THE ROLE OF ANTIFREEZE COMPOUNDS IN INHIBITING ICE CRYSTAL GROWTH ACROSS THE SKIN OF MARINE FISHES

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BY

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ABSTRACT

It has been well established that antifreeze peptides are responsible for the ability of many marine teleosts to survive in icy sea water at temperatures below the colligative freezing points of their blood. However the *in vivo* site of antifreeze action has yet to be determined. The "ice-exclusion hypothesis" suggests that antifreeze proteins act within epithelial membranes (skin, gills, gut) and block the entry of external ice crystals. This hypothesis was examined by measuring ice propagation temperatures across isolated skin samples from the winter flounder (*Pleuronectes americanus*). The results obtained were consistent with the hypothesis, indicating that fish skin is an effective barrier to ice propagation and that the effectiveness of this barrier improves with the addition of antifreeze proteins. The results also demonstrated that the skin ice propagation temperatures were substantially lower than the lethal freezing temperatures of the fish. Therefore, some other epithelial tissue (possible gill) must be less effective than the skin in blocking ice crystal penetration into the fish.

Analysis of tissues from the cunner, *Tautogolabrus adspersus*, revealed the presence of a thermal hysteresis compound in aqueous extracts of the skin. The apparent absence of this substance from cunner plasma provided further support for the hypothesis that antifreeze proteins block the entry of external ice crystals into the fish.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AF	-	Antifreeze
AFP	-	Antifreeze protein
AFGP	-	Antifreeze glycoprotein
BSS	-	Blind Side Skin
CPMs	-	Counts per minute of isotope
CUN	-	Cunner, *Tautogolabrus adspersus*
DPMs	-	Disintegrations per minute (isotope)
ECS	-	Extracellular space
ECF	-	Extracellular fluid
FP	-	Freezing point
FPD	-	Freezing point depression (FPD = 0°C - FP)
FW	-	Freshwater
ICS	-	Intracellular space
LH	-	Longhorn sculpin, *Myoxocephalus octodecemspinosus*
MP	-	Melting point
OSS	-	Ocular Side Skin.
OP	-	Ocean pout, *Macrozoarces americanus*
SW	-	Seawater
SH	-	Shorthorn sculpin, *Myoxocephalus scorpius*
TH	-	Thermal hysteresis (TH = FP - MP)
Tht	-	Heterogeneous nucleation temperature
Thm	-	Homogeneous nucleation temperature
Tp	-	Temperature at which ice propagates across a barrier (Ice propagation temperature)
¦Tp¦	-	Absolute value of Tp
TR	-	Rainbow trout, *Oncorhynchus mykiss*
WF	-	Winter flounder, *Pleuronectes americanus* (formerly *Pseudopleuronectes americanus*)

CHAPTER ONE

GENERAL INTRODUCTION

A. Ice in Aquatic Habitats

B. The Freezing Process

   (i) Pure Water and Aqueous Solutions

   (ii) Gels and Porous Solids

   (iii) Biological Tissues

      a. The Freezing of Cells - Membrane Barriers to Intracellular Freezing
      b. The Freezing of Organisms - Tissue Barriers to Extracellular Ice Propagation

C. Freezing Tolerance vs Susceptibilty

D. The Role of Ice Nucleating Agents in the Freezing of Organisms

E. Freezing Avoidance Strategies in Freezing Susceptible Organisms

   (i) Freezing Avoidance by Undercooling
   (ii) Freezing Avoidance by Elevation of Solute Concentrations
   (iii) Freezing Avoidance by Migratory Movements

F. Freezing Avoidance in Marine Fishes

G. Antifreeze Compounds in Marine Fishes

   (i) Types of Biological Antifreeze Compounds
   (ii) Antifreeze Regulation
   (iii) Tissue Distribution of Antifreeze Proteins
   (iv) The <u>In Vitro</u> Mechanism of Thermal Hysteresis in Antifreeze Solutions: Adsorption-Inhibition

H. Tissue Barriers and Ice Propagation In Fish Tissues

I. Objectives of the Present Study

GENERAL INTRODUCTION

A. ICE IN AQUATIC HABITATS

Although subpolar marine waters usually do not reach their freezing temperatures, it is not unusual to find an abundance of surface ice that is in the process of melting. Estuarine and atmospheric sources of ice, as well as drifting pack-ice and icebergs continually supply many regions with ice crystals (Sverdrup et al., 1942).

These conditions present a danger to marine organisms such as marine teleost fishes whose tissue fluids freeze at relatively high temperatures (-0.50 °C to -0.65 °C) compared to the freezing point of seawater (DeVries, 1971a,b).

B. THE FREEZING PROCESS

(i) Pure Water and Aqueous Solutions

The equilibrium freezing point of pure water, Tf, is defined as 0°C under standard conditions (Franks, 1985).

The exotherm, the latent heat of fusion, produced by freezing water is substantial, and can be measured to provide a record of the freezing process.

It is not sufficient to lower the temperature to 0°C for water to freeze. A necessary requirement for the formation of

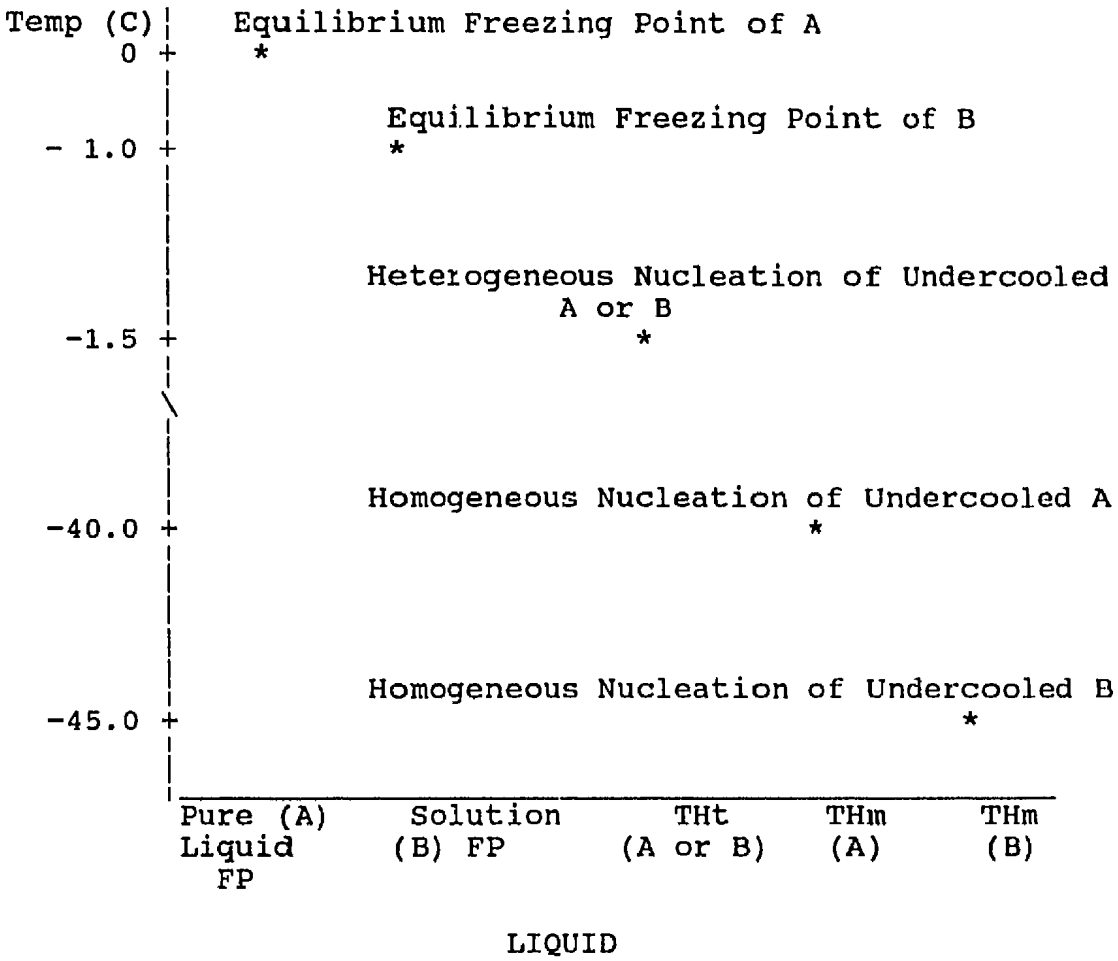
ice crystals is the presence of ordered clusters of water molecules, termed nuclei, upon which further aggregation can occur. Nucleation may be facilitated by seed ice crystals, mechanical factors, or the classical homogeneous and heterogeneous mechanisms (Knight,1967).

Once initiated, freezing generally proceeds by growth of the ice crystal in a thermodynamically preferred direction relative to the crystal lattice, parallel to the a-axis (Fletcher, 1970). The c-axis, and the axis perpendicular to the c-axis, the a-axis, are defined according to the orientation and spacing of oxygen atoms in the lattice, which are measured by x-ray diffraction (Fletcher, 1970).

To illustrate these differences in the temperature at which fluid freezing may be initiated, Figure 1.1 compares two fluids, pure water (A), and water containing a solute (B). It may be emphasized that the temperature at which freezing is initiated is often far below the actual ('equilibrium') freezing point of a sample, unless ice crystals are in contact with the fluid while it's temperature is lowered, or nucleation is facilitated by other factors (see below).

In most situations of biological interest, it is likely that water freezes by crystal seeding (the initiation of freezing by ice contact, or secondary nucleation) or by a heterogeneous mechanism, since the homogeneous nucleation temperature is about -40°C for pure

Figure 1.1: The Initiation of Freezing in Pure Water (A) and an Aqueous Solution (B)



Fp = Freezing point

THt = Heterogeneous nucleation temperature

THm = Homogeneous nucleation temperature

water (Figure 1.1), and substantially lower for solutions and body fluids (Angell, 1988; Franks, 1985; Mackenzie, 1977).

Biological fluids generally contain solutes whose effect is to elevate the boiling point (bp.) and depress the freezing point (fp.) of the solvent. Solution properties (such as bp. or fp.) that depend on the concentration of solute particles are termed colligative properties.

(ii) Gels and Porous Solids

A considerably more complex mechanism of freezing point depression is observed in polymer solutions and hydrated gels, where liquids are entrapped in small spaces. Occasionally, a non-colligative freezing point depression, due to a so-called thermal hysteresis, is observed (Solms and Rijke, 1971).

Thermal hysteresis may be defined as the magnitude of the difference between melting and freezing points (TH = FP-MP), and indicates a non-colligative mechanism of freezing point depression. Thermal hysteresis has also been demonstrated in gels of biological origin (Bloch et al., 1963), and in aqueous solutions of the so-called antifreeze (thermal hysteresis) proteins (see below).

The growth of an ice 'front' through a channel of small diameter containing an aqueous solution is limited not only by the freezing point of the liquid phase as predicted from

colligative effects due to dissolved solutes, but also depends on the channel's diameter or radius. This effect has been described by the so-called Kelvin equation, which describes the vapor pressure of a fluid as a function of the radius of curvature of the phase interface. This phenomenon may be readily understood if it is appreciated that the difference in chemical potential between the solid and liquid phases for a curved surface differs from that for a planar surface. Thus, since the curvature of the solid/liquid phase interface increases as the pore diameter decreases, the freezing point of the liquid phase is reduced at smaller pore diameters, and ice propagates through the pore at a temperature somewhat below the freezing point of the solution in 'bulk' form (as for example in a beaker of fluid with an approximately planar solid/liquid interface).

According to Mazur (1966), the Kelvin equation may be used to describe the freezing point depression of an aqueous solution entrapped in a pore of small radius as,

$$FPD = \Delta T = \frac{2 v \sigma T_f}{r L_f}$$

where σ = **Interfacial tension between ice and water**

T_f = **Equilibrium freezing point of solution**

L_f = **Molar heat of fusion of water**

v = **Molar volume of water**

r = **Radius of curvature of ice/water interface**

By estimating the approximate values of these parameters, Mazur (1966) concluded that the FPD is approximately 300/r, where r is the pore radius in Ångstroms. This conclusion indicates that for a channel of 200 Å diameter (r=100 Å), an FPD of about 3°C (300/100) may be expected.

Accurate quantitative predictions of pore-entrapped fluid freezing points, or of pore radius from apparent fluid freezing points have, however, had limited success, probably in part due to the approximate values estimated for the Kelvin equation parameters, (Mazur, 1966).

