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Does fish from the Disko Bay area of Greenland possess antifreeze proteins during the summer?

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Abstract The blood of 21 teleosts and 1 elasmobranch was analysed for antifreeze-protein activity by determining the thermal hysteresis. The fish were caught in the summertime at different locations in West Greenland (Disko Bay area). The difference between the melting and hysteresis freezing point (thermal hysteresis) is a numerical indication of the presence of antifreeze-protein activity.

No thermal hysteresis was detected in the blood of the elasmobranch, *Raja radiata* (thorny skate) and, as expected, its blood was isosmotic to seawater. Of the 21 teleost species examined, 11 were found to have a thermal hysteresis greater than 0.1°C, an indication of the presence of substantial amounts of antifreeze. The remaining 10 species had a hysteresis less than 0.1°C, and thus their summertime possession of antifreeze protein was concluded to be very low or absent. No hysteresis was detected in *Gadus morhua* (Atlantic cod), but there was a slight faceting of the seed crystal, indicative of a low, possibly physiologically unimportant, level of antifreeze protein.

This study is the first time antifreeze-protein activity has been detected in the species *Stichaeus punctatus* (Arctic shanny).

Introduction

The body fluid of most marine teleosts is strongly hypoosmotic to seawater (Eastmann 1993). While seawater freezes at −1.8 to −1.9°C, depending upon the salinity, the blood of marine teleosts freezes at between −0.5 and −0.9°C. In the absence of ice, they often survive in a supercooled state, but in the presence of ice they are susceptible to freezing and thus cannot survive in the supercooled state.

Antifreeze proteins allow marine fish to exist at temperatures below their equilibrium freezing point (supercooled) by preventing growth of ice crystals that occasionally enter their circulation (DeVries 1982).

The Arctic and Antarctic marine environments differ, in that the Arctic experiences much larger seasonal temperature fluctuations and less ice cover than the Southern Ocean. As a consequence, yearly fluctuations in the circulating levels of antifreeze proteins are seen in many of the Arctic fishes. Also, populations of a given species inhabiting both temperate and Arctic waters may show yearly variations in antifreeze-protein levels following the regional temperature changes, so that one species in one area might posses antifreeze protein for a longer period than the same species in another area, both populations following the regional temperature fluctuations of their specific habitat. An example of this is seen in *Pseudopleuronectes americanus* (winter flounder) (Fletcher 1977; Fletcher and Smith 1979).

Yearly fluctuations in the hydrography of the study area are given in Nielsen and Hansen (1999) and Levinsen et al. (2000).

The aim of this study was to examine the antifreeze activity in a wide range of fish species in west Greenland during the summer (July), a couple of months after the ice breakup. Knowledge of the yearly temperature fluctuations of the area and our measured summertime levels of antifreeze-protein activity in the fish species could provide indications of whether or not an overall seasonal variation might be present.

Materials and methods

Fishes were caught by hook and line, trawling, traps and set line in July 2000. A Seabird Electronic SBE-25 Sealogger CTD was, at selected locations of catch, used to measure salinity and temperature down through the water column.

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Blood samples were drawn from the caudal vein and allowed to coagulate for 20 min or longer. The samples were then centrifuged at 3000 RPM and the blood serum was frozen at −20°C. Serum osmolality was measured on a Wescor vapour pressure osmometer (Wescor model 5520) and the osmolality was converted to serum melting points.

A Chiron model 664 ion analyser was used to determine the concentrations of sodium, potassium and chloride in the serum samples. In many cases, the serum was diluted one- to twofold with distilled water to bring the ion concentrations into the range of the ion analyser.

The melting point (MP) and hysteresis freezing point (HFP) were measured using a Clifton nanolitre osmometer (Clifton Technical Physics, Hardford, N.Y.). This instrument accurately controls the temperature of nanolitre samples of serum so that the melting and hysteresis freezing point can be determined through observation of the ice-crystal growth in a microscope on a single, small (2-μm) crystal. The MP was taken as the temperature at which disappearance of the last small ice crystal upon slow warming was observed, while the HFP was taken as the temperature at which the first ice growth of the seed crystal could be observed. If substantial difference was observed between the melting and freezing processes, the morphology of habit of the ice crystal was recorded. In some cases, hexagonal bipyramids were observed, while in others no change of shape was observed in the hysteresis gap and the first growth was in the form of small spicules. The melting and hysteresis freezing temperatures were calculated from the respective osmolality values by multiplying by −0.001858°C/mOsm (DeVries 1986).

