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Protein content and freezing avoidance properties of the subdermal extracellular matrix and serum of the Antarctic snailfish, *Paraliparis devriesi*

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Abstract

The Antarctic snailfish, *Paraliparis devriesi* (Liparididae), occupies an epibenthic habitat at a depth of 500–650 m in the subzero waters of McMurdo Sound, Antarctica. This species has watery (97%) gelatinous subdermal extracellular matrix (SECM) comprising a mean of 33.8% of the body weight, the largest known proportion of any adult fish. The protein concentration of the SECM was found to be 6–7 mg ml^{-1} (0.6–0.7% w/v). Separation of the polypeptides of the SECM by SDS-PAGE revealed 11 polypeptides ranging in relative molecular mass (M_r) from 67,000 to 13,000, with other unresolved polypeptides of less than 13,000. The isoelectric points of these proteins ranged from 4.85 to 8.05. Partial N-terminal amino acid sequence data were obtained for four of the major SECM polypeptides. The N-terminal amino acid sequences of three of these were not identical to or homologous with any other known sequences, whereas the N-terminal sequence of one polypeptide (M_r 51,000) was identical to partial sequence from the apolipoprotein A-I precursor of Atlantic salmon (*Salmo salar*). Although not isolated from either SECM or serum, melting point-freezing point behavior of body fluids suggest that *Paraliparis* possess modest amounts of a noncolligative antifreeze compound. Since relatively small amounts of antifreeze are present in the serum and even less in the SECM, freezing avoidance results from the combined effects of antifreeze and the elevated osmolality of body fluids. There are no special adaptations to prevent freezing in the superficially located high water content SECM.

Introduction

With 31 known species, scorpaeniform teleosts of the family Liparididae (snailfishes) are exceptionally diverse in the Southern Ocean around Antarctica (Stein and Andriashev 1990). Since there are an additional 20–30 undescribed species, the Liparididae will eventually surpass the endemic Nototheniidae as the most speciose fish family in the Southern Ocean (DeWitt *et al.* 1990; Stein *et al.* 1991). *Paraliparis devriesi* Andriashev (1980) is an epibenthic snailfish living at 500–650 m in McMurdo Sound, Antarctica. It has the most southerly distribution of any liparidid, inhabiting subzero water continuously near its freezing point of −1.91°C. Although it lacks a swim bladder, *P. devriesi* is

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neutrally buoyant through the combined effects of reduced skeletal ossification and an extensive low density subdermal extracellular matrix (SECM) (Eastman *et al.* 1994). The SECM is a clear gelatinous substance comprising 33.8% of the body weight and containing 97% water. Histochemistry suggests that hyaluronic acid is the dominant glycosaminoglycan in the SECM of *P. devriesi* (Eastman *et al.* 1994). This anionic molecule is probably responsible for the water binding properties of the SECM.

An expanded SECM is a convergent features in a number of teleostean lineages including other scorpaeniforms like *Cyclopterus lumpus* (Davenport and Kjørsvik 1986), zoarcids (McAllister and Rees 1963), mesopelagic salmoniforms and stomiiforms (Yancey *et al.* 1989) and various larval anguilliforms and elopiforms (Pfeiler 1986, 1991). Histochemical, monoclonal antibody and electrophoretic techniques have been used to determine the glycosaminoglycan composition of connective tissues in teleosts (Banerjee and Yamada 1985; Benjamin 1988; Benjamin and Ralphs 1991; Pfeiler 1993). The protein content of the SECM is 2.9% of wet weight of mesopelagic *Bathylagus pacificus* (Yancey *et al.* 1989), but there is no information on the protein composition of the SECM.

The investigations reported here provide the first information on the nature of the proteins of the expanded SECM of any adult teleost. We sought to determine: 1) the levels of protein (high or low) and the composition of proteins in the SECM; 2) the complexity of the protein spectrum in the SECM and 3) the similarities, if any, between the SECM proteins/polypeptides and other known proteins. Because the SECM of *P. devriesi* is unique among polar fishes in its high water content and liability to freezing, we also examined ion levels and melting point-freezing point behavior of the SECM and serum. Although we did not isolate an antifreeze, the freezing behavior suggests that freezing avoidance in *P. devriesi* is the result of the combined effects of modest concentrations of a noncolligative antifreeze and elevated osmolality.

