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Antifreeze Proteins in the Arctic Shorthorn Sculpin *(Myoxocephalus scorpius)*

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ABSTRACT. The plasma of shorthorn sculpin caught at Grise Fiord (Southern Ellesmere Island, arctic Canada) during late August contained antifreeze proteins which were essentially identical, with respect to molecular weight, number of components and amino acid composition, to the antifreeze proteins found in Newfoundland populations of shorthorn sculpin. The concentration of antifreeze protein in the plasma of the arctic sculpins during the summer was similar to that observed in the plasma of Newfoundland sculpin during the winter. The results suggest that unlike their Newfoundland counterparts, the plasma of sculpin residing in the High Arctic contains high concentrations of antifreeze protein all year round.

Key words: shorthorn sculpin, *Myoxocephalus scorpius*, antifreeze proteins

RÉSUMÉ. Le plasma de chaboisseaux à épines courtes capturés à Grise Fjord (Territoire du Nord-Ouest) à la fin d'août contenait des protéines résistantes au froid. Celles-ci étaient essentiellement identiques, en considérant le poids moléculaire, le nombre d'éléments et la composition de l'acide aminée, aux protéines résistantes au froid trouvées chez des populations de chaboisseaux à épines courtes de Terre-Neuve. La concentration de protéines résistantes au froid dans le plasma des chaboisseaux à épines courtes de l'Arctique durant l'été, était similaire à celle observée dans le plasma des chaboisseaux à épines courtes de Terre-Neuve durant l'hiver. Les résultats suggèrent que le plasma des chaboisseaux résidant dans le Haut Arctique contient toute l'année de plus hautes concentrations de protéines résistantes au froid.

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INTRODUCTION

The blood plasma freezing point of most teleost fishes which inhabit temperate marine waters is approximately −0.6°C to −0.8°C (Holmes and Donaldson, 1969). Such fish are capable of surviving supercooling to temperatures of −2° to −3° in the absence of ice. However, if ice is present, ice crystals appear to penetrate the epithelium and immediately cause the supercooled fish to freeze and die (Scholander *et al.*, 1957). The polar oceans are characterized both by subzero water temperatures (−1.4°C to −2°C) and the presence of ice. Since many marine fish spend their lives in these regions they must differ from the temperate fish species by possessing specialized means of protecting themselves from freezing.

Extensive investigations carried out over the past decade have established that many of these polar and subpolar fish possess antifreezes which depress the freezing temperature of the blood plasma below that of the surrounding environment (DeVries, 1974, 1980; Feeney and Yeh, 1978). To date these antifreezes have not been found in either fresh-water fishes or in temperate fishes which never inhabit ice-laden sea water. It is believed that these antifreeze proteins are a major means by which many polar and subpolar fish are protected from freezing.

Two general classes of macromolecular antifreezes have been described in fishes, namely the polypeptides and the glycoproteins. These two classes differ in their primary structures, notably the absence of carbohydrate in the polypeptide antifreezes. The antifreeze polypeptides (AFP) have been described in two members of the family Pleuronectidae (winter flounder *Pseudopleuronectes americanus*, and Alaskan plaice *Pleuronectes quadritaberculatus*) and three cottids (Bering Sea sculpin *Myoxocephalus verrucosus*, shorthorn sculpin *Myoxocephalus scorpius*, and the sea raven *Hemitripterus americanus*). The molecular weights of these peptide antifreezes range from 3000 to 15 000, and with the exception of the sea raven antifreeze, alanine accounts for approximately 60% of their amino acid residues (Raymond *et al.*, 1975; Duman and DeVries, 1976; Hew and Yip, 1976; DeVries, 1980; Hew *et al.*, 1981; Slaughter *et al.*, 1981).

Glycoprotein antifreezes (AFGP) have been described in two members of the family Nototheniidae, *Trematomus borchgrevinki* and *Dissastichus mawsoni* from the Antarctic, and five members of the family Gadidae, saffron cod (*Eleginus gracilis*), polar cod (*Boreogadus saida*), Labrador rock cod (*Gadus ogac*), Atlantic cod (*Gadus morhua*) and frostfish (*Microgadus tomcod*) (DeVries *et al.*, 1970; Raymond *et al.*, 1975; Van Voorhies *et al.*, 1978; Osuga and Feeney, 1978; Hew *et al.*, 1981; Fletcher *et al.*, 1982). In contrast to the structural diversity exhibited by the polypeptide antifreezes, all of the glycoprotein antifreezes isolated have similar, if not identical, structures in which the basic repeating unit is a glycotripeptide of alanine-alanine-threonine with a disaccharide linked to the threonine residue. This tripeptide unit is repeated to produce polypeptide polymers of molecular weights ranging from 2500 to 33 000. In the smallest glycopeptides a few of the alanine residues are replaced by proline, and in the frostfish and saffron cod arginine replaces one or two threonine residues (Fletcher *et al.*, 1982).

