirmed that ice propagation was minimal after the initial formation of ice in the endolymph, cerebrospinal fluid and urine.

Appearance of Notothenia gibberifrons after death by freezing. There were no external signs of freezing and there was no indication of restriction of peripheral circulation. Touching the hearts of the fish 60 min. after death did not cause them to beat.

Death by freezing of Notothenia gibberifrons—summary. *N. gibberifrons* died very quickly when supercooled in the presence of ice. It is not known whether "gill-seeding" or "body-seeding" occurred, but the speed of death suggested that both could be lethal. The most probable cause of death was freezing of part of the central nervous system. Post-mortem propagation of ice in the fish was similar to that in *N. neglecta*. Ice formed in the eyes, urine, endolymph and cerebrospinal fluid but little or no freezing of the tissues occurred and no ice was found in the blood.

Trematomus newnesi

The results of a freezing experiment carried out on some small specimens of *T. newnesi* have been reported previously (Smith, 1970). The fish were able to survive continual contact with ice at -1.8°C for 60 min., after which the experiment was discontinued. It appeared that *T. newnesi* was at least as resistant to freezing as *N. neglecta* which it resembled in that "seeding" through the skin or muscular tissue did not occur under the experimental conditions. It was uncertain whether the gills of *T. newnesi* came into contact with ice so more detailed comparison with *N. neglecta* was not possible. The small size of the fish and the few specimens available precluded more detailed experiments.

Comparison of skin and gills

Skin (including the underlying tissue) and gill samples from *Notothenia neglecta*, *N. rossii* and *N. gibberifrons* were embedded in wax, sectioned at 6–10 μm ., stained with haematoxylin and eosin, and examined under the microscope. Differences between the three species occurred but bore no obvious relationship to variations in freezing resistance of the fish.

Skin thicknesses of several hundred fish (16–51 cm. long) were measured, scatter diagrams of skin thickness vs body length were drawn and regression curves were fitted by eye. The mean skin thicknesses were calculated. The regression curves and the calculated means showed that *N. neglecta* had the thickest skin (mean thickness 0.080 cm., 148 specimens), *N. rossii* had a less thick skin (mean thickness 0.070 cm., 76 specimens) and *N. gibberifrons* had the thinnest (0.050 cm., 70 specimens). The freezing resistance of *N. neglecta* cannot, therefore, be attributed to an unusually thick skin.

DISCUSSION

The experiments showed that the freezing resistance of the Signy Island fish varied considerably. *Trematomus newnesi* and *Notothenia neglecta* were found to be far more resistant to freezing than *N. rossii* or *N. gibberifrons*. There is also evidence that, like *N. rossii* and *N. gibberifrons*, *Chaenocephalus aceratus* at Signy Island has a low resistance to freezing (Twelves, 1970).

All the species studied are unusual in that they can survive indefinitely at temperatures below the lower lethal limit for many temperate species (Prosser and Brown, 1961). They can also survive in the supercooled state, and supercooling itself is not dangerous if contact with ice is avoided (Smith, 1972a).

The above facts raise the following three questions: how do fish survive low temperatures, how do supercooled freeze-resistant fish survive contact with ice, and how does the freezing resistance of a species affect its distribution?

Survival of fish at low temperatures

Many papers have been published on the phenomenon of temperature acclimation in poikilotherms (e.g. Scholander and others, 1953; Wohlschlag, 1963; Somero and DeVries, 1967; Roots and Johnston, 1968; Morris and Schneider, 1969) and numerous problems remain unsolved. On the basis of the information available, speculation is unwarranted as to why Signy Island and South Georgia fish can survive at temperatures lethal to many temperate species.

Survival of supercooled fish in contact with ice

Three main factors appear to be involved in the survival of supercooled fish in contact with ice. The first of these is the presence of "protective" solutes in the blood (and possibly in other body fluids) that prevent or retard ice formation (Lusena, 1955; Smith, 1972a). This is not restricted to freeze-resistant fish only, since, after death by freezing, ice was not found in the blood of any of the Signy Island species which were studied.

The second factor is the possibility that a structurally caused freezing-point depression (Bloch and others, 1963) may lower the temperature at which "seeding" and propagation of ice occurs in the tissues of the fish. This effect may also be combined with the presence of "protective" solutes in the tissue fluids. Evidence that a structurally caused freezing-point depression occurred to varying extents in the three *Notothenia* species which were studied was provided by the post-mortem temperature measurements and observations on the formation of ice in the fish.

The third factor that must be considered is the behaviour of the fish under potentially lethal conditions. The survival of *N. neglecta* was undoubtedly aided by very shallow respiratory movements that excluded ice crystals from the mouth and gills. In addition, it is possible that less resistant species could escape lethal contact with ice by quickly swimming away if localized concentrations of ice crystals were encountered in the sea, near grounded icebergs for instance.

These three factors partially account for the observed variations in freezing resistance

between the species studied, but several problems remain unsolved. In particular, the site of the initial, lethal freezing is unknown, though it is probably some part of the central nervous system. Also unknown is the route by which ice is propagated to the lethal site from the surface of the fish where "seeding" occurs, nor is it known for certain whether "body-seeding" and/or "gill-seeding" occurs in species which have a low resistance to freezing. It is to be hoped that techniques required to solve these remaining problems will be devised and employed.