The reader is referred to the discussions by Everett (1961), Franks (1982, 1985), Jackson and Chalmers (1958), Knight (1967) and Puri and co-workers (1954) for a detailed discussion of ice growth in capillaries of small diameter. The application of the so-called Kelvin equation describing freezing point depression imposed by low porosity to biological systems is discussed by Bloch and co-workers (1963) for tissues, and by Mazur (1966) for cell membranes.

(iii) Biological Tissues

(a) The Freezing of Cells - Membrane Barriers to Intracellular Freezing

Mazur (1966) concluded that ice crystals are prevented from entering cells due to the absence of pores with

appropriate dimensions for ice growth. This situation, however, can be altered by extreme conditions (eg. very low temperatures or rapid cooling rates) that change pore dimensions (Fennema et al., 1973).

Mechanisms of freezing point depression in biological fluids may therefore be categorized as either effects due to colligative mechanisms, thermal hysteresis proteins, or structural freezing point depression due to low porosity, although both structural and hysteresis mechanisms appear to be based on similar principles (Table 1.1).

(b) The Freezing of Organisms - Tissue Barriers to Extracellular Ice Propagation

Although several investigators have reported a structural FPD in biological tissues and porous solids (see below), exact measurements of porosity are rarely available. Ultrastructural measurements of biological tissues are often based upon analysis of photographs made from processed tissues, whose shrinkage during various stages of processing prevents an accurate measurement in the required range (within a few Ångstroms).

Many aquatic poikilotherms possess a surface layer of mucous secretions that may contribute to freezing protection. Such a role has been attributed to the mucus of some molluscs (Hargens and Sabica, 1973).

Table 1.1.

***Major Mechanisms of Freezing Point Depression**

Mechanism	**Examples**
A) Colligative Solutes	Salt ions, most other solutes. (FPD = 1.86 times molar particle concentration)
B) Non-colligative Solutes	Thermal hysteresis or antifreeze proteins. (TH = FP - MP) Protein adsorbs to ice crystal.
C) Structural	Micro-porous membranes or soils, where spatial constraints limit ice crystal growth by a 'Kelvin' effect.

FPD = Freezing point depression.
TH = Thermal Hysteresis.
FP = Freezing point.

* Mechanisms A and B are probably strictly additive.
i.e. Σ (Solute FPD) = (Colligative FPD) + (Antifreeze FPD).

C. FREEZING TOLERANCE VERSUS SUSCEPTIBILITY

It is possible to categorise organisms in terms of their response to freezing conditions. On this basis, it has been suggested that organisms are either freezing - susceptible or freezing - tolerant (see reviews by Storey and Storey, 1988; Zachariessen, 1985).

It is generally considered likely that most, if not all, fishes are freezing susceptible, and must avoid ice contact or lower the freezing point of their tissue fluids if they cool below their plasma freezing point. Strategies of freeze tolerance have been reviewed by Storey and Storey (1988), Baust (1973) and Asahina (1966).

D. THE ROLE OF ICE NUCLEATING AGENTS IN THE FREEZING OF ORGANISMS

When cells, intact tissues or whole organisms are cooled below the freezing point of their extracellular fluids, there are generally two possible results :

1. Undercooling of the tissue fluids in the absence of ice crystals, with or without subsequent nucleation and freezing.
2. Initiation of freezing at the equilibrium freezing temperature by contact with exogenous ice crystals. If

seed crystals of ice are absent from the surrounding medium, the presence of ice nucleating agents in the extracellular fluid may initiate freezing somewhat below the freezing point of the fluid (Zachariassen, 1985).

E. FREEZING AVOIDANCE STRATEGIES IN FREEZING-SUSCEPTIBLE ORGANISMS

It may be noted that freezing susceptible organisms that come into contact with ice in their habitat have essentially three strategies available to them, namely the three mechanisms of freezing point depression discussed above.

(i) Freezing Avoidance by Undercooling

Although undercooling would appear to be a somewhat risky venture, it is a surprisingly common strategy among marine fishes (Gordon et al., 1962; Umminger, 1969; Scholander and Maggert, 1971; Green, 1974; Clarke, 1983a,b;1987), terrestrial arthropods (Somme, 1982), intertidal invertebrates (Aarset, 1982), and terrestrial plants (George et al., 1982).

(ii) Freezing Avoidance by Elevation of Solute Concentrations

The most widespread strategy for freezing avoidance among organisms is colligative freezing point depression of the tissue fluids by soluble metabolites.

The solutes employed for this purpose are generally salt ions or polyhydroxy derivates of starch or glycogen, such as sorbitol, trehalose and glycerol although other soluble metabolites may be employed (Layne and Lee, 1988; Levitt, 1966; Yingst, 1978; Storey and Storey, 1985; Somme, 1982).

(iii) Freezing Avoidance by Migratory Movements

Numerous examples of seasonal migration by fishes exist in the literature, including long-range latitudinal migrations (Leim and Scott, 1966) and short-range migrations between local habitats (Leim and Scott, 1966; Smith and Paulson, 1977; Van Guelpen, 1974) in response to changing day length, temperature or weather conditions such as wind-induced turbulence (Van Guelpen, 1974). Micro-habitat selection, often accompanied by states of metabolic torpor may also be included in this category, although organisms may remain in close proximity to regions subjected to ice contact (Green and Farwell, 1971; Olla et al., 1979).

F. FREEZING AVOIDANCE IN MARINE FISHES

Several studies have found fishes to be intolerant of freezing (Brett and Alderdice, 1958; DeVries, 1982; 1983, Fletcher et al., 1986; 1988, Gordon et al., 1962; Leivestad, 1965; Scholander and Maggert, 1971; Scholander et al., 1957).

Migratory strategies of freezing avoidance by fishes in the Newfoundland region are summarized in Figure 1.2 (data primarily from Leim and Scott (1966) and Templeman (1966).

Around the shores of Newfoundland, it has been noted that the cunner, Tautogolabrus adspersus, the only member of the primarily tropical Labridae that is found commonly in icy waters, enters rock caves when water temperatures drop to about 5°C. They remain within this microhabitat in the inshore region without feeding until temperatures rise again in the spring (approximately from November to May). This metabolic state has been referred to as a form of seasonal torpor (Green and Farwell, 1971). That these fish survive in an undercooled state has been verified by Green (1974).

Many northern marine teleosts also exhibit a slight elevation of plasma sodium and chloride during winter (Umminger, 1969; 1970; DeVries, 1971b; Duman, 1974; DeVries, 1975; Fletcher, 1977; 1981; 1979; Fletcher and Smith, 1980; Petzel et al., 1980; Fletcher et al., 1982) that makes a minor contribution to the tissue fluid freezing point depression.

Figure 1.2 Freezing Avoidance Strategies in Marine Fishes of the Newfoundland Region

Southerly Migration
1. Atlantic Mackerel (*Scomber scombrus*)
2. Bluefin Tuna (*Thunnus thynnus*)

Offshore Migration
1. Winter Flounder (*Pseudopleuronectes americanus*)
2. Atlantic Cod (*Gadus morhua*)
3. Sculpins (various cottidae)

Microhabitat Selection
1. Cunner (*Tautogolabrus adspersus*)

Antifreezes (various species)
1. Pleuronectidae (AFP)
2. Cottidae (AFP)
3. Zoarchidae (AFP)
4. Gadidae (AFGP)
5. Stichaeidae
6. Clupeidae
7. Osmeridae

Freshwater Migration
1. Arctic char (*Salvelinus alpinus*)

Although Scholander and others (Gordon et al., 1962) had suspected that the anomalously low freezing points of tissue fluids sampled from many northern marine fishes was due to a high molecular weight solute, it was not until DeVries and Wohlschlag (1969) isolated an antifreeze glycoprotein from the blood of Antarctic fishes in the Ross Sea that it became obvious that several marine fishes have these unique compounds in their tissue fluids.

G. ANTIFREEZE COMPOUNDS IN MARINE FISHES

(i) Types of Biological Antifreeze Compounds

Currently, it is believed that fish antifreezes are water-soluble proteinaceous compounds that are restricted to a few groups of terrestrial arthropods and marine fishes.

On the basis of structural characteristics, fish antifreezes may be categorized into 4 groups (Table 1.2), including 3 groups of antifreeze proteins (AFP), found in a variety of teleost families, and a fourth group of antifreeze glycoproteins (AFGP), restricted to the gadids and nototheneid fishes.

Antifreeze glycoproteins (AFGPs) occur as a group of 8 components (Molecular weight = 2500-3300), and typically consist of a repeating (Ala-Ala-Thr)n tripeptide sequence with a threonyl-linked disaccharide unit. In contrast to

Table 1.2

***Structural Basis of Antifreeze Classification**

(1 kDa = 10^3 Daltons = 1,000 molecular weight)

Type	Taxonomic Distribution	Primary Structure	Secondary Structure	Size (kDa)
AFP I	Right-eyed flounders and most sculpins.	Repeating sequences of 11 amino acids, containing 60 mol % alanine.	Amphiphilic α-helix.	3.3-4.5
AFP II	Sea Raven (Cottidae)	Cystine-rich.	ß-structure	14
AFP III	Eel Pouts (Zoarcidae)	Neither alanine nor cystine-rich.	Compact, with little α-helix or ß-structure.	6.5
AFGP	Nototheniidae and Gadidae	[Ala-Ala-Thr-disaccharide] repeating unit.	Expanded	3-26

* Modified from Davies et al., 1988 (also see Hew et al., 1986).

AFGPs, AFPs are considerably more variable in structure and may be classified into 3 groups on the basis of relative amino acid content and secondary structure (Table 1.2).

(ii) Antifreeze Regulation

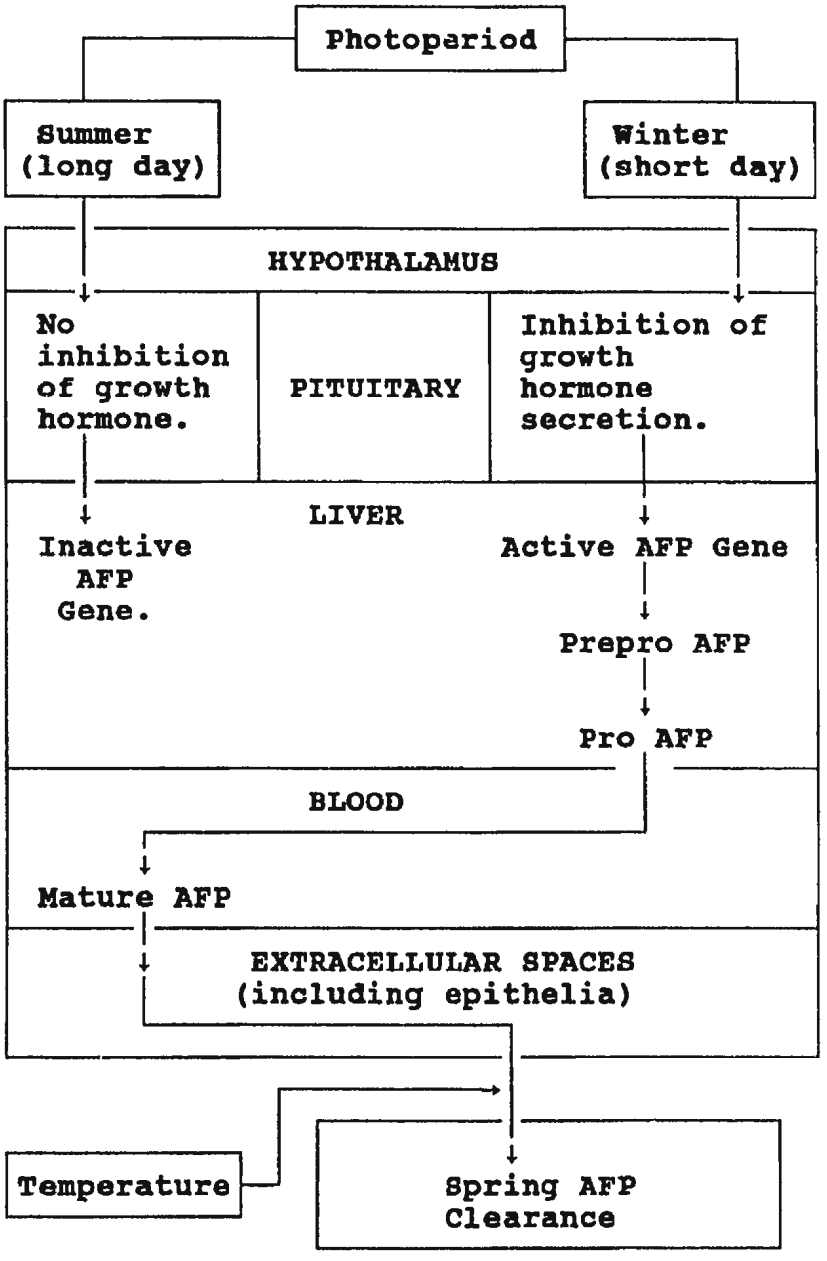
In the winter flounder, Pseudopleuronectes americanus, antifreeze synthesis is initiated in the fall when the release of growth hormone, which inhibits AFP gene transcription (Figure 1.3), is suppressed by decreasing day-lengths (Hew and Fletcher, 1985). Hypophysectomy may be used to experimentally activate antifreeze production, while treatment of fish with long day length during the winter allows continued release of growth hormone and the plasma remains free of antifreeze (Hew and Fletcher, 1985; Fletcher et al., 1989b).

