COPY NUMBER VARIATION OF GENES ENCODING ANTIFREEZE PROTEINS IN WINTER FLOUNDER (*PSEUDOPLEURONECTES AMERICANUS*)

by

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Dedicated to

My mother Edith for always encouraging me

My husband Marc for understanding and speaking the science lingo

My son Jake who is my inspiration

My sisters Wendy and Isi for all their love, and knowing how to push my buttons

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Abstract

Winter flounder (*Pseudopleuronectes americanus*) survive in icy seawater at temperatures reaching -1.9 °C by producing antifreeze proteins (AFPs). AFPs bind to ice crystals and lower the freezing point of fish blood in a non-colligative manner. Previous studies have shown that AFP levels vary among populations of winter flounder, even when fish are raised in the same location, suggesting a genetic rather than an environmental mechanism. Other studies have shown that AFP gene copy numbers vary among locations and that AFP activity in plasma is proportional to gene copy number. The goal of the current study was to determine the AFP gene copy numbers in winter flounder from several locations along the eastern coast of North America, and to determine whether gene dosage is related to antifreeze activity. We designed a quantitative PCR (qPCR) assay to determine the copy numbers of genes encoding the four major AFPs in winter flounder plasma (wflAFP-6, wflAFP-8, wflAFP-9 and the hyperactive AFP Maxi). AFP gene copy numbers were analyzed in flounder from Newfoundland, St. Lawrence Estuary, Baie des Chaleurs, Passamaquoddy Bay and Georges Bank. Winter flounder from Newfoundland had considerably higher AFP gene copy number for each of the genes tested compared to Georges Bank fish. Flounder from the other locations had intermediate gene dosages, differing from Georges Bank fish for some genes and differing from Newfoundland fish for others. There was a relationship between AFP gene copy numbers and antifreeze activity at the population level. We found the hyperactive gene Maxi to be in considerable lower numbers in comparison to the smaller AFPs wflAFP-6 and 8. We attribute this to the protein size and activity. In order to better understand the gene organization of Maxi, we performed Southern blot analysis on samples from each of the locations studied and found that Maxi is represented by a multi-gene family. The antifreeze activity analysis also showed that some fish which did not have any copies of Maxi still had Maxi-like antifreeze activity. This suggests there might be other genes encoding AFPs with high activity similar to maxi that are contributing to winter flounder freeze resistance.

List of Abbreviations Used

AFGP	Antifreeze glycoprotein
AFPs	Antifreeze proteins
BC	Baie des Chaleurs
bp	Base pair
CCAC	Canadian Council on Animal Care
cDNA	Complementary deoxynucleic acid
Ct	Cycle threshold
DNA	Deoxynucleic acid
DIG	Digoxigenin
DMSO	Dimethyl sulfoxide
gDNA	Genomic deoxynucleic acid
GB	Georges Bank
g	Gram
GH	Growth hormone
kb	Kilobase
Tm	Melting temperature
mRNA	Messenger ribonucleic acid
µg	Microgram
µl	Microliter
mol	Mole
ng	Nanogram
NRC	National Research Council
NL	Newfoundland
PB	Pasamaquoddy Bay
PGP	Permeability glycoprotein
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
SDS	Sodium dodecyl sulfate
ES	St Lawrence Estuary
SEM	Standard error of the mean
TBE	Tris Boric acid EDTA
UV	Ultraviolet
U.S.A.	United States of America
Maxi	Winter flounder hyperactive antifreeze protein
wflAFP-6	Winter flounder liver antifreeze protein 6
wflAFP-8	Winter flounder liver antifreeze protein 8
wflAFP-9	Winter flounder liver antifreeze protein 9
21a	Winter flounder antifreeze protein gene encoding wflAFP-9

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Chapter 1

Introduction

Sub-polar and temperate regions have large seasonal fluctuations in temperature. In cold oceans such as the North Atlantic, the water can reach its freezing point in winter resulting in ice formation, particularly in coastal areas. In full-strength seawater with a salinity of 32 parts per thousand, the freezing temperature is -1.9 °C (DeVries 1982). This is only moderately cold, compared to terrestrial temperatures at similar latitudes, which can reach -50 °C in the Arctic and -80 °C in the Antarctic winters; however, a temperature of -1.9 °C can be lethal to teleost fish. The freezing temperature of a solution is a colligative property, resulting from the presence of solutes and is proportional to their concentration. Since most cold-water marine teleost fish have lower concentrations of solute particles in their body fluids than seawater, they are hyposmotic to seawater and therefore their colligative freezing point is higher than that of the surrounding seawater. In most marine fish species inhabiting the North Atlantic ocean, NaCl is the main blood electrolyte, conferring 85% of the freezing point depression. Other electrolytes, which also contribute to lowering the freezing point of fish blood are potassium ions, calcium ions, urea, glucose and amino acids (DeVries 1983). Together, these result in blood plasma freezing points ranging from -0.6 to -0.8 °C. Therefore, with no further freezing point depression, fish would freeze to death in freezing seawater. Nonetheless, fish species that inhabit cold oceans have evolved the means to avoid freezing.

North temperate fish species exhibit several survival strategies that reduce the risk of freezing in icy seawater. Some fish such as Arctic char (*Salvelinus alpinus*) migrate to fresh water (Dempson and Kristofferson 1987), while others such as the halibut (*Hippoglossus hippoglossus*) migrate to deeper waters (30-200 m) offshore to

avoid contact with ice (DeVries 1983). In the sea, ice formation does not occur at depths below 30 m due to the hydrostatic pressure. Therefore, although fish that live at those depths are supercooled (i.e. below their freezing point) by approximately 1 °C, they do not freeze over the course of their lifespan (DeVries 1982). In contrast, there are fish species that remain in icy shallow waters during winter. A few species avoid freezing by producing high concentrations of osmolytes; such is the case of the rainbow smelt (*Osmerus mordax*), which produces high concentrations of glycerol during the winter (Raymond 1992). The high concentrations of glycerol in the serum depress the freezing point colligatively. A larger number of species produce antifreeze proteins (AFPs) that lower the freezing point of the blood in a non-colligative matter (DeVries et al. 1970, Fletcher et al. 2001).

AFPs lower the freezing point very efficiently (DeVries et al. 1970, Feeney and Yeh 1978, Feeney et al. 1986). They are defined as proteins that have an affinity for ice and that can therefore bind to the surface of seed ice crystals (Raymond and Devries 1977). AFPs are found in bacteria (Gilbert et al. 2004, Gilbert et al. 2005), fungi (Duman and Olsen 1993, Robinson 2001), plants (Griffith and Yaish 2004, Moffatt et al. 2006), insects (Graether et al. 2000, Liou et al. 2000, Tomchaney et al. 1982) and fish, where they were first discovered.

1.1 Interaction of AFPs with ice

1.1.1 Freezing point depression

The freezing point of a solution at a fixed pressure is the temperature at which the liquid and solid phases have the same Gibbs free energy. If the solution is in thermal

equilibrium, then by definition the freezing point of the solution is the same as the melting point of the solid phase (Gibbs, J. W. 1906 cited by Kristiansen and Zachariassen 2005). The freezing point of a solution can be determined by observing the temperature at which a small seed ice crystal begins to melt as the temperature is gradually raised. The temperature at which melting occurs is the equilibrium melting-freezing temperature. If the temperature is decreased below the melting-freezing equilibrium and before the ice crystal is completely melted, the ice crystal will suddenly grow. The equilibrium freezing point of pure water is 0 °C.

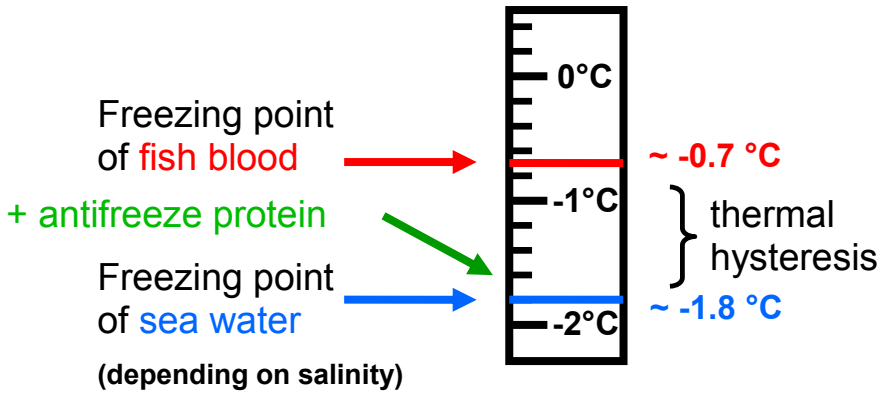


Figure 1.1 Schematic representation of Thermal Hysteresis

Depression of the equilibrium freezing point (which is also the melting point) is a thermodynamic phenomenon in which the freezing temperature is lowered by an amount proportional to the molar concentration of the solute molecules or segments of a polymer in a solution (Poynting, J. H. 1881 cited by (Kristiansen and Zachariassen 2005). As a colligative property, the freezing point depression depends on the mole fraction of solute

particles present, but not on the molecular mass or chemical properties of the same particles. This effect will depress the melting point of the solution by the same amount (Poynting, J. H. 1881 cited by (Kristiansen and Zachariassen 2005). AFPs depress the freezing point of a solution far more efficiently on a molar basis than would be predicted by their colligative properties alone (DeVries et al. 1970, Feeney and Yeh 1978, Feeney et al. 1986). Binding of AFPs to ice surfaces results in a non-colligative freezing point depression with a thermal hysteresis, which is a gap between the freezing and melting points (Figure 1.1). The activity of AFPs can be measured as thermal hysteresis by determining the melting and freezing points of seed ice crystals. The magnitude of the thermal hysteresis is then calculated by measuring the difference between the melting point and the freezing point of the solution and its presence indicates a non-colligative mechanism (Kao et al. 1986).

1.1.2. Adsorption- inhibition mechanism of AFPs

At the molecular level, AFPs bind to distinct and specific surfaces of ice crystals (Figure 1.2). The binding surfaces appear to be different for some of the different AFPs, resulting in impedance of crystal growth in specific directions and distinct ice morphologies (Chao et al. 1995, Raymond and Devries 1977).

The adsorption-inhibition hypothesis postulates that AFPs bind to ice and thereby impede ice growth. Binding of AFPs to ice surfaces would need to be quasi-irreversible in order to stop crystal growth, as reversible equilibrium binding to ice would lead to gradual crystal growth, which in turn would be lethal to fish (Hew and Yang 1992, Knight and DeVries 1994, Yeh and Feeney 1996). Recent experiments using fluorescently

labeled AFPs on ice crystals have shown binding to be essentially permanent (Pertaya et al. 2007).

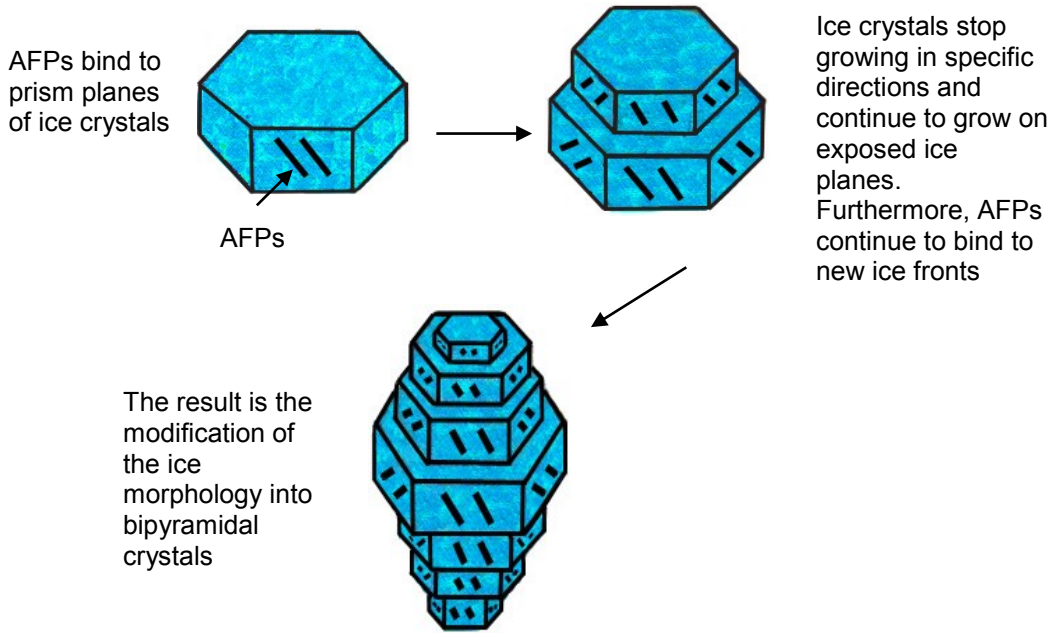


Figure 1.2 Schematic representation of interaction of AFPs Type I with ice. Adapted from Davies and Hew (1990).

The mechanism by which adsorbed AFPs are assumed to cause thermal hysteresis is by limiting ice growth to convex zones at the exposed areas between the adsorbed AFPs (Figure 1.3A). The curvature would make it thermodynamically unfavourable for other water molecules to be added to the ice lattice. In this manner, ice growth ceases within the temperature interval below the equilibrium freezing temperature. When the temperature is lowered below this non-equilibrium freezing point, the ice crystal suddenly grows in a manner that appears explosive, as supercooled water

rapidly joins the ice (Knight and DeVries 1994, Raymond and Devries 1977, Wilson 1993).

AFP binding causes ice crystals to adopt distinct morphologies such as bypiramids, depending on the type of AFP acting of the crystals (Figure 1.3B). The modification of ice crystal morphology results from the direct interaction of the AFPs with the ice crystal surface. When solutions of AFPs are partially frozen, AFPs are not excluded from the frozen fraction like other proteins (Kuiper et al. 2003, Raymond and Devries 1977). Interestingly, AFPs have been shown to be located at the interface between the liquid phase and the growing ice crystal (Brown et al. 1985, Wilson et al. 1993). Scanning tunneling microscopy has also shown adsorption of AFP to ice crystals, providing direct evidence of AFP interaction with specific ice surfaces (Grandum et al. 1999).

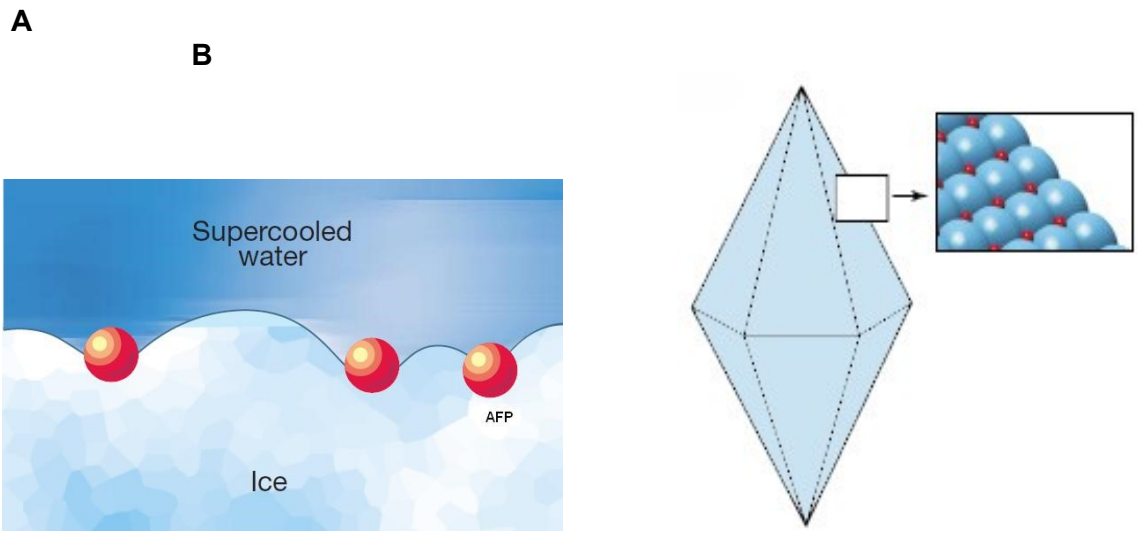


Figure 1.3 Schematic representation of AFP-ice interaction. Panels are (A) the adsorption of AFPs to ice, and (B) the effects of AFPs on ice growth morphology. Adapted from (Knight 2000) and (Jia and Davies 2002).

1.2 Antifreeze protein diversity in fish

In fish, five types of AFP have been identified to date. The first such protein discovered was a highly glycosylated mucin-like polymer from Antarctic notothenioids. It is called antifreeze glycoprotein (AFGP) to reflect its extensive glycosylation. The other four types (I-IV) are distinct from the AFGP and from one another. All fish AFP types differ in protein sequence, secondary and tertiary structures; however, they share similarities in the way they interact with ice. Some AFPs are nearly identical and have evolved in unrelated taxa, whereas others that are completely different are present in closely related taxa. This diversity of AFPs demonstrates the endless possibilities of independent and convergent evolution (DeVries et al. 2005).

1.2.1 Type I

Type I AFPs are found in righteye flounders including winter flounder (Duman and Devries 1976, Marshall et al. 2004), yellowtail flounder (*Limanda ferruginea*) (Scott et al. 1987), plaice *(Pleuronectes quadritaberulatus, Hippoglossoides platessoides)* (Gauthier et al. 2005), sculpins (*Myoxocephalus octodecimspinosis, M. scorpius, M. aenaeus*) (Chakrabartty et al. 1988, Hew et al. 1985, Low et al. 2001), and snailfish (*Liparis atlanticus*) (Evans and Fletcher 2001). The short 3-4-kDa Type I AFPs (Figure 1.4) are the ones that were first discovered in flounder and have been best studied. There are two forms of short Type I AFPs, classified according to their primary site of production. Serum AFPs, which are generally produced in the liver of the fish and secreted to the blood plasma, and skin AFPs, which are produced in multiple tissues, notably the skin (Gong et al. 1995).

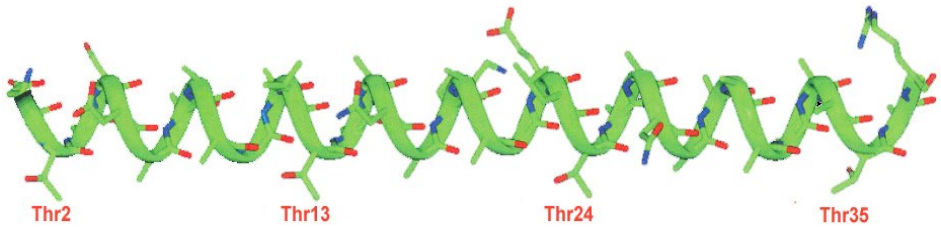


Figure 1.4 Structure of a Type I AFP. The winter flounder wflAFP-6 peptide backbone is shown in a ribbon diagram with amino acid side chains shown in stick form. Adapted from (Harding et al. 1999).