Materials and methods

Collection of specimens

Field work and some phases of laboratory work were conducted at the U.S. McMurdo Station on Ross Island in the southwestern Ross Sea. We captured *Paraliparis devriesi* in the Erebus Basin of McMurdo Sound during November, 1991 and 1992. The collection site was approximately 22 km from McMurdo Station (77° 47′ 2134 S, 166° 09′ 5400 E – GPS coordinates). *Paraliparis* were taken in large, conical wire mesh bottom traps fished at a depth of 635 m through holes drilled in the annual sea ice. The traps were baited with chopped fish, left on the bottom for 3–5 days and retrieved with an oceanographic winch.

We used six specimens in the studies described below. We used syringes to withdraw blood and coelomic fluid. We obtained SECM by placing the fish on Parafilm, removing the skin and then scraping the SECM into 20 ml centrifuge tubes. Care was taken to avoid scraping the underlying muscle. Under warm conditions, as in contact with human fingers, the SECM exudes a watery fluid which was included in the sample.

Materials

ProBlott membranes were purchased from Applied Biosystems, and PhastGels (IEF 3–9) were purchased from Pharmacia LKB. Coomassie-based Protein Assay solution was a product of Bio-Rad. All chemicals used in these studies were of analytical grade or better.

Electrophoresis

Protein samples (20 μg) were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) in a Hoefer SE 250 apparatus using a 10% running gel and the ammediol buffer system of Wyckoff *et al.* (1977). For analysis of protein samples (20 μg) by isoelectric focusing (IEF), the Pharmacia PhastSystem ap-

paratus was used with pre-formed PhastGels that had a pH range of 3–9. In each system, electropherograms were calibrated in terms of relative molecular mass (M_r) or isoelectric point (pI) values by the inclusion of standard marker proteins in a gel lane.

Column chromatography

Samples of SECM (300 µl) were diluted with three volumes of buffer A (0.1 M NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.01% NaN_3, 50 mM Tris pH 7.6) and then dialyzed extensively against buffer A using Spectrapor dialysis membrane with a 3,500 kDa cutoff. Using a Pharmacia FPLC system, the sample was applied to a Mono Q HR 5/5 column which had been equilibrated with buffer A, and elution was performed using a 30 ml 0.1–1.0 M NaCl gradient in buffer A at a flow rate of 0.5 ml min^{-1}, with 0.5 ml fractions collected.

Electrotransfer and amino acid sequence determination

Protein samples (approximately 100–300 µg) in fractions obtained from multiple Mono Q column runs were concentrated by precipitation in the presence of 7.5% trichloroacetic acid, and after washing first with 5% trichloroacetic acid and then with acetone, the samples were dissolved in SDS-PAGE sample buffer and ran on a preparative-scale SDS-PAGE system (1.5 mm gel thickness with 1.2 cm lanes). Proteins were then electrotransferred to a ProBlott membrane and stained protein bands were cut out of the ProBlott membrane and sequenced in an Applied Biosystems Model 477A Protein Sequencer as described previously (Johnson and Saulinskas 1993).

Amino acid sequence comparisons

The sequences of the SECM proteins were analyzed for identities with known protein sequences and with DNA sequence information using the current Swiss Protein and GenBank databases of the Intelligenetics Gene Works program. Searches for sequence identities of less than five amino acid residues in length were not performed as these are not considered statistically meaningful by the Intelligenetics search algorithm.

Ion concentrations and freezing point-melting point determinations

Cation concentrations in body fluids were determined with a Perkin-Elmer model 4000 atomic absorption spectrometer. Samples for spectroscopy were digested for 14 hr in concentrated nitric acid at 80°C. Chloride concentrations were obtained with a Buchler-Cotlove direct readout chloridometer. Osmolality of serum was measured in triplicate with a Wescor 5100C vapor pressure osmometer. Values for coelomic fluid and SECM are from Eastman *et al.* (1994).