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Both classes of antifreeze (glycoprotein and polypeptide) appear to function in the same way in that they lower the freezing point of the plasma in a non-colligative fashion. That is, they lower the temperature at which ice will form, but do not lower the melting temperature of the ice (DeVries, 1980). The difference between the freezing and melting temperatures is termed thermal hysteresis. This non-colligative lowering of the freezing temperature is unique to the antifreeze proteins, and forms the basis for their identification, purification and quantification in plasma. The mechanism(s) whereby these antifreeze proteins prevent ice crystal growth is not well understood. However, it appears that the antifreeze proteins bind to the ice surface and somehow prevent the addition of water molecules to the ice lattice (DeVries, 1980). DeVries (1980) has suggested that these antifreeze proteins may act at the epithelium and prevent the entry of ice crystals into the fish.

The seawater temperatures of coastal Newfoundland range from approximately −1.5°C during the winter to 12 to 16°C in summer. Fishes inhabiting these waters thus only experience subzero temperatures and ice-laden seawater for a maximum of four to five months each year. As might be expected, plasma antifreeze levels in such fish correlate with the seasonally cycling water temperatures. However, the degree of correlation varies with species. Antifreeze proteins appear in the plasma of the winter flounder (*Pseudopleuronectes americanus*) and the shorthorn sculpin (*Myoxocephalus scorpius*) during November when the water temperatures approximate 4-6°C and reach peak values during January to March. The winter flounder begin to lose their antifreeze in May when the water temperatures usually rise above 0°C, and they exhibit negligible values during late June. In contrast, Newfoundland populations of shorthorn sculpin possess measurable quantities of antifreeze during July and early August, long after any danger from freezing has passed (Fletcher, 1977; Hew *et al.*, 1980).

Since the evolutionary origins of the shorthorn sculpin are in the Arctic, it was reasoned that the retention of the antifreeze by the Newfoundland populations of shorthorn sculpin during a large part of the summer was a reflection of this origin (Cowan, 1972; Hew *et al.*, 1980). However, we know nothing about the plasma antifreeze levels in shorthorn sculpin residing in the Arctic. Do they change seasonally, or are high levels maintained throughout the year? This report documents our findings on the plasma of an arctic population of shorthorn sculpin, and presents evidence suggesting that they maintain high levels of antifreeze protein in their plasma throughout the year.

MATERIALS AND METHODS

Arctic shorthorn sculpin (*Myoxocephalus scorpius*) (identified using Leim and Scott, 1966) were caught by jigging on 22 and 24 August 1980 at depths of 1-10 m at Grise Fiord (76°10′N, 83°15′W), Ellesmere Island, Northwest Territories. They were maintained outside, on the deck of the CSS *Hudson* in 100-*l* aquaria at ambient temperature (~0°C) and photoperiod for 4 to 48 h prior to blood sampling. The fishing area had a patchy cover of fresh-water ice (0.5 cm thick), and several small icebergs were grounded near by. Surface sea water temperatures were approximately 1.5°C.

The Newfoundland shorthorn sculpin were collected by SCUBA divers from Witless Bay during August and September. They were maintained in 40 000-*l* aquaria at seasonally ambient conditions of photoperiod and water temperature (Fletcher, 1977; Table 1).

Blood samples were collected from a caudal blood vessel using plastic 3-cc syringes with 21-gauge needles and stored in test tubes containing sodium heparin. The plasma was separated from the cells by low speed centrifugation (4000 G). Plasma freezing point depression was determined using a freezing point osmometer (Model 3D or 3R, Advanced Instruments Inc., MA, USA). Plasma antifreeze protein activity was determined by measuring thermal hysteresis (difference between freezing and melting temperatures) using the method of Slaughter and Hew (1981). Plasma Cl^- was determined coulometrically using a chloride titrater (Model CMT 10, Radiometer Copenhagen) and plasma Na^+ by atomic absorption spectrophotometry (Varian-Tectron Model AAS) (Fletcher, 1975). Plasma Na^+ values were corrected for the amount of Na^+ in the heparinized test tubes.