Type I AFPs also exist in a much larger form. The more recently discovered 32-kDa hyperactive AFP (Maxi) is much larger and approximately 10-fold more active than the short AFPs (Marshall et al. 2004). The short AFPs are typified by the well-studied 37-residue alanine-rich peptide existing as single, long amphipathic, α-helix, whereas Maxi comprises two extended alpha helices of approximately 195 residues forming an amphipathic homodimer with a series of linked Ala- and Thr-rich patches on the surface, each resembling the ice-binding sites of the short Type I AFPs (Graham et al. 2008c).

The winter flounder provides a remarkable example of AFP diversity. In winter flounder, the most abundant AFPs are the shorter form consisting of three 11-residue sequence repeats. Examples of the short three-repeat Type I AFPs are wflAFP-6 and wflAFP-8 found in the blood serum, and several distinct AFPs found in the skin (Gong et al. 1996, Gronwald et al. 1998, Harding et al. 1999, Marshall et al. 2004, Sicheri and Yang 1995). They are referred to as skin-type AFPs and they are a subset of the short Type I AFPs. In addition, the 4-repeat AFP (wflAFP-9) encoded by the gene 21a (Chao et al. 1996) and the much longer Maxi AFP (Marshall et al. 2004) are also present in the serum. The snailfish provides a contrasting example, in which the shorter AFP form is

present in plasma and skin and this AFP appears to be produced in the skin and not the liver (Evans and Fletcher 2005a).

The relationship among the diverse Type I AFPs is interesting. For example, the snailfish and sclupin produce Type I AFPs, but the proteins from snailfish have higher alanine content (79-83% depending on the species) than those found in sculpin. Also, snailfish AFPs contain proline, which is known to disrupt the helical structure (Chakrabartty et al. 1995, Evans and Fletcher 2001). Moreover, the short flounder AFPs are the only ones with highly regular repeated sequence elements. Therefore, there are notable differences and similarities among these related proteins. The sequence, compositional and structural similarities among the Type I AFPs in snailfish, sculpins, flounder and another species, the cunner (*Tautogolabrus adspersus*) are not reflected at the nucleotide level in the genes that encode them, which indicates that the AFPs arose in these different groups by convergent evolution (Graham et al. 2013).

AFP-ice interaction has been the focus of much work and the Type I AFPs have provided the basis for the most definitive studies of ice recognition. The molecular mechanism by which these proteins adsorb to ice has only recently begun to emerge. In the initial hypothesis, the hydrophilic amino acid side chains were proposed to mediate binding to ice. Specifically, regularly spaced hydrophilic Thr and Asp/Asn residues were suggested to form hydrogen bonds with oxygen atoms on the prism planes of ice (Devries and Lin 1977). Another hypothesis proposed that the16.5 Å spacing of the Thr residues at the start of each 11 amino acid repeat in the wflAFP-6 was the key binding feature to ice and binding was proposed to occur by ordered interaction with all four Thr hydroxyls. The interaction of the protein to ice was assumed to be through hydrogen bonding. The 4 Å spacing of water molecules along the ridges appeared to provide accessible hydrogen bonding targets for both ice-binding groups (Harding et al. 1999, Houston et al. 1998).

In other studies, the hydrophilic surface of the helix was noted to be highly variable while the hydrophobic face consisting of spaced Ala and Thr was found to be highly conserved. Furthermore, the Asn or Asp residues are not conserved among type I AFPs. Based upon these observations, amino acid substitution studies were performed. Ala residues on different surfaces of the helix were substituted with Leu, which would sterically impede interaction with ice if located at the position of an Ala on the ice-binding surface. Activities of the mutants revealed that the surface comprising the conserved Ala residues and Thr residues binds ice, and not the Asn/Asp-containing surface (Baardsnes et al. 1999). In subsequent studies, substitution of Thr with Val (retaining the methyl group) or with Ser (retaining the hydroxyl group) revealed a far stronger effect of methyl group loss on thermal hysteresis activity (Baardsnes et al. 1999, Chao et al. 1997, Haymet et al. 1998, Zhang and Laursen 1998). A further study supported the role of methyl groups by showing that a single substitution of Ala was sufficient to decrease thermal hysteresis activity, revealing the importance of Ala for ice binding, especially those Ala found immediately next to the Thr methyl group (Baardsnes et al. 1999).

These studies led to the conclusion, that the ice-binding site of the small Type I AFPs is the flat hydrophobic side of the protein (which is oriented towards the ice surface in the ice/water interface) by means of Van der Waals force surface complementarity and hydrophobic interactions (Kwan et al. 2005). This new hypothesis, supported by empirical studies, was in sharp contrast to earlier hypotheses that focused exclusively on hydrogen bonds.

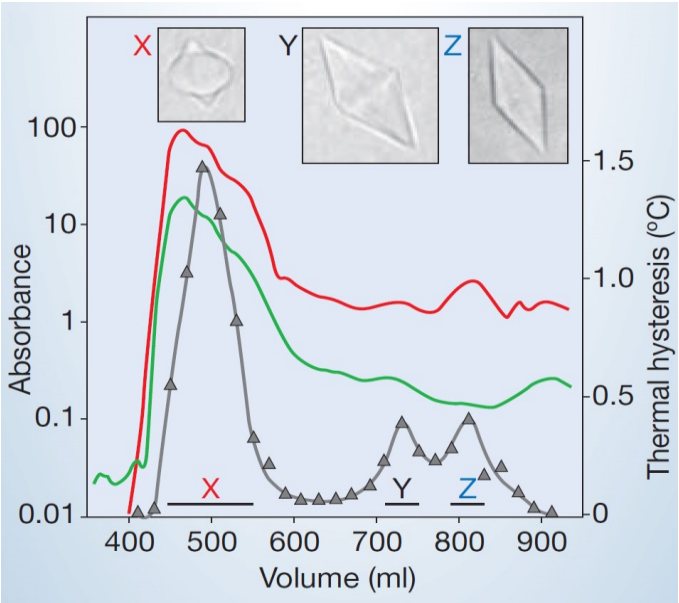


Figure 1.5 Fractionation of winter-flounder plasma by gel permeation chromatography on Sephadex G-75. Thermal hysteresis activity (black triangles) are shown for fractions across the chromatogram. Insets are showing the morphology of the ice crystals produced from peak fractions X, Y and Z: Peak X contains the 5a-like AFP; peak Y contains Type-I isoform AFP-9; peak Z contains Type-I isoforms wflAFP-6 and wflAFP-8, respectively. Adapted from Marshall et al. (2004).

Since ice has many surfaces for binding, it is not surprising that AFPs evolved to bind to different planes. The discovery of the Maxi AFP suggested binding to distinct ice surfaces (Marshall et al. 2004). In that study, size-exclusion chromatography was used to separate AFPs in plasma from winter flounder and MALDI-TOF mass spectrometry was used to identify the AFPs present in each peak (Figure 1.5). The results revealed that the new hyperactive fish AFP produces different ice morphology (lemon shaped) compared to the other AFPs (wflAFP-6 - wflAFP-8). Further studies revealed that the Maxi forms a dimer and that it has a large combined surface area of ice binding sites,

recognition of multiple planes of ice, and protection of the basal plane from ice growth (Graham et al. 2008b).

1.2.2 Type II

Type II AFPs are cysteine-rich globular proteins containing five disulfide bonds that are homologous to the carbohydrate recognition domain Ca^{2+}-dependent (C-type) animal lectins (Figure 1.6). As such, they are members of the C-type lectin superfamily. These AFPs are found in sea raven (*Hemitripterus americanus*) (Slaughter et al. 1981), smelt (*Osmerus mordax*) (Ewart et al. 1992), and herring (*Clupea harengus harengus*) (Ewart and Fletcher 1993), and consist of over 110 amino acids with molecular masses of approximately 14 kDa (herring and sea raven) to 24 kDa (smelt). Cloning of AFPs from liver cDNA libraries show that they are synthesized in the liver (Ewart et al. 1992, Ewart and Fletcher 1993, Ng et al. 1986) and Northern blot hybridization has confirmed this for the sea raven AFP (Gong et al. 1992). The sea raven, AFP mRNA has been found in other tissues including the stomach mucosa, skin and pancreas, although levels were lower than in liver (Evans and Fletcher 2004, Gong et al. 1992).

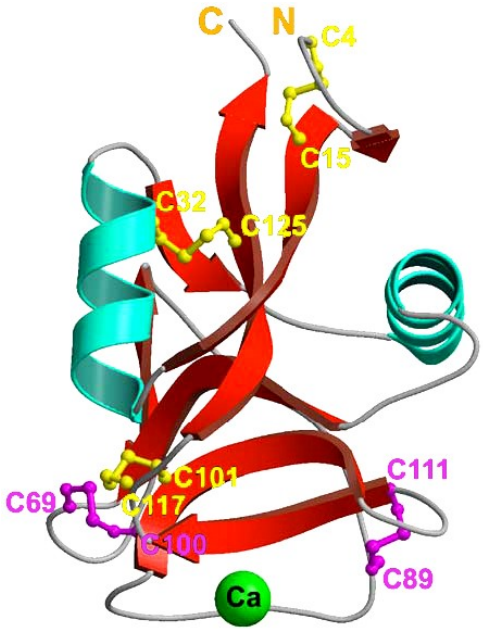


Figure 1.6 Structure of a Type II AFP. The sea raven AFP structure is shown in a ribbon diagram, with alpha helices in aquamarine, beta sheets in red and unstructured and loop regions in gray. Adapted from (Liu et al. 2007).

The ice-binding site of the herring AFP was shown by site-directed mutagenesis to correspond to the Ca^{2+}-binding site of this protein (Ewart et al. 1998). This is also the location of the carbohydrate-binding site of homologous lectins (Ewart et al. 1998). Type II AFPs from smelt and herring require Ca^{2+} for antifreeze activity and they have retained the critical Ca^{2+}-coordinating residues for the Ca^{2+}-site that are conserved among the lectins. The ice surface may be recognized as a variant of galactose at the molecular level by the lectin-like herring AFP. This concept might also be useful to consider in seeking to understand protein-ice interactions in general (Ewart et al. 1999). The ice-binding site of the herring was mapped to a higher resolution by further mutagenesis and

it was confirmed to correspond to that site. The interactions of the four residues (Asp94, Thr96, Thr98 and Glu99) on the binding face of the protein and the direct Ca^{2+}-coordination with ice results in inhibition of crystal growth. The binding site of the herring AFP has two additional disulfide bonds on both sides of the Ca^{2+} site that appear to stabilize the binding surface and helps project the ice-binding residues to engage with the ice lattice (Liu et al. 2007).

In contrast to the AFPs of smelt and herring, the sea raven AFP is Ca^{2+}-independent (Sonnichsen et al. 1995). The ice binding site of the sea raven AFP appears to be located separately from the ice-binding site of the other AFPs Type II on a different surface from the location of the conserved C-type lectin Ca^{2+} binding site. Sterically, this region seems to be ideally suited for interaction with ice due to the position of surface-accessible side chains. Furthermore, this ice-binding site corresponds to the location of the proposed $CaCO_3$-binding surface of the pancreatic stone-binding protein, lithostathine. The role of T23 of the sea raven AFP, which is equivalent to the putative $CaCO_3$-binding residue in lithostathine, has been studied by site-directed mutagenesis revealing that mutant T23H retains 92% of the wild type antifreeze activity, and indicating that the binding site of the sea raven AFP does not correspond to the $CaCO_3$-binding of lithostathine (Loewen et al. 1998). It is interesting that 65% percent of the accessible surface area of the sea raven AFP II is non-polar (Sonnichsen et al. 1995). Therefore, this is a hydrophobic protein with a relative non-polar surface area close to those observed for water-insoluble proteins. This may be relevant to antifreeze activity, as hydrophobic residues could prevent ice from growing around the protein by repelling liquid water from the ice lattice in their vicinity, thereby contributing to its effectiveness as an AFP (Sonnichsen et al. 1995).

1.2.3 Type III

The Type III AFP is found in eel pouts such as ocean pout (*Macrozoarces americanus*) (Hew et al. 1984), wolffish (*Anarhichas lupus*) (Scott et al.. 1988) and Antarctic species (*Austrolycicthys brachycephalus, Lycodichthys dearborni)* (Wang et al. 1995). This globular AFP type exhibits a compact fold with a relatively large hydrophobic core and a dozen homologuous components with molecular masses of 7 kDa (Figure 1.7). The fish Type III AFP appears to be homologous to the C-terminal region of mammalian sialic acid synthase (Baardsnes and Davies 2001). Type III AFPs are synthesized in the liver, but mRNA has been found in several tissues such as fins and gills, including red blood cells (in lower amounts) and rich-blood tissues such as kidney, spleen, heart and gills. mRNA has also been found in the intestinal tract, with the highest proportion occurring within the intestinal mucosa and in the pancreas of *L.dearborni* (Gong et al. 1992).

The overall fold of the ocean pout AFP comprises numerous short irregular beta-strands and one α-helix turn. It has 65 amino acids with no particular bias. The ice-binding site is the amphipathic surface of the protein, a flat and relatively non-polar region encompassing a loop and parts of the C-terminal sheet. The non-polar groups accessible to the surface area fall within the range observed in most proteins and the folds of the protein bury an unusually high proportion of the hydrophobic residues in the core, limiting access of the extended hydrophobic areas to the solvent (Chao et al. 1994, Sonnichsen et al. 1993, Sonnichsen et al. 1996).

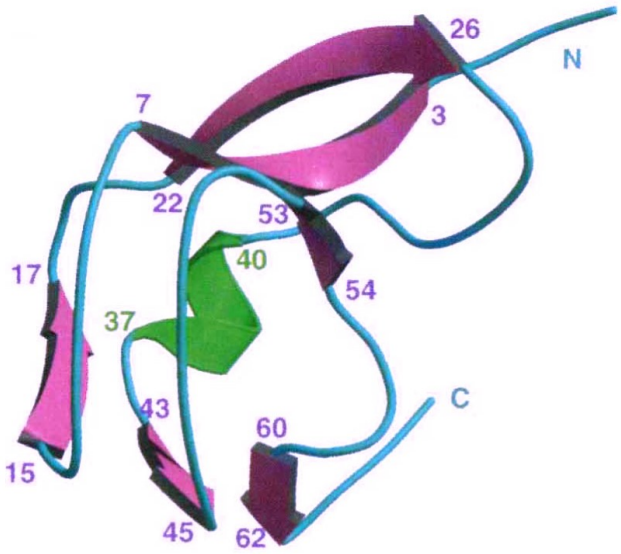


Figure 1.7 Structure of a Type III AFP. The ocean pout AFP structure is shown in a ribbon diagram with secondary structure elements labeled by amino acid number. β-sheets are showing in magenta, the one turn of α-helix is showing in green and unstructured and loop regions in aquamarine. Adapted from (Jia et al. 1996).

The ice-binding site is composed of residues 9, 15–18 and 44. The protein binds on the prism planes of ice, shaping the ice into a hexagonal bipyramid (Li et al. 1985, Sonnichsen et al. 1993). The varied residues of the binding site suggest that the high affinity of the Type III AFP binding for ice nuclei requires dual characteristics of the protein ice-binding surface, i.e. polar groups in proximity to non-polar groups, and non-polar-like hydration properties (Yang and Sharp 2004).

1.2.4 Type IV

The 12 kDa Type IV AFP is found in the longhorn sculpin (*M. octodecimspinosus*). It is produced by the liver and found in the serum and it is an α-helical protein with a 26% content of Glu/Gln and no Cys residues (Deng et al. 1997). It has been proposed that the protein has a four-helix bundle structure (Figure 1.8), based upon its alignment with plasma apolipoproteins to which it is homologous (Deng et al. 1997, Deng and Laursen 1998).

Type IV AFPs cause ice crystals to grow as hexagonal bipyramids or trapezohedra, presumably by binding to and inhibiting growth on the hexagonal bipyramidal surface, which is nearly identical to that seen for the Type I AFPs (Wen and Laursen 1992). The halves of the bipyramid are slightly rotated (20º) with respect to each other, consistent with a lattice match unlike any other AFP studied to date (Deng and Laursen 1998). Ice etching studies also showed that the protein produces sausage-shaped ice crystals, suggesting that the orientation of this protein on ice is unique among known AFPs.

Previous investigations of longhorn sculpin Type IV AFP, did not reveal sufficient freezing point depression to confer meaningful protection from freezing; serum concentrations of this AFP were estimated to be less than 10 μM. In previous studies, AFP activity was not directly evident in serum. Concentration of the chromatographic fractions during the purification procedure was required before antifreeze activity could be detected (Deng and Laursen 1998). A recent study of the Type IV AFP has questioned whether it functions as an AFP to depress the freezing point *in vivo*. The low concentrations of Type IV AFP together with the presence of substantial Type I AFP in the plasma of shorthorn and longhorn sculpin suggest that the Type I AFP is likely the protective AFP in these fish (Gauthier et al. 2008, Low et al. 2001).

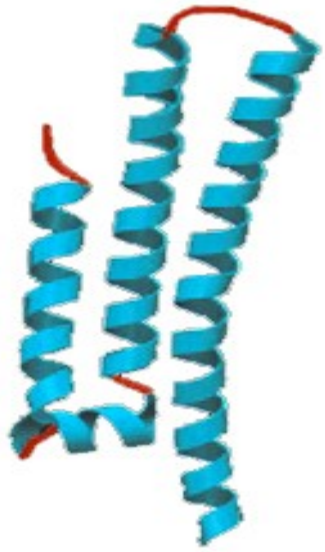


Figure 1.8 Structure of the Type IV AFP. Ribbon diagram of a model structure showing alpha helices in blue and loops and unstructured regions in red. Adapted from (Jia and Davies 2002).

1.2.5 Antifreeze glycoproteins

Antifreeze glycoproteins (AFGP) are found in the blood serum of Antarctic notothenioids such as *Pagothenia borchgrevinki* and Arctic and northern cods from the genera *Microgadus*, *Eleginus, Boreogadus, Gadus* and *Arctogadus* in the family Gadidae. They consist of a repeated tri-glycopeptide unit $(Ala\text{-}Ala\text{-}Thr)_n$, with minor sequence variations and the disaccharide β-D-galactosyl-(1→3)-α-*N*-acetyl-galactosamine joined as a glycoside to the hydroxyl oxygen of the Thr residues (DeVries 1971, Lin et al. 1972) (Figure 1.9).

