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Reports from the research projects:

Antifreeze activity in body fluids of some marine fishes from the Disko Bay and Disko Fjord

By Karin Holk, Tiina Lecklin, Eyofinn Magnussen and Peter Rask Møller

Supervised by Art. L. DeVries and Jørgen Schou Christiansen

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Abstract

The presence of macromolecular antifreezes (thermal hysteresis factors) in blood serum and gut fluids of marine teleost fishes from West Greenland waters was investigated in August 1997, during a NorFa Fish Physiology Course.
Thermal hysteresis in °C in the serum of five shallow water (depth < 50 m) species was: *Anisarcus medius* (1.27), *Pholis fasciatus* (0.51±0.27), *Gymnachantus tricuspis* (0.50±0.11), *Myoxocephalus scorpius* (0.42) and *Gadus ogac* (0.40±0.09). Only two of the species caught in deep water (depth 300-500 m), *Boreogadus saida* and *Leptoclinus maculatus*, showed considerable thermal hysteresis of 0.87 °C and 0.98 °C, respectively. No thermal hysteresis was found in three shallow water species (*Ammodytes dubius, Gadus morhua* and *Anarchias lupus*) and eight species caught in deep water (300-500 m): *Mallotus villosus, Artediellus atlanticus, Leptagonus decagonus, Sebastes sp., Liparis gibbus, Caraproctus fabrici, Lycodes seminudus* and *Lycodes vahli*).
This is probably the first time that thermal hysteresis has been reported for *Leptoclinus maculatus, Anisarcus medius, Pholis fasciatus* and *Gymnachantus tricuspis*.

Introduction

The osmolality of marine fish species is much lower than that of the surrounding seawater. Thus, compared to the seawater osmolality of approximately 1000 mOsm/l the osmolality of temperate marine teleost fish is around 350 mOsm/l. For species inhabiting more polar areas the osmolality may be as high as 600 mOsm/l (Christiansen et al., 1995). For polar species this may cause problems during winter when the water temperature decreases to subzero levels. This means that the fish becomes supercooled and that the appearance of ice crystals in the water could lead to an immediate freezing and therefore death of the fish.

This problem is in some species solved by the presence of special organic compounds (antifreeze glycopeptides and peptides) (DeVries, 1982). The antifreezes bind to the surface of ice crystals and prevent further growth of the crystals. In that way the freezing point of the solution is depressed to a lower value than can be predicted from the osmolality of the solution. However, they do not change the melting point of the solution significantly since they are high molecular (Eastman & DeVries, 1984). The antifreeze agents are primarely synthesized in the liver and are present in the extracellular fluids within a range of tissues, e.g. in the blood and the gut (DeVries, 1982). Antifreeze molecular weights range between 2600 and 34000 Da (DeVries, 1982). The presence of antifreeze compounds can be detected by measuring the melting and freezing points of the solution as milliosmoles and from this difference the thermal hysteresis can easily be calculated (DeVries, 1982).

The purpose of our study was to analyse the serum and gut fluid in a range of different fish species living in Disko Bay, Greenland, for the possible presence of antifreeze compounds. Additionally, from some of the species we wished to separate the antifreeze agents by means of gel filtration.

Material

A total of 19 marine fish species were caught in Disko Bay and Disko Fjord by traps, trawls and lures in August 1997. The approximate catch depths are given in Appendix 1. The single *Ammodytes dubius* was taken from the stomach of a *Gadus ogac*. The fish were kept alive in large buckets, at the Arctic Station, Godhavn until the blood sampling. Blood samples from specimens used for gut fluid measurements (*G. ogac* and *M. scorpius*) were, however, taken right after capture. The length and weight of each fish were measured. Blood samples were taken from the caudal vein and clotted for a few minutes before centrifugation. Serum was transferred to separate tubes and stored at 5°C. The gut content from two species (*Myoxocephalus scorpius* n=6 and *G. ogac* n=7) was sampled and processed as the clotted blood.