Isolation of Antifreeze Protein

Plasma samples (4-5 ml) were dialyzed against 2 *l* of 0.1 M NH_4HCO_3 buffer, pH 8.0, using a dialysis membrane with a molecular weight cut-off of 3500. The samples were then applied to a Sephadex G75 column (2.6 x 86 cm) in 0.1 M NH_4HCO_3. Individual fractions collected from the column were lyophilized and assayed for antifreeze activity as described by Hew *et al.* (1980). Those fractions

TABLE 1. A comparison of arctic and Newfoundland shorthorn sculpin plasmas

	Water temp. (°C)	N	Freezing point depression (°C)	Thermal hysteresis (°C)	Na^+ (mM/l)	Cl^- (mM/l)
Arctic						
August	1.5	16	1.34 ±0.012	0.518 ±0.030	225 ±6.2	192 ±1.23
Newfoundland						
August	14	6	0.741*** ±0.019	0.05*** ±0.02	174*** ±1.88	166*** 0.57
February	−0.5	7	1.24** ±0.03	0.445 ±0.019	215 ±3.66	194 ±3.02
		P	<0.01	<0.001	<0.001	<0.001

N = Number of fish. Water temp = water temperature at the time of sampling. Thermal hysteresis is the difference between the freezing and melting temperatures of the plasma. P = level of statistical significance between August and February samples from Newfoundland sculpin. ** = P<0.01, *** = P<0.001, levels of statistical significance between Newfoundland and arctic sculpins. Values are expressed as means ± one standard error.

containing antifreeze activity were pooled and lyophilized. The molecular weights of the active components were determined on a high-performance liquid chromatography (HPLC) system (Model 334, Beckman Instruments, with a Hitachi variable wave length detector and a BioRad dual I-125 column) using a 0.1 M sodium sulfate, 0.1 M Tris buffer, pH 6.8). Following Sephadex gel filtration the antifreeze proteins were further fractionated on reverse-phase HPLC using an Ultrasphere - ODS column (particle size 5μ, Altex Instruments, USA). The gradient used was triethylamine phosphate buffer pH 2.1 with acetonitrile. Fractions delineated in Figure 2 (appearing elsewhere in this report) were dialyzed against 2 *l* 0.1 M NH_4HCO_3 buffer. Following lyophilization the fractions were hydrolyzed under vacuum in 6 N HCL at 110°C for 24 h. Amino acid analyses were performed on the hydrolysates using a Beckman 121 amino acid analyzer.

Acetonitrile was purchased from Burdick and Jackson Laboratories Ltd., Muskegon, Michigan, USA. Triethylamine (Eastman Kodak) was redistilled and stored under nitrogen. Distilled water used with HPLC was purified on a milli Q™ water purification system. All solutions used with HPLC were filtered through a millipore filter (HA 0.45μ) and bubbled with N_2 for at least 10 min prior to use. All other chemicals were reagent grade.

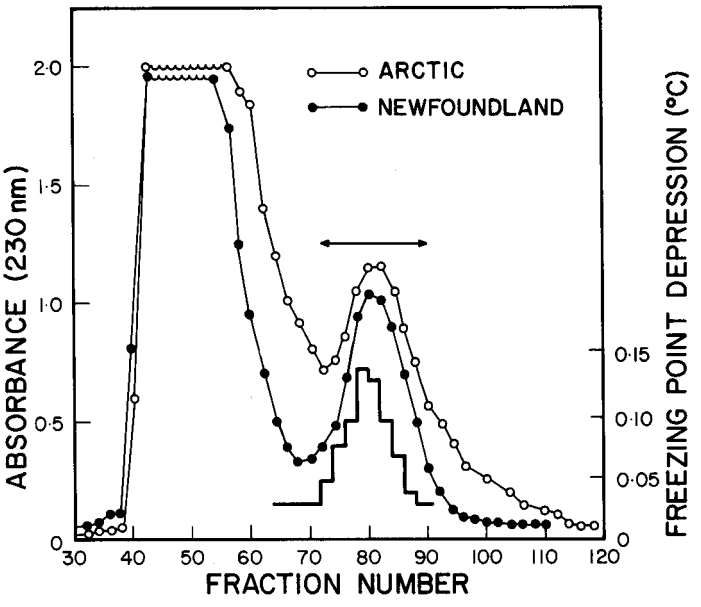

FIG. 1. Elution profile of arctic and Newfoundland shorthorn sculpin antifreeze proteins on Sephadex G75. Individual fractions within the region indicated by the arrow were lyophilized and redissolved in 0.01M NH_4HCO_3 and their freezing points determined. The lowest curve (histogram) represents the freezing point depression of the arctic shorthorn sculpin. The values for the Newfoundland sculpin (not shown) peaked in the identical fractions.