There are 8 distinct classes of glycopeptides in notothenioids, which range in relative molecular mass from 33.7 kDa to 2.6 kDa and that are termed AFGPs 1-8. Furthermore, these AFPs have been classified as large AFGPs (1-5) and small AFGPs (6-8). Small AFGP 6-8 show minor variation in which the first Ala in some repeats is replaced by proline (Pro) (Burcham et al. 1984). Although the AFGPs have a similar primary structure, they exhibit significant size and sequence variation. In Northern cods, glycoproteins are remarkably similar to those present in notothenioids; however, Thr residues are occasionally replaced by Arg residues, resulting in the lack of a disaccharide in the glycopeptide in that position (Geoghegan et al. 1980, Hew et al. 1981). AFGPs are synthesized in the exocrine pancreas of Antarctic notothenioids, polar cods (*Boreogadus saida*) and Arctic cods (A*rctogadus glacialis*) from where they are secreted into the blood. Northern blot studies revealed the anterior stomach was the only additional site of AFGPs production in these fish (Cheng et al. 2006).

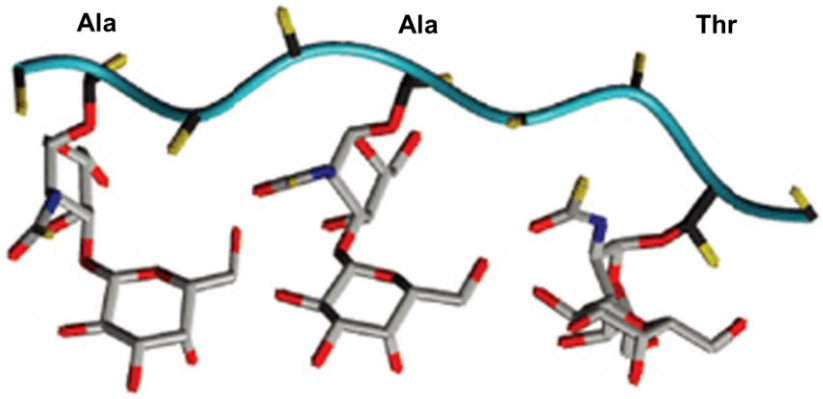


Figure 1.9 Structure of an AFGP. Diagram of a model of the of the tri-glycopeptide unit of AFGP showing the disaccharide attached to the Thr residue in stick form. Adapted from (Wilson 2004).

The molecular masses of AFGPs are relevant to their activity, since the longer polymers (AFGP 1-5) generate greater activity on a molar basis than the shorter polymers (AFGP 6-8). The small molecular mass forms (AFGP 7-8) comprise most of the circulating AFGP in nototheniids, but only show to contribute two thirds of the measurable antifreeze activity in the fish (Knight et al. 1984).

AFGPs appear to adopt a three-fold left-handed helix (also called polyproline Type II) with all the sugars aligned on one side. The hydrophobic peptide backbone and methyl groups form the other side of the unit (Bush et al. 1981, Bush et al. 1984, Bush and Feeney 1986, Feeney et al. 1986, Feeney et al. 1994). It has also been proposed that AFGPs can form a *γ*-turn structure. In this conformation, all the disaccharides are also on one side of the molecule; however, the opposite side of this structure is not hydrophobic, as it is in the case of the polyproline Type II model (Drewes and Rowlen 1993). Both *γ*-turn and polyproline Type II helix models have been proposed to account for an ordering of the carbohydrate moieties along the face of the protein to facilitate ice binding, and both solution-phase structures suggest that the AFGP molecule "pre-exists" in a conformation that allows maximum interaction between the disaccharide hydroxyls and the ice surface (Drewes and Rowlen 1993, Feeney et al. 1994). However, the relevance of hydroxyls in ice binding by AFGP may need to be revisited, given the discovery that methyl groups play the predominant roles in Thr binding to ice in Type I AFP (Baardsnes et al. 1999).

AFGPs accumulate on specific surfaces of ice crystals, modifying the rate and shape of crystal growth. Ellipsometry studies have shown that AFGP7 and AFGP8 accumulate on both the basal and prism planes of single ice crystals (Knight and DeVries 1994, Raymond et al. 1989). To date, the ice binding mechanism of AFGPs has not been determined. AFGPs have been studied very little in comparison to other AFPs with regard to ice binding. One reason for this is that it is impractical to produce

appropriate analogs for structural studies of AFGPs comparable to the mutational studies that have been performed for the other AFPs.

1.3 Antifreeze protein regulation

1.3.1 Seasonal variation in expression

The seasonal fluctuations of environmental conditions of a region are often reflected in the physiological responses of its inhabitants. For fish inhabiting freezing conditions, it is crucial to mirror the conditions they face. For instance, seasonal cycles of antifreeze protein production in fish inhabiting the North Atlantic are common among species. As a general rule, the levels of AFPs rise during the fall reaching a peak during February and March and decreasing during the spring months to reach a low in summer (Fletcher et al. 2001). The mechanisms that control AFP expression throughout the year are distinct in different species. The winter flounder and shorthorn sculpin, for example, produce AFPs well before seawater reaches its freezing point in the fall whereas cod begin producing their AFGP later in the season in response to temperature.

In the winter flounder, the liver-type Type I AFPs are produced in hepatocytes and the proteins secreted into the blood for transport to other tissues (Fletcher et al. 2001). Liver-type AFPs are under the inhibitory control of the growth hormone (GH) which indirectly blocks the transcription of AFP genes. During the warmer seasons, GH is released from the pituitary under the control of hypothalamic hormones, which are regulated by the central nervous system. In response to the shortening photoperiod during the fall, growth hormone ceases to be produced and liver AFP genes expression begins, continuing through the winter season (Fletcher et al. 2001). The AFP mRNA is also most stable at low, winter temperatures, allowing AFPs to build up in the plasma

during the winter season (Fletcher et al. 1985a, Idler et al. 1989). The negative regulation of AFP gene expression by GH implies that fish are only freeze resistant when they are not growing, which could present a challenge in aquaculture.

Seasonal variations in plasma AFP levels must be considered in the context of the tissue expression pattern of AFPs in general. In winter flounder, plasma (circulating) AFPs are produced primarily in the liver; however, as noted above, AFPs can also be found in other tissues such as skin, gills and pancreas (Cheng et al. 2006). Flounder skin AFPs, unlike liver AFPs, lack pre- and pro-sequences, suggesting that these AFPs could be intracellular (Murray et al. 2003). The skin has the highest level of detectable expression when compared to other exterior tissues such as gills and fins. Nonetheless, other studies support the contention that these AFPs are secreted through an atypical pathway to the extracellular spaces (Gong et al. 1992, Gong et al. 1996, Mignatti et al. 1992). The peptide profile of the plasma AFPs in winter flounder did not show any detectable skin type AFPs, which argues against the secretion of the skin type AFPs (Gong et al. 1996); however, in the snailfish, skin-expressed AFPs appear to make their way to the blood, implying secretion (Evans and Fletcher 2005a).

Interestingly, the expression of AFPs in the skin of winter flounder is not seasonally regulated, but constitutively expressed and thus inherently different from the expression of the liver type AFPs (Gong et al. 1995). Constitutive expression of plasma AFP's is also suggested in other species. In the sea raven and ocean pout, for example, AFPs fluctuate only marginally in response to sea temperature. And despite ocean pout showing a marked seasonal cycle of AFP in plasma, levels remain substantial all year round (Fletcher et al. 1985b, Slaughter et al. 1981).

The other species in which regulation has been well studied is the Atlantic cod (*G. morhua*). In contrast to the flounder, adult cod produce AFGPs in response to decreasing temperatures in the fall and early winter (Fletcher et al. 1987, Goddard and

Fletcher 2002). This correlation with water temperature has been demonstrated by a study that involved wild and cultured cod in a laboratory setting in which both types of cod produced equivalent AFGP levels in response to lower water temperatures as the winter season progressed (Purchase et al. 2001).

1.3.2 Geographic variation in expression

The levels and duration of AFPs in the plasma of fish during the winter season reflect the severity, duration and regularity of freezing conditions that the fish encounter. Therefore there are differences in the levels of AFPs between fish from different locations (Fletcher et al., 2001). In species such as flounder, there is a clear relationship between antifreeze proteins levels and freezing conditions. Winter flounder from Newfoundland exhibit high concentrations of AFPs in their plasma compared to winter flounder from Passamaquoddy Bay, New Brunswick and Nova Scotia, which produce lower concentrations (Fletcher et al. 1998). All flounder appear to respond to annual photoperiod variation influencing the timing of AFP production; however, there are differences among populations in their response to photoperiod cues. Flounder from Newfoundland develop measurable levels of AFP two months earlier than flounder from Nova Scotia, even when housed in adjacent aquaria under identical light and temperature conditions (Fletcher and Smith 1980). A further study indicated that flounder from Newfoundland and Long Island NY began producing AFP approximately two months earlier in the fall season than Passamaquoddy Bay NB flounder, which correlates with the timing of AFP requirement in the three locations with Passamaquoddy Bay water temperatures declining far more slowly than those at the other locations (Fletcher et al. 1985a).

Another example of antifreeze levels corresponding to water conditions is found in Atlantic cod (*G. morhua*). Higher levels of plasma AFGPs are found in juvenile cod populations from St. Lunaire, Newfoundland compared to three more southern populations (Goddard et al. 1999). It is obvious that all populations inhabiting the northeastern coast of Newfoundland are well equipped to deal with extreme winter conditions; however, the St. Lunaire population showed an earlier onset of AFGP production and greater levels of thermal hysteresis (Goddard et al. 1999). These observations can be explained by the fact that the most northerly populations are exposed to an extended period of exposure to lower water temperatures, reduced annual insulation, and later retreat of ice cover which involves more vertical mixing of the water column that carries ice crystals (Goddard et al. 1999).

1.3.3 Ontogenic variation in expression

Extreme environments would exert the greatest selection pressure, and thus, one might expect to see environmental and physiological coupling in terms of winter severity and levels of AFP production. For example, juvenile cod overwinter in shallow waters to avoid being eaten and by doing so they are exposed to colder temperatures, floating ice and ice crystals from the water column. In contrast, adult cod migrate to deeper waters during winter. So, it is not surprising that juvenile cod produce higher levels of AFGP than adults (Goddard et al. 1992, Goddard and Fletcher 1994). Furthermore, the production of AFGPs in juvenile cod occurs earlier in the season compared to the adults. Interestingly, while adult cod AFGP production is correlated with water temperature, juvenile AFGP levels are linked to photoperiod (Goddard et al. 1997). These ontogenetic

differences in AFGP production between adult and juvenile cod are related to their overwintering behaviours and the environments they inhabit.

A different example of ontogenetic variation occurs in the winter flounder. Murray et al. (2002) showed that the epithelial AFP gene expression in winter flounder occurs as early as hatching. This expression occurs in association with two types of larval integumental cells. A band of paired spindle-shaped cells that run along the lateral surface of the larva have detectable expression until the end of yolk sac absorption, suggesting that they are important during the early period of development. Also, epidermal mucous cells are produced during this time, suggesting that these cells could be secreting AFPs together with acid glycoproteins. These cells continue to show AFP expression until the time of metamorphosis (Murray et al. 2002).

In a different study, a period of development in which skin type AFPs cannot be detected in epidermal cells was reported (Murray et al. 2003). This observation suggests a lag time in the expression of skin type AFPs following the changes involved in the process of metamorphosis. Murray et al. (2003) also suggest that the initiation of epidermal skin type AFP gene expression may be regulated developmentally, rather than by environmental cues, as a preparation for exposure to cold water and ice.

1.4 The biological roles of antifreeze proteins

Despite their different structures and modes of regulation, the common role of the diverse AFPs is to prevent the freezing and subsequent death of the fish that produce them. AFPs recognize ice crystals, bind to them and prevent them from growing within the temperature range in which the fish live. The purpose is to keep the body fluids of the

fish in the liquid state by an efficient mechanism that does not require high concentrations of solute that could cause physiological perturbation.

There are two hypotheses put forth to explain how AFPs protect fish from freezing. One hypothesis, named ice-exclusion, suggests that fish employ a barrier protection system in their skins and gills including their digestive systems. This barrier protects the tissues from ice crystals in seawater by stopping them from growing across the tissues. This hypothesis is based on the presence of AFPs in intracellular and extracellular spaces in the skin and other tissues of winter flounder (Gong et al. 1996). It is also supported by experimental work. In fish that live in icy waters, epithelia were found to contain elevated concentrations of electrolytes, which would act colligatively, as well as the non-colligative AFPs at levels sufficient to reduce the freezing point and impede ice propagation (Valerio et al. 1992). However, even if this barrier protects the fish from ice crystals, the skin would have to be in perfect condition for it to be fully effective, meaning that damaged skin would bring the risk of freezing. Also, it seems unlikely that this barrier would fully protect the skin and tissues from ice crystals which can form inside the organism. Whether or not freezing takes place is determined to a large extent by ice nucleation that initiates ice growth.

The other hypothesis suggests that ice crystals do enter the fish body or form within it and that the AFPs would bind to them to prevent them from growing further, thus preventing freezing of the fish. The binding of AFPs to small ice crystals within the fish would be expected to stabilize them, preventing their growth. In the presence of AFP, ice crystals remain the same size for hours or days at temperatures between the colligative freezing point (melting point) and the lower non-equilibrium freezing point (Knight and Wierzbicki 2001, Knight et al. 2001, Raymond and Devries 1977, Wilson 1993). Therefore, long-term protection from freezing could result from AFP-ice interaction within the fish. Two lines of evidence support this hypothesis. First, it was shown that ice-

exposed fish have an immune response to ice crystals that is evident from the presence of immunoglobulins in their serum that recognize ice (Verdier et al. 1996). In contrast, immunoglobulins from tropical and freshwater species that would not have been exposed to ice did not recognize ice (Verdier et al. 1996). An immune response to ice would necessitate the presence of ice within the tissues or the circulation of the fish. Further support for this hypothesis was drawn from freezing experiments in which Antarctic fish were shown to freeze to death when placed in ice-free water below their freezing points. This could not occur if ice were not already present within the body of the fish (Cheng and DeVries, 1991 cited by (Ewart 2002)).

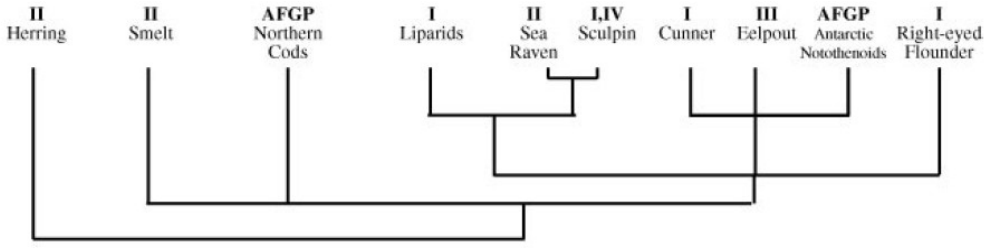
It has been suggested that AFPs also prevent ice formation (ice nucleation) in fish. Wilson and Leader (1995) have suggested that AFPs act by binding directly to heterogeneous ice nucleation sites and thereby masking them. Furthermore, they suggest that in fish, supercooling of only 1-2 °C below the freezing point would make homogenous nucleation unlikely. Thus, AFPs would stabilize supercooled fluids of the fish by binding to both ice nucleators and pre-existing ice crystals (Wilson and Leader 1995).

Roles at cold temperatures above the freezing point have been suggested for AFPs. The AFPs are suggested to interact with cell membrane components, thereby stabilizing them and reducing cold damage at low temperatures above 0 °C. Ion channel leakage normally occurs upon cooling of cells beyond their limits of adaptation (Hochachka 1988). Interaction of AFPs with ion channels was reported to be a mechanism of cell protection (Hochachka 1988, Rubinsky et al. 1992). It was subsequently suggested that AFPs interact directly with membrane phospholipids to minimize packing irregularities during phase transition in cell membranes, thereby preserving the stability of the cell membranes mechanisms at low temperatures (Wu and

Fletcher 2000). Activities of AFP at cold, but non-freezing, temperatures remain unclear and will likely be an ongoing area of investigation.

1.5 Origins of AFP genes in fish

Teleost fish represent the most recently evolved group of fish, originating during the Triassic period, 200 million years ago. It is believed that they arose from the holosteans, replacing them by the beginning of the Cretaceous, approximately 130 million years ago. Teleost fish resembling their modern counterparts appeared to have existed since the Eocene about 40 million years ago (Greenwood et al. 1966). The teleost emergence occurred during an era in which temperatures fluctuated between 6 °C and 14 °C (Kerr 1984). Later, a gradual cooling occurred around the Cretaceous period, with rapid intensification that lasted until the Eocene and Oligocene eras. The first hemisphere glaciation in the North Atlantic appeared 2.5 million years ago during the Cenozoic (Shackleton et al. 1984). With the approach of the cooling era, teleost fish were exposed to water temperatures that were below their serum freezing levels and this environment would have imposed strong selection for mechanisms of freezing point depression. It appears logical that AFP function would have evolved in a variety proteins during this time (Cheng 1998, Greenwood et al. 1966). The recent evolution of AFPs is known as the “sea level glaciation hypothesis”, and accounts for the diversity of AFPs due to the strong selective pressure brought by cold exposure (Scott et al. 1986) (Figure 1.10).



AFP type	Ancestral protein function
I	unknown
II	C-type lectin
III	unknown
IV	apolipoprotein
AFGP	Trypsinogen-type serine protease

Figure 1.10 Distribution of teleost AFPs. Adapted from (Fletcher et al. 2001).

1.5.1 Mechanisms of AFP emergence and evolution

Genes can acquire new functions by acquiring changes in their amino acid coding sequence, and also by changes in gene regulation which can lead to expression of proteins in different tissues or stages of development (Carroll 2001). The phenomenon of gene duplication, resulting in two or more separate copies is often essential in the creation of new functions, allowing at least one of the gene copies to evolve while preserving the essential function of the original gene. Therefore, the duplication of pre-existing genetic information provides the raw material from which new genes and its functions can evolve, contributing to the evolution of genetic complexity and the evolution of new sophisticated life forms. Genes derived from a common progenitor will still share regions of sequences in terms of gene identity and the order in which such sequences

are located within the chromosome of their ancestral gene (Taylor and Raes 2004). However, it is also argued that the evolution of new gene functions can take place with or without gene duplication and that the manner in which duplication and acquaintance of novel functions takes place vary according to the evolutionary trajectory (Long 2001).