Analytical procedures

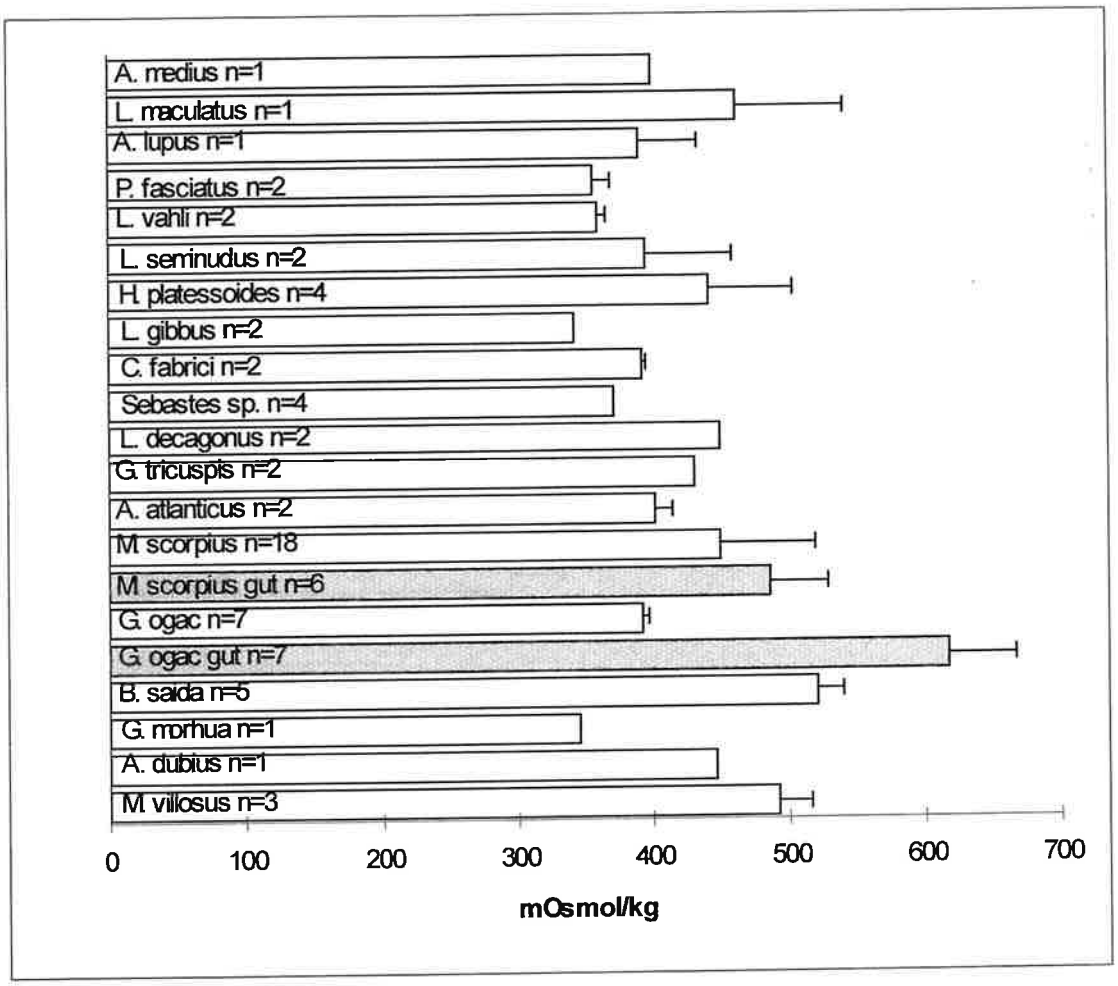
A Wescor 5100C vapour pressure osmometer and an Advanced Micro-osmometer (Model 310) were used to measure vapour pressure (adequate melting point) and freezing point, respectively. However, in some samples an error occurred, probably due to the presence of antifreeze compounds. Therefore, the melting and freezing point of a number of samples was additionally measured by a Clifton Nanolitre Osmometer. The melting process of c. 30 nl samples was observed in a microscope, while the temperature was regulated with an accuracy of ± 0.001°C. The temperature at which the last tiny ice crystal disappeared during slow warming of the prefrozen sample was taken as the melting point (MP). Serum samples containing a tiny seeding ice crystal were then cooled, and the temperature at which a rapid growth of ice crystals took place was taken as the hysteresis freezing point (HFP). The depression of the freezing point was calculated by multiplying the difference in molality between the freezing and melting point by -0.001858 ^{0}C/mOsmol.