Effect of freezing resistance on distribution

Chaenocephalus aceratus (Twelves, 1970) and *Notothenia gibberifrons* have a low resistance to freezing and live at depths (>40 m.) where contact with ice is unlikely to occur. *N. rossii* also has a low resistance to freezing but is found at depths of 2–40 m. *N. neglecta*, which has a partial resistance to freezing, is also found at depths of 2–40 m. Contact of *N. rossii* and *N. neglecta* with ice is possible at the depths in which they live, but neither species is found in the vicinity of anchor ice or in other potentially dangerous areas. *Trematomus newnesi*, however, which is at least as resistant to freezing as *N. neglecta* if not more so, does not avoid anchor ice and is found in shallow water (0–20 m. approximately; personal communication from I. Everson).

It is also noteworthy that, at Signy Island, *N. neglecta* is much more abundant than *N. rossii*, whereas at South Georgia, where less extreme sea temperatures are encountered, *N. rossii* is the abundant inshore species. In addition, few species of the genus *Notothenia* are found in higher latitudes (Andriashev, 1965), whereas *Trematomus* species predominate at McMurdo Sound (DeVries, 1970).

Despite these apparent correlations of freezing resistance and distribution, however, it would be unwise to predict the general distribution of a species on the basis of its freezing resistance since this can vary with locality. For example, at Signy Island, *N. neglecta* is partially resistant to freezing and *N. gibberifrons* has a very low freezing resistance, whereas DeVries (1969) found that *N. coriiceps* (= *neglecta*) and *N. gibberifrons* caught off the Antarctic Peninsula were both highly resistant to freezing.

"Biological freezing point"

The freezing point of a solution is generally defined as the temperature at which the vapour pressure of the solution and the vapour pressure of pure frozen solvent are equal, i.e. the temperature at which the solution is in equilibrium with solid solvent. However, the effect of solutes on the growth of ice crystals in solution (Tamman and Büchner, 1935; Lusena, 1955) and the possibility of a structurally caused freezing-point depression in tissue (Bloch and others, 1963) raise the question of whether this definition is meaningful when applied to biological materials. In the study of freezing resistance particularly, the temperatures at which ice formation may be initiated and at which crystal growth may occur are of immediate relevance to survival but are not incorporated in the above definition of freezing point. At present, there is little information available on the magnitude of the effects concerned, but it is probable that further study will lead to the definition of a "biological freezing point" in terms applicable to practical situations.

SUMMARY OF RESULTS

The results of this study may be summarized as follows:

Trematomus newnesi and *Notothenia neglecta* were more resistant to freezing than *N. rossii* or *N. gibberifrons*. *T. newnesi* appeared to be extremely resistant, whereas *N. neglecta* was only partially resistant to freezing and owed its survival under the experimental conditions to shallow respiratory movements that excluded ice from the gills and/or buccal cavity. "Seeding" of *T. newnesi* and *N. neglecta* by ice did not seem to occur through the skin or muscular tissue. In *N. neglecta* the gills were implicated as the site where "seeding" was most liable to occur. *N. rossii* and *N. gibberifrons* died quickly when supercooled in the presence of ice, and it appeared that "seeding" could occur anywhere on the body surface of these fish.

Death of the fish was thought to be due to freezing of part of the central nervous system, with respiratory paralysis as a contributing factor in the case of *N. rossii*.

Serum analysis showed that "antifreeze" was not released into the blood of *N. neglecta* in response to potentially lethal conditions.

Temperature measurements showed that metabolic heat production did not contribute significantly to the freezing resistance of the three *Notothenia* species. Death by freezing in these species was accompanied by a small rise in body temperature, and equilibrium post-mortem body temperatures showed that post-mortem ice formation was slow, particularly in *N. neglecta* and *N. gibberifrons*, implying that a structurally caused depression of tissue freezing point occurred.

Ice formed in the eyes of *N. neglecta*, *N. rossii* and *N. gibberifrons* before or at death, but the times at which this occurred were unrelated to the survival times of the fish. The initial site of lethal ice formation was not found, but, soon after death, ice was found in the urine, endolymph and cerebrospinal fluid. Ice propagation was subsequently very slow in *N. neglecta* and *N. gibberifrons*, and freezing of the viscera or muscular tissue did not appear to occur, thus confirming the results of the temperature measurements. In *N. rossii*, however, post-mortem ice formation was less protracted and freezing of the gall bladder, viscera and muscular tissue occurred. In no case was ice found in the blood, heart or pericardium.

There were no external signs of freezing on any of the fish, and restriction of peripheral blood circulation did not occur in response to the experimental conditions.

Skin and gill sections of the *Notothenia* species revealed no obvious differences that could account for variations in the freezing resistance of the species.

Variations in freezing resistance were related to the effect of blood solutes on ice-crystal growth, the presence of a structurally caused depression of tissue freezing point and the behaviour of the fish. These factors provided a partial explanation of the experimental observations, but several features remain unexplained.

The freezing resistance of the various species appeared to be correlated with their distribution at Signy Island and South Georgia, but prediction of distribution on the basis of freezing resistance is unwise, since there is evidence that freezing resistance varies with locality.

The need was indicated for an extension of the normal definition of freezing point to take into account factors that affect the temperatures at which ice formation may be initiated and at which ice-crystal growth may occur.

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