A clear example of endogenous change within a gene to produce a completely new protein was discovered in *Dissostichus mawsoni* by Chen et al. (1997b). The novel portion of the AFGP gene, which encodes the ice-binding function, was derived from the recruitment and iteration of a small region spanning the boundary between the first intron and second exon of the trypsinogen gene. Later, this novel region was expanded and duplicated, possible by replication slippage or unequal crossing over, producing 41 tandemly repeated segments, with sequences at either end that are nearly identical to the trypsinogen gene (Chen et al. 1997a). The small sequence divergence between notothenioid AFGP and trypsinogen genes indicates that the evolution of this gene into the novel ice-growth inhibition gene occurred about 5–15 million years ago, which is consistent with the estimated times of freezing of the Antarctic Ocean.

The AFGPs also present a remarkable case of convergent evolution. Cloning and sequencing of the AFGP gene of Arctic cod (*Boreogadus saida*) revealed that it bears no relationship with the *D. mawsoni* gene for AFGP and that it contains no trypsinogen elements (Chen et al. 1997b). However, the proteins are so similar that a DNA probe from the Antarctic AFGP protein-coding sequence was used to retrieve the Arctic cod AFGP gene. More subtle convergent evolution in pre-existing C-type lectins was postulated to explain the emergence of the Type II AFPs in herring, smelt and sea raven, which are fish belonging to separate orders. They appeared to have undergone minor changes allowing the encoded proteins to bind ice instead of carbohydrate or $CaCO_3$ crystals. These proteins may also represent unusual evolution, as examination of the proteins and gene organization in these fish revealed that these three AFPs share

a common origin separate from the fish C-type lectins and further analysis suggested their emergence in these disparate orders by lateral gene transfer (Graham et al. 2008a).

Besides the sea raven Type II AFP, the cottid AFPs appear to involve both convergent and parallel gene evolution. Both the shorthorn sculpin (*M. scorpius*) and its relative, the sea raven are cottids; however, they produce different types of AFPs as noted in section 2 and above (Figure 1.10). Furthermore, the co-occurrence of Types I and IV AFP in the longhorn sculpin reveals that multiple ice-active proteins can be present within a species, although it is not clear that all contribute to meaningful freeze protection. The evolutionary origins of Type I AFPs remain unresolved, although evolution of the snailfish AFPs has been postulated to result from frameshift mutations in related Ala-rich genes, as noted in an earlier section (Evans and Fletcher 2005b). Given the diversity of the Type I AFPs, they may have evolved by convergent evolution in different fish orders, similarly to the AFGPs described above. (Hew et al. 1980, Low et al. 1998, Low et al. 2001).

The Type I AFP poses multiple questions with respect to evolution, as there are liver-type and skin-type short AFPs, and the Maxi AFP in flounder. The skin and liver AFPs were both characterized by a high degree of Ala residue conservation and Thr-based sequence repeats, as is the AFP of shorthorn sculpin (Low et al. 1998) . It has been hypothesized that skin-derived AFPs originated first and subsequently gave rise to the liver derived plasma AFPs (Cheng and DeVries 2002). Evans et. al (2005b) proposed that the same evolutionary mechanism described for snailfish AFPs may be applicable to Type I AFPs of other fish species. Furthermore, the snailfish AFP-encoding genes shared sequence characteristics with structural proteins found in fish eggs, as well as epithelial proteins such as fish type II keratin; and it was suggested that these proteins are the progenitor proteins of Type I AFPs in snailfish and that the Type I AFP arose in these fish by a frameshift mutation (Evans and Fletcher 2005b). Whether

winter flounder and sculpin AFPs are derived through convergent evolution from two unrelated ancestral sequences remains uncertain. At the genomic level, there is evidence of multiple isoforms which only differ by a few amino acids, suggesting these small differences may be the result of genetic drift. However, the isoforms from the winter flounder show greater divergence including contrasting tissue specificity, indicating functional divergence. The tandem amplification found in winter flounder also suggests a relatively rapid evolutionary event contributing to the dominance of isoforms wflAFP-6 and wflAFP-8 in the serum (Scott et al. 1985). These isoforms are encoded by 1 kb genes in 7-8 kb direct tandem repeats suggesting they were kept homogenous by unequal crossing over or gene conversion; however, length polymorphisms within the tandem repeats indicate that the homogenization or conversion between repeats is not absolute (Davies 1992).

Types III and IV AFPs are the ones most likely to have arisen by straightforward duplication and divergence, as both have clear homologs in fish that predict selection on a duplicated gene leading to ice-binding activity in one copy. Therefore, overall, the evolution of the AFPs has no common thread except their being relatively recent. It would appear that there are many ways of evolving an AFP for freeze protection.

1.5.2 AFP gene amplification

The levels and cycles of AFPs in the plasma in a fish species generally reflect the severity, extent and regularity of freezing conditions that the fish encounter through its life history. In nature, fish that show significant thermal hysteresis activity, have large gene families encoding their AFPs, which translates to higher production of AFPs. It seems logical to explain this gene amplification in response to intense evolutionary

selective pressure driven by northern hemisphere Cenozoic cooling (Scott et al. 1985). The production of high levels of a protein can occur by two means. One is to have a single gene with a very strong promoter region, driving high levels of transcription. The other possibility is the presence of multiple copies of a gene that are all transcribed at once, such that levels of mRNA are high even if transcription from any particular gene copy is low or moderate. Fish that produce AFPs have evolved to have very large multi-gene families encoding the small AFPs in their genome, allowing them to produce high levels of AFP in the liver, which is secreted into the plasma, as well as skin AFP in lower amounts (Scott, 1988; Gong et al., 1995). Since AFPs lower the freezing point of fish blood by binding to ice crystals, it is particularly important that they are efficient in doing so. Flounders have also evolved to produce the Maxi AFP that is more efficient, producing higher thermal hysteresis activity (Graham et al. 2008c, Marshall et al. 2004), hence eliminating the need of high amounts of protein. As a result, this protein may require fewer gene copies to confer an effective advantage.

Modest semi-quantitative studies have suggested that AFP gene copy numbers (i.e. gene dosages) differ widely among populations inhabiting different locations along the east coast of North America (Hayes et al. 1991, Scott et al. 1985). Overall, those early results sug gest that maximal winter AFP activity in plasma is directly proportional to the dosage of the liver-expressed AFP genes, which directly relates to the severity of winter temperatures in different locations(Hayes et al. 1991, Scott et al. 1985). In species such as winter flounder and ocean pout, there is a clear relationship between antifreeze gene dosage, antifreeze proteins levels and freezing conditions (Fletcher et al. 2001, Hew et al. 1988).

In winter flounder, genes encoding the liver and skin-type AFPs, which are all Type I AFPs, are estimated to have copy numbers ranging from approximately 10 AFP genes in yellowtail flounder (Scott et al. 1988) to 30-40 copies in the winter

flounder(Gourlie et al. 1984, Scott et al. 1985). Liver AFP genes appear to be tandemly repeated and skin-type AFP genes appear to be linked but regularly spaced (Davies et al. 1984, Gong et al. 1992, Scott et al. 1985). Gene duplication of the primordial skin-type AFP gene and subsequent divergence may have given rise to the two distinct genes: liver type and skin type. The skin type appears to have gained a signal peptide sequence (pre-sequence), pro-sequence and liver regulatory elements to become a liver-specific gene encoding secreted AFPs (Cheng and DeVries 2002).

Winter flounder populations from Newfoundland waters generate high concentrations of AFPs in their plasma compared to yellowtail flounder populations inhabiting the same region. This difference corresponds to their different overwintering biology. Winter flounders live in shallow waters making them more susceptible to freezing conditions, whereas yellowtail flounder live in deeper waters where ice crystals grow and accumulate less readily (Fletcher et al. 1985a).

A further example of multigene families and relationships among antifreeze gene dosage, antifreeze proteins levels and freezing conditions is found in the eel pouts. In the ocean pout, high levels of plasma AFPs are found during winter in populations of Newfoundland compared to low levels of plasma AFPs found in ocean pout populations from New Brunswick. This difference corresponds with the ice exposure of these fish; New Brunswick populations are in warmer waters with less ice exposure than their counterparts in Newfoundland (Fletcher et al. 1985b, Hew et al. 1988). Furthermore, gene copy numbers found in ocean pout (Type III AFPs) for example, belong to multigene families with approximately 40 copies in the New Brunswick fish and approximately 150 copies in the Newfoundland fish (Hew et al. 1988). These genes are not organized in tandem repeats, but are linked and irregularly spaced. The antifreeze protein genes of the wolffish (*A. lupus*) constitute a large multigene family of 80 to 85 copies, which can be classified into two sets. One-third of the genes are linked but

irregularly spaced, the other two-thirds are organized as 8 kb tandem direct repeats with each containing two genes in inverted orientation (Scott et al. 1985).

In the sea raven (AFPs Type II), 12-15 copies of the AFP genes have been identified. There is evidence that these genes contain 5 introns spanning 2.2 kb and based on restriction maps of genomic clones, a second copy is found within at least 25 kb of the flanking DNA (Hayes et al. 1989). The smelt and herring Type II AFPs are also encoded by multigene families, as shown by protein and cDNA microheterogenity (Ewart, unpublished results).

The amplification of genes encoding multiple AFP types and in distinct species of fish suggests that there has been strong selection for freeze resistance in north temperate fish species exposed to ice crystals. The best known example of this sort of gene amplification is the esterase genes of insects exposed to organochloride pesticides (Guillemaud et al. 1999) and the salient feature of the esterase example is the high intensity of the selective pressure.

1.6 Summary and key questions

Teleost fish that inhabit polar and north temperate oceans rely on the protection of AFPs to avoid freezing in icy seawater. AFPs lower the melting point of a solution colligatively according to their concentration; however, the interaction of AFPs with ice crystals also generates a non-colligative freezing point depression that far exceeds the depression predicted from the colligative properties alone. AFPs bind to ice crystals and inhibit crystal growth by limiting ice growth to convex zones at the exposed areas between the adsorbed AFPs. This results in a difference between the freezing and melting points that is called thermal hysteresis.

The primary goal of this study was to determine the copy numbers of the genes encoding plasma AFPs in winter flounder populations and examine related questions in order to more fully understand the predictive value of gene copy number data. Previous semi-quantitative studies have shown that AFP gene copy numbers vary among populations and that AFP activity in plasma is proportional to gene dosage. However, little is known about the diversity and gene dosage of AFPs within or between fish populations. In order to begin understanding the gene dosage variation within a species for AFP, we will determine gene copy numbers for geographically distinct populations using a quantitative method and consequent plasma AFP levels.

This research project comprises four objectives: (1) to develop qPCR assays for the winter flounder AFP genes that could be implemented in a laboratory setting, (2) to determine the AFP gene copy numbers in winter flounder populations along the Western Atlantic Ocean using the qPCR assay, (3) to measure AFP activity in a set of the same fish to determine whether the gene dosages predict plasma antifreeze activities, and (4) to gain insight into the gene organization of the newer hyperactive AFP (Maxi) by Southern blotting, in order to more fully interpret our results. The findings generated in this study will improve our knowledge on freeze resistance in winter flounder. This study will provide the molecular assays for future testing by other groups seeking to assess genetic freeze resistance capacity in winter flounder and, more importantly, it will broaden our understanding on antifreeze gene evolution and the genetic adaptation of winter flounder to diverse environments.

Chapter 2

Design and implementation of a set of quantitative PCR assays for determining antifreeze protein gene dosage in winter flounder (*Pseudopleuronectes americanus*)

In winter flounder, there are a variety of distinct AFPs and these are encoded by genes that occur in multiple copies in the genome (Gong et al. 1996, Scott et al. 1988, Yun et al. 2006). The gene copy status of AFP genes in these fish is of interest because it is believed to be an important factor in their survival, as suggested by their apparent copy numbers in fish inhabiting colder waters. Initial studies in winter flounder in which AFP gene copy number, plasma antifreeze activity levels and regional environmental freezing conditions were compared, suggested that AFP activity in plasma is proportional to gene dosage and that this dosage reflects different selection intensity in different locations (Fletcher et al. 2001, Scott et al. 1988). In these studies, gene copy numbers were determined using genomic Southern blots which are technically demanding and require considerable amounts of DNA. Therefore, sample numbers were very small, with as few as 1-2 fish being used. In addition, Southern blotting analysis offers only semi-quantitative analysis of gene amplification over a modest dynamic range. Thus, although the discovery of copy number elevation and variation in flounder was ground-breaking, further investigation of copy number variation required a more quantitative and efficient process for gene copy measurement.

In the decades since AFP gene copy numbers were first investigated in flounder, new technology has become available for these measurements: the quantitative polymerase chain reaction (qPCR). Quantitative PCR (qPCR) is a method used to detect and quantify changes in gene expression (by cDNA abundance) or gene copy number (by gene abundance), and has been employed in a wide variety of studies in disciplines ranging from medicine to ecology.

The process central to qPCR is the PCR reaction. Sample preparation, controls and the detection of amplified products are conducted differently in order to allow the measurement of a quantity of a specific DNA molecule present in a sample with a high degree of sensitivity and accuracy (Ding and Cantor 2004). As for regular PCR, qPCR relies on the amplification of a gene of interest with target-specific primers. In its simplest form, the fluorescence resulting from double stranded DNA is measured after each amplification cycle of PCR by the use of fluorescent markers such as SYBR Green I (Morrison et al. 1998), although technologies exist for more complex product detection employing, for example, labeled primers or probe sequences (Gasparic et al. 2010). Under ideal conditions the target products accumulate exponentially in the reactions. Thus, qPCR can be applied to determine a fixed threshold at which the accumulation of PCR product is first significantly detectable over a real-time measurement background signal (Higuchi et al. 1993). The fractional cycle number at which PCR product accumulation passes this fixed threshold is called the threshold cycle (Ct) (Livak et al. 1995, Sambrook and Russell 2001) and this value can then be used to determine the initial concentration of a DNA target from an unknown sample.

qPCR quantitation methods are usually classified as relative or absolute (Wong and Medrano 2005). Relative quantitative PCR measures the differences in abundances of the target DNA between samples without determining the actual abundance of the target, by focusing only on determining the relative change between the reference and the target sample (Livak and Schmittgen 2001). For this reason, relative quantitation is mostly used in expression studies, where the changes in gene expression of a sample are calculated based on external standards or reference samples also called calibrators (Livak and Schmittgen 2001). The results are expressed as a target/reference ratio.

On the other hand, absolute qPCR allows the precise quantitation of the target DNA. The standard curve method employs a standard curve which is generated by

amplifying a dilution series of known concentrations of a plasmid containing the target DNA or gene; these internal standards can also be made of a PCR amplicon, a synthesized oligonucleotide, or cDNA (Whelan et al. 2003). Plasmid DNA is the preferred standard in qPCR assays due to its high stability and reproducibility (Wong and Medrano 2005). The standard curve produces a linear relationship between Ct and initial amounts of total DNA allowing the determination of the concentration of the unknowns based on their Ct values. The plasmid copy numbers can be calculated based on the sequence of the plasmid and target insert using the number of base pairs, the mass of the DNA, and the AVOGADRO's number (Whelan et al. 2003, Yun et al. 2006). For the determination of the final target copy numbers, a normalization between the control and the target gene can be used as long as the PCR efficiencies are similar, but in cases where this normalization cannot be applied because of different PCR efficiencies between standards and targets, or other PCR artifacts, the calculation can be done on a per genome basis, assuming that the genome size of the species studied is known. Because the concentration of the standard is represented as copies per microliter, it is possible to estimate the absolute number of target genes in the sample (Wilhelm et al. 2003, Yun et al. 2006).

In this study, assays for the absolute quantification of four AFP genes in winter flounder were implemented by applying the standard curve method using a standard curve of known quantities and the genome size (representing the total number of base pairs in the genome) for calibration. Our aim was to enable fast and accurate assessment of AFP genes in winter flounder samples.

2.1 Materials and Methods

2.1.1 Sample collection

For AFP gene amplification and cloning, Nova Scotia winter flounder blood samples were obtained from fish kept in tanks at seasonally ambient conditions of temperature and photoperiod at the former NRC Institute for Marine Biosciences Marine Research Station near Ketch Harbour, Nova Scotia. Prior to sampling, the fish were anaesthetized with MS-222 (tricaine methane sulfonate) according to the CCAC guidelines. Blood was withdrawn by needle from the caudal blood vessel into a vacutainer containing heparin. All samples were collected at 4 °C, centrifuged to separate red blood cells and plasma and stored at -80 °C until their use.

For DNA genome size determination, winter flounder were sampled from Eastern Newfoundland, St. Lawrence Estuary, Baie des Chaleurs, Passamaquoddy Bay, and Georges Bank.

2.1.2 DNA Isolation

Total flounder DNA was isolated using a commercially available Puregene Tissue DNA isolation kit (Gentra) which was adapted for the purification of DNA from 15 μL of nucleated red blood cells. Samples were thawed quickly and then subjected to DNA extraction according to the manufacturer's protocol. In brief, the cells were lysed and then subjected to RNAse treatment, followed by protein and DNA precipitation and DNA hydration. DNA concentrations were determined spectrometrically using a NanoDrop ND-1000 UV spectrophotometer (NanoDrop Technologies) and aliquots of 100 and 50

ng were prepared for use in qPCR analysis. Purified genomic DNA (gDNA) was stored at 4 °C until further use. All gDNA was further analyzed by gel electrophoresis.

2.1.3 Oligonucleotide primer design and gene cloning

Suitable primers for amplification of genes encoding the four major serum AFPs (wflAFP-6, wflAFP-8, wflAFP-9 encoded by the gene 21a, Maxi) were designed using the software program Primer3 (Rozen and Skaletsky 2000). The sequences of each target gene were obtained from GenBank, a database provided by the National Center for Biotechnology Information (see Table 2.2 in the Results). Primers for AFP genes were obtained from Integrated DNA Technologies.

Individual reactions to test for each AFP gene type were done following the conditions used in qPCR analysis. The reactions were performed in a 96-well plate, with a volume of 20 µL per reaction. Individual reaction mixtures contained 10 µL of iQ SYBR Green Supermix (Bio-Rad), 6 µL water (Sigma, molecular biology grade), 1 µL of forward primer at 10 µM, 1 µL of reverse primer at 10 µM and 2 µL of gDNA at 50 ng/µL. The cycling parameters were adjusted according to base composition and GC content from target genes. The reaction conditions are shown in the table below:

Table 2.1 PCR conditions for amplification of each type of AFP gene.