Using a method outlined by DeVries (1986), we determined the freezing points of body fluids with a cryoscope. This involves slowly lowering the temperature of a sample of the fluid in a 10-µl capillary tube in the presence of a small seed crystal. The freezing point or freezing temperature is the temperature at which ice propagates from the seed crystal. The melting point is the temperature at which the frozen fluid sample melts as it is slowly rewarmed, and it is the same as the melting temperature of the seed crystal in the fluid. Values for osmolality of the various fluids, as obtained by osmometry, were multiplied by −0.001858°C mOsm kg^{-1} to obtain calculated melting points (DeVries 1986, 1988). The calculated and experimentally determined melting points were identical. Differences between melting and freezing points indicate the presence and relative concentration of glycopeptide or peptide antifreezes which lower the freezing point of body fluids in a non-colligative manner (DeVries 1986, 1988).

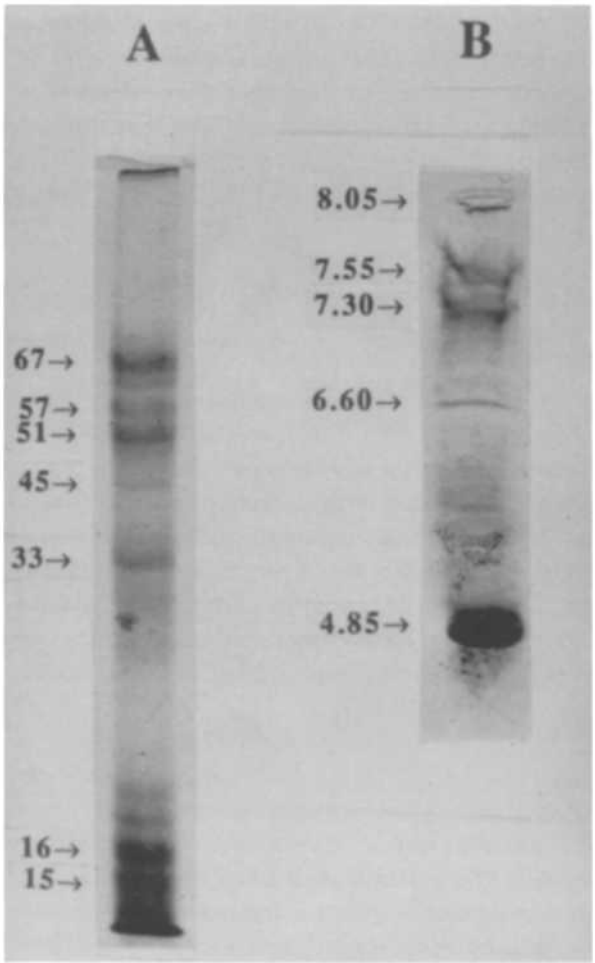

Fig. 1. Electrophoretic analyses of *Paraliparis devriesi* SECM polypeptides and proteins. SDS-PAGE analysis of the polypeptide components of SECM is shown in gel A, with the approximate M_r values for major polypeptides shown multiplied by 10^{-3}. IEF analysis of SECM proteins is shown in gel B, with approximate pI values indicated for the major proteins in the sample. Approximately 20 μg of protein were used in each analysis.

Results

Characterization of protein components of SECM

The protein content of both the intact SECM and the supernatant after centrifugation was found to be about 6–7 mg ml^{-1} (0.6–0.7% w/v) as determined by Coomassie assay. SDS-PAGE of this material revealed that the SECM contained 11 polypeptide components with M_r values ranging from 67,000 to 13,000 (Fig. 1A), in addition to the presence of lower molecular weight polypeptides which were not resolved in the gel. The major polypeptide species in SECM appeared to be the components with M_r values of approximately 67,000, 57,000, 51,000, 33,000, 16,000 and 15,000. As shown in Fig. 1B, the proteins of the SECM had pI values ranging from 8.05 to 4.85, with the protein band at pI 4.85 as the major component.