A CMT 10 Chloride titrator (Radiometer, Copenhagen) was used to measure the chloride concentration of the samples.

For gel filtration serum from *Gadus ogac* was pooled. The serum was deproteinized by 5% trichloroacetic acid (TCA), centrifuged and the clear supernatant transferred to a dialysis tube and dialysed in distilled water for 5.5 h to remove the TCA. To concentrate the content of the tube, the dialysis tube was covered by Aqualite, and this compound was allowed to soak water for approximately 3 h. The sample was placed on the top of a 1.5 m gel filtration column (Sephadex G75 column), and was washed through the column with a 5 M NH_4HCO_3 solution (flow rate c. 20 ml/h). The N-terminal of the peptides was marked with a fluorescence compound (flourescamine 4mg/ml acetone, 100ml/5 ml serum). When the fluorescence approached the bottom of the column, the outflow was sampled in 10 ml tubes for photometric measurements. The absorbance of the samples were measured at two wavelengths (230 and 280 nm) on an ultraspec 2000 (Pharmacin, Biotech).

Results and discussion

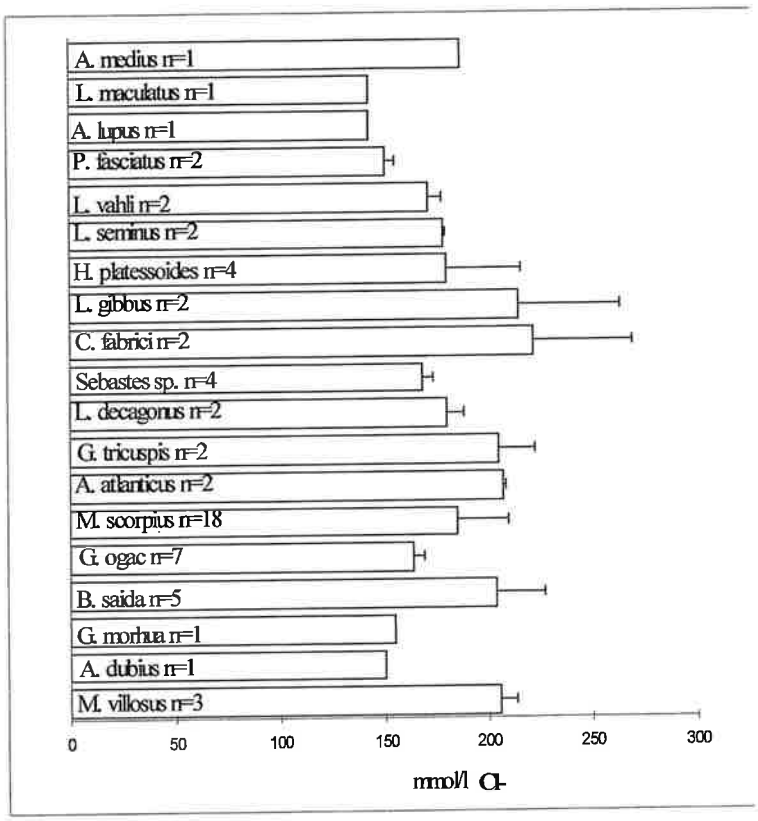
Body fluid osmolality



Figur 1. The osmolality of the serum and gut samples from the 19 species. Values are given as mean + S.D.

The highest blood serum osmolalities were found in the pelagic species *Boreogadus saida* and *Mallotus villosus* (Fig. 1, Appendix 1). The values of around 500 mOsm/l found for *B. saida* is slightly higher than the value (c. 440 mOsm/l) given by Christiansen et al., 1995.
The blood serum and gut fluid osmolality of *M. scorpius* did not differ, whereas the gut fluid osmolality of *G. ogac* exceeded that of the blood serum.