Number of Cycles	Temperature	Time	Step
1	95 °C	30 sec	
2	95 °C	30 sec	Well factor collection
1	95 °C	10 min	Denature
40	95 °C	30 sec	Denature
	70 °C	30 sec	Annealing for wflAFP-6*
	72 °C	1 min	Extend
	92 °C	30 sec	Data Collection
1	95 °C	1 min	Melt All
1	55 °C	30 sec	Re-anneal All
84	55 °C + 0.5 °C/cycle until 95 °C	10 sec	Melt Curve Analysis

*Annealing temperatures for other genes were as follows: 70 °C (wflAFP-6), 65.7 °C (wflAFP-8); 70 °C (wflAFP-9); 62 °C (Maxi).

Following the amplification of templates, a melt curve analysis starting at 55 °C with the addition of 0.5 °C every cycle until reaching 95 °C was performed to confirm the amplification of single products. During the melt curve analysis, the SYBR Green I dye binds to double stranded DNA in a sequence-independent way and fluoresces, so there is a risk that non-specific products can be detected; however, the melting process of double stranded DNA causes a sharp reduction in the fluorescence signal around the melting temperature (Tm) of the PCR product, resulting in a clear peak in the negative derivative of the melting curve. This results in different fragments with specific melting temperatures appearing as separate peaks, which indicate the presence of multiple products if they occur.

The acquired DNA fragments were then cloned into pCR4 TOPO vector, plasmid purified with a QIAprep Miniprep kit (Qiagen) and confirmed by sequencing (GENEWIZ) both strands of at least 10 clones. Sequences were aligned using Sequencher software (Gene Codes Corporation) and the BLAST algorithm (Altschul et al. 1990) was used to

compare all sequences with those found in the GenBank database. Dilution curves were generated and used to assess efficiencies of the reactions for each gene with the appropriate primer sets.

2.1.4 Standard curves in qPCR

Plasmid constructs were made by inserting a single fragment of target AFP gene (wflAFP-6, wflAFP-8, wflAFP-9, Maxi). The PCR products from all target genes were individually ligated into a pBluescript II KS plasmid vector according to the manufacturer's instructions. The ligation mix was then transformed into competent *E. coli* (DH5α) and positive transformants were identified by blue/white screening. Five mL LB-broth cultures containing single colonies were grown overnight, with shaking at 200 rpm at 37 °C. The resulting recombinant plasmids were purified from the transformed *E. coli* (DH5α) cell cultures using a Qiagen Plasmid Purification Kit (Qiagen, Carlsbad, California). Plasmid DNA was quantified with a NanoDrop ND-1000 UV spectrophotometer (NanoDrop Technologies), and diluted in water to generate samples ranging from approximately 100 to 5 ng/μL. The 5 ng/μL dilutions were stored at -20 °C in single-use aliquots. The size of the cloned amplicons was confirmed by analysis of extracted plasmid DNA using *Eco*RI digestions and gel electrophoresis. All plasmids with inserts were sequenced for confirmation (GENEWIZ). Supercoiled plasmids were used to create standard curves for qPCR by creating a ten-fold dilution series starting at 1 ng. All products synthesized by plasmid standards were verified by electrophoresis in a 2% agarose gel containing ethidium bromide.

The copy number of each plasmid construct was calculated using the OD_{260} value and the DNA sequence. The molar concentration of the standard DNA was then

calculated using the OligCalc oligonucleotide properties calculator (Kibbe 2007), and then converted into copy number of DNA molecules per unit volume (Whelan et al. 2003).

2.1.5 qPCR analysis of AFP genes

After confirmation of DNA products, qPCR analysis was performed using an iCycler IQ real time PCR instrument (Bio-Rad). The qPCR parameters followed were those outlined in the iCycler PCR instrument's manual under universal cycling conditions of 40 cycles with SYBR Green-based detection of double stranded DNA. The reactions were performed in a 96-well plate, with a volume of 20 µL per reaction. PCR conditions were the same as mentioned in the primer design and gene cloning section (Table 2.1). All reactions were performed in triplicate, except for the standards which were prepared as a single dilution series. Initial data organization and Ct determination was performed using iCycler software. Ct values were exported to an Excel spreadsheet (Microsoft) for further data processing.

2.1.5.1 Determination of flounder genome size by flow cytometry

Five winter flounder blood samples from each location studied (Eastern Newfoundland, St. Lawrence Estuary, Baie des Chaleurs, Passamaquoddy Bay, and George's Bank) were obtained. The 500µL of whole blood was collected from the caudal blood vessel of the fish with a vaccutainer and needle and immediately mixed with an equal volume of dimethyl sulfoxide (DMSO) solution (CycleTEST™ PLUS DNA Reagent

Kit, Becton Dickinson). Five aliquots of 200 µL were separated from the main sample obtained from each fish sampled and kept at -80 °C until final use.

At the laboratory, samples were thawed and immediately prepared for propidium iodide staining following the manufacturer's instructions manual (CycleTEST™ PLUS DNA Reagent Kit, Becton Dickinson). In summary, blood cells were resuspended three times in 1 mL of Buffer Solution (containing DMSO), counted using a hemacytometer and adjusted with Buffer Solution to 1 x 10^6 cell/mL. All samples were centrifuged for 6 minutes at 1600 rpm at room temperature (20 °C to 25 °C) in swinging bucket centrifuge and without disturbing the pellet the supernatant was removed. The last drop was used to resuspend the blood cells a little bit by tapping with fingers. The cells were then resuspended with cold 70% EtOH by gently vortexing while adding the EtOH. All samples were kept at -20 °C until Flow Cytometry analysis.

Samples of blood cell suspensions of 70% EtOH were centrifuged at 1600 rpm for 6 minutes at room temperature (20 °C to 25 °C) in swinging bucket centrifuge. All the supernatant was carefully removed including the last drop by tapping it off onto a tissue. The blood cells were then treated for 10 minutes with 250 µL of Solution A (containing trypsin and spermine tetrahydrochloride detergent buffer) and gently mixed. This step allows for the enzymatic disaggregation of the solid tissue fragments and digestion of cell membranes and cytoskeletons. Without removal of Solution A, 200 µL of Solution B (containing trypsin inhibitor and ribonuclease A in citrate stabilizing buffer with spermine tetrahydrochloride) was added, gently mixed, and left to work for 10 minutes to inhibit the trypsin activity and to digest the RNA. After this step, without removal of Solutions A and B, the remaining nuclei were stained for flow cytometric analysis by adding 200 µL of Solution C (containing propidium iodide stain solution). The solution was gently mixed and incubated in the dark at 2-8 °C and analyzed by flow cytometry in order to determine staining intensity (Hickey and Clements 2005, Peruzzi et al. 2005).

Chicken blood cell nuclei (BioSure®) were used as a reference standard, as those cells have a known genome size. Equal numbers of cells of each fish and chicken reference nuclei were filtered through a 35 µm nylon mesh (Round-Bottom Tube, BD Falcon™). The DNA content of the winter flounder nuclei was determined by comparing their staining intensity to that of the chicken nuclei in a FACSAria II (BD Biosciences) flow cytometer (fluorescence-activated cell sorter, FACS). All preparations were used without delay (Hickey and Clements 2005, Peruzzi et al. 2005). Samples were excited at a wavelength of 488 nm and emission was measured at 617 nm. Fluorescence was measured on 10,000 cells per sample and the cell fluorescence values representing each winter flounder sample were compared to the chicken cell fluorescence values by using this formula:

DNA Index= (Mean unknown X 1.25 pg chicken) / Mean chicken

Data obtained was further analyzed in MiniTab to check the cell distributions. In addition, a Kruskal-Wallis test was performed using the R statistical analysis program to determine if there were differences in genome size among locations, using $p < 0.5$ as the significance threshold.

2.1.5.2 AFP gene copy number on a per genome basis

AFP gene dosage was achieved by calibration of the data using a plasmid construct (which included the target gene insert) as the internal control (Whelan et al. 2003, Yun et al. 2006). This allowed the determination of the starting gene copy number in winter flounder gDNA samples on a per genome basis (Wilhelm et al. 2003, Yun et al.

2006). To follow this approach we used the calculated winter flounder genome size obtained by flow cytometry, the starting quantity which represents the number of amplicons in the reaction, and the Ct value (both calculated by iCycler iQ software).

During qPCR the threshold cycle number (Ct) is automatically calculated by the iCycler iQ program under the ‘‘PCR baseline subtracted’’ option, the standard curve is generated as linear regression between Ct and $\log_{10}$ starting copy number of standard DNA, the iCycler iQ program also calculates the amplification efficiency (E) of the standard DNA from the slope of the standard curve: $E=10^{(-1/slope)}-1$, and the differences in Ct values are calculated as the average Ct difference across serial dilutions

Plasmid copy number was calculated using the following equation (Whelan et al. 2003):

$$= \frac{6.02 \times 10^{23} \text{ (copies per mol)} \times \text{DNA amount (g)}}{\text{DNA length (bp)} \times 660 \text{ (g/mol/bp)}}$$

Where 6.02×10^{23} is the Avogadro number and 660 is the mean molar mass of a base pair.

Absolute AFP gene copy number was calculated as follows:

1. Genome copies in reaction =

$$\frac{\text{100 ng genomic DNA}}{\text{wf genome size (ng)}}$$

2. Quantity of amplicon per genome copy (ng) =

$$\frac{\text{mean of starting quantity of amplicon in reaction (ng)}}{\text{genome copies in reaction}}$$

3. Gene copy number =

$$\frac{\text{quantity of amplicon per copy (g)} \times 6.02\text{X}10^{23} \text{ copies per mole (Avogadro)}}{\text{molecular mass of amplicon (g)}}$$

2.2 Results

2.2.1 qPCR primer design and evaluation

The first goal of the project was to develop a robust primer system for the precise and reliable quantitation of AFP genes. Special attention was paid to the melting behaviour of each amplicon representing each type of AFP target gene with regards to temperature inhomogeneities. Primers were designed within the second exon sequence and the periphery of the only intron of the AFP genes. Each set of primers produced a single product as shown by the gel electrophoresis and melting curve analysis. Additionally, each product obtained by PCR using each set of primers designed for specific AFP genes was cloned and sequenced (20 clones, both strands) for verification. The sequences showed that, in every case, only specific products corresponding to each target AFP gene were obtained with these primers. Primers created for AFP target genes are shown in Table 2.2.

Table 2.2 AFPs gene primers for qPCR. F: Forward primer, R: Reverse primer.

Target Gene	Primer Sequence (5'→3')	Length (nt)	Product Size (bp)
wflAFP-6	F: GCAGAATCACTGAAGCCAGCCC	22	151
	R: GTGAGTTCGGCAGCGGCTTT	20	
wflAFP-8	F: AATCACTGAAGCCAGACC	18	213
	R: CATCAAGACGACCACGATCC	20	
wflAFP-9	F: GCCCCAGACACCGCCTCTGATG	22	103
	R: CTGCGGCTTTGGCAGCGGTGACTG	24	
Maxi	F: CATCACTGAAGCCAACAT	18	180
	R: GCGTTGATGGTTGCTATGG	19	

Figure 2.1 show an example of the melting peaks of a primer set (wflAFP-6) and Figure 2.2 shows the gel band picture of each set of primers. All primer sets showed single sharp melting peaks for the quantitative standards as well as for the total DNA samples, at the corresponding melting temperature. Every PCR product also generated clear bands with expected sizes in the gel electrophoresis analysis. The identity of amplified products was additionally confirmed by sequencing analysis. These results indicated that non-specific PCR products with the primer sets were not detected in the analyzed temperature range.

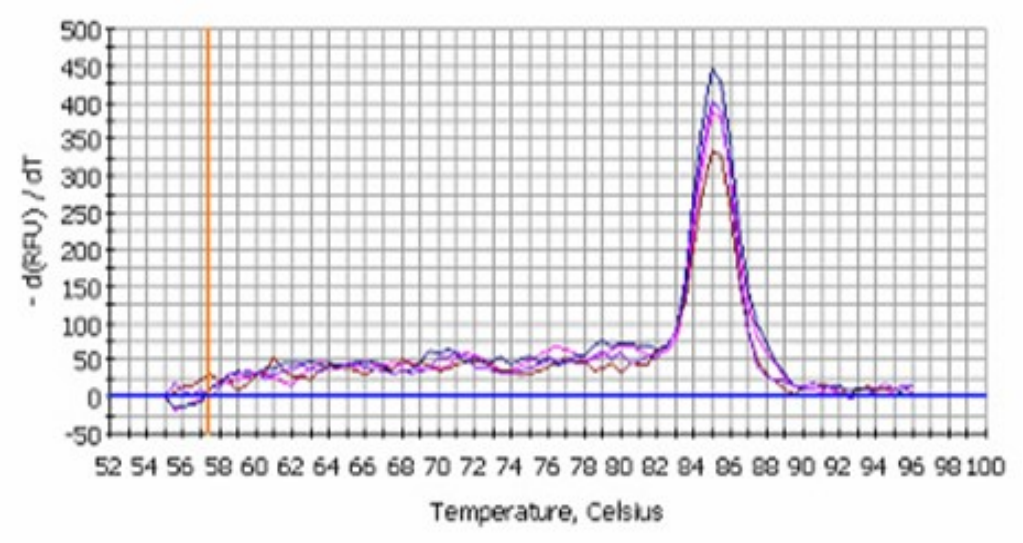


Figure 2.1 Melting curve analysis after amplification with wflAFP-6 specific primers

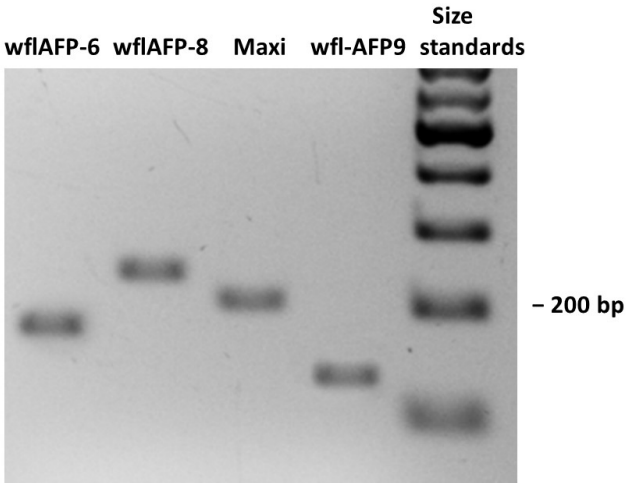


Figure 2.2 Gel analysis shows single products obtained for key genes encoding serum AFPs.

2.2.2 Standard curves and amplification efficiencies

A requirement for quantitative analysis in this study was accurate standard curves for the determination of gene concentration. The AFP gene standards were generated using the same primers as were used in qPCR quantitation and cloned into the pBluescript ± KS vector.

Undiluted plasmid DNA from each plasmid construct representing each target gene was measured by using a NanoDrop ND-1000 (NanoDrop Technologies). Plasmid DNA concentrations ranged from 1 to $1X10^{-7}$ ng/µl. The samples were free of non-DNA impurity with a 260/280 ratio of 1.85, which exceeds the ratio of 1.80 that indicates a high level of purity (Sambrook and Russell 2001). All curves were linear ($R^2 > 0.99$) in the range tested (Figure 2.3 and 2.4) demonstrating the accuracy of amplification over a wide range of concentrations. Measurements for each plasmid were reproducible with no difference in slopes between different reaction runs within a single assay and remained the same after storage of the standards at -20 °C for up to a year. The log-linear standard curves generated from these plots were precise ($R^2 > 0.99$).

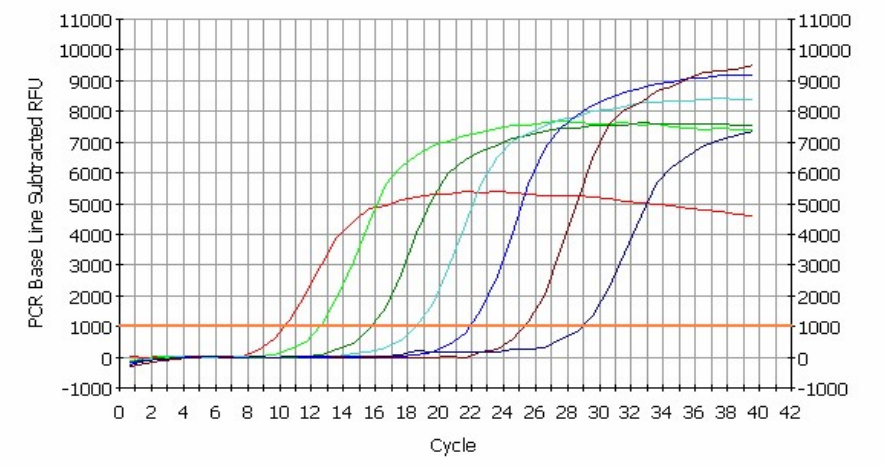


Figure 2.3 Standard curves for target genes showing 1/10 serial dilutions (1-1X10^{-7} ng/µl) of plasmid DNA containing the single products of serum AFP genes.

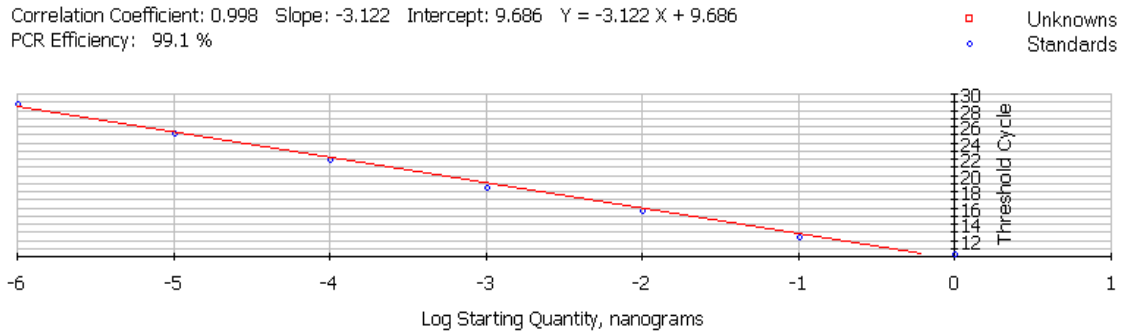


Figure 2.4 A plot of the log of the initial plasmid DNA concentration vs. the Ct value yields a straight line.

2.2.3 qPCR analysis of AFP genes and control genes

2.2.3.1 Standard curve method – Single gene reference method for calibrating gene copy number

Calibration using the genome size and plasmid DNA that included the target gene as the internal control allowed straightforward determination of AFP gene copy number on a genome basis (Wilhelm et al. 2003, Yun et al. 2006).