RESULTS AND DISCUSSION

The plasma freezing point depression, thermal hysteresis and major electrolyte concentrations (Na^+ and Cl^-) of arctic sculpins caught during late summer closely resembled the concentrations observed in the plasma of Newfoundland sculpins during the winter (Table 1). In contrast, the plasma of Newfoundland sculpin caught during late August exhibited significantly lower freezing point depression and Na^+ and Cl^- concentrations and only a slight amount of thermal hysteresis (Table 1). The 0.5°C of thermal hysteresis found in the arctic sculpin plasma indicates that a high concentration of protein antifreeze is present during the summer months.

The antifreeze component was isolated from the plasma of the arctic sculpin in order to determine how closely it resembled the antifreeze protein isolated from the Newfoundland shorthorn sculpin sampled during winter.

A single active component was isolated from the plasma of the arctic and the Newfoundland shorthorn sculpins indicating that they were of similar molecular weights (10 000) (Fig. 1). When the antifreeze proteins (AFP) from both the arctic and the Newfoundland sculpin were analyzed on gel filtration high-performance liquid chromatography (HPLC), a broad single peak of approximately 12 000 daltons was observed. The width of the peak suggested that the AFP may be mixtures of several components of slightly differing molecular weights. When the antifreeze component, isolated following Sephadex G75, was fractionated on reverse-phase HPLC (Fig. 2), at least four major components were evident in both the arctic and Newfoundland sculpins. Three of these (A,B,C) were hydrolyzed and found to have a similar amino acid composition with alanine accounting for approximately 60% of the residues (Table 2). These results indicate that the antifreeze proteins isolated from Newfoundland and arctic populations of shorthorn sculpins are very similar with respect to molecular weight, number of components and amino acid composition and could well be identical.

In a previous study it was shown that plasma antifreeze concentrations in a Newfoundland population of shorthorn sculpin declined to very low levels and disappeared altogether during the period of peak water temperatures (August to September) (Hew *et al.*, 1980). Since Grise Fiord seawater temperatures would be at essentially peak values at the date of sampling (late August), it would appear that unlike their more southerly counterparts in Newfoundland, high arctic populations of shorthorn sculpin retain high concentrations of antifreeze proteins throughout the year. Although we have not sampled arctic populations of sculpin during the winter, it seems reasonable to assume that plasma antifreeze concentrations at this time of year would be at least as high as those observed during the summer.

The maintenance of high levels of plasma antifreeze proteins throughout the year appears to be a feature common to a number of fish species residing in polar and sub-polar oceans. Antarctic fish (*Trematomus borchgrevinki* and *Rhigophila dearborni*), inhabiting waters which are near the freezing point all year round, have high levels of plas-

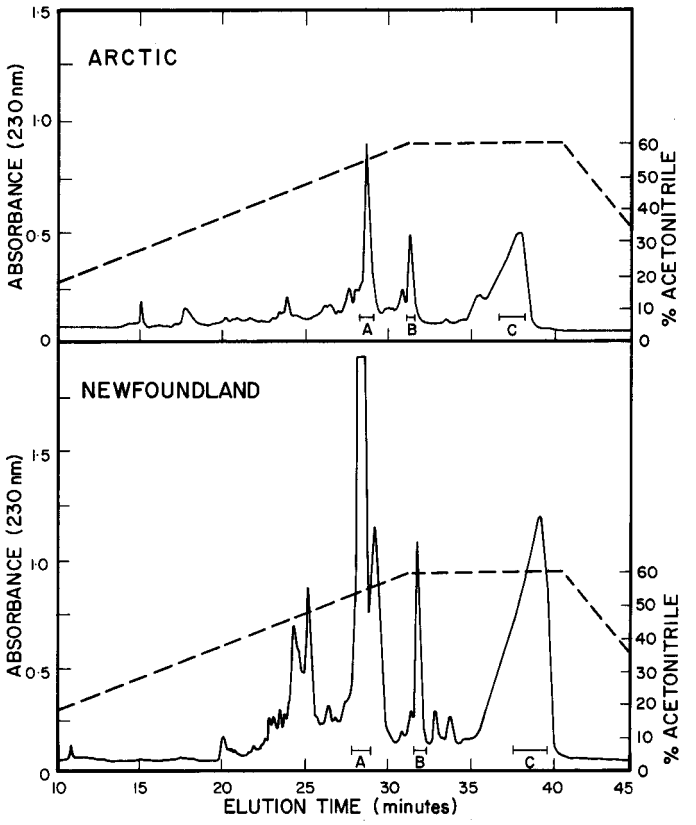

FIG. 2. Purification of arctic and Newfoundland shorthorn sculpin antifreeze proteins on reverse-phase high-performance liquid chromatography (HPLC). The antifreeze proteins purified on Sephadex G75 (Figure 1) were further fractionated on reverse-phase HPLC using an Ultrasphere-ODS column.