Body fluid Cl^- concentration



Figur 2. The [Cl^-] in the serum from the 19 species. Values are given as means + 1 S.D.

The Cl^- concentration varie between 140 and 225 mmol/l (Fig. 2., Appendix 1). Thus, all values were much lower than the concentration of sea water, which is around 535 mmol/l (Sverdrup et al., 1942). Highest values were found in the two liparids, but otherwise no general trends were found. In this study no attempt was done to determine the total inorganic ion concentration and therefore the organic fraction could not be calculated.

Freezing point - Melting point (Thermal hysteresis)

Some of the freezing point measurements of *B. saida*, *G. ogac*, *M. scorpius*, *Gymnacanthus tricuspis*, *Pholis fasciatus* and *Anisarcus medius* measured with the advanced Micro-osmometer, were affected by an error message (values given in bold in Appendix 1). This is generally known

to be an indication of antifreeze agent, but since some samples without error message actually showed thermal hystersis when using the Clifton Nanolitre Osmometer, we did not pay attention to the advanced Micro-osmometer values.

Taxa	Sample size n	HFP	MP mOsmole	D	HFP °C	MP °C	MP-HFP °C	S.D.
Osmeridae								
Mallotus villosus	3	575	540	35	-1,07	-1,00	0,07	0,01
Ammodytidae								
Ammodytes dubius	1	567	485	82	-1,05	-0,90	0,15	
Gadidae								
Gadus morhua	1	325	320	5	-0,60	-0,59	0,01	
Gadus ogac	3	631	414	217	-1,17	-0,77	0,40	0,09
Gadus ogac (gut)	1	803	988	185	-1,83	-1,49	0,34	
Boreogadus saida	1	971	503	469	-1,80	-0,93	0,87	
Scorpaeniformes								
Myoxocephalus scorpius	1	664	440	224	-1,23	-0,82	0,42	
M. scorpius (gut fluid)	1	523	597	75	-1,11	-0,97	0,14	
Artediellus atlanticus	1	487	444	43	-0,90	-0,82	0,08	
Gymnacantus tricuspis	2	755	485	270	-1,40	-0,90	0,50	0,11
Leptagonus decagonus	1	445	410	35	-0,83	-0,76	0,07	
Sebastes sp.	2	420	385	35	-0,78	-0,72	0,07	0,03
Liparidae								
Caraproctus fabricii	2	501	434	67	-0,93	-0,81	0,12	0,04
Liparis gibbus	1	605	583	22	-1,12	-1,08	0,04	
Pleuronectiformes								
Hippoglossoides platessoides	1	513	430	83	-0,95	-0,80	0,15	
Zoarcoidae								
Lycodes seminudus	2	420	377	44	-0,78	-0,70	0,08	0,02
Lycodes vahli	1	427	409	18	-0,79	-0,76	0,03	
Pholis fasciatus	2	689	413	277	-1,28	-0,77	0,51	0,27
Anarhichas lupus	1	418	365	53	-0,78	-0,68	0,10	
Leptoclinus maculatus	1	984	457	527	-1,83	-0,85	0,98	
Anisarchus medius	1	1.126	440	686	-2,09	-0,82	1,27	

Table 1. The serum hysteresis freezing point (HFP) and melting point (MP) given as mOsmol,

The blood serum hysteresis freezing points (HFP), melting points (MP) and magnitude of thermal hysteresis (MP-HFP) are shown in table 1. Significant thermal hysteresis were found in *Anisarcus medius* 1.27 °C, *Pholis fasciatus* 0.51±0.27 °C, *Gymnachantus tricuspis* 0.50±0.11 °C, *M. scorpius* 0.42 °C and *Gadus ogac* 0.40±0.09 °C. All these species were caught in relatively shallow water and may experience contact with ice crystals during winter. Thermal hysteresis of equal size has previously been reported for *M. scorpius* caught off Spitsbergen in August (Denstad et al., 1987) and it is actually known to posses antifreeze peptide (AFP) type I (Logsdon and Doolittle, 1991). Anti-freeze glycopeptides (AFGP) have been described from *G. ogac* (Van Voorhies et al., 1987). According to our knowledge the three other species have not previously been examined for thermal hysteresis. Three of the shallow water species (*Ammodytes dubius, Gadus morhua* and *Anarchias lupus*), showed no thermal hysteresis.