2.2.3.2 Winter flounder genome size

The DNA isolated from winter flounder was quantified and then evaluated for integrity. All DNA concentrations were derived from the absorbance at 260 and 280 nm and yields ranged from 450 ng/μL to 1500 ng/μL per every 15 μL of fish blood.

The determination of AFP gene copy number using total genomic DNA as a calibrator required a measure of the genome size. The genome size of the flounder has not been previously reported, necessitating its measurement for the current study. Since there are substantial geographical differences in flounder gene organization, five samples were analyzed from each location to determine whether the genome size was homogenous.

All flow cytometric sample cell distributions based on fluorescent intensity showed normal distributions. The repeated analysis of a single sample held on ice after all samples were analyzed (approximately 1 hour) revealed no detectable variation. Size distribution plots and typical fluorescence histograms obtained from target fish erythrocytes nuclei and chicken nuclei are reported in Figure 2.5 and 2.6. Mean genome size values (mean 0.67 pg per haploid genome) in winter flounder from different locations are shown in Figure 2.7. The genome size of winter flounder is in line with the

broad range of genome sizes reported in various teleost species (0.89-3.5 pg per nucleus) (Ciudad et al. 2002, Zhu et al. 2012), which is attributed to the plasticity of fish genomes compared to other vertebrate genomes (Venkatesh 2003).

Analysis of the data by Kruskal-Wallis test showed no significant differences among locations. Therefore, genome size values from all locations were used to obtain a single mean. The mean nuclear DNA content in winter flounder red blood cell nuclei using chicken cell DNA as a reference was 0.672 ± 0.009 pg.

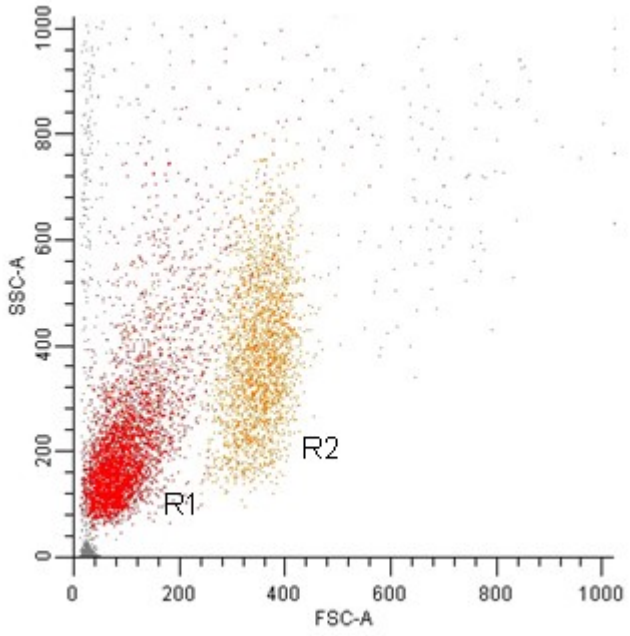


Figure 2.5 FACS analysis of nuclei from winter flounder RBC cells stained with propidium iodide (PI). Two distinct populations of cells (each dot represents a cell) termed R1 (red) and R2 (orange) are clearly defined according to the fluorescence intensity. R1: winter flounder, R2: chicken.

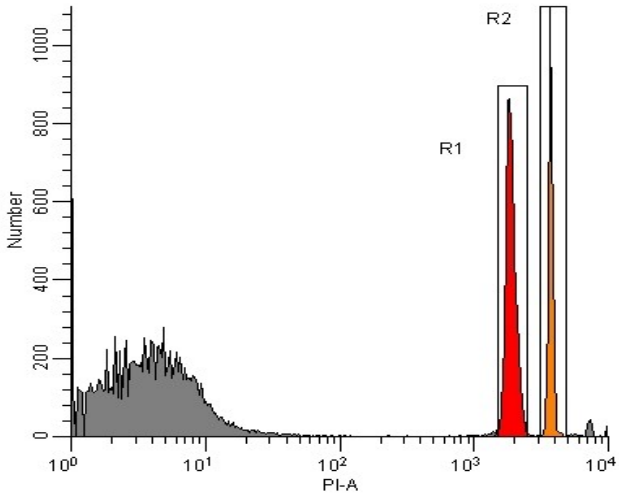


Figure 2.6 Flow cytometric histogram of relative fluorescence intensity after simultaneous analysis of winter flounder nuclei and internal standard chicken nuclei. Peaks are marked R1 for winter flounder and R2 for chicken.

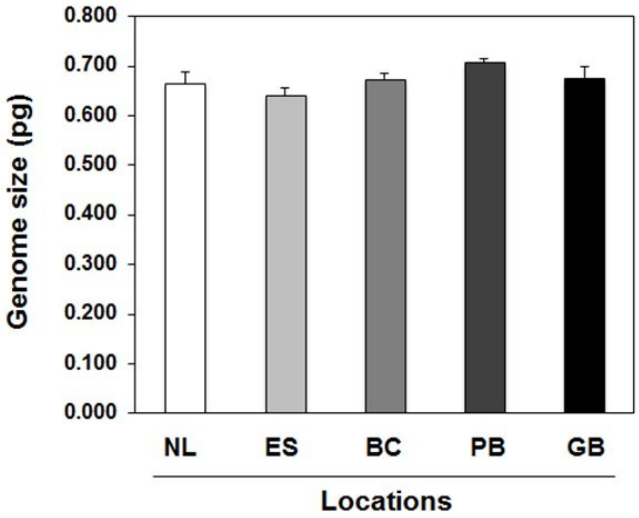


Figure 2.7 Genome sizes in winter flounder from different locations. The locations are Newfoundland (white), St. Lawrence Estuary (light grey), Baie des Chaleurs (medium grey), Passamaquoddy Bay (dark grey) and Georges Bank (black) and they are indicated by NL, ES, BC, PB and GB, respectively in the legend. The values are means ± SEM for each location.

2.2.4 AFP gene copy number on a genome basis

A pilot run of the assay was carried out for genes encoding the Maxi hyperactive AFP and wflAFP-9 gene. These were analyzed in genomic DNA from an individual winter flounder from the coast of Nova Scotia. A total of 1.49 X 10^5 genomes equivalents were analyzed during each qPCR reaction. The assay results indicated that the fish had two copies of the Maxy hyperactive gene and a single copy of the wflAFP-9 gene. Only values in which one of the triplicates had Ct variability of 1 Ct were used to minimize starting quantity calculation errors. The melting curve analysis also confirmed that only specific products of Maxi and wflAFP-9 were amplified during runs.

2.3 Discussion

The method used for AFP gene copy number determination to date has been Southern blot hybridization (Hayes et al. 1991). Although the method was informative in revealing the presence of large AFP gene copy numbers in fish genomes (Fletcher et al. 2001, Scott et al. 1988), it limited the possibilities for further investigation. Southern blotting requires large quantities of DNA and it is semi-quantitative and can only be used for limited numbers of gene copies. While Southern blot is very good for analyzing low-copy genes and their organization, the method is of limited practicality for analyzing large numbers of samples and it is difficult to apply in a quantitative manner to large copy-number genes. Early determination of fish AFP gene copy numbers have therefore only been performed in a few fish samples (Hayes et al. 1991) and the gene dosages determined were approximate. In order to examine the gene copy numbers more closely, a quantitative alternative to Southern blotting was required. The implementation of a

quantitative PCR assay offered an alternative that met the requirements of this study, while retaining the advantages of the Southern blot.

Since there were no suitable genes from flounder with reliable known copy numbers to use as calibrators in our study, we calibrated our data by using plasmid DNA that included the target gene in the standard, and obtained the final copy numbers on a per genome basis. Calibration using plasmid DNA as the standard has been used successfully in qPCR since it allows straightforward determination of gene copy number (Whelan et al. 2003, Wilhelm et al. 2003). For this to take place, it is required to calculate the amount of DNA corresponding to one haploid genome with precision. This is necessary because the genome size of an organism describes the DNA content in picograms and it represents the number of base pairs present. As it is not known if there was variation in the winter flounder genome size between populations, we calculated the winter flounder genome size in all populations targeted using flow cytometry (Lamatsch et al. 2000). This method allows for precise genome size determination by normalizing results using a reference genome with a known size (Lamatsch et al. 2000). Chicken cells provided a convenient reference, as the genome size is well established and the data did not overlap with those from flounder, allowing each peak of fluorescence to be distinguished clearly. The calculated haploid genome size of winter flounder determined by flow cytometry, was consistent with those previously estimated by bulk fluorometric assay (Hinegardner and Rosen 1972) and Feulgen image analysis densitometry (Hardie and Hebert 2003).

Calibration to genome size proved to be reliable, without great variation between samples as it was in other studies (Yun et al. 2006), and since PCR is a very sensitive technology, this method was able to detect single copy genes, as does the Southern blot technique. Any well characterized gene could potentially be quantified in the genome with the added advantage of requiring very small quantities of DNA. The accuracy of the

method strongly depends on the specificity of the PCR assay and the precision of the UV spectrophotometric determination of the DNA concentrations. The amplification of non-specific PCR products during the generation of standard curves is very important, since it could lead to an underestimation of gene copy numbers; however this can be overcome by the use of SYBR Green I which has several advantages over sequence-specific probes. The accumulation of primer dimers and non-specific products for example can be analyzed only in SYBR Green detection by the melting curve step (Deprez et al. 2002, Wilhelm et al. 2003). Also, errors from UV absorption measurements can cause both an underestimation and an overestimation of PCR products, which is why this measurement is so important in the quantification of DNA concentrations and the estimation of quality DNA.

Although the absolute standard curve method is the assay designed to measure DNA copy numbers, both relative and absolute quantification methods can be used. In the end, the ideal method to use for a qPCR given study depends on the scientific question being asked, and whether the study will be focused on measuring mRNA expression levels by quantitative reverse transcriptase PCR (qRT-PCR) or DNA copy number. Both methods have their advantages and disadvantages, in the relative quantification method one must assume that the control gene used to normalize the final copy numbers does not vary in copy number or expression levels amongst the samples, while the absolute standard curve method requires amplification of the target sequence from a series of external standards of known quantities to generate a standard curve from which the quantity of the target sequence in the unknown sample can be calculated. This method is more time consuming and requires more materials (Schmittgen 2001).

Overall, the quantification technique used here for AFP gene copy determination in winter flounder has broad applicability. It can be standardized and easily adapted to study the copy number of other genes in other species. The success of this technique is

dependent on the availability of the genome size and a cDNA or genomic clone corresponding to the target gene inserted into a plasmid and which can serve as standard in the qPCR analysis described. Other studies in the literature also demonstrated the usefulness of using the genome size in qPCR assays (Yun et al. 2006). Since all gene sequences for a given species are represented in gDNA it could even serve as a universal standard for the absolute quantification of gene copy numbers or any expressed gene.

Chapter 3

Antifreeze gene copy numbers in winter flounder (*Pseudopleuronectes americanus*) in the western North Atlantic ocean

Fish have evolved to occupy almost every aquatic habitat in the planet. The success of teleost fish has depended on their level of adaptation. The winter flounder is a species that has adapted to the risk of freezing in seawater during winter by producing antifreeze proteins (AFPs). These proteins bind to the surfaces of ice crystals and restrict their growth, which in turn prevents damage to cells (Fletcher et al. 2001). By producing AFPs the winter flounder has adapted to withstand the constant exposure of icy waters from the North Atlantic Ocean, but to do so winter flounder must produce enough AFPs to lower the freezing point of its blood. In winter flounder from Newfoundland, AFPs are produced in the liver as soon as the water temperature is lower than 8 °C (around November), and during the winter the fish are known to produce 10-15 mg/mL of AFPs (Fletcher et al. 1985a). Studies have shown that 10-15 mg/mL of AFPs in the blood reduce the plasma freezing temperature to approximately −1.7 °C (Fletcher et al. 1986)

To produce enough AFP, winter flounder produce more of the AFP genes. With greater numbers of the AFP genes, the fish can produce more of the proteins and thereby further reduce their freezing points. It has been postulated that selective pressure imposed by extreme exposure to ice led to the emergence of the tandem amplification of liver AFP genes in these fish (Scott et al. 1988). This is further supported by the discovery that the closely related yellowtail flounder also has multiple copies of AFP genes in its genome (Scott et al. 1988), but not to the extent found in the winter flounder. Among the pleuronectids, both AFP gene amplification and antifreeze activity in the serum correlate with depth at which they live and exposure to ice (Goddard and Fletcher 2002, Scott et al. 1988). Furthermore, in winter flounder, differences of AFP

gene dosage have been observed between individuals from different populations in winter flounder (Hayes et al. 1991).

The genetic potential for freeze resistance in winter flounder is of interest to the growers in the aquaculture industry because they wish to select broodstock from maximally freeze resistant populations (Litvak 1999). Where possible, they would also choose the most freeze-resistant individuals. That would allow them to most confidently avoid freezing of their fish in the more risky locations and open the opportunity of flounder culture to a greater part of Atlantic Canada. To better understand the differences among flounder from separate geographic locations and to begin observing individual variation, AFP copy numbers must be quantified. Furthermore, the relationship between the gene copy numbers and plasma antifreeze activity would allow some measure of the predictive value of the gene dosage determination.

In the current study, the gene copy numbers were determined by qPCR for the four major forms of AFP in winter flounder serum. The plasma antifreeze activities of flounder from two populations held under identical conditions were also determined for a preliminary assessment of the predictive value of qPCR gene copy determination.

3.1 Materials and Methods

3.1.1 Sample Collection

Winter flounder (*P. americanus*) were obtained from five geographic locations. Thirty flounder were sampled from Georges Bank, 22 from the St. Croix estuary in Passamaquoddy Bay, New Brunswick, 25 from the Baie des Chaleurs near Carleton, Quebec, 14 from the St. Lawrence estuary near St. Luce, Quebec, and 26 from Conception Bay near the town of Conception Bay South, Newfoundland and Labrador,

Canada (Figure 3.1). Fish kept in tanks were maintained at seasonal ambient conditions of temperature and photoperiod. The fish were anaesthetized with MS-222 (tricaine methanesulfonate) according to the Canadian Council on Animal Care guidelines. Blood was withdrawn from the caudal artery with a Vacutainer tube (Becton Dickinson) containing heparin and a 20-21 G needle. All samples were collected at 4 °C and centrifuged to separate red blood cells and plasma. Both red blood cells and plasmas were kept in storage at -80 °C until their use at the laboratory.

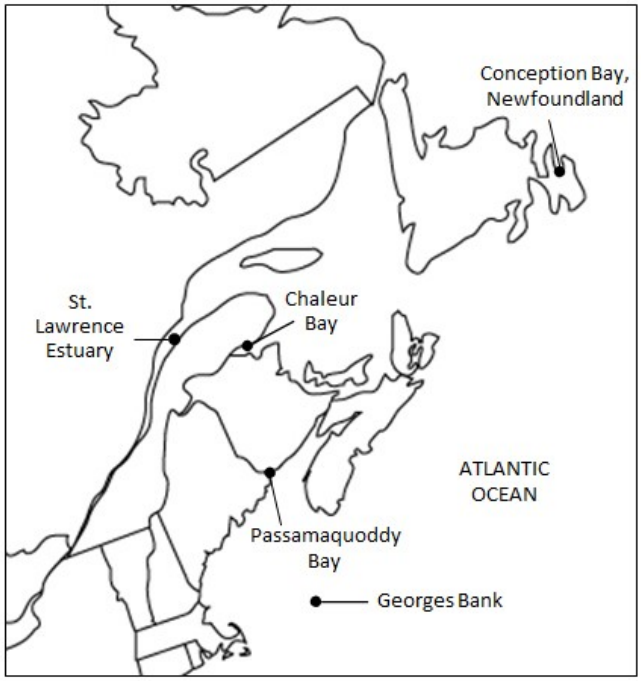


Figure 3.1 Geographic locations from which winter flounder samples were taken.

3.1.2 Laboratory Methods

All laboratory methods involving sampling, DNA purification, oligonucleotide primer design, quantitative PCR methodology and analysis of data were undertaken precisely as described in Chapter 2.

3.1.3 Antifreeze Activity

Flounder from Passamaquoddy Bay and Baie des Chaleurs were held in aquaria in a single location and under the same conditions of temperature and photoperiod at the Station aquicole de l'Institut des sciences de la mer de Rimouski in Rimouski, Quebec, Canada (48° 31' N; 68° 28' W). Therefore, environmental parameters that are known to affect AFP accumulation in the plasma were the same for these two groups of fish. For this reason, the conditions were appropriate for sampling plasma and comparing antifreeze activities among individual fish and between the geographic locations. Ice crystal morphology assessments and triplicate measurements of thermal hysteresis were performed on plasma samples obtained from these fish using a Clifton nanoliter osmometer with an Olympus compound microscope fitted with a camera (Moticam) according to previously described procedures (Ewart et al. 2000, Kao et al. 1986). Briefly, samples were frozen in a small holder and allowed to thaw until a single crystal remained in each of them. By binding ice crystals, AFPs generate observable crystal morphologies and a thermal hysteresis, which is a quantifiable gap between the melting and freezing point and a measure of AFP activity. The crystals were photographed and monitored for morphology in the temperature interval where they were stable. The melting and freezing points of the plasma samples were determined by the temperatures at which the crystal began to grow smaller and larger, respectively. In order to preserve the activity of the Maxi antifreeze protein, which is thermolabile (Marshall et al. 2004,

Marshall et al. 2005), samples were kept below 5 °C at all times, except for a few seconds when each sample was loaded onto the nanolitre osmometer sample holder using a glass needle.

3.1.4 Determination of AFP gene copy number

As described in Chapter 2, the copy number of AFP genes encoding the four major serum antifreeze proteins in winter flounder was quantitated on a per haploid genome basis, using standard curves created with circular plasmid that included the target gene insert as internal control. For this, the amplicon size, the winter flounder genome size (0.67 pg), the genomic DNA concentrations (100 ng/μL) per reaction and the starting quantities which were calculated by the iCycler software were used. Molecular weight of all bases corresponding to the amplicon was calculated with the OligoCalc oligonucleotide properties calculator (Kibbe 2007), the winter flounder genome size was calculated by Flow Cytometry (described in detail in Chapter 2) and the genomic DNA concentrations were acquired by spectrophotometry. Based on the OD_{260} value and the DNA sequences of both the amplicon and the plasmid vector, the molar concentration of the standard DNA was calculated using the OligoCalc oligonucleotide properties calculator, and then converted into copy number of DNA molecules per unit volume (in the order of magnitudes of $1X10^{-1}$ to $1X10^{-7}$ starting at 1 ng/μL).