Amino acid sequence analysis of SECM proteins

In order to determine if the SECM proteins had identifiable sequence identities or homologies with other known proteins, the major proteins of SECM were isolated and N-terminal amino acid sequence data were obtained. SECM was initially fractionated using a Mono Q anion exchange column (Fig. 2), and the major proteins which were partially purified by the chromatography were then further purified by preparative SDS-PAGE, and subjected to N-terminal sequence analysis (20 cycles) after electrotransfer to ProBlott membranes.

Of the six polypeptides which were subjected to sequence analysis, four gave acceptable sequence data (Table 1) whereas two (M_r values of 57,000 and 33,000) gave no sequence data, presumably because of N-terminal blockage. Although the data for the four sequencable polypeptides were clear for the initial six cycles, sequence data after this step became unreliable because of high backgrounds, and it was not possible to assign sequences beyond this point with any certainty. For one of the polypeptides (M_r 15,000 in Table 1), two amino acids were identified at residue position 3. As the sequence analysis in subsequent residue positions 4–6 showed single residues, the heterogeneity at position 3 is suggestive of polypeptide polymorphism rather than a background problem. In the case of another polypeptide (M_r 51,000 in Table 1), two residues were identified at position 6, after which the sequence analysis became uninterpretable. In this case, it is therefore possible that the heterogeneity may have been related to background problems in the sequence run.

Analysis of the Intelligenetics data bank of pro-

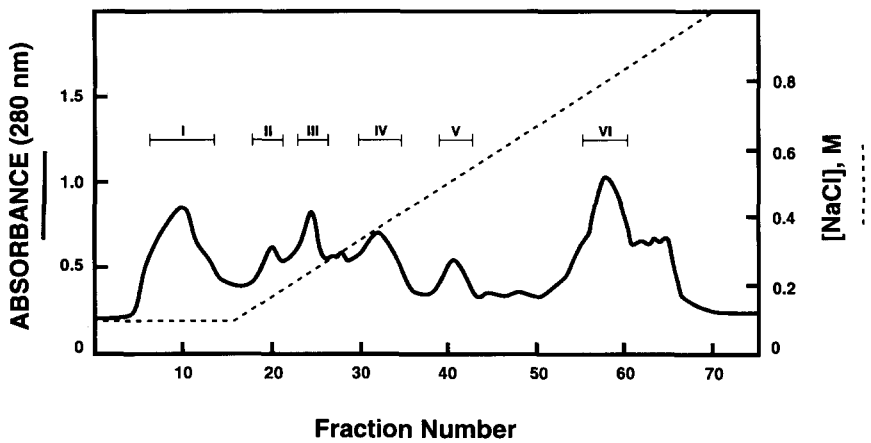

Fig. 2. Fractionation of SECM proteins of *Paraliparis devriesi* by FPLC Mono Q column chromatography. A 300 µl sample of SECM was prepared and applied to a Mono Q column as described in the Methods and the column was eluted with a 0.1–1.0 M NaCl gradient as shown. Major peaks in the chromatograph which contained SECM polypeptides (identified by SDS-PAGE) are shown with bar lines and are labeled with Roman numerals.

Table 1. N-terminal amino acid sequence analyses of the SECM polypeptides of *Paraliparis devriesi*

Polypeptide M_r from SDS-PAGE[1]	Purified from Mono Q Peak[2]	N-terminal sequence[3] Residue position					
		1	2	3	4	5	6
67,000	III	A	P	A	T	N	V
65,000	VI	G	P	N	T	A	E
51,000	IV	D	A	P	S	Q	L P
15,000	I	V	A	P	D E	P	S

[1]See Fig. 1; [2]See Fig. 2; [3]Protein samples were sequenced with initial amounts of approximately 20 pmol based on the initial yield of the PTH-amino acid at residue 1, and repetitive yields were above 80%. At residue position 6 of the 51,000 polypeptide and position 4 of the 15,000 polypeptide, two amino acids (shown above and below each other) were identified in the sequence analysis.

tein and DNA sequences for identities of six-residue sequences revealed that only one of the possible *P. devriesi* sequences was identical to a known protein sequence. This identity was between one of the two possible sequences for the *P. devriesi* SECM M_r 51,000 polypeptide (DAPSQL) and residues 24–29 of apolipoprotein A-I precursor of Atlantic salmon (Powell *et al.* 1991).