H. platessoides were found in both shallow and deeper water, but the single examined specimen showed only a small trace of antifreeze activity. Eight of the nine deep water (300-500 m) species (*M. villosus, Artediellus atlanticus, Leptagonus decagonus, Sebastes sp., Liparis gibbus, Careproctus fabrici, Lycodes seminudus* and *Lycodes vahli*) showed no thermal hysteresis in their blood plasma in August, which for *M. villosus, H. platessoides, Sebastes sp.* and *L. decagonus* is in accordance with the results of Denstad et al., (1987). These species are probably unlikely to experience ice crystals in their deep habitat, even if the water get supercooled. It is, however, possible that some of them will generate antifreezes during autumn, as seen in the winter flounder off the coast of North America (Petzel et al., 1980). The deep water in Disko Bay does usually not get below 0 °C even in the winter (Andersen, 1981), but some of the species (e.g. L. seminudus, *L. decagonus, A. atlanticus* and *C. fabrici*) are indeed known to occur in subzero temperatures.
Only two of the ten species caught in deep water, *Boreogadus saida* and *Leptoclinus maculatus*, showed pronounced thermal hysteresis of 0.87 °C and 0.98 °C, respectively. *B. saida* is known to occur close to the ice in some parts of the year and its AFGPs have been described by Chen et al. (1997). Specimens of *B. saida* caught in deep water (180 m) off Spitsbergen also showed thermal hysteresis (Denstad et al. 1987). The high thermal hysteresis of *Leptoclinus maculatus* is extremely interesting and has not been reported previously. This species is generally thought to be bentic, but is not rarely caught in pelagic trawl hauls in West Greenland waters (Pers. obs.).

The gut fluid from *M. scorpius* showed only very little hysteresis, whereas the gut fluid of *G. ogac* had hysteresis of the same level as in the blood serum of the species.

Gel filtation of AFGPs from *G. ogac*

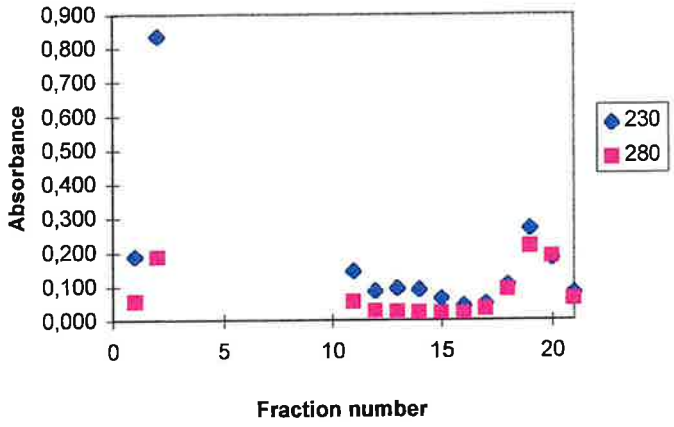


Fig. 3. Elution profile of Greenland cod, *Gadus ogac*, antifreeze on Sephadex G-75. 5 ml serum was applied and fraction read at absorbances of 230 and 280 nm.

The column was running a little faster than we expected. For this reason, unfortunately, the first heavy band came out in the pooled 100 ml wash-through, before collection of 10 ml fractions was started. The fluorescence was, however, very intense in the bottom of the 100 ml fraction, therefore a sample from this layer was taken and the absorbance measured. This sample was named fraction two. Additionally a sample was taken just above the fluorescent layer - this fraction was named fraction one. The collected fractions was afterwards numbered from 10 and up. From fig. 3 it appears that the serum had two absorbance peaks indicating the presence of at least two antifreeze agents with different molecular weight.

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