3.1.5 Data analysis

The AFP gene copy numbers per genome encoding the major AFPs from the five locations studied (Eastern Newfoundland, St. Lawrence Estuary, Baie des Chaleurs, Passamaquoddy Bay, and Georges Bank), which were obtained by qPCR as described in Chapter 2 were subjected to Kruskal-Wallis using the R statistical analysis program for each AFP gene in order to detect significant differences ($p < 0.05$) in copy numbers among locations. In addition, the mean antifreeze activities of sera obtained from fish from Baie des Chaleurs and Passamaquoddy Bay were analyzed using a two-tailed Mann-Whitney U test ($p < 0.05$ indicating significance) using Excel (Microsoft) and the mean gene copy numbers from the same individuals were analyzed in the same manner.

3.2 Results

3.2.1 AFP gene copy number in winter flounder populations

Copy numbers for the four genes tested, encoding wflAFP-6, wflAFP-8, wflAFP-9 and Maxi, were determined by qPCR. Results obtained for each gene in the separate locations are shown in Figure 3.2. Data for individual fish are shown in Tables 3.1 to 3.5. There was variation among the AFP copy numbers among locations and among fish within locations. However, some features of gene dosage were common. The most abundant gene measured was wflAFP-6 (Figure 3.2). Mean copy numbers of this gene per genome exceeded 30 in flounder from Newfoundland, the St. Lawrence Estuary and Baie des Chaleurs, while fish from Passamaquoddy Bay and Georges Bank had less than 15 on average (Figure 3.2). The wflAFP-8 gene showed a similar pattern among

locations, but with considerably lower numbers in each case overall, as mean copies did not exceed 10 in any location.

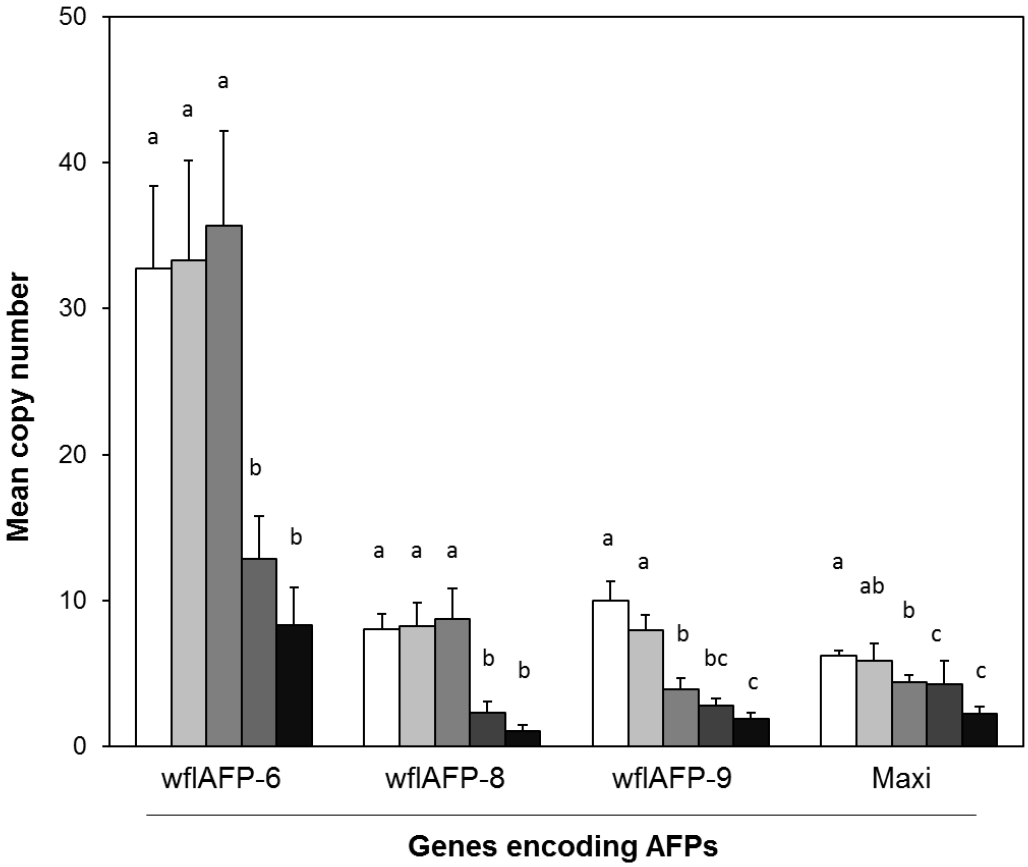


Figure 3.2 Copy numbers of each AFP gene in winter flounder from five locations determined by qPCR. Genes are wflAFP-6, wflAFP-8, wflAFP-9 and Maxi as shown. Locations are Newfoundland (white), St. Lawrence estuary (light grey), Baie des Chaleurs (medium grey), Passamaquoddy Bay (dark grey) and Georges Bank (black). The values are means ± SEM per genome in all flounder samples collected from each location. Means that do not share a letter are significantly different ($p<0.05$).

3.2.1.1 Copies of the genes encoding the shorter AFPs

The wflAFP-6-encoding gene appeared to be the most abundant and most wide ranging in number among those measured, with copy numbers in individual fish up to 122 per genome (Table 3.1). It was present in all fish from all locations. Fish from Newfoundland, the St. Lawrence Estuary and Baie des Chaleurs had greater than 30 copies of the gene for wflAFP-6 on average, whereas the mean copy numbers were 13 and 8 in those from Passamaquoddy Bay and Georges Bank, respectively. Differences were significant, as shown (Figure 3.2). The flounder had far fewer gene copies encoding wflAFP-8 than wflAFP-6. Winter flounder from Newfoundland, the St. Lawrence River and Baie des Chaleurs had, on average, 8-9 copies of the wflAFP-8-encoding gene, while those from Passamaquoddy Bay and Georges Bank had two copies and one, respectively (Figure 3.2).

3.2.1.2 Copies of the genes encoding the longer AFPs

Results for the gene encoding wflAFP-9 indicated similar mean numbers in the genome to those for wflAFP-8. Mean copy numbers were significantly higher in the most northerly locations (Newfoundland and the St. Lawrence River) than elsewhere. Copy numbers of the gene encoding Maxi appeared to be generally homogenous, with means ranging from two to six copies in the five locations sampled (Figure 3.2). Nonetheless, a few fish from Passamaquoddy Bay had highly elevated Maxi gene copy numbers ranging from 17 to 24 per genome along with modest copy numbers of the other AFP genes studied here.

Table 3.1 AFP gene copy numbers in individual winter flounder from Newfoundland determined by qPCR. Numbers shown are copies per genome.

Location	wflAFP-6	wflAFP-8	wflAFP-9	Maxi
NF01	20	8	7	6
NF02	19	0	8	8
N0F3	12	15	2	5
NF04	17	10	5	9
NF06	8	3	5	9
NF07	83	13	10	6
NF08	101	18	9	6
NF09	33	2	10	3
NF10	81	18	5	7
NF11	89	10	6	5
NF12	12	3	10	6
NF13	16	9	3	8
NF14	16	7	8	8
NF15	17	7	11	4
NF16	16	3	15	2
NF18	11	9	10	6
NF19	47	8	10	7
NF20	32	4	21	6
NF21	13	14	8	6
NF22	12	11	7	8
NF23	19	5	12	7
NF24	30	5	35	6
NF25	50	5	13	7
NF26	27	7	10	6

Table 3.2 AFP gene copy numbers in individual winter flounder from the St Lawrence Estuary determined by qPCR. Numbers shown are copies per genome.

Location	wflAFP-6	wflAFP-8	wflAFP-9	Maxi
ES01	23	17	5	15
ES02	44	6	6	9
ES03	3	8	3	4
ES04	12	11	2	12
ES05	22	13	6	7
ES06	58	13	8	6
ES07	52	2	8	2
ES08	65	5	10	2
ES09	42	2	10	1
ES10	19	1	16	2
ES11	89	19	11	6
ES12	1	1	4	1
ES13	21	5	11	6
ES14	15	13	10	10

Table 3.3 AFP gene copy numbers in individual winter flounder from Baie des Chaleurs determined by qPCR. Numbers shown are copies per genome.

Location	wflAFP-6	wflAFP-8	wflAFP-9	Maxi
BC01	15	1	11	6
BC02	0	0	0	0
BC04	0	0	1	0
BC05	47	8	3	5
BC06	87	47	3	4
BC07	20	5	2	3
BC08	122	27	1	2
BC09	43	15	1	2
BC10	50	10	1	3
BC11	72	4	3	3
BC12	94	6	1	3
BC13	10	2	1	3
BC14	7	3	1	4
BC15	11	12	6	4
BC16	17	2	3	7
BC17	9	2	6	5
BC18	8	3	6	9
BC19	52	13	6	9
BC20	18	3	10	5
BC21	39	2	1	4
BC22	52	16	11	8
BC23	32	12	9	6
BC24	40	8	6	6
BC25	12	9	1	5

Table 3.4 AFP gene copy numbers in individual winter flounder from Passamaquoddy Bay determined by qPCR. Numbers shown are copies per genome.

Location	wflAFP-6	wflAFP-8	wflAFP-9	Maxi
PB01	16	3	3	0
PB02	0	2	2	1
PB03	2	1	3	2
PB04	0	0	4	0
PB05	45	7	3	0
PB06	6	0	9	3
PB07	8	3	4	0
PB08	38	0	3	0
PB09	37	0	3	0
PB10	22	9	2	2
PB11	28	2	0	1
PB12	19	2	3	23
PB13	3	0	1	13
PB14	3	0	0	24
PB15	2	1	2	0
PB16	5	4	1	17
PB17	3	1	0	0
PB18	9	0	4	0
PB19	20	0	0	1
PB20	2	0	2	0
PB21	2	1	5	3
PB22	12	14	8	3

Table 3.5 AFP gene copy numbers in individual winter flounder from Georges Bank determined by qPCR. Numbers shown are copies per genome.

Location	wflAFP-6	wflAFP-8	wflAFP-9	Maxi
GB03	0	0	0	1
GB04	1	0	1	1
GB06	1	0	2	2
GB07	5	2	3	3
GB08	0	0	0	0
GB10	7	0	1	1
GB11	11	0	5	3
GB14	36	2	6	8
GB15	7	0	4	4
GB16	10	2	0	0
GB17	5	1	0	0
GB18	4	0	4	6
GB19	1	0	0	0
GB20	52	2	4	4
GB21	1	0	0	1
GB22	3	0	3	2
GB23	7	9	1	2
GB24	18	1	4	5
GB25	3	1	1	1
GB26	13	1	0	0
GB27	5	1	4	5
GB28	0	0	0	0
GB30	2	0	0	0

3.2.2 Gene copy number and serum antifreeze activity

Plasma samples were obtained from fish collected in Baie des Chaleurs and Passamaquoddy Bay and were held in a single location under ambient conditions in Rimouski to allow comparison of antifreeze activity. A number of samples obtained for the measurement of antifreeze activity (thermal hysteresis) were not analyzed, as they formed gels that were unsuitable for measurement. Nonetheless, measurements were completed on 13 samples from Passamaquoddy Bay and 12 from Baie des Chaleurs. When comparing mean values of antifreeze activity and AFP gene copy number for the two groups of flounder from the two locations, we observed a higher plasma antifreeze activity in fish from Baie des Chaleurs which correlated with a higher copy number of wtlAFP-6, wtlAFP-8 and total AFPs in fish from this location (Figure 3.3), suggesting a predictive value of copy number determination at the level of population or geographic location.

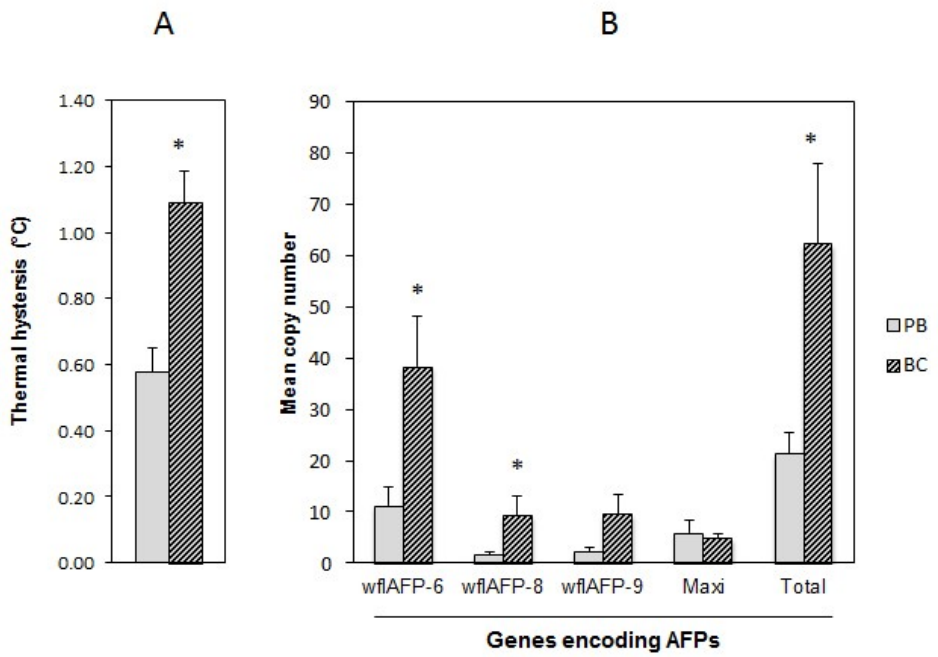


Figure 3.3 AFP gene copy numbers and thermal hysteresis for subsets of winter flounder measured from the Passamaquoddy Bay (light grey) and Baie des Chaleurs (light grey with black hatch marks) locations. Data are means ± SEM. (A) Plasma thermal hysteresis showing significantly higher mean activity in Baie des Chaleurs samples, (B) wflAFP-6, wflAFP-8 and total (combined) gene copy number showing significantly higher mean activity in Baie des Chaleurs samples ($p<0.05$).

3.3 Discussion

Winter flounder seasonally produce a set of closely related α-helical AFPs, which can reach serum concentrations of 15 mg/mL (Fletcher et al. 1985a). These AFPs are present in the form of multigene families with genes tandemly repeated in the genome and encoding the bulk of the circulating AFPs in fish blood (Scott et al. 1985).

The initial method used for winter flounder AFP gene copy number determination was Southern blot hybridization (Hayes et al. 1991, Scott et al. 1985, Scott et al. 1988). Although the method was useful in revealing the presence of large AFP gene copy numbers in fish genomes and their arrangement in the genome, it limited the possibilities for quantitative results and larger sampling sets. For those reasons, early determination of fish AFP gene copy numbers were only performed in a few fish samples (Hayes et al. 1991) and the gene dosages determined were approximate. In order to examine the gene copy numbers more closely, a quantitative approach was required. The current study employed qPCR to quantify AFP gene copy numbers in winter flounder, using a targeted primer set and specific assay parameters that gave specific results for distinct AFPs.

The geographic variation among gene copy numbers for wflAFP-6 and -8 together was similar to that predicted by Southern analysis in previous studies (Hayes et al. 1991, Scott et al. 1985, Scott et al. 1988). The results also showed that winter flounder from Newfoundland have significantly greater gene dosage for each of the AFPs tested than the flounder from Georges Bank and Passamaquoddy Bay. The other three populations have gene dosages that are intermediate in general, being significantly different from the Georges Bank fish for some genes and significantly different from Newfoundland fish for others. Both latitude and water depth affect the prevalence of ice and consequent freezing risk. At the lowest latitudes sampled and in what is likely the deepest water, the Georges Bank and Passamaquoddy Bay fish had the lowest gene copy numbers, which may have been the result of weaker selection pressure for freeze resistance than in the other locations. These results correspond to previous findings of shallow- and deep-water populations of winter flounder in the North Atlantic (Hayes et al. 1991) and in a study that compared copy number and circulating AFP levels as an indication of the species exposure to ice (Scott et al. 1985). Investigations into the genomic organization

of liver-type AFPs in the winter flounder revealed that fish in colder locations (Newfoundland and North Sydney, Nova Scotia) had high AFP gene copy number and a lengthy tandem component, compared to fish in warmer locations such as Passamaquoddy Bay, Bay of Fundy, Browns Bank and Georges Bank, which showed a reduced tandem component and a significant reduction in the overall copy number of AFP genes (Hayes et al. 1991). Related studies were also done on gene dosage between different groups of eel pouts and its relation to their antifreeze activity. Genomic Southern blotting showed that within the Newfoundland ocean pout genome, the number of antifreeze protein genes is approximately four to five times greater than the antifreeze gene dosage in the ocean pout from New Brunswick (Hew et al. 1988). The more southerly New Brunswick pout population had 30-40 copies of the AFP gene and the Newfoundland population had approximately 150 copies (Hew et al. 1988). Similar Southern blotting results have been obtained in species of wolffish (*Anarhichas sp.*); *A. lupus*, which inhabits shallow icy waters, has greater AFP copy numbers than its deeper water counterpart *A. minor* (Desjardins et al. 2012). Results of those studies suggested that winter flounder and pouts from regions with greatest freezing risk should be capable of producing significantly higher levels of antifreeze over a winter cycle compared to their counterparts from less ice-exposed areas of the distributional range, when held under identical conditions.

The finding in this study that copy numbers of wflAFP-6 were, on average, higher than those for wflAFP-8 was consistent with earlier reports (Fourney et al. 1984). In flounder from four locations, ranging from Long Island, U.S.A. to Newfoundland, wflAFP-6 was the predominant AFP in serum. Genes encoding these two AFPs have been considered as interspersed and they have always been quantified together due to their sequence similarity (Davies 1992), which made it impossible to distinguish them from one another by Southern blotting. Based on sequence and restriction analyses, it was

suggested that wflAFP-6 and wflAFP-8 genes occurred at a ratio of 1.5:1 in the genome, coincident with the ratio of the protein products in plasma (Davies 1992), whereas the current quantitative analysis of mean copy numbers revealed ratios of wflAFP-6 to wflAFP-8 ranging from 3.8:1 (Baie des Chaleurs) to 8:1 (Georges Bank). The reason for lower numbers of isoform 8, on average, in flounder genomes is unclear. It is possible that during duplication of wflAFP-6 in tandem repeats, one copy underwent a mutation to generate a slightly modified gene (wflAFP-8) that continued to increase in copy number without reaching the numbers of the wflAFP-6, thus creating multiple copies of wflAFP-6 and wflAFP-8 genes.