TABLE 2. Amino acid composition of the various antifreeze components found in the arctic and Newfoundland shorthorn sculpin

	(mole %)					
	Arctic			Newfoundland		
	A	B	C	A	B	C
Aspartic acid	9.3	6.7	5.2	9.7	7.8	5.4
Threonine	7.1	5.7	4.9	6.3	8.0	4.8
Serine	1.2	1.9	2.5	0.5	0.7	2.8
Proline	1.4	0.5	2.4	2.0	1.4	1.5
Glutamic acid	3.4	6.5	4.8	1.3	2.6	5.0
Glycine	1.5	3.4	2.8	0.6	1.2	2.8
Alanine	60.0	64.0	58.6	62.5	62.0	59.5
Methionine	1.3	0.7	1.5	2.3	2.0	1.7
Isoleucine	—	—	2.4	—	—	2.3
Leucine	3.4	3.4	4.9	3.1	2.8	4.7
Lysine	8.3	4.9	7.4	8.0	6.0	7.3
Arginine	2.8	2.5	2.3	3.4	4.3	2.0

Components A, B and C were isolated using reverse-phase high-performance liquid chromatography (Fig. 2).

ma antifreeze during the summer period. In addition, these levels do not decline when the fish are acclimated to 4°C for 60 days (DeVries and Lin, 1977). During the summer months the Alaskan sculpin (*Myoxocephalus verrucosus*) only loses 50% of its winter levels of antifreeze, despite the fact that the water temperature rises to approximately 7°C (DeVries, 1980).

The concentration of any protein in the blood plasma is the result of an interplay between biosynthetic and degradation (turnover) rates (Putnam, 1975). Therefore the means by which arctic and Antarctic fish maintain high levels of antifreeze during the summer could involve one, or both of these processes.

Antifreeze degradation rates (biological half life) have been estimated to be approximately four weeks in an Antarctic fish (*Trematomus hansoni*) maintained at −1.5°C (Haschemeyer and Mathews, 1980). A similar estimate can be made from data obtained from winter flounder at −1°C (Fletcher, 1981: Fig. 4). These estimates indicate that although antifreeze proteins are degraded rather slowly, significant amounts would disappear from the blood within a short period of time following the cessation of biosynthesis.

Little is known concerning seasonal changes in antifreeze biosynthetic rates in polar fishes. However, the fact that the Antarctic species and the Alaskan sculpin do not lose their antifreeze proteins even when acclimated to relatively warm water suggests that they are synthesized all year round (DeVries and Lin, 1977; DeVries, 1980). With this in mind, it is possible that the arctic population of shorthorn sculpin sampled in the present study also synthesize the antifreeze proteins throughout the year. Antifreeze biosynthesis is a seasonal event in fish such as the winter flounder which inhabit more temperate waters (Fletcher, 1981). Since Newfoundland populations of shorthorn sculpin lose their antifreeze during the late summer, it would appear that synthesis is also a seasonal event in this population.

The apparent difference between arctic and Newfoundland populations of shorthorn sculpin with regard to their annual cycles of plasma antifreeze levels is consistent with results obtained for winter flounder. In this species, Newfoundland populations synthesized antifreeze proteins one month earlier and cleared them from the plasma two months later than did the more southerly Nova Scotian populations. Moreover, when Nova Scotian winter flounder were transferred to Newfoundland, and maintained under Newfoundland conditions of temperature and photoperiod, they retained their plasma antifreeze cycle (Fletcher and Smith, 1980). Therefore for winter flounder it appears that the timing and length of the antifreeze biosynthetic period is matched to the annual water temperature cycle, and is to some extent genetically determined. Recent evidence indicates that in winter flounder the onset of antifreeze biosynthesis in the fall is influenced by photoperiod and comes under the control of the pituitary gland (Fletcher *et al.*, 1978; Hew and Fletcher, 1979).

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