In other fishes that make antifreeze compounds, different regulatory mechanisms may operate. In the Atlantic cod, Gadus morhua, for example, low temperatures in fall appear to initiate antifreeze production (Fletcher et al., 1987).

(iii) Tissue Distribution of Antifreeze Proteins

In the fishes, antifreezes are known to be synthesized by the liver, and are secreted extracellularly.

Figure 1.3 Regulation of Winter Flounder Antifreeze Production and Clearance



Few studies have examined the distribution of antifreeze in solid tissues, since, as noted by DeVries (1971b), the isolation of antifreezes from tissues is complicated by problems associated with their separation from other components in extracts. Although DeVries has suggested that antifreezes in some non-hepatic tissues such as muscle were intracellular, there is no clear indication that extra-hepatic antifreeze is found within cells (DeVries, 1974).

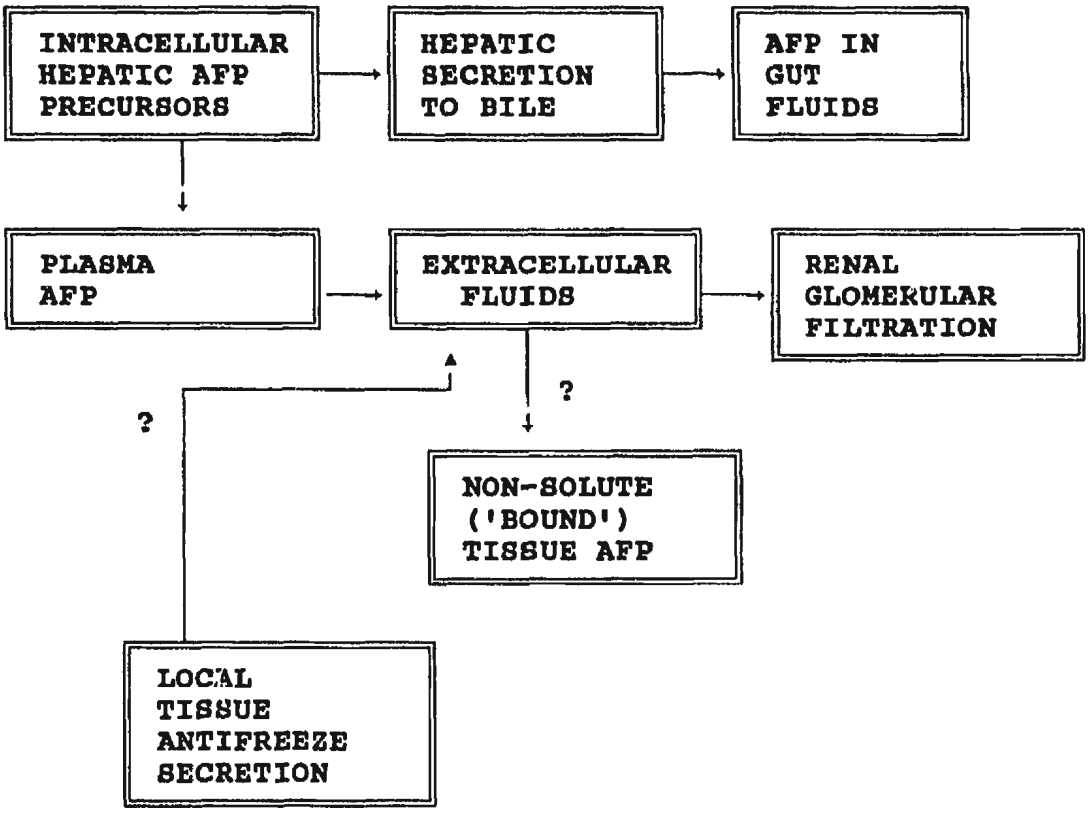
Apparent routes of antifreeze secretion and clearance are summarized in Figure 1.4.

(iv) The In Vitro Mechanism of Thermal Hysteresis in Antifreeze Solutions : Adsorption-Inhibition

The so-called "poisoning" of crystal growth (Sears, 1958) by adsorption of contaminants to crystals was a well-established concept when antifreezes were discovered. Thus most models of antifreeze function have tended to emphasize the role of antifreezes as compounds that act by aligning with atoms in the ice lattice (DeVries, 1984; Raymond and DeVries, 1977; Raymond, 1976) - the so-called 'adsorption-inhibition' model.

The effects of antifreeze on solution freezing behaviour include the following features -:

Figure 1.4 Apparent Routes of Antifreeze Distribution.



1. Hysteresis between melting and freezing points, as a result of a non-colligative freezing point depression.
2. Hysteresis increases in a non-linear, asymptotic fashion with increasing antifreeze concentration, implying saturation of active sites.
3. Colligative and non-colligative depression of the freezing point are strictly additive for solutions containing colligative solutes and antifreeze.
4. Ice recrystallization is strongly inhibited by antifreeze compounds.
5. Incorporation of antifreeze into the solid ice phase.
6. Thermal hysteresis is proportional to antifreeze molecular weight.
7. Ice crystals in antifreeze solutions exhibit a modified crystal habit, with propagation occurring preferentially parallel to the c-axis, rather than parallel to the a-axis, often resulting in the formation of bipyramidal-shaped ice crystals.

Observations such as the above, prompted DeVries and others to suggest that antifreeze compounds exert a freezing point depression by coating the "prism face" surface of ice crystals parallel to the c-axis, thereby inhibiting growth of the basal plane (along the a-axes) of the ice crystals (Raymond and DeVries, 1977).

According to the adsorption-inhibition model proposed by Raymond and DeVries (1977), antifreeze molecules possess hydrophilic amino acid residues that are appropriately spaced to align with atoms in the ice crystal lattice for non-covalent bonding to occur. The exposed ice fronts between adsorbed antifreeze molecules would therefore be restricted in size, with a highly convex surface, and the reduced radius of curvature of the ice/solution interface would result in an increased freezing point depression, the so-called Kelvin effect, similar to that reported for fluids in porous solids (section B.ii, above).

H. TISSUE BARRIERS AND ICE PROPAGATION IN FISH TISSUES

In multicellular animals, tissue barriers often consist of a layer of epithelial cells (which may be secretory, as with mucosal epithelia) resting on a thin gel-like layer of connective tissue, the basement membrane. The basement membrane itself may have a thicker layer of connective tissue attached to it's non-epithelial surface. It would therefore seem reasonable to assume that ice propagation across these complex barriers is partly determined by the porosity of their constituent layers, specifically the intercellular gaps in cell layers and the interfibril spacing in connective tissue layers.

Fletcher and coworkers (Fletcher et al., 1988) have

determined that the lethal freezing temperature of salmonid fishes is slightly lower than the freezing point of their plasma, suggesting that, although a factor other than colligative freezing point depression of tissue fluids may be involved, for the most part fish freezing temperatures are close to plasma freezing points. This implies that fishes freeze when ice propagates either directly into plasma, or into tissue fluids that have a freezing point close to that of plasma. Similar experiments conducted with Antarctic (marine nototheniid) fishes (DeVries and Wilson, 1988) have revealed that freezing occurs at about -2.2°C, but that ice-free fish may be cooled to at least -8°C before heterogeneous nucleation occurs. In these fish ingested ice does not, apparently, cross the intestinal wall, and it has been assumed that ice will only penetrate the epithelial surfaces when temperatures reach the freezing point of the tissue barrier (in the presence of ice).

Although some workers have shown that touching the skin of undercooled fish with ice will result in freezing of the fish (Scholander et al., 1957), others have speculated that ice may be more likely to seed tissues via the gills (Gordon et al., 1962; Smith, 1970). According to Schrag (1984), nototheneids challenged with ice when submerged will respond by convulsing, and this has been interpreted by this author as an attempt to reduce ice contact with the gills by frequent "back- flushing" of the gill chamber.

Other workers have questioned whether ice propagates across the gills or integument of fishes when they are cooled in the presence of ice (DeVries, 1986; Eastman and DeVries, 1986b), although no clear consensus has been reached. As DeVries (1986) has noted, however, gill capillaries are not shielded by an extensive layer of connective tissue and scales, as is fish skin, and thus may represent the site of minimum freezing resistance.

If, however, whole-fish freezing points are equivalent to plasma freezing points, it is reasonable to assume that structural barriers do not generally play a role in freezing resistance in fishes.

I. OBJECTIVES OF THE PRESENT STUDY

The present investigation was designed to examine possible roles played by skin in preventing ice from entering fish. The results are presented in two chapters. In the first of the chapters (Chapter 2), <u>in vitro</u> studies of isolated skin samples from winter flounder indicate that the skin can be an effective barrier to ice propagation, and that this barrier improves with the addition of antifreeze proteins. In the second chapter (Chapter 3), the potential value of antifreeze proteins in preventing ice propagation across fish skin is further underlined by the discovery that they are present in the skin, but not in blood plasma of cunner.

CHAPTER TWO

FISH SKIN IS AN EFFECTIVE BARRIER TO ICE CRYSTAL PROPAGATION

A. Introduction

B. Materials and Methods

C. Results

D. Discussion

INTRODUCTION

FISH SKIN IS AN EFFECTIVE BARRIER TO ICE CRYSTAL PROPAGATION

Despite the considerable body of literature on antifreeze proteins and their role in improving the freezing resistance of fish, it remains unclear as to how these proteins protect fish from freezing (DeVries, 1984; Feeney et al. 1986; Fletcher et al. 1986, 1991; Davies et al. 1988; Davies and Hew, 1990). Two hypotheses have been proposed to explain the in vivo mechanism of antifreeze action (DeVries 1986) and they can be termed (a) the "ice-modification hypothesis" and (b) the "ice-exclusion hypothesis".

The "ice-modification hypothesis" is based on the mechanism proposed for antifreeze action in vitro. Detailed observations of ice crystal growth under the microscope reveal that antifreeze proteins not only lower the freezing point of an aqueous solution, they modify the growth habit of the ice. The antifreezes are believed to do this by preferentially binding to the prism faces of ice crystals and thereby inhibit growth along the favoured direction (a - axis) by blocking access of water to the ice lattice (Scholander and Maggert, 1971; DeVries and Lin, 1977; Knight et al. 1984; Yang et al. 1988; Davies and Hew, 1990). The "ice-modification hypothesis" suggests that small, possibly

embryonic, ice crystals can be present within the body fluids of fish, but are prevented from growing by the antifreezes that are bound to them (Franks et al. 1987).

The "ice-exclusion hypothesis" argues that by inhibiting the growth of ice crystals, the antifreeze proteins prevent them from entering the fish by blocking their propagation across epithelial membranes (skin, gills, gut).

DeVries (1986) argues in favour of the "ice-exclusion hypothesis" and cites two lines of evidence in support of his argument. One piece of evidence stems from the fact that antifreeze glycopeptides are absent from the urine and ocular fluids of Antarctic nototheniids residing in the ice laden seawater. Thus antifreezes are unavailable to prevent ice crystal growth in these undercooled fluids (DeVries, 1982, Eastman et al. 1987). The second line of evidence comes from unpublished experiments cited by DeVries (1986) in which fish survived undercooling to -6°C. As DeVries (1986) pointed out, microscopic ice crystals could not have been present in the fish during the course of these experiments otherwise they would have propagated and killed the fish at -2.2°C; the lowest effective temperature of the plasma antifreeze proteins.