Results

Study site

Figure 1 shows the vertical profile of salinity and temperature in a 300-m-deep sampling station approximately 2 nautical miles south of the island of Disko (approximately 69°N) in early July 2000.

Mean thermal hysteresis and the growth of ice crystals

The melting point, hysteresis freezing point and thermal hysteresis (MP-HFP) for the various Disko Bay area specimens are given in Table 1. Depth of catch locations was also noted for each fish caught.

The freezing point, melting point and thermal hysteresis recorded from the Clifton are listed in degrees Celsius. The MP ranged from −2.13 to −0.69°C. The HFP ranged from −2.15 to −0.74°C. The thermal hysteresis ranged between 0.02 and 1.35°C, with *Pholis fasciata* (banded gunnel) having the highest thermal hysteresis.

In addition to measuring the melting and hysteresis freezing point, the Clifton cryoscope also allows one to describe the ice morphology within the hysteresis gap and the growth habit at the hysteresis freezing point.

Distinct and sudden spicule formation was seen at the HFP in *Myoxocephalus scorpius* (Shorthorn sculpin), *Myoxocephalus scorpioides* (Arctic sculpin), *Gymnacanthus tricuspis* (Arctic staghorn sculpin), *Gadus ogac* (Greenland cod), *Boreogadus saida* (polar cod) and *Pholis fasciata*. In *M. scorpius* and *M. scorpioides*, the crystal growth in the hysteresis gap was observed to be in the form of a hexagonal bipyramid. In *G. tricuspis*, the round seed-ice crystal grew into a flat hexagonal plate, which enlarged into a hexagonal bipyramid. The hexagonal bipyramid grew longer and longer until coarse spicules grew from its surface, as the temperature was lowered further. The five samples of *G. ogac* showed the largest individual variation in the amount of hysteresis (SD 0.17), and at the HFP the ice crystal in some samples grew into a hexagonal plate and eventually into a hexagonal bipyramid, while in other samples the ice crystal did not change shape at all. At the HFP, many very thin spicules grew which, together with the difference between MP and HFP, indicated that the given species had high levels of antifreeze proteins in its blood. In *B. saida*, the crystal growth in the hysteresis gap was in the formation of hexagonal bipyramids, and spicules were formed at the HFP. In *P. fasciata*, no change in the shape of the crystal was observed in the hysteresis gap, and spicules were formed at the hysteresis freezing point.

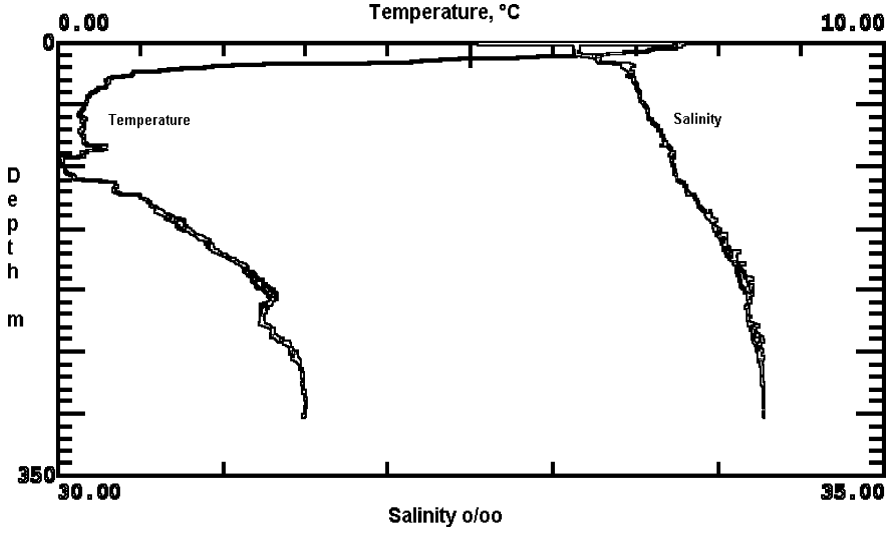

Fig. 1 CTD-profile from the Disko Bay area in early July. Location of catch of *Boreogadus saida*