Freezing behavior of body fluids

When observed in a cryoscope, body fluids of *P. devriesi* undergo multiple growth events as the temperature is lowered by 0.01 °C min^{-1}. This unusual pattern of freezing is summarized in Table 2. Initial ice growth is in the form of plates or spicules, growth then proceeds in a series of stops and starts. Finally, with progressive lowering of the temperature, rapid crystal growth occurs and the sample becomes completely frozen, the ice often appearing granular. Samples from individual fish are also variable; a serum sample might freeze at a higher temperature during one observation but not during another. We conclude that the serum of *P. devriesi* contains modest amounts of a noncolligative antifreeze based on the presence of a measurable (0.4 °C) thermal hysteresis; the SECM and coelomic fluid show considerably less hysteresis (Table 3).

Ionic content of body fluids

The osmolality of the body fluids of *P. devriesi* is elevated (Table 3) compared to mean value of 350 mOsm kg^{-1} typical for temperate marine teleosts (Holmes and Donaldson 1969; Olson 1985). The osmotic strength is attributable primarily to inorganic ions like sodium and chloride (Table 4). The ionic

Table 2. Freezing point determinations by cryoscopy for some body fluids of *Paraliparis devriesi*

Specimen number	Serum			Coelomic fluid			SECM		
	1[1]	2[1]	3[1]	1	2	3	1	2	3
PDE 15	−1.24	−1.70	−1.76	−1.14	−1.24	−1.55	−1.17	−1.28	−1.35
PDE 16	−1.60	– –	−1.71	−0.96	– –	−1.15	−0.35	−1.20	−1.92
PDE 17	−1.25	−1.32	−2.50	– –	– –	– –	−1.20	– –	−1.94
PDE 18	−1.28	– –	−1.38	−0.47	−0.95	−1.10	−1.28	– –	−1.48
PDE 19	−1.19	−1.43	−1.70	– –	– –	– –	−1.34	−1.90	−2.79
PDE 20	−1.10	−1.27	−1.31	−1.10	−1.27	−1.31	– –	– –	– –
Mean	−1.28	−1.43	−1.73	−0.92	−1.15	−1.28	−1.07	−1.46	−1.90
SEM	0.07	0.10	0.17	0.15	0.10	0.10	0.18	0.22	0.25
N	6	4	6	4	3	4	5	3	5

[1]Refers to stages of freezing as follows: 1 = initial growth of plates or spicules of ice, 2 = resumption of ice growth, 3 = final rapid growth of ice (often granular in appearance) to complete freezing of sample.

Table 3. Contributions of osmolality and hysteresis (suggestive of presence of antifreeze) to the freezing point depression of some body fluids of *Paraliparis devriesi*

Fluid	Osmolality (mOsm kg^{-1})	Calculated MP[3] (°C)	Observed initial FP[4] (°C)	MP−FP (°C)	Contrib. osmolality to FP depression (%)	Contrib. hysteresis to FP depression (%)
Serum	474[1]	−0.88	−1.28	0.40	69	31
Coelomic fluid	444[2]	−0.83	−0.92	0.09	90	10
SECM	513[2]	−0.95	−1.07	0.12	89	11

[1]Mean for specimens PDE 92-1 to 92-4 (Table 4) as determined by vapor pressure osmometry; [2]From Eastman *et al.* (1994); [3]Calculated from value for osmolality as follows: FP = osmolality × (−0.001858 °C mOsm kg^{-1}) (DeVries 1988, p. 612); [4]See Table 2.