Although the arguments presented by DeVries (1986) support the "ice-exclusion hypothesis", there is no direct evidence demonstrating that antifreeze proteins can prevent ice propagation across epithelial tissues. The present study

examines the role of antifreeze proteins in blocking the movement of ice across fish skin.

MATERIALS AND METHODS

General procedures

Winter flounder (Pseudopleuronectes americanus Walbaum) and ocean pout (Macrozoarces americanus) were caught in Conception Bay, Newfoundland by SCUBA divers. Most of the fish were maintained in 250 l aquaria at seasonally ambient seawater temperatures and photoperiod. This ensured a normal annual plasma antifreeze cycle (Fletcher, 1977).

Blood samples were collected from a caudal blood vessel using plastic syringes equipped with 21 gauge needles and transferred to Vacutainers containing sodium heparin (Becton Dickinson). Plasma was separated from red cells by low speed centrifugation (3000 x g) and stored at -20°C until analysis.

Mucus samples were obtained from intact fish by enclosing them in plastic bags at 2°C for a few minutes (Shephard, 1981). The mucus was removed from the bags and centrifuged to remove traces of cellular debris.

The fish were killed by a blow on the head. Skin samples from the main trunk of the fish were dissected free of muscle tissue and either frozen (liquid N_2) for later analysis or, if they were to be used immediately, placed in

$$\text{inulin space (\% tissue water)} = \frac{\text{skin inulin (dpm/kg water)}}{\text{saline inulin (dpm/l)}} \times 100$$

Tissue and plasma water was measured by drying samples to constant weight at 50°C. Skin samples were digested in nitric acid prior to chloride determinations. ^{14}C-inulin levels were determined in skin samples solubilized by Protosol. Radio-active determinations were carried out using a liquid scintillation counter (Minaxi ß Tri-Carb 4000 series). Protosol and ^{14}C-inulin were purchased from New England Nuclear.

Chloride analyses were carried out on plasma and digested skin samples using a Radiometer CMT 10 chloride titrator. Freezing point depression was determined using a freezing point osmometer requiring small samples (50 μl) (Fiske OR, Fiske associates). Antifreeze activity was measured using a nanolitre osmometer (Clifton Technical Physics, Hartford, NY). In this method the melting and freezing behaviour of a small ice crystal is observed under a microscope. Thermal hysteresis, a measure of antifreeze activity, is the difference between the freezing and melting temperatures (Kao et al., 1986).

The fish saline (pH 7.8, freezing point depression approximately 0.630°C) used in the following experiments consisted of (mM): NaCl, 175; KCl, 2.7; $MgCl_2.6H_2O$, 0.64; $CaCl_2.2H_2O$, 2.74; glucose, 2.22; Trizma, 3.0.

a Petri dish at 4°C with the dermal surface in contact with filter paper moistened with saline. Considerable care was taken not to damage the scales or the epithelial cell layer of the skin samples. Muscle tissue was removed from the anterior dorso-lateral region of the fish.

Extracellular fluid volumes of muscle and ocular (dark upper side) and blind (lower light side) skin were estimated from the chloride space (Manery, 1954).

$$Cl^- \text{ space} = \frac{Cl^-_t \cdot H_2O_p \cdot r_{Cl^-}}{Cl_p}$$

where t = tissue concentration in mM/kg tissue water; H_2O_p = plasma water, g/100g; Cl^-_p = plasma Cl^-, mM; r = Gibbs-Donnan equilibrium ratio = 0.977.

Extracellular space was also measured in blind side skin by incubating samples in solutions containing ^{14}C-inulin (Lutz, 1972). In this method approximately 3 g of skin was placed in 25 ml saline containing 2 x 10^6 DPM ^{14}C-inulin and incubated at 4°C for 80 h. Sub-samples of the skin and incubation medium were removed at various times following the initiation of incubation. Equilibrium between the incubation medium and the skin extracellular space occurred between 60 and 80 h. Inulin space was computed at equilibrium.

Antifreeze extraction from skin

All samples (2-4 g) were homogenized under liquid N_2 using a mortar and pestle. 30 ml distilled water was added to the resulting powder and incubated for 1 h at 2°C. Following incubation the suspension was filtered under vacuum through Whatman No. 1 filter paper to remove suspended debris. The filtered debris was washed with 10 ml of distilled water and the original filtrates along with the washings were pooled and lyophilized. The resulting material was reconstituted to its original concentration by adding a volume of saline equivalent to the estimated extracellular fluid volume of the skin (Cl^- space). This method appeared to remove all of the antifreeze proteins from the skin since no antifreeze activity was found associated with the skin tissue debris.

Skin freezing points

Skin freezing points were determined by placing an amount of tissue (approximately 50 mg) in the Fiske osmometer tube sufficient to cover the thermistor probe. These samples were briefly rinsed in isotonic saline, blotted on filter paper and lightly coated in mineral oil to prevent water loss by evaporation.

Stability of undercooling in fish tissues

In order to preclude the possibility of spontaneous (hetero-geneous) nucleation during ice propagation experiments, and to test the hypothesis that fish tissues are capable of significant undercooling in the absence of seed ice crystals, experiments were designed to test the ability of tissue homogenates to undercool without freezing.

Winter flounder tissues, consisting of whole blood (2 ml), muscle (5 g), gonad (2 g), liver (2.5 g), kidney (2 g), gill (2 g), dermis (2 g) and epidermis (0.2 g) as well as the tissue fluids, plasma (2 ml), bile (2 ml) and skin mucus (2 ml) were homogenized in saline (total volume 10ml) and placed in 50 ml plastic centrifuge tubes. The test tubes containing the homogenates were then cooled to, and held at -4°C for 30 minutes when they were given a sharp tap to initiate freezing. The temperature of each homogenate was monitored using a digital thermometer (Model 8502-25 Cole Palmer, Chicago, Ill.) coupled to a Varian A-25 strip chart recorder.

Ice propagation experiments

Ice propagation through dialysis membranes (Spectra/por, Spectrum Medical, L.A., USA), Millipore filters and fish epithelial tissues was investigated using a modified Ussing chamber (Fig. 2.1) (Dobson and Kidder, 1968). The membrane

of interest was placed between the two plexiglass chambers, each of which was enclosed by a jacket containing circulating coolant. The temperature of the coolant was controlled by a Neslab cooling unit (Neslab Instruments Inc.). One of the chambers was filled with saline and the other with seawater (32‰) both of which were filtered through a 0.45 μm cellulose acetate filter. Each chamber was continuously agitated using a modified stirring bar and the temperature monitored using a digital thermometer as previously described.

In experiments using dialysis membranes or Millipore filters the chambers were undercooled to -2°C and ice crystals introduced into the seawater filled side by touching the surface of the liquid with a wooden splint that had been pre-soaked in saline and frozen. The resulting suspension of ice crystals acted as a source of seed crystals for propagation across the membrane separating the two chambers. The initiation of freezing in the second chamber was recorded by the thermistor probe registering an exotherm.

In experiments using fish skin the saline was added to the chamber on the dermal side of the skin, while seawater was added to the mucosal side. During each experimental run the temperature control unit was programmed to reduce the temperature in the chambers linearly from 1.0°C to -3.0°C over a period of 30 minutes. Reported ice crystallization rates are rapid (approximately 10 mm/s) (Blond, 1988). Therefore the time lag between seeding the skin's epidermal

extracellular fluids and subsequent freezing of the undercooled saline compartment would be negligible. When the temperature of the chambers had reached the freezing point of the saline (dermal side) the frozen, water-soaked, wooden splint was placed in contact with the mucosal surface of the skin where it remained until ice propagated across the skin. The temperature at which this occurred is termed the ice propagation temperature.

Initial experiments indicated that isolated skin samples were very susceptible to mucosal surface damage resulting in highly variable and unrepeatable ice propagation temperatures. This damage was prevented by placing a layer of nylon netting (150 μm pore diameter) over the mucosal surface of the skin.

Preliminary observations with fish skin established that the highly water soluble antifreeze proteins rapidly diffused from the dermal surface of the skin into the saline filled chamber. In order to retard the rate of loss of antifreeze proteins, a layer of surgical gauze soaked with blood plasma (0.4 ml) from the fish was placed on the dermal surface of the skin. The gauze was then covered by a layer of dialysis membrane with a molecular weight cut-off point that was freely permeable to ice crystal propagation (50 kd) (see Results).

Two lines of evidence indicated that the dialysis membrane did not retard ice crystal propagation across the

skin preparation. (1) The skin sample showed no evidence of being frozen until ice propagated from the seawater into the saline. That is, ice did not form in the skin or gauze solution prior to propagation. (2) Skin ice propagation temperatures were not altered when a small incision was made in the dialysis membrane when the skin was undercooled.

At the completion of each experimental run the tissue and gauze were removed for analyses, and the chambers were cleaned and rinsed with ethanol, to ensure the removal of ice crystals, and then dried.

RESULTS

Stable undercooling of fish tissues and seawater

All tissue homogenates and tissue fluids from winter flounder could be undercooled to -4°C for at least thirty minutes without spontaneous freezing occurring. In all cases freezing was immediately initiated by a sharp tap on the side of the test tube containing the undercooled sample.

Ice propagation across dialysis membranes and filters

Dialysis membranes with a molecular weight cut-off of 25 kd or less, effectively blocked the propagation of ice from one chamber to the other even when exposed to ice (-2°C) for

periods of up to two hours (Table 2.1). Ice propagation rapidly occurred across membranes of 50 and 100 kd and across Millipore filters with a pore diameter of 0.45 μm. The addition of mineral oil to the Millipore filters blocked ice propagation.

Ice propagation across fish skin

Experiments were performed on winter flounder at two times of the year: spring, when blood plasma antifreeze levels would be expected to vary widely between fish, and summer, when antifreeze proteins are absent.

In most cases ice propagation temperatures for ocular and blind side skin samples from each flounder were essentially identical, differing by less than 0.2°C ($r = 0.93$, $N=11$, spring fish).

Experiments carried out during spring revealed that ice propagation temperatures were correlated with skin freezing points (Fig. 2.2) which were in turn, correlated with blood plasma freezing points (Fig. 2.3). Ice propagation temperatures were also correlated with plasma freezing points (Fig. 2.3). Thus it would appear that some of the variance observed in skin freezing and ice propagation temperatures can be accounted for by the variance in plasma freezing temperatures.

It is pertinent that antifreeze protein levels (thermal

hysteresis) in the plasma samples ranged from 0 to 0.67°C and were highly correlated with plasma freezing points ($r = 0.93$, $N=11$) suggesting that a component of the skin freezing point and ice propagation temperature was attributable to the antifreeze proteins (Fig. 2.3). There was a significant correlation between skin freezing points and plasma antifreeze thermal hysteresis; r values equalling 0.66 ($N=11$) and 0.76 (ln thermal hysteresis). However the correlation between ice propagation temperatures and thermal hysteresis was not significant ($r = 0.46$, $N=11$). This lack of correlation seems to be attributable to the high variation in propagation temperatures when antifreeze proteins were absent or present at very low levels in the blood. Propagation temperatures were consistently low when plasma thermal hysteresis values exceeded 0.15°C (Fig. 2.3). The mean propagation temperature (-2.74 ± 0.15 (SE) °C, $N = 4$) for the skin samples with the higher plasma thermal hysteresis values (> 0.15°C) was significantly lower than that of the samples with little or no plasma antifreeze (-1.81 ± 0.21 (SE) °C, $N=7$). Propagation temperatures for the three samples with no detectable plasma antifreeze averaged -1.72 ± 0.3°C.

Experiments carried out during the summer months revealed that antifreeze proteins can decrease ice propagation temperatures. In these experiments the addition of blood plasma containing antifreeze proteins or an antifreeze protein solution to the skin preparation resulted

in significant reductions in the propagation temperatures (Table 2.2).

Ice propagation temperatures were always lower than the respective skin freezing points with the mean difference between the two being 0.96 ± 0.11 (SE) °C, N=22. The differences between ice propagation temperatures and skin freezing points are illustrated in Figure 2.3 where they are plotted against the plasma freezing points. Both regressions intercept the Y axis at approximately the same point (0.4°C). However this intercept value only differs significantly from zero in the case of the skin freezing temperature. It is apparent that although ice propagation temperatures are lower than skin freezing points, the slopes of the regressions are such that the difference between the two increases as the plasma freezing points decline. The regression relating differences between propagation temperatures and skin freezing points to the plasma freezing points was significant at the $P<0.05$ level (Fig. 2.4).