Table 1 Freezing point, melting point and thermal hysteresis in degrees Celsius. *Right column* gives the depth, in metres, at which the fish were caught

Family	Sample size (*n*)	MP (°)	HFP (°)	MP-HFP (°)	SD	Caught at depth (m)
Agonidae (Poachers)						
Leptagonus decagonu	2	−0.84	−0.86	0.02	0.01	200–240
Anarhichadidae						
Anarhichas lupus	3	−0.92	−1.39	0.47	0.08	20–30
Anarhichas minor	7	−0.81	−0.88	0.07	0.03	100–150
Cottidae (Sculpins)						
Artediellus atlanticus	1	−0.83	−0.99	0.16	–	240–260
Myoxocephalus scorpius	19	−0.84	−1.59	0.75	0.27	2–30
Myoxocephalus scorpioides	1	−0.69	−1.54	0.85	–	5–10
Gymnacanthus tricuspis	5	−0.90	−1.62	0.72	0.33	26–55
Triglops murrayi	1	−1.10	−1.11	0.01	–	20–30
Triglops nybelini	3	−0.85	−0.86	0.01	0	300
Cyclopteridae (lump & snailfishes)						
Eumicrotremus derjugini	1	−1.10	−1.11	0.01	–	15–40
Liparis tunicata	2	−1.15	−1.43	0.28	0.01	240–260
Gadidae (codfishes)						
Gadus morhua	4	−0.70	−0.74	0.04	0.03	2–5
Gadus ogac	5	−0.94	−1.66	0.72	0.17	10–30
Boreogadus saida	9	−1.24	−2.11	0.87	0.15	300
Pholidae (Gunnels)						
Pholis fasciata	6	−0.84	−2.19	1.35	0.3	5–10
Pleuronectidae (Righteye flounders)						
Reinhardtiushippoglossoides	2	−0.76	−0.78	0.02	0.02	20–150
Hippoglossoidesplatessoides	2	−0.80	−0.89	0.09	0.02	100–150
Salmonidae (Salmonids)						
Salvelinus alpinus	1	−0.81	−0.90	0.09	–	10–15
Stichaeidae (Pricklebacks)						
Lumpenus lampretaeformis	1	−0.89	−1.04	0.15	–	30–90
Stichaeus punctatus	1	−1.09	−1.76	0.67	–	50
Zoarcidae (Eelpouts)						
Lycodes seminudus	1	−0.99	−1.03	0.04	–	240–260
Rajidae (Skates)						
Raja radiata	2	−2.13	−2.15	0.02	0.01	100–300

Five species [*Anarhichas lupus* (wolf-fish), *Liparis tunicata* (kelp snailfisch), *G. morhua*, *Lumpenus lampretaeformis* (snakeblenny) and *S. punctatus*]showed some kind of spicular growth. Freezing of *A. lupus* serum produced coarse spears. In *L. tunicata*, similar coarse spears were seen. The ice crystal grew from round into a hexagonal bipyramid and then, at the HFP, spicules were formed. These spicules were thicker in shape than those seen in, for example, the cod species. The crystal growth in *G. morhua* was observed to be somewhat continuous, and no spicule formation was seen. However, in one of the samples, some faceting and hexagonal bipyramid formation of the ice crystal was seen. In *L. lampretaeformis* and *S. punctatus*, the crystal growth was not entirely continuous, and at the HFP, a somewhat explosive growth was seen, but no spicules were observed.

In *Leptagonus decagonus* (Atlantic poacher), *Anarhichas minor* (spotted wolf-fish), *Artediellus atlandicus* (Atlantic hookear sculpin), *Triglops murrayi* (moustache sculpin), *Triglops nybelini* (bigeye sculpin), *Reinhardtius hippoglossoides* (Greenland halibut), *Hippoglossoides platessoides* (American plaice), *Salvelinus alpinus* (Arctic charr), *Lycodes seminudus* (longear eelpout), *Eumicrotremus derjugini* (leatherfin lumpsucker) and the elasmobranch *R. radiata*, no spicular growth was observed.