Table 4. Ionic content (mmol l^{-1}) of body fluids and subdermal extracellular matrix in *Paraliparis devriesi* as determined by atomic absorption spectroscopy and chloridometry[1]

Specimen No.	Serum					Coelomic fluid				SECM			
	Na^+	Cl^-	K^+	Ca^{2+}	Mg^{2+}	Na^+	K^+	Ca^{2+}	Mg^{2+}	Na^+	K^+	Ca^{2+}	Mg^{2+}
PDE 92-1	– –	228	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –
PDE 92-2	– –	247	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –	– –
PDE 92-3	253	254	14	6	3	– –	– –	– –	– –	355	14	6	3
PDE 92-4	224	234	11	4	2	– –	– –	– –	– –	360	13	5	2
PDE 92-5	242	– –	14	3	5	212	36	2	4	– –	– –	– –	– –
PDE 92-6	252	– –	11	3	4	181	9	2	4	– –	– –	– –	– –
Mean	243	241	12	4	4	196	22	2	4	358	14	6	2
SEM	6.7	5.9	0.9	0.7	0.6								
N	4	4	4	4	4	2	2	2	2	2	2	2	2

[1]Except for chlorides, numbers are means of two determinations for each specimen.

content of all sampled fluids is similar (Table 4), with the exception of elevated values for Na^+ in the SECM. Since glycosaminoglycans are fully ionized under physiological conditions (Comper and Laurent 1978), Na^+ probably serves as a counterion to the anionic hyaluronic acid in the SECM. The relatively low values for K^+ indicate that the SECM, like serum and coelomic fluid, is an extracellular fluid with few cells. Values for divalent ions, considerably lower than levels in seawater (McDonald and Milligan 1992), are similar among the various body fluids.

Discussion

SECM proteins

The present studies show that the SECM of *P. devriesi* contains a significant protein concentration (6–7 mg ml^{-1}), in addition to its previously reported complex polysaccharide content (Eastman *et al.* 1994). The constituent polypeptides of these proteins have diverse M_r values ranging from 67,000 to below 13,000, and the most abundant species appear to be those with M_r values of 16,000 and 15,000. The native proteins of SECM show a wide variety of pI values (from 8.05 to 4.85), although the majority of SECM protein is acidic (pI value of 4.85).

In an attempt to address the question of the possible identity and functions of the SECM proteins, sequence data obtained from some of the polypeptides were compared to known protein and structural gene DNA sequences in a data bank. This search revealed that only one of the SECM polypeptides has N-terminal sequence which shows a possible identity with other known sequences, namely with a six-residue sequence from apolipoprotein A-I precursor of Atlantic salmon (Powell *et al.* 1991).

Further sequence studies on this SECM protein will be required to unequivocally establish this sequence homology, as it has been suggested that longer sequence identities or similarities need to be established before familial or structural relationships between different polypeptides can be assigned (Argos 1989).

It is interesting to note that none of the SECM sequences showed homology with known antifreeze protein sequences, although this does not exclude the possibility of the presence of antifreeze proteins in the SECM as not all of the SECM proteins have been sequenced. Further work will therefore be necessary to characterize the SECM proteins, including amino acid sequence and immunological analyses, in order to investigate the functions and origins of these proteins and to determine if they are uniquely expressed in SECM or are also found in other tissues in *P. devriesi* such as serum.

Antifreeze and the deepwater habitat

Glycopeptide and peptide antifreeze compounds have evolved in a number of unrelated lineages of cold water teleosts including notothenioids, zoarcids, cottids, gadids, pleuronectids, clupeids and osmerids (Davies *et al.* 1988; DeVries 1988; Cheng and DeVries 1991; Kao *et al.* 1986; Ewart and Fletcher 1990). Like liparidids, the cottid, *Hemitripterus americanus*, is also a member of the teleostean order Scorpaeniformes. This species possesses a well characterized antifreeze polypeptide with an M_r value of 14,000 (Ng *et al.* 1986; Hayes *et al.* 1989; Ng and Hew 1992). Phylogenetic relatedness, however, is no assurance that the antifreeze of *P. devriesi* will be similar in size to that of *Hemitripterus*.