Skin freezing temperatures were slightly lower than plasma freezing temperatures; the mean difference between the two being 0.156 ± 0.038 (SE) °C (N=11). The differences between skin and plasma freezing points were not correlated with plasma freezing points. This indicates that the difference between these two measurements is essentially the same over the range of plasma freezing points observed.

Ice propagation temperatures were always lower than the

respective blood plasma freezing temperatures (Fig. 2.3). The mean difference between average (ocular side and blind side) ice propagation temperatures and plasma freezing points was 1.11 ± 0.16 (SE) °C during the spring. There was no correlation between this difference and antifreeze protein levels in the blood. The low ice propagation temperatures relative to plasma freezing points were also evident from experiments carried out during the summer when antifreeze proteins had completely cleared from the blood (mean difference = 0.80 ± 0.066 (SE) °C (Table 2.2).

Experiments carried out on ocean pout epithelia indicated that they were equally as effective as winter flounder skin in blocking ice crystal propagation (Table 2.3). All three epithelial membranes, skin, urinary bladder, gall bladder, had similar ice propagation temperatures, ranging from -2.5 to -2.9°C. Ocean pout skin differs from that of the winter flounder in that the scales are small and completely buried leaving the mucosal surface smooth and slippery. Despite these anatomical differences there were no differences between the skins in terms of ice propogation. As was the case with winter flounder skin, ice propagation temperatures across ocean pout skin were considerably lower than blood plasma freezing points, the difference between the two being approximately 1.1°C (Table 2.3).

Freezing behaviour of skin mucus

The freezing points of the gel and sol phases of winter flounder skin mucus did not differ significantly (sol = -1.64 ± 0.057°C, gel = -1.65 ± 0.064°C, N=5) and both were slightly higher than the freezing point of seawater (-1.75°C). There was no seasonal cycle in the mucus freezing points.

There was no evidence of antifreeze activity in most of the samples examined. In the occasional sample that did exhibit antifreeze activity the values were very low (<0.05°C) and were associated with cellular debris indicative of epithelial damage.

Antifreeze proteins in skin

Preliminary analyses revealed that winter flounder skin contained substantial amounts of antifreeze proteins. This prompted measurement of the skin extracellular space in order to estimate skin antifreeze protein concentrations. Ocular side and blind side skin chloride spaces were essentially identical (approximately 69%) and substantially greater than muscle chloride space (approximately 8.8%). Inulin space, as determined by the incubation method gave a value that was approximately 10% greater than the chloride space. The chloride space was used to calculate skin antifreeze protein concentrations.

Antifreeze proteins were extracted from ocular and blind side skins of selected flounder sampled during winter (February, March, April) and during July. Skin antifreeze activity was highly correlated with plasma antifreeze activity (Fig. 2.5).

Antifreeze activity in the winter skin samples did not differ significantly from antifreeze activity in the blood plasma (plasma thermal hysteresis = 0.569 ± 0.12°C, N=5; ocular skin = 0.568 ± 0.18°C, N=3; blind side = 0.681 ± 0.17°C, N=5).

There was no evidence of antifreeze activity in July plasma samples, however traces of antifreeze activity could be detected in the skin extracts (thermal hysteresis = 0.03 ± 0.008°C). The presence of low levels of antifreeze proteins in these summer skins was confirmed by the fact that ice crystal growth, as observed under the microscope, was in the form of long spicules rather than hexagonal as would be the case with salt solutions (DeVries 1982).

DISCUSSION

The results of this study demonstrate conclusively that fish skin is an effective barrier to ice propagation and that the barrier is enhanced by the addition of antifreeze proteins. This conclusion is consistent with the "ice-exclusion hypothesis" in that antifreeze proteins can act

within epithelial tissues and assist in blocking ice movement from the external environment into the fish.

Additional evidence that the skin may be an important *in vivo* site of antifreeze action comes from studies on the European shorthorn sculpin (*Myoxocephalus scorpius*) and the cunner (*Tautogolabrus adspersus*). Schneppenheim and Theede (1982) found no evidence for antifreeze peptides in the blood plasma of shorthorn sculpin exposed to freezing conditions. However they were able to isolate and purify these proteins from aqueous extracts of skin. Similar observations have been made on cunner, during the winter, where antifreeze activity was found in skin but not in blood (Chapter 3).

The present study establishes that antifreeze proteins are also present in the skin of winter flounder during winter. However, in contrast to the above observations on sculpin and cunner, the concentration of antifreeze proteins in flounder skin extracellular space appears to mirror blood plasma concentrations. This is in accord with our current hypothesis that the liver is the source of antifreeze proteins in winter flounder (Davies *et al*. 1988, Fletcher *et al*. 1989b). However, the presence of antifreeze peptides in the skin of sculpin and cunner lacking measurable levels of plasma antifreeze suggests the possibility that these proteins may also be synthesized in the skin (Schneppenheim and Theede, 1982; also see chapter 3 of the present study).

One striking feature of the present study was the

observation that skin ice propagation temperatures were, on average, considerably lower than skin freezing points (spring X = approximately 0.96°C). This suggests that some component of skin, in addition to antifreeze proteins and colligatively active solutes, participates in blocking ice propagation. A possible candidate for such a function is the structure of the skin itself.

Spatial restriction of ice crystal growth in small diameter pores has been well established in a number of non-biological (Solms and Rijke 1971, Viaud, 1972) and biological systems (Bloch et al. 1963, Yingst 1978). In the present study the spacial restriction of ice growth across dialysis membranes with a molecular weight cut-off of 25 kd illustrates that this phenomenon can operate at pore diameters of 50-70Å (based on Spectra/por product information). Reported values for cell junction intercellular spacing in vertebrate epithelia (desmosomes, 100-200 Å; gap junctions 20-40 Å (Novikoff and Holtzmann, 1976) are within this range, thus ice propagation across fish skin may well be restricted by intercellular spacing.

It is evident from the literature that biological membranes can be very effective at blocking ice propagation. Turner et al. (1985) demonstrated that both the cornea and the corneal epithelium effectively block ice crystal propagation to the undercooled ocular fluids of Antarctic nototheniids. Similarly Davenport et al. (1979) found that

the chorion of capelin eggs (Mallotus villosus) allowed the eggs to be undercooled (-12°C) even when they were in close contact with ice. Harvey and Ashwood-Smith (1982) also observed that rainbow trout eggs could remain supercooled in the presence of ice for a period of time at -19°C.

Histological studies of fish skin reveal that it consists of a layer of epithelial cells resting on a thin gel-like layer of connective tissue; the basement membrane. The basement membrane itself overlies a relatively thick dermal layer of largely non-cellular connective tissue (Burton and Fletcher 1983). The large extracellular space observed for the winter flounder skin (approximately 70-80%) indicates clearly that the bulk of this tissue is non-cellular.

The spacing between vertebrate connective tissue collagen fibers varies considerably (80-950Å) (Fuji 1968) and does not appear to be small enough to exert a structural freezing point depression. One likely location for an ice barrier in fish skin is within the epithelial cell layer. This argument is supported by the results of a recent study by Eastman and Hikida (1991) who found that the epithelial cell membranes of an Antarctic notothenioid exhibited more extensive membrane specializations in terms of tight junctions, interdigitations and desmosomes than do other species of teleost fishes.

The hypothesis that the epithelial cell layer may

constitute a barrier to ice propagation is in accordance with the observations of the present study indicating that skin freezing points were more similar to those of the blood plasma than they were to the ice propagation temperatures. In other words the major exotherm observed during measurements of skin freezing points was attributable to the large extracellular space of the dermal connective tissue compartment, rather than to the small cellular compartment.

The lower freezing temperatures observed for the skin relative to the plasma (approximately 0.16°C) suggest that the solute concentration in the skin extracellular space may be higher than the plasma by approximately 80 mOsm. However since the external surface of the skin was not washed extensively it is possible that the samples were contaminated to a small degree by seawater. 80 mOsm represents less than 8% of the dissolved solutes in seawater. This argument is supported by the observation that the regression relating skin freezing temperatures to plasma freezing points intercepted the Y axis at a value greater than zero (approximately 0.4°C) (Fig. 2.3).

If the differences between ice propagation temperatures and skin freezing points are attributable to a structural component of the skin, it is evident from the results that the magnitude of this structural freezing point depression is directly affected by the dissolved solute concentration in the skin; antifreeze proteins and electrolytes. The slope of

the regression (Fig. 2.2) relating ice propagation temperatures to skin freezing points suggests that for every increase in skin solute concentration there is a doubling of the structural freezing point depression. We are unaware of an explanation that would account for this interesting phenomenon.

One argument counters the hypothesis that the low ice propagation temperatures observed were attributable to a structural component of the skin. The Y-intercept of the regression relating propagation temperatures to skin freezing temperatures did not differ from zero, (Fig. 2.2) suggesting that, in the absence of dissolved solutes, there would be no structural freezing point depression. A Y-intercept value greater than zero would have been more consistent with the existence of such a phenomenon.

If there is no simple structural barrier to ice propagation across the skin, what accounts for the observed differences between propagation temperatures and skin freezing points? Perhaps the dissolved solute concentration in a minor compartment of the skin exceeds, but stays in equilibrium with the bulk of the extracellular space. One possible candidate for such a compartment would be the interstitial space of the epithelial cell layer. Evidence for hypertonic cellular interspaces has been reported for fluid transporting epithelia (Gupta and Hall 1979). However, nothing is known about the interstitial space in fish skin.

There is no evidence that antifreeze proteins can concentrate in interstitial spaces. Clearly the presence or absence of structural or concentrated solute barriers to ice propagation across fish skin will have to await further experimentation.

In previous experiments it was observed that the lethal freezing temperatures of winter flounder and other fish species in the presence of ice were consistently lower (0.05 to $0.2^{0}C$) than their blood plasma freezing points. This suggested that the epithelium was acting as a barrier to ice crystal propagation (Fletcher et al. 1986; 1988). The discovery in the present study, that ice propagation across the skin could only occur when the water temperature was 0.8 to $1.11^{0}c$ below the plasma freezing points indicates that the skin could not have been the route of ice crystal propagation into the intact fish.

Two other potential sites of ice propagation into the fish are the gut and gill epithelia both of which have only a single layer of epithelia cells lying between the environment and the blood. Of the two the most likely one would be the gills because of the relatively large volumes of water pumped across them during ventilation.

Table 2.1

Ice propagation across dialysis membranes and millipore filters

Barrier (size)	Extent of undercooling (°C)	Duration of undercooling in presence of ice	Ice propagation (time)
Dialysis membranes (kd)			
1.0	-2.0	0.1 - 2 h	No
3.5	-2.0	0.2 - 0.3 h	No
12-14	-2.0	0.4 - 1.0 h	No
25	-2.0	0.5 - 2.0 h	No
50	-2.0	<1 min	Yes (<1 min)
100	-2.0	<1 min	Yes (<1 min)
Millipore filter (μm)			
0.45	-2.0	< 1 min	Yes (<1 min)
0.45 (+ mineral oil)	-2.0	0.2 - 1 h	No

Ice propagation time is the time between introduction of ice crystals to the seawater side, and the observation of a freezing exotherm on the saline side of the membrane. Size is molecular weight cutoff in kilo Daltons for dialysis membranes, or pore size in μm for millipore filters. One millipore filter was presoaked in mineral oil. Four to five separate experiments were carried out using each membrane except the 25 and 50kd sizes where 10 experiments were conducted.

Table 2.2

Effects of antifreeze proteins on ice propagation temperatures (°C)

	Solution added to skin		
Fish #	AFP-free Plasma	AFP plasma (difference)	AFP saline (difference)
1	-1.4	-	-2.02 (0.62)
2	-1.53	-	-1.81 (0.28)
3	-1.38	-	-1.80 (0.42)
4	-1.19	-1.38 (0.19)	-
5	-1.56	-1.99 (0.43)	-
Mean (± SE) propagation temp (°C)	-1.41 ±0.066	-1.69 (0.25 ±0.19 ±0.05)	-1.88 (0.44 ±0.053 ±0.1)

Experiments were carried out on winter flounder skin samples during July and August when antifreeze proteins were absent from the blood. AFP-free plasma was pooled from a number of fish during July (plasma freezing point = -0.61°C). AFP plasma was obtained from flounder during winter (freezing point = -1.5°C, thermal hysteresis = 0.9°C). AFP saline was a 5 mg/ml winter flounder antifreeze solution dissolved in saline (freezing point = -1.12, thermal hysteresis = 0.5°C). Winter flounder AFP was purified as described in Kao et al. (1986). Differences = difference between propagation temperatures using AFP-free plasma and propagation temperatures using the AFP test saline.