Osmolality and ion concentration

Table 2 lists the mean osmolality and ion concentration measured in each species except *B. saida*, where the sample volume was insufficient for ion determination. The osmolality in the teleosts ranged from 342 mOsm in *S. alpinus* to 587 mOsm in *B. saida*. The elasmobranch, *R. radiata*, had an osmolality of 1031 mOsm, identical to the seawater from which it was collected.

The chloride concentration ranged from 125 to 225 mmol/l, and the sodium concentration from 160 to 240 mmol/l. Potassium contributed very little to the total osmolality, and was highly variable. It ranged from under 0.5 to 10 mmol/l. The highest values were found in *S. alpinus*, *H. platessoides* and *L. tunicata*.

Discussion

From our findings, we can conclude whether the different species of fish possessed antifreeze protein at the

Table 2 Serum osmolality and ion concentration

Species	Sample size (*n*)	Osmolality (mOsm)	Na^+ (mmol/l)	Cl^- (mmol/l)	K^+ (mmol/l)
Leptagonus decagonus	2	449 ± 0	214 ± 5.7	204 ± 5.7	4.17 ± 1.68
Anarhichas lupus	3	448 ± 25.5	169 ± 37.7	148 ± 26.6	4.59 ± 1.03
Anarhichas minor	7	390.9 ± 32.4	192.7 ± 11.0	177.1 ± 15.8	3.60 ± 0.94
Artediellus atlanticus	1	435	212	200	2.76
Myoxocephalus scorpius	19	427.1 ± 48.0	205.4 ± 12.3	187.9 ± 11.8	4.16 ± 4.89
Myoxocephalus scorpioides	1	382	199	173	0.67
Gymnacanthus tricuspis	5	433.6 ± 8.9	209.1 ± 3.6	183.2 ± 4.8	3.61 ± 0.78
Triglops murrayi	1	447	195	196	< 0.5
Triglops nybelini	3	461 ± 20.7	206 ± 10.39	190 ± 15.6	2.21 ± 1.10
Eumicrotremus derjugini	1	443	224	214	2.02
Liparis tunicata	2	489.5 ± 53	214 ± 11.3	222 ± 19.8	9.13 ± 12.2
Gadus morhua	4	348.8 ± 8.5	170.6 ± 7.2	146.1 ± 8.8	2.16 ± 1.54
Gadus ogac	5	437.8 ± 41.8	195.6 ± 17.8	175.2 ± 16.7	4.82 ± 3.58
Boreogadus saida	9	587.9 ± 21	–	–	–
Pholis fasciata	6	379 ± 29.4	191 ± 7.73	154 ± 9.14	2.42 ± 3.49
Reinhardtius hippoglossoides	2	410 ± 49.5	184 ± 39.6	186 ± 36.8	2.4 ± 2.69
Hippoglossoides platessoides	2	433.5 ± 61.52	198 ± 19.8	191 ± 24	8.26 ± 4.89
Salvelinus alpinus	1	342	158.5	131.5	9.76
Lumpenus lampretaeformis	1	434	191	166	2.1
Stichaeus punctatus	1	423	> 200	167	1.5
Lycodes seminudus	1	498	238	230	5.3
Raja radiata	2	1031 ± 35.4	197.5 ± 121.7	197 ± 117.4	1.77 ± 1.80

temperature, location and time of year they were caught. Based on what we know about the behaviour and habitat of the different species, we can make assumptions on the likelihood that these species possess antifreeze protein in the winter or not. High summertime levels of antifreeze protein in a species can be an indication of no or little seasonal variation.

Mean thermal hysteresis and the growth of ice crystals

If the hysteresis freezing point was nearly equal to the melting point, we concluded that no antifreeze protein was present, but if a gap between these two was seen, we could conclude that antifreeze protein was present. A thermal hysteresis greater than 0.1°C was taken as an indication of the presence of antifreeze proteins. This limit of above and below 0.1°C does not exclude the possibility that serum with values between 0 and 0.1 contain low concentrations of antifreeze protein. But it gives a margin of error and, also, such low hysteresis values probably will not have any particular physical effect for the fish, in the sense of resistance of freezing. Recrystallisation might, though, be prevented by very small concentrations.