The measurable thermal hysteresis (0.4°C) suggests that the serum of *P. devriesi* contains modest amounts of a noncolligative antifreeze, but that this does not sufficiently lower the freezing point of serum to protect *P. devriesi* from freezing at the usual −1.91°C surface temperature in McMurdo Sound. Coupled with elevated osmolality of body fluids, a widespread phenomenon in polar teleosts (O'Grady and DeVries 1982), the freezing point depression of serum is only −1.28°C. The less marked hysteretic effect (Table 3) in the SECM and coelomic fluid indicates that antifreeze may not be present in these body fluids at a level equivalent to serum, and as noted earlier, none of the available SECM polypeptide sequences showed identity or homology with antifreeze proteins (Davies and Hew 1990). At this

stage however, we cannot definitely exclude the possibility of the presence of antifreeze proteins in SECM because not all SECM polypeptides were sequenced.

The freezing behavior of the serum of *P. devriesi* is similar to that reported for some northern populations of the Pacific herring, *Clupea harengus pallasi* (Raymond 1989). In this species, suspected to have a noncolligative antifreeze providing between 0.28 and 0.61°C of protection, freezing points of serum are not well defined, and ice growth in the serum progresses through periods of stops and starts when the temperature is lowered.

While freezing resistance protection to −2.2°C is necessary for fishes inhabiting relatively shallow ice-laden water in polar regions (DeVries 1988), *P. devriesi* lives at 500–650 m, a habitat with different physical parameters and possibly less ice. Seawater at the surface of McMurdo Sound freezes at −1.91°C (Littlepage 1965). Because there is a pressure effect on the freezing point of seawater (0.00753°C 10 m^{-1}) (Lewis and Perkin 1985), the *in situ* freezing point is lower at depth. For example, at 600 m the *in situ* freezing point of seawater with a salinity of 34.7‰ is −2.36°C. Based on the freezing point of the serum (Table 3), the freezing point of *P. devriesi* at 600 m would be −1.73°C.

Presumably fishes living at these depths and temperatures are in little danger of freezing because there is no ice in the water, hence the body cannot be seeded by ice crystals. *P. devriesi* was thought to lack antifreeze, remaining instead in a supercooled state in this supposedly ice-free habitat (DeVries and Lin 1977). However, there is now evidence that water at the undersurface of ice shelves is at its equilibrium freezing point (Foldvik and Kvinge 1977). As this water flows out from under the shelf and rises, it becomes supercooled and the potential exists for ice formation at considerable depths. For example, large masses of single-crystal ice platelets were discovered at 250 m near the Filchner Ice Shelf in the Weddell Sea (Dieckmann *et al.* 1986). The undersurface of the Ross Ice Shelf in McMurdo Sound is at a depth of about 500 m (Lewis and Perkin 1985), therefore below 500 m there is probably no ice formation. Since the typical habitat of *P. devriesi* is greater than 500 m and at some distance from the Ross Ice Shelf, modest levels of antifreeze may offer sufficient protection from freezing.

Ice conditions in deep water are not as well understood, and it is not known whether antifreezes are an absolute necessity for fishes living in this habitat. In notothenioids living in shallower water in McMurdo Sound, antifreeze accounts for a 3-fold greater freezing point depression of the serum (−1.2°C) than in *P. devriesi* (DeVries 1988). It is remarkable that a superficially located, high water content tissue like the SECM does not require additional protection against freezing in this subzero environment. For example, trunk skin is a barrier to entry of ice crystals into the body of some polar fishes. *In vitro* experiments on the skin of winter flounder (*Pseudopleuronectes americanus*) from Newfoundland support the hypothesis that epithelia exclude the entry of ice, probably through the action of antifreeze contained in the interstitial fluid and by the narrowness of the intercellular space (Valerio *et al.* 1992). Based on light and electron microscopic appearance and the thinness of the dermis (Eastman *et al.* 1994), the skin of *Paraliparis* presents a less substantial barrier to ice entry or propagation than the skin of Antarctic notothenioids, a group protected by thicker skin and by higher levels of antifreeze (Eastman and Hikida 1991).

In conclusion, it may be that in species experiencing only sporadic exposure to ice, adequate protection involves elevated osmolality of the body fluids and relatively low levels of an antifreeze that does not completely inhibit the growth of ice. It is also possible that we are viewing *P. devriesi* at a point in its evolutionary history when it does not possess a fully effective level of antifreeze.

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