Table 2.3

Ice propagation temperatures across ocean pout tissue barriers

Barrier	Gauze solution	Solution freezing point (°C)	Ice propagation temperature (°C)
Skin	plasma	-1.52	-2.66 ± 0.26 (4)
Urinary bladder	urine	-1.47	-2.55
Gall bladder	bile	-1.1	-2.88

Four experiments were carried out on ocean pout skin and one on each of the bladders. In experiments using skin, seawater was added to the chamber on the mucosal side while saline was added to the dermal side. Ocean pout plasma was added to the gauze on the dermal side. In experiments using urinary and gall bladder epithelia, saline was added to both chambers and urine and bile collected from the respective bladders added to the gauze on the serosal side of the membranes.

Table 2.4

Extracellular fluid volumes in winter flounder skin and muscle

	Ocular Side skin	Blind Side skin	Muscle	Plasma
Water (g/100g)	70.6 ± 0.68	74.7 ± 0.47	81.2 ± 0.35	97.3 ± 0.28
Cl (mM/kg tissue H_2O)	132 ± 3.5	131 ± 4.1	16.9 ± 1.6	182 ± 1.3
Cl space (% tissue water)	69.1 ± 1.9	68.5 ± 2.1	8.82 ± 0.81	-
N	4	4	4	4
Inulin space (% tissue water)	-	78.2 ± 1.25	-	-
N	-	2	-	-

Plasma water was measured as g/100 ml.

Figure 2.1 Ice propagation apparatus. Drawing not to scale. Each chamber holds approximately 130 ml. C1= sea water (sw) chamber. C2= saline chamber (sal). CJ= cooling jacket. The diameter of the membrane (skin sample etc.) exposed to the two chambers is 2.6 cm. therefore the area of ice contact is 5.31cm²

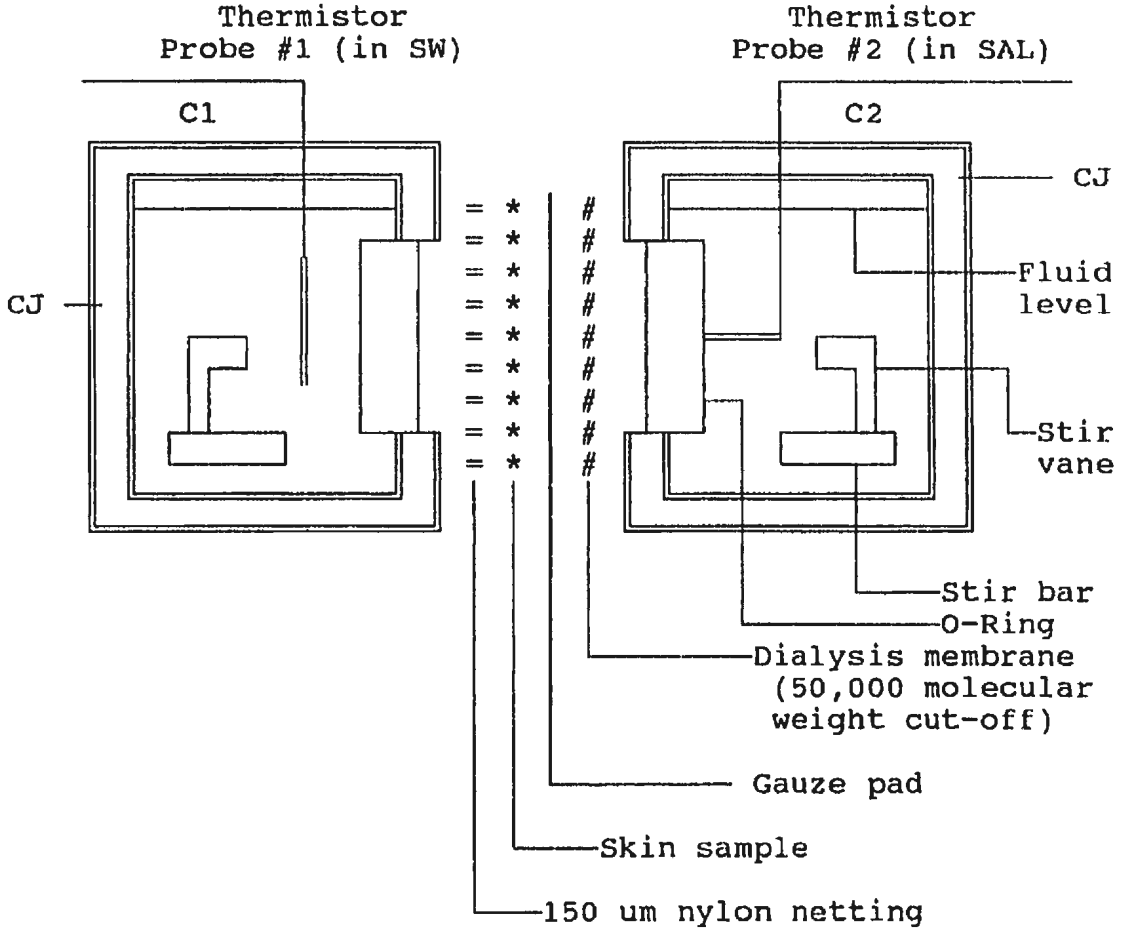


Figure 2.2 Relationship between the skin ice propagation temperature (T_p) and the skin freezing point (FP). Each point represents a single measurement using an ocular side or blind side skin sample. Regression line Y = 2.15 X + 0.417 ($r=0.739$ $n=22$, $P<0.01$). Intercept does not differ significantly from 0. ($P<0.05$). Y= skin T_p, X= Skin FP.

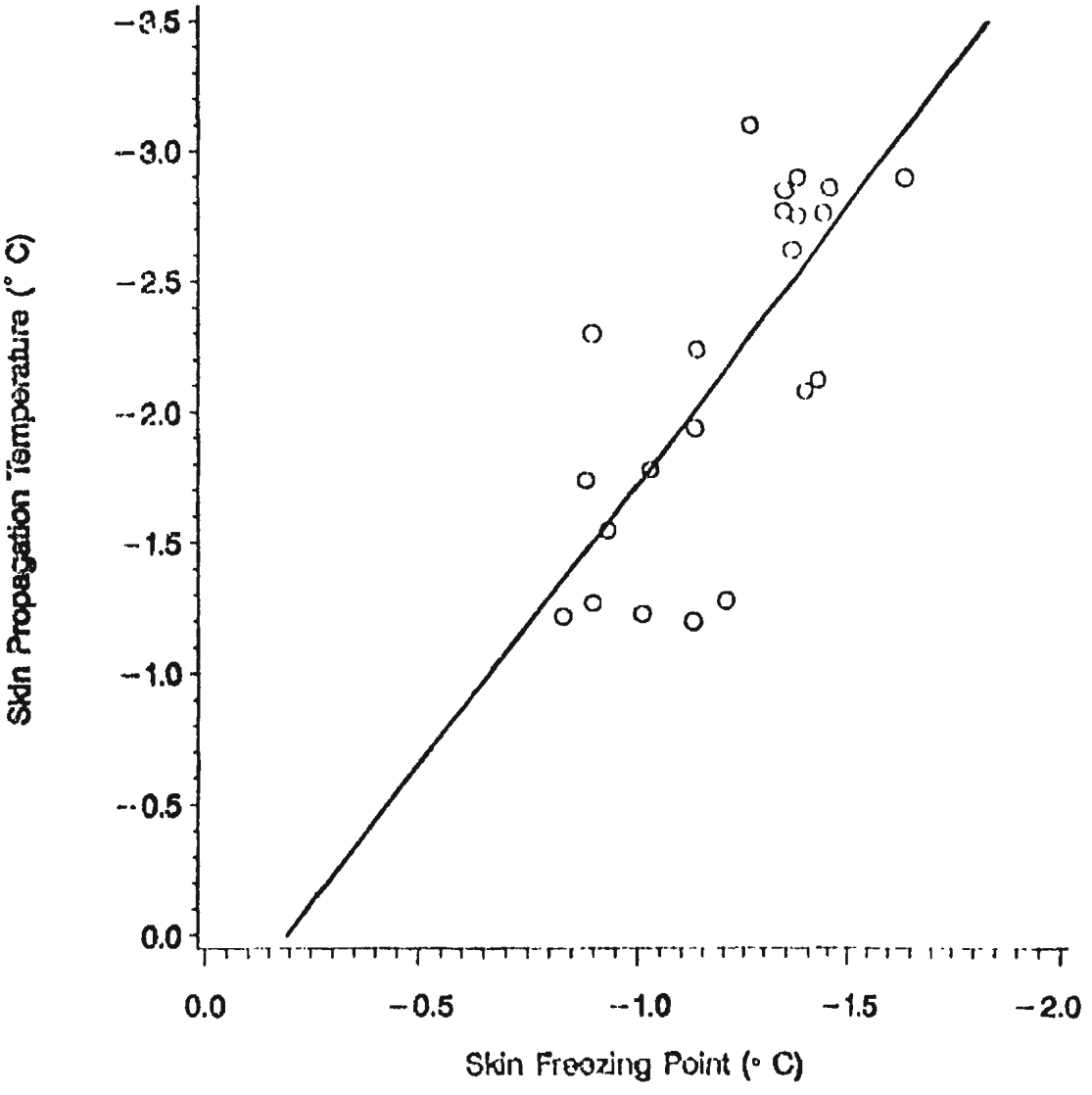
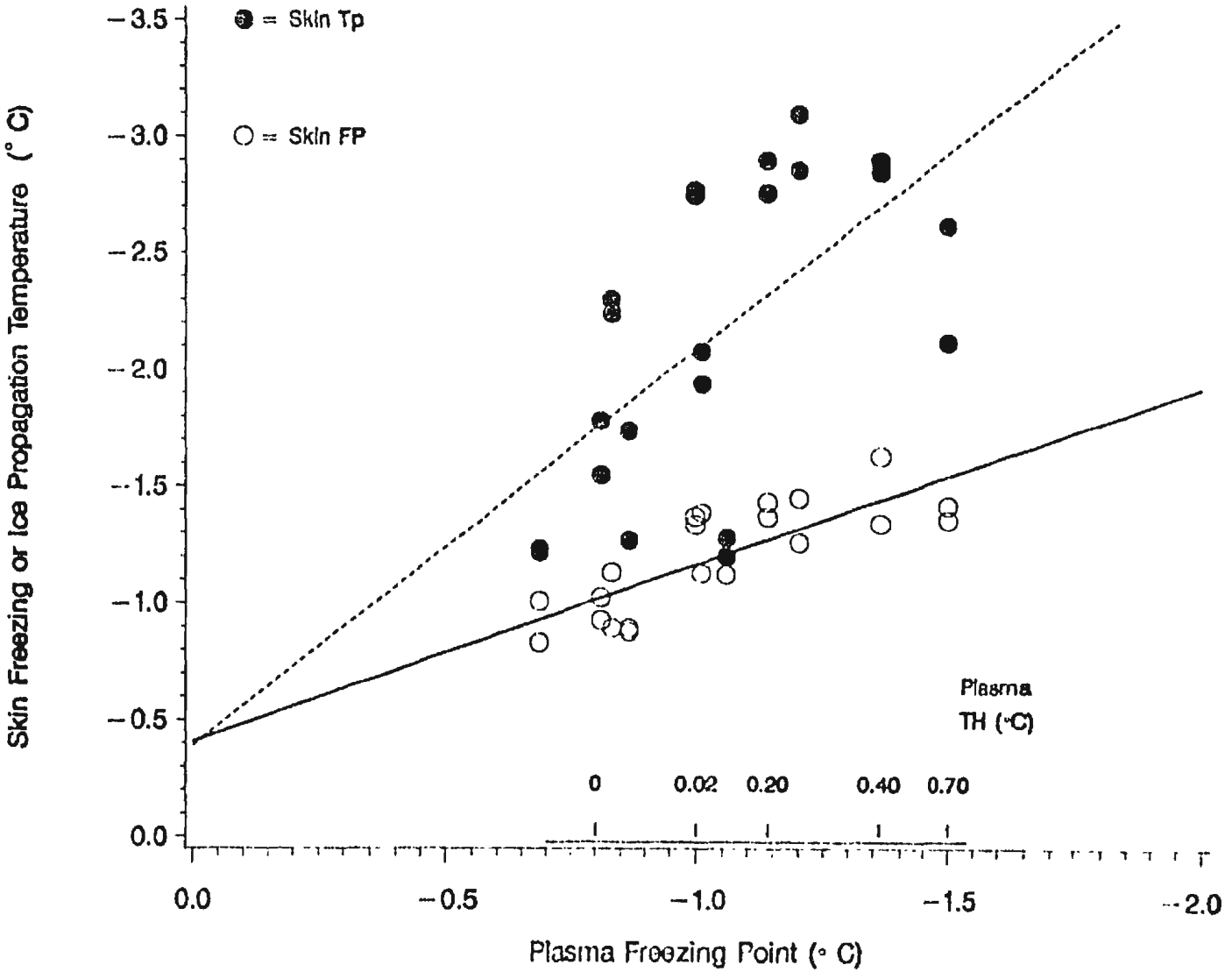