The habit of growth of the ice crystal in the hysteresis gap and at the hysteresis freezing point was taken as indication of presence or non-presence of antifreeze protein. Non-continuous ice-crystal growth in the hysteresis gap and/or spicule formation at the hysteresis freezing point were taken as indications of presence of antifreeze protein. But no conclusions on the type of antifreeze were made from these data.

Our study confirms the fact that *R. radiata* does not need any antifreeze protein because it is isosmotic to seawater and therefore does not face the same problems of freezing as the teleosts.

No significant thermal hysteresis was found in *L. decagonus*, *S. alpinus* and *L. seminudus*. *Leptagonus decagonus* and *L. seminudus* were caught in quite deep waters where ice is rarely present, so these species probably do not posses antifreeze protein at all. *S. alpinus* belongs to the salmonids, which are semi-freshwater species and in which, to our knowledge, no antifreeze has ever been found. *S. alpinus* was caught at shallow depth (10–15 m), where it might need antifreeze protein. But the water was low in salinity because of being supplied with freshwater; therefore, it is likely that this species does not possess antifreeze protein at all due to its semi-marine habitat.

We concluded that *R. hippoglossoides* and *H. platessoides* did not posses any antifreeze protein. Antifreeze proteins have been observed in other pleuronectids, e.g., in *P. americanus* (Duman and DeVries 1974). Pleuronectids (righteye flounders) have also been observed to have seasonal antifreeze-protein variation in other areas (Fletcher and Smith 1979). *R. hippoglossoides* and *H. platessoides* therefore might also have seasonal variation, which could be why no antifreeze protein was observed by us during summertime.

Eumicrotremus derjugini (leatherfin lumpsucker) and *L. tunicata* belong to the family Cyclopteridae (lump- and snailfishes). We found a small amount of hysteresis in *L. tunicata*, but none at all in *E. derjugini*. Although *E. derjugini* was caught in shallow water (15–40 m), it normally lives in deep waters where ice is rarely present. So *E. derjugini* probably does not posses antifreeze protein at all. In *L. tunicata*, we found some evidence of some form of antifreeze protein. The mean thermal hysteresis in the two individuals examined was not very

large (0.28°C), and the spicules formed at HFP did not show the otherwise typical threadlike structure, but were wider. Antifreeze proteins have previously been found in other species of liparids, e.g., in *Liparis liparis* (Denstad et al. 1987). In the summer of 1997, in the same area, two other species of liparids were found not to have any antifreeze protein (Holk et al. 1997). Seasonal variation and/or different habitats could explain why a somewhat low concentration of antifreeze protein was found in *L. tunicata*, but no antifreeze protein was found in *E. derjugini* and in the two species of liparids examined in 1997.

L. lampretaeformis and *S. punctatus* belong to the family Stichaeidae (pricklebacks). No, or only a little, antifreeze protein was observed in *L. lampretaeformis*: its habitat of deep waters would suggest that it does not need antifreeze. But in 1997, in the same waters, antifreeze was found in two species, closely related to *L. lampretaeformis*, so there is a possibility that this species may have antifreeze if caught in the winter (Holk et al. 1997). *S. punctatus* was observed to have antifreeze protein, which is consistent with its shallow-water habitat. To our knowledge, this is the first time antifreeze protein has been detected in this species.

A. lupus and *A. minor* belong to the family Anarhichadidae (wolf-fishes). No antifreeze protein was found in *A. minor*, but some form of antifreeze was found in *A. lupus* ($n=3$). This last discovery is contrary to what has been observed earlier in the same area, when *A. lupus* ($n=1$) was examined and found not to possess any antifreeze protein (Holk et al. 1997). Further investigations need to be done on these fishes to conclude anything final on whether this is due to seasonal variation or sample size. It should be noted that we, in all cases, caught *A. lupus* at much shallower depths than *A. minor*, so if this is a general characteristic of the habitats of these two species, it would logically follow that *A. lupus* would need antifreeze protein while *A. minor* would not. However, since these two species are quite closely related, it would likely follow that both either possessed antifreeze protein or did not.