Skin Propagation Temperature (° C)
−3.5
−3.0
−2.5
−2.0
−1.5
−1.0
−0.5
0.0
0.0
−0.5
−1.0
−1.5
−2.0
Skin Freezing Point (° C)

Figure 2.3 Relationship between skin freezing point (FP), ice propagation temperature (T_p) and plasma freezing point. Closed circles = propagation temperature, open circles = skin freezing temperature. Each point represents a single measurement using an ocular side or blind side skin sample. Plasma TH (thermal hysteresis) (°C) indicates the amount of antifreeze activity observed in the plasma. Regression lines. Y=skin T_p or FP, X= plasma FP. Dashed line (T_p) Y= 1.70X - 0.39 (r=0.612, n= 22, P<0.01). Solid line (FP) Y= 0.761X-0.407 (r= 0.797, n=22, P<0.01). The intercept for the solid line (FP) differed significantly from 0 (P<0.05).


● = Skin Tp
○ = Skin FP
Skin Freezing or Ice Propagation Temperature (° C)
−3.5
−3.0
−2.5
−2.0
−1.5
−1.0
−0.5
0.0
Plasma
TH (°C)
0
0.02
0.20
0.40
0.70
0.0
−0.5
−1.0
−1.5
−2.0
Plasma Freezing Point (° C)

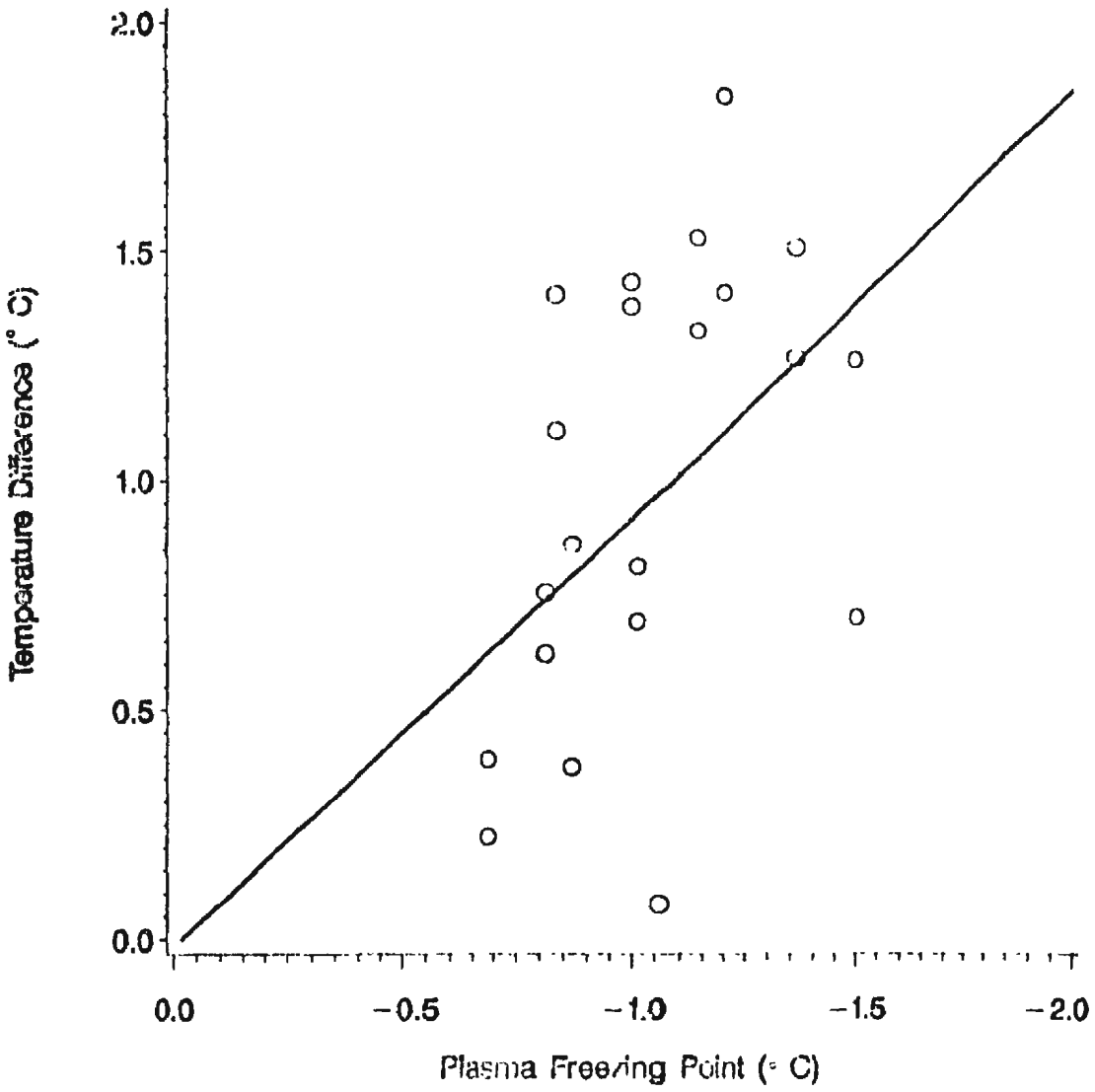
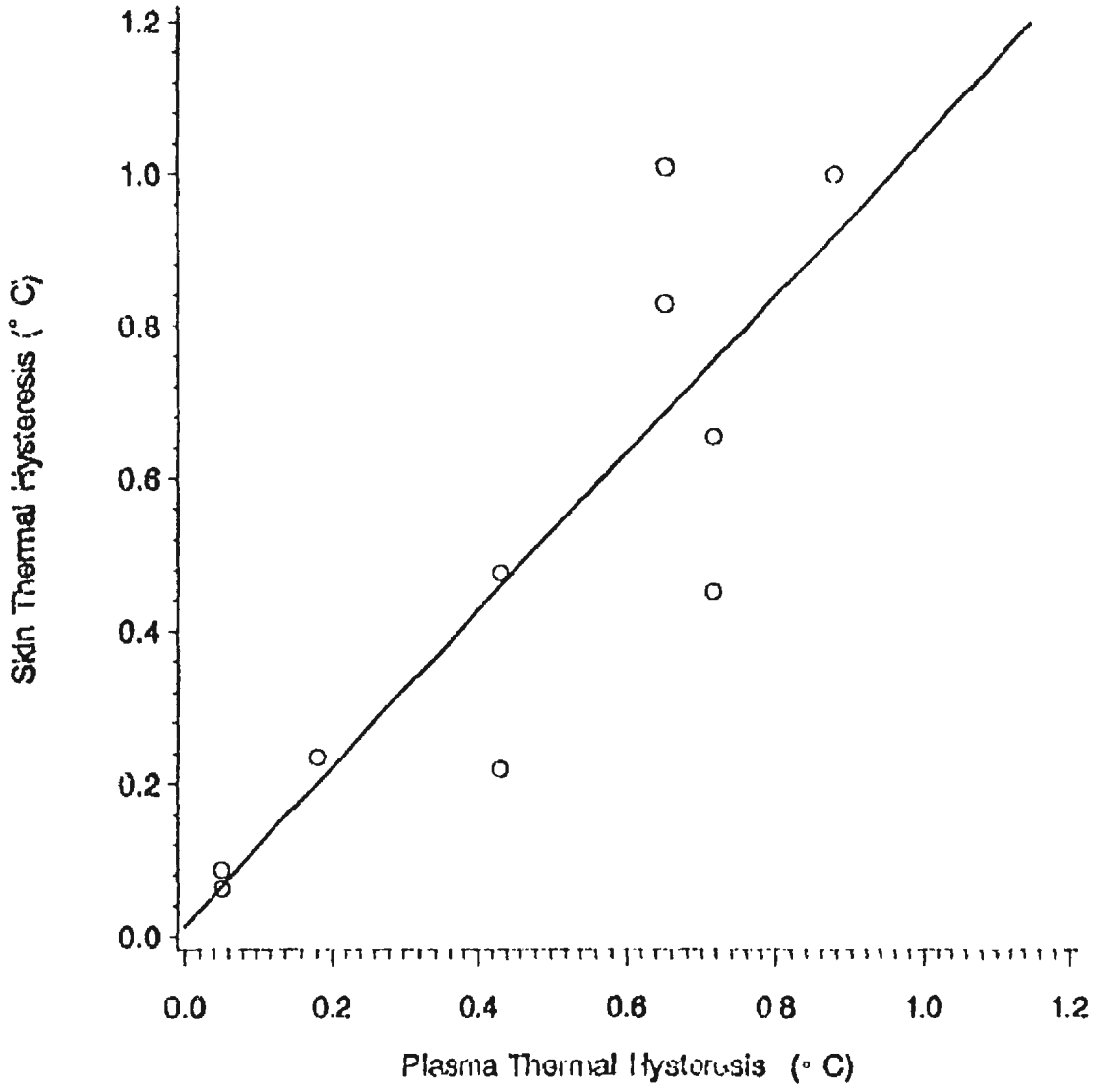

2.0
1.5
1.0
0.5
0.0
Temperature Difference (° C)
0.0
−0.5
−1.0
−1.5
−2.0
Plasma Freezing Point (° C)

Figure 2.4 Differences between the skin ice propagation temperatures (T_p) and the skin freezing points (FP). Each point represents the difference between the two temperatures for a single skin sample (ocular side or blind side). Regression line. Y= T_p or FP and X= plasma FP. Y= 0.935X - 0.017 (r=0.432 n=22, P<0.05).

Figure 2.5 Relationship between skin antifreeze activity and plasma antifreeze activity. Thermal hysteresis (TH)= measure of antifreeze activity. Each point represents a single skin sample. Regression Y=skin TH, X=plasma TH. $Y=0.0135 +1.037X$. ($r=0.882$, $n= 11$, $P<0.01$).


Skin Thermal Hysteresis (° C)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.2
0.4
0.6
0 8
1.0
1.2
Plasma Thermal Hysteresis (° C)

CHAPTER THREE

PRESENCE OF ANTIFREEZE ACTIVITY IN THE SKIN OF CUNNER, *TAUTOGOLABRUS ADSPERSUS*

A. Introduction

B. Materials and Methods

C. Results

D. Discussion

INTRODUCTION

PRESENCE OF ANTIFREEZE ACTIVITY IN THE SKIN OF CUNNER, TAUTOGOLABRUS ADSPERSUS.

In the natural habitat of cold-water marine teleosts, it is likely that ice crystals will first contact the fish at peripheral tissue sites, such as the skin, gill and corneal epithelia (see Chapter 2).

In most species possessing antifreeze compounds, such tissues would probably rely on a vascular supply of antifreeze that is secreted by the liver.

In all but one instance, antifreeze compounds have been reported to occur throughout most of the tissue fluids of fish and are apparently always secreted by the liver in these cases. Hepatic antifreeze secretion may occur by way of both the vascular system and the common bile duct, since antifreeze occurs in the gall bladder and intestine (O'Grady et al., 1983).

An exception to this pattern of distribution has been described for European populations of Myoxocephalus scorpius, a species of marine cottidae. Although several western Atlantic cottidae (including M. scorpius) are known to possess antifreezes that are generally distributed through all tissues, the M. scorpius antifreeze appears to be restricted to the skin in the European populations. Aqueous

extracts of skin samples from this species exhibit a thermal hysteresis, yet other fluid samples lack antifreeze activity (Schneppenheim and Theede, 1982). Since antifreeze proteins function by preventing ice crystals from propagating across fish skin and other external epithelial tissues, these observations raise the possibility that the cold water-adapted fish species increase their freeze-resistance by possessing antifreeze proteins that are restricted to the skin.

Cunner, Tautogolabrus adspersus, inhabit the shallow coastal waters off Newfoundland year round. To date antifreeze proteins have not been detected in the blood of this species, thus they are forced to overwinter at temperatures below their body freezing points. The mechanism by which this species survives in icy seawater is not well understood.

In the present study cunner skin is examined for the presence of antifreeze proteins in order to determine whether such compounds could play a role in the winter survival of this species.

MATERIALS AND METHODS

Cunner were obtained from Conception Bay, Newfoundland, and held in flowing seawater at ambient temperature and photoperiod. Fish were killed by a blow on the head. Skin samples were removed from the main trunk of the fish and dissected free of adherent muscle tissue.

The skin was separated into 2 fractions by scraping away the scales, epidermis and mucus. The samples were then weighed and divided into 3 groups for extracellular space determinations, extractions, and measurement of total tissue water. Skin samples from Atlantic salmon (Salmo salar) were used as a control group.

Mucus samples were obtained by a modification of the method of Shephard (1981), by placing fish in plastic bags for about 3 min. at 2°C, then collecting mucus with a pipette. Mucus was centrifuged to check for epithelial contamination. After centrifugation, it was noted that samples separated into a gel and sol phase. Freezing points of some samples were determined separately for sol and gel fractions. Plasmas were collected by centrifuging heparinized blood samples collected with a syringe.

Extracellular space was determined by chloride titration of skin digests (Lutz, 1972). Total tissue water was measured by drying preweighed samples in an oven at 50°C for 24 hr. Plasma chlorides were measured by the same procedure

as for extracts, and plasma water was determined by oven drying known volumes of plasma.

Samples were extracted by grinding under liquid nitrogen and then adding 30 ml of chilled water. The samples were incubated at 2°C for 1 hr and filtered to remove debris. The debris was further washed with 10 ml water and the filtrates were pooled. The filtrates were then freeze-dried and reconstituted to their original concentrations based on an estimate of extracellular fluid volume. Re-extraction of the solid debris after freeze-drying was performed to check whether extraction was complete.

Thermal hysteresis measurements for plasma, mucus and extracts were performed using the method of Kao and co-workers (1986), while freezing points were measured as described by Slaughter and Hew (1981), and all measurements were corrected with saline blanks.

RESULTS

Total water content of cunner skin (as a % of skin wet wt. was 68.56 ± 0.74 (N=4) (standard error, SE) (WSK = whole skin), 65.84 ± 0.45 (D = dermal fraction), and 70.94 ± 0.87 (R = remainder; epidermis, scales).

Extracellular spaces were 63.31 ± 3.80 (WSK) (N=4), 50.95 ± 2.54 (D) and 74.17 ± 2.30 (R), as g per 100 g total water.

Plasma freezing points were lower in winter than they were during summer (Table 3.1). This difference was attributable, at least in part, to the increased plasma chlorides during winter. No antifreeze activity (thermal hysteresis) was found in any plasma sample.

Mucus freezing points were significantly lower than plasma samples and seawater (Table 3.1). Traces of antifreeze activity (<0.01°C) were only observed in two of the winter samples. In contrast to solutions containing antifreeze proteins (Kao et al. 1986), mucus samples exhibited a linear relationship between concentration and freezing point ($r = 0.9997$, $N=11$). In addition, freezing points of the gel and sol phases of centrifuged mucus did not differ significantly (gel = -1.95 ± 0.0058°C, $N = 4$; sol = -1.94 ± 0.043°C, $N=4$). This is consistent with mucus being a water-saturated gel that freezes according to its colligative properties.

The freezing point of seawater during winter was -1.75 ± 0.004°C (SE) ($n=6$).

All skin extracts showed significant levels of thermal hysteresis, and the activity was greater in winter than during the summer (Table 3.2).

DISCUSSION

The possibility that mucoproteins were responsible for

the thermal hysteresis observed was ruled out by the observation that activity was generally absent from mucus samples. The occasional occurrence of thermal hysteresis activity in mucus is more often observed in mucus samples from fish where samples are heavily contaminated with sloughed epithelial cells (Chapter 2) and since cunner skin is protected by a relatively thick layer of mucus, such contamination is less frequent. However, the occurrence of a thermal hysteresis in other components of the skin does point to an active water-soluble compound.

The occurrence of thermal hysteresis in both the dermal and the remaining dermal/epidermal fractions is interesting, since it would imply that the limits of antifreeze activity in cunner skin occur from the epidermis to the hypodermis (where blood vessels occur). If this were not true, activity would be expected in the blood or mucus. This distribution pattern lends further support to the idea that the peripheral tissues of fishes are the sites where ice seeding occurs, and therefore are the most critical site for freezing protection.

The absence of thermal hysteresis activity from other tissues would suggest 3 possibilities: 1) that an antifreeze is secreted by cells in the skin and diffusion into deeper tissues is blocked or slowed by a tissue barrier, 2) that antifreeze is secreted elsewhere and activated only in the skin, or 3) that plasma antifreeze activity is undetectable due to low concentrations or a brief period of secretion.

The third possibility may be the most reasonable, since winter flounder (Pseudopleuronectes americanus) skin retains some antifreeze activity after spring clearance from the plasma (Chapter 2), and previous studies have revealed that some cottidae secrete plasma antifreeze, while others possess an extractable skin antifreeze (Schneppenheim and Theede, 1982). Further seasonal sampling is required to clarify this point, and to determine whether skin and plasma antifreeze are derived from the same (hepatic) source. Although the absence of activity from skin mucus suggests that the compound responsible is not a mucoprotein, it is not clear how a water-soluble compound could exist in the dermis without entering the vascular compartment or being diluted by adjacent tissue fluids. Since the extracts were reconstituted by assuming a uniform distribution throughout the ECS, the possibility remains that the cunner antifreeze is actually more concentrated in a local region of the skin. Again, however, the solubility of the compound would tend to argue against this.

If the localized distribution is due to a compartment barrier, this may occur at the level of the capillary wall, since the lowest layer of fish skin, the hypodermis, is usually the only vascularized region of the skin (Whitear, 1984).

The reduced level of thermal hysteresis activity in summer samples would appear to indicate a seasonal cycle.

The use of activity curves to estimate antifreeze concentration on the basis of molecular weight depends on a reliable estimate of the latter (Kao et al., 1986). It is therefore anticipated that future investigations of local tissue 'antifreezes' will concentrate on the isolation and characterization of these compounds, so that their sites of synthesis and true function may be determined.

The fact that the Labridae are a tropical group of teleosts (Scott and Scott, 1988) may imply that thermal hysteresis activity in cunner skin is a recent evolutionary development. Another interesting possibility is that skin antifreezes exist in other teleosts, and may even be masked by the activity of the more prevalent plasma antifreezes. These results indicate that the sampling of bulk tissue fluids may not be sufficient to determine the presence of antifreeze compounds, since there is a possibility that a local tissue distribution would prevent their detection in some species.

Around the shores of Newfoundland, the extreme northern range of the species, it has been noted that cunner enter rock crevices when water temperatures drop to about 5°C, and that they remain within this microhabitat in the inshore region without feeding until water temperatures rise again in the spring (approximately from November to May). This metabolic state has been referred to as a form of seasonal torpor (Green and Farwell, 1971). That these fish survive in

an undercooled state has been verified by Green (1974), who noted that a local winterkill of cunners in the region coincided with turbulent inshore conditions, when vertical mixing of suspended ice crystals was maximal. The experimental introduction of ice crystals to rock crevices resulted in the freezing and mortality of cunners (Green, 1974), suggesting that undercooled fish survive by avoiding ice contact.

Cunners kept in aquaria over the winter displayed a seasonal cycle of behaviour similar to tnat reported by Green and Farwell (1971), huddled in groups in the corners during the winter months, but resuming active swimming and feeding in the spring-time. Such fish have been maintained at the marine laboratory for several years.

The cunner's behaviour and physiology during the winter can be viewed as tactics for survival during freezing conditions. Since ice contact is lethal to supercooled fish it is evident that cunner reduce the possibility of such a circumstance by "hiding" huddled in groups deep in rock crevices. Moreover by ceasing feeding and entering a near torpor state their requirements for oxygen and thus gill ventilation by water laden with ice crystals would be minimized.

Cunner also reduce ice contact by secreting copious amounts of mucus at low temperatures (unpublished observations). It is evident that a mucus layer would hinder

contact between ice crystals and the undercooled epithelial tissue fluids. In addition, since skin mucus had a lower freezing temperature than seawater, ice crystals in contact with the mucus would be in the process of melting. In the event of ice crystal contact with epithelial cells, the presence of antifreeze compounds within the skin provides a further small barrier to ice crystal propagation to the undercooled blood and other body fluids.

Although the mechanisms displayed by the cunner to avoid ice contact may only delay freezing under the worst conditions, it should be noted that seawater temperatures rarely approach the freezing point in the Avalon area of Newfoundland (lowest observed at the marine laboratory -1.6°C). Therefore the danger from suspended ice crystals would be confined to sporadic, but relatively brief episodes of stormy weather.

Table 3.1

Freezing points of plasma and mucus

	Plasma		Mucus
	Freezing Point (°C)	Cl (mM)	Freezing Point (°C)
Summer	-0.678 ± 0.012	157 ± 1	-1.93 ± 0.033
	(4)	(10)	(4)
Winter	-0.768 ±0.02	180 ± 2	-2.08 ± 0.12
	(5)	(10)	(6)
t	3.59	8.75	1.15
df	7	18	6
p	0.0089	0.0000	0.2962

Values expressed as means ± SE. Numerals in parentheses indicate the number of fish. Plasma and mucus freezing points measured in March and June. Plasma Cl- measured in March and August. Comparisons between summer and winter were made using Student's t test. NS = not significant.

Table 3.2

Thermal hysteresis activity in cunner skin extracts

	Fish (skin fraction)	Skin Wet Weight (g)	Thermal Hysteresis (°C)
MARCH	1 (D)	1.7982	0.115 ± 0.003
	2 (D)	2.1980	0.143 ± 0.003
	3 (D)	2.5669	0.198 ± 0.011
	4 (D)	3.4353	0.093 ± 0.006
	3 (R)	2.9144	0.347 ± 0.011
	4 (R)	5.0472	0.861 ± 0.006
	Mean March		0.293 ± 0.12
JUNE	5 (D)	2.7258	0.130 ± 0.006
	5 (D)	2.1699	0.025 ± 0.002
	6 (D)	2.5262	0.028 ± 0.002
	6 (D)	3.3633	0.031 ± 0.000
	7 (D)	2.2094	0.028 ± 0.002
	7 (D)	3.3309	0.096 ± 0.003
	5 TO 7 (R)	2.4200	0.075 ± 0.002
	Mean June		0.059 ± 0.016
JUNE	SALMON	2.1792	0.006 ± 0.002

All values presented as means ± S.E.

Thermal hysteresis determinations carried out in triplicate.

D = dermis R = scales plus epidermis

CHAPTER FOUR

CONCLUSIONS

The main conclusions from the present study are

1. The tissues of winter flounder may be undercooled without spontaneously freezing, indicating an absence of endogenous nucleating sites in the tissues that operate in the normal range of temperatures experienced by fish. Consequently, fish may avoid freezing by avoiding ice contact in deep water at sub-freezing temperatures.
2. The relatively large extracellular space of skin allows fishes to maintain a significant reservoir of antifreeze at the body surface.
3. Ice crystals do not enter the tissues of fishes before the tissues reach their freezing point. The latter is determined by at least 2 factors: the concentration of colligative solutes and the concentration of antifreeze proteins in the tissue fluids.
4. The temperature at which ice propagates across marine teleost skin is lower than the skin freezing point.
5. The presence of antifreeze proteins in the skin lowers the skin ice propagation temperature.
6. Ice crystals probably enter fish at some site other than the skin in healthy fish, possibly the gills.

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