A quite high thermal hysteresis was found in *P. fasciata*. It can be concluded that this species possesses antifreeze protein, which is consistent with its habitat of cold and shallow waters. Although all individuals were acclimated at approximately 10°C, which was warmer than where they were caught, they all still had quite high antifreeze-protein activity. Maybe this is an indication of little or no seasonal variation.

Six different species of the family Cottidae (sculpins) were sampled. We found that *A. atlanticus*, *T. murrayi* and *T. nybelini* did not have any antifreeze protein. This is consistent with the habitats of these three species: deep waters, where ice is rare. Antifreeze-protein activity was observed in *M. scorpius*, *M. scorpioides* and *G. tricuspis*. These three species are closely related and share the same habitat, i.e. shallow and cold waters both during winter and summer. A general trend for the sculpins examined here is that the species we caught in shallow water had the highest thermal hysteresis. Sculpins have been examined before and are known to posses AFP type I (Hew et al. 1980). Probably great seasonal variations of antifreeze-protein concentrations will show up in these species, since their environment is so changeable in temperature. Maybe the antifreeze-protein evidence we have observed is the remainder of a higher concentration seen in the wintertime.

Three species of the family Gadidae (cods) were examined: *G. morhua*, *G. ogac* and *B. saida*. In *G. morhua*, we only found what we concluded to be traces of antifreeze protein in one of the individuals. We know that AFGP has been found in these fish before (DeVries 1980), so this indicates that *G. morhua* does not synthesise antifreeze protein in the summer, or that they synthesise very little and that they have seasonal variation in the Disco Bay area. Seasonal change in antifreeze-protein activity has been recorded in *G. morhua* inhabiting other geographical areas (Fletcher et al. 1982). We caught *B. saida* in deep waters (Fig. 1), which does not fit into the general trend of high hysteresis being correlated with shallow-water species. But *B. saida* seeks deeper/colder waters during summer because low temperature is optimal for other physiological reasons (Craig et al. 1981). *G. ogac* is solely a northern species, and *B. saida* is solely an Arctic species, which lives near the ice lattice and is often seen in close contact with the ice (Craig et al. 1981). So these two species live in habitats that require some form of protection toward the ice and, as expected, we found antifreeze-protein activity.

Osmolality and ion concentration

R. radiata showed the highest osmolality (1031 mOsm), but the sodium, potassium and chloride content are not significantly higher than in the teleost species. Most of *R. radiata*'s higher osmolality is due to urea (Goldstein and Forster 1971). The osmolality of the teleost species lies between 350 and 600 mOsm. Generally, fish that live in waters where fresh water is constantly supplied have a lower osmolality, e.g. *S. alpinus*. *B. saida* had the highest osmolality of the teleost fish. It is known that this species lives near the ice in very cold waters. It has been observed that serum ion concentrations increase in marine fishes and decrease in freshwater species, as environmental temperature is lowered (O'Grady and DeVries 1982).

When looking at the melting point (mOsm) measured by the Wescor and the Clifton, we saw a difference. This difference was probably due to the fact that it was very difficult to melt down a small enough ice crystal and still be able to observe it, so therefore most of the time a higher osmolality of the melting point is seen when measuring on the Clifton.

The sodium, chloride and potassium ion concentrations are much the same as the results found by O'Grady and DeVries (1982) on other Arctic species. A general trend is that no difference between fishes with or without

antifreeze is seen with regards to the ion concentrations. The large variations seen in the potassium-ion concentrations might be due to haemolysis in some samples (O'Grady and DeVries 1982).

Generally, we see that the species of fish that need antifreeze, based on the habitat in which they live, do possess antifreeze protein. A general trend is that the species inhabiting shallow waters also have a higher antifreeze-protein activity, expressed as a higher thermal hysteresis. For most of the fishes examined here, further experiments are needed to make final conclusions on the types and seasonal variations in concentration of their antifreezes.

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