The Maxi-encoding gene was found in low numbers in the genome compared to the gene for wflAFP-6, but its mean numbers in comparison to the mean overall total AFP gene copies was a lot higher than anticipated, given its approximately 50-fold lower concentration than shorter AFPs in plasma (Marshall et al. 2004). The lability of the Maxi protein (Marshall et al. 2005) may reduce circulating levels of native Maxi AFP in the fish, particularly in locations where temperature fluctuates. Nonetheless, Maxi may have as much impact on freeze resistance as the other AFPs, as it has far greater thermal hysteresis activity than do its shorter AFP counterparts (Marshall et al. 2004). Therefore, a small number of genes encoding Maxi, and its accumulation in modest concentrations, could still lead to a proportionally substantial freezing point depression.

The study relating antifreeze activity and gene copy number revealed that there were significant differences ($p<0.01$) (Figure 3.3) in thermal hysteresis activities and copy numbers when comparing flounder grouped by locations, and this suggests a predictive value of copy number determination at the level of population or geographic location. This suggests that gene copy numbers in populations can be employed to determine the potential for freeze resistance. Samples used in the thermal hysteresis study came from two of the five locations studied (Baie des Chaleurs and from

Passamaquoddy Bay) and they were intended to represent colder and warmer locations. Flounder from these sites were kept under the same conditions of temperature and photoperiod, which is very important when measuring thermal hysteresis since antifreeze activity can be influenced by either parameter (Fletcher et al. 2001). The quantification of gene copy numbers of a location could be used to determine the overall potential for freeze resistance of the fish, and farmers could thereby select a particular population to use as their most freeze-resistant brood stock. In order to apply this approach at finer resolution, a clear understanding of the relationship between each known AFP type and overall freeze resistance would be helpful. Furthermore, considering the relative importance of Maxi because of its antifreeze activity compared to other AFPs, an increased understanding of the gene organization, variation and possible variant copy numbers for Maxi would be required, as earlier studies on AFP gene organization were performed prior to the discovery of the Maxi protein.

Chapter 4

Hyperactive antifreeze protein gene organization and arrangement in winter flounder (*Pseudopleuronectes americanus*)

In the North Atlantic, a number of marine fish species, such as winter flounder, can survive icy seawater at temperatures reaching as low as -1.9^{o}C by producing high levels of alanine-rich α-helical AFPs seasonally (Davies et al. 1982, DeVries 1982). AFPs bind to ice crystals, thereby preventing ice growth in a non-colligative fashion. There are three categories of AFP isoforms in winter flounder encoded by distinct genes with different tissue distributions and differential expression: two of these are the short AFPs consisting of three (and occasionally 4) sequence repeats. Each isoform type has several variants with the same overall structure and regulation, but they differ slightly in sequence. The two main liver-expressed isoforms are wflAFP-6 and wflAFP-8 containing three 11-amino acid repeats and molecular masses of 3.4 kDa. Additionally, there is an isoform called wflAFP-9, encoded by the gene 21a, which contains a fourth repeat motif, resulting in a molecular mass of 4.3 kDa (Chao et al. 1996, Gauthier et al. 1990, Gourlie et al. 1984). The latest addition to the AFP isoforms is a much larger hyperactive form called Maxi. This protein is a 32-kDa AFP that is approximately 10-fold more active than the smaller AFPs on a concentration basis (Marshall et al. 2004). In contrast to the liver and skin isoforms, the hyperactive isoform has a less defined sequence repeat pattern, although it is highly Ala-rich (Graham et al. 2008c). The Maxi AFP exists in solution as a homodimer (Marshall et al. 2005). It appears that the two straight helices of the protein form an anti-parallel dimer, in such a way that both polypeptide side chains can interact with the ice by using their ice-binding faces at once (Graham et al. 2008c). It is believed that this characteristic is what makes this AFP much more active than the other AFPs, since it has been shown that AFPs with a larger ice-binding face are more active

(Baardsnes et al. 2003, Chao et al. 1996, Knight et al. 1984, Nishimiya et al. 2003, Schrag et al. 1982).

Previous studies established that the liver-expressed AFPs are encoded by a gene family consisting of 30-40 members (encoding predominantly the wflAFP-6 and wflAFP-8 peptides), most of which are arranged in direct tandem repeats in the genome(Davies et al. 1984, Scott et al. 1985). The skin isoform is also encoded by a large multi-gene family comprising of 30-40 gene copies which are linked but irregularly spaced (Gong et al. 1996). The maximal levels of AFPs in the winter flounder varies among populations, even when fish are raised in a single location, suggesting that AFP production is governed by genetic rather than environmental differences (Fletcher and Smith 1980). Semi-quantitative studies of the liver isoforms have shown that in fact AFP gene copy numbers vary among populations and that AFP activity in plasma is proportional to gene dosage (Hayes et al. 1991, Scott et al. 1985).

Because the small AFPs are represented by multi-gene families, it is possible that the hyperactive Maxi is also encoded by a multigene family similar to the liver and skin types. In order to determine whether this is the case and to better understand the gene arrangement of Maxi, genomic Southern blot hybridizations were performed on several samples spanning a wide geographic range on the Western Atlantic coast.

4.1 Materials and Methods

4.1.1 Genomic DNA Purification

Five samples of winter flounder (*P. americanus*) blood were obtained previously from five geographic locations (refer to Chapter 3).

Genomic DNA (gDNA) was isolated from 15 μL of nucleated red blood cells from winter flounder using a commercially available Gentra Puregene Tissue Kit (Qiagen). Samples were thawed quickly and then subjected to DNA extraction according to the manufacturer's protocol. In brief, the cells were lysed and then subjected to RNAse treatment, followed by protein and DNA precipitation and finally DNA hydration. DNA concentrations were determined spectrometrically using a NanoDrop ND-1000 UV spectrophotometer (NanoDrop Technologies). Purified genomic DNA (gDNA) was stored at 4 ºC until further use. All gDNA was further analyzed by gel electrophoresis for confirmation of high molecular DNA.

4.1.2 Probes

A portion of the Maxi-encoding cDNA that was previously used for primer design (Chapter 2) was labelled with digoxigenin (DIG) by PCR according to the PCR Labeling of DNA Probes method of the DIG Application Manual for Filter Hybridization (Roche). A second probe from P-glycoprotein B (PGP-B) (F: CCATAGGGCTGGATTCAACTCGTG, R: GCCCACGGTCGGTAGCCATTGTTC) (199 bp) cDNA (single copy gene in the genome of the winter flounder) (Chan et al. 1992) was also labeled in the same fashion. One ng of plasmid DNA was used to obtain the PCR products of Maxi and PGP-B, which were then eletrophoresed and gel purified to use in DIG labeling. The labelling of both

probes by PCR was confirmed by gel electrophoresis as described in the DIG Application Manual for Filter Hybridization (Roche). Additionally, concentrations of each probe were optimized for Southern blotting as described in the manufacturer's instructions.

4.1.3 Southern blotting

Ten µg of each sample of winter flounder genomic DNA was digested separately with HindIII (Fermentas) restriction enzyme as directed by the manufacturer protocol. Additionally, two more aliquots of 10 µg were digested with PstI (Fermentas) restriction enzyme. One of these aliquots contained zebrafish genomic DNA (to use as DNA carrier) plus plasmid DNA corresponding to a single copy of the Maxi hyperactive gene, while the other aliquot contained 10 µg of zebrafish genomic DNA and plasmid DNA corresponding to a single copy of PGP-B. These two aliquots were used as single gene copy controls in Southern blots. All reactions were incubated overnight at 37 °C without shaking.

For each Southern blot, digested DNA was separated by electrophoresis (10 µg/lane) on a 0.7% agarose gel cast in 0.5x TBE at 25 V. Alkaline transfer buffer was used for capillary transfer of genomic DNA to a positively charged nylon membrane (Roche) by the alkaline transfer method (Sambrook and Russell 2001). The air-dried, UV-crosslinked blot was pre-hybridized for 30 minutes at 52 ºC in DIG Easy Hyb buffer following the manufacturer's recommended protocol (Roche). Labeled probe was boiled for five minutes at 95 ºC in order to denature it and it was then added to the fresh hybridization buffer. Hybridization was performed overnight (16 hrs) at 52 ºC. All washes were done in 0.1x SSC with 0.1 % SDS at 68 ºC for 30 minutes according to the

Hybridization of DNA Probes to the method recommended by the manufacturer (Roche). Blots were exposed to Kodak BioMax light film for periods ranging from 15 to 45 min.

4.2 Results

The distribution of Maxi AFP genes in the winter flounder genome of several fish carried out by Southern blotting using a Maxi cDNA probe is shown in Figure 4.1. Several bands with different degrees of intensity were visible on most lanes. The lightest bands appeared to correspond to genomic fragments containing one copy of the Maxi AFP gene, as indicated by the single control lane (Maxi ctrl). Therefore, the more intense bands corresponding to the 2.5 kb, 5 kb and 10 kb fragments appear to contain two or more copies of the Maxi AFP gene.

When comparing the banding pattern of samples from Newfoundland and elsewhere, all Newfoundland samples were missing the 5 Kb band found in the other locations, and the relative intensity of the bands were different. Since signal strength is a function of many factors (such as elution of target DNA from the gel during transfer, binding of target DNA to the membrane during blotting, retention of target DNA during hybridization and stringency washes, and similarity between the sequences of the target DNA and the probe molecule) (Sambrook and Russell 2001), patterns of relative intensity within lanes can be compared in a blot. Comparison of the intensity of bands in digested samples to the intensity of the signal in the single copy control lane (Maxi 1 copy ctrl) suggests there are at least five copies of the Maxi gene in the genome of the winter flounder. A few lanes (e.g. NL16, ES4 and GB30) appeared to have hybridization bands lighter than the single copy control. This appears to have been a loading effect, as shown below.

To verify that sufficient DNA was used in each lane and to allow more objective comparison among them, the blot was reprobed using PGP-B in order to determine whether equal quantities of DNA were loaded in each lane and that similarly hybridizable bands were evident in all. Results of the blot probed with PGP-B are shown in Figure 4.2. Most of the bands in the blot have similar intensities and clearly show single copies in all lanes including the single copy control lane corresponding to PGP-B (PGP-B I copy ctrl). However, it is notable that the lanes with somewhat lighter bands than the single-copy maxi, such as NL7 or GB19, also had correspondingly lighter bands for PGP, indicating that those maxi bands could be considered as valid single genes. As expected, no PGP-B band was visible in the single copy control lane for the Maxi gene. This supports the contention that the banding pattern obtained with the Maxi cDNA probe accurately represents the minimum gene copy number and distribution of Maxi in the genome of winter flounder.

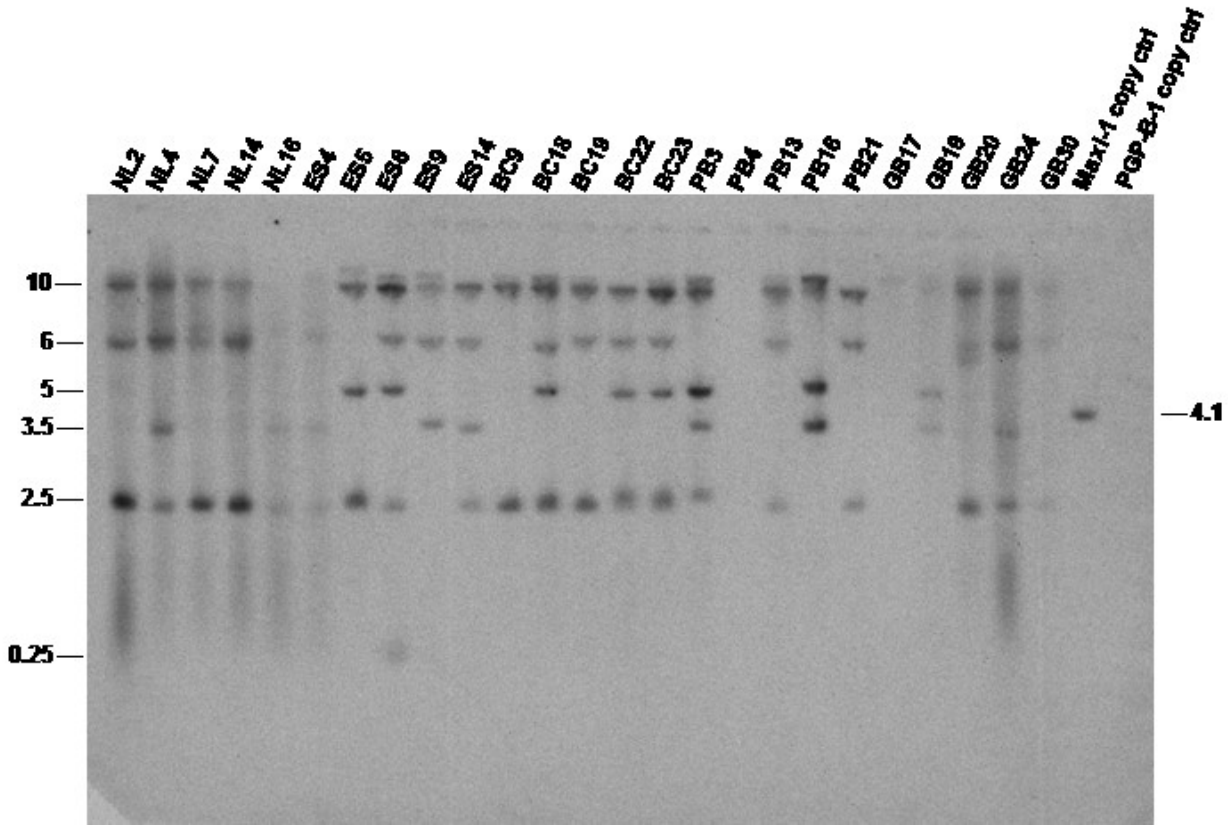


Figure 4.1 Winter flounder genomic Southern blot probed with a gene fragment encoding Maxi. Samples of 10 µg of genomic DNA from individual winter flounder from several locations were digested with HindIII. The digested DNA was electrophoresed on a 0.7% agarose gel cast in 0.5x TBE, blotted onto a positively charged nylon membrane and probed with the hyperactive isoform Maxi DIG Probe (180 bp). The positions of the DNA size markers are given in Kb and indicated by bars on the left side of the blot. The size of the plasmid containing one copy of the Maxi gene is indicated on the right right side of the blot. Abbreviations are the following: NL, Newfoundland; ES. St. Lawrence Estuary; BC, Baie des Chaleurs; PB, Passamaquoddy Bay; GB, Georges Bank.

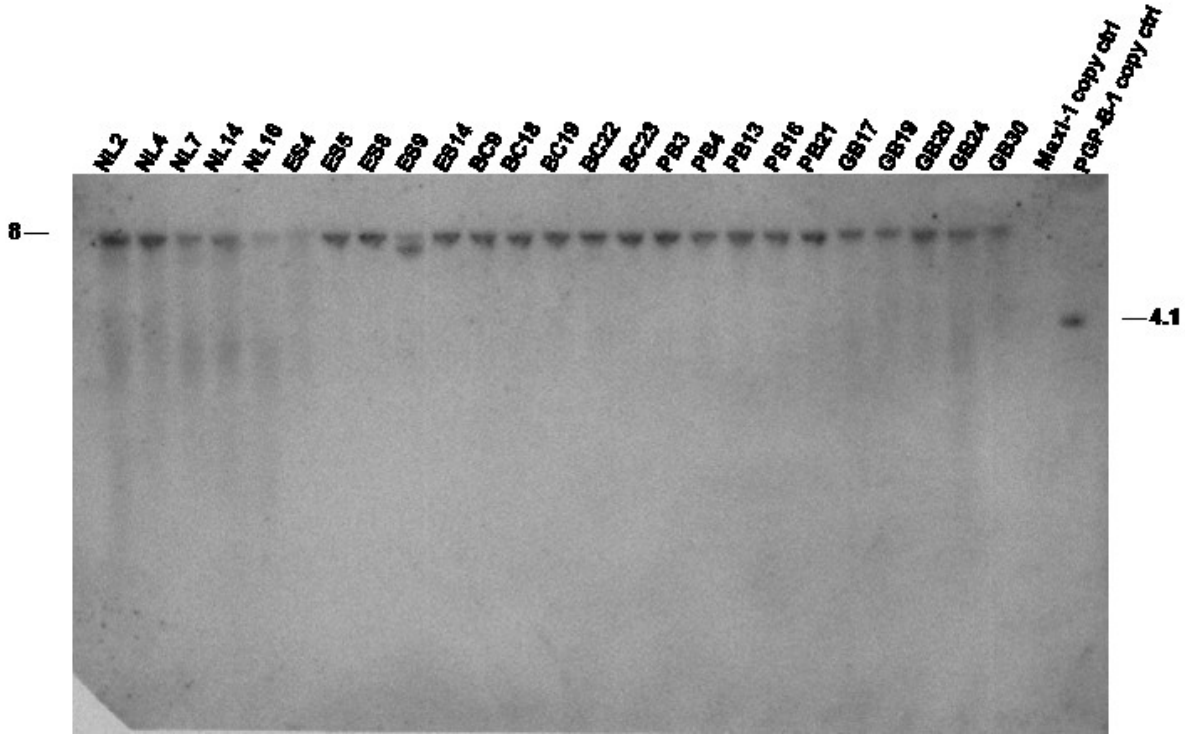


Figure 4.2 Winter flounder genomic Southern blot probed with a gene fragment encoding PGP-B. Samples of 10 µg of genomic DNA from individual winter flounder from several locations was digested with HindIII. The digested DNA was electrophoresed on a 0.7% agarose gel cast in 0.5x TBE, blotted onto a positively charged nylon membrane and probed with PGP-B DIG Probe (199 bp). The positions of the DNA size markers are given in Kb and indicated by bars on both sides of the blot. The size of the plasmid containing one copy of PGP-B is indicated on the right side of the blot. The size of the plasmid containing one copy of the Maxi gene is also indicated. Abbreviations are the following: NL, Newfoundland; ES. St. Lawrence Estuary; BC, Baie des Chaleurs; PB, Passamaquoddy Bay; GB, Georges Bank.

4.3 Discussion

Until recently, the liver and skin isoform set of AFP encoding genes representing two of the most abundant AFPs (wflAFP-6 and wflAFP-8) in the serum of the winter flounder were the only ones known to contribute to the freeze protection of the fish(Davies 1992). However, it is now known that the Maxi AFP is also present in the serum contributing with more than 1 ºC of the thermal hysteresis required (at least 2 ºC) for freeze protection. No analysis has been published to date on the gene organization and arrangement of the Maxi AFP.

The goal of this study was to analyze the gene arrangement and distribution of the newly discovered Maxi AFP gene. DNA fragments ranging from 2.5 kb to 10 kb and containing one or more copies of the Maxi gene were present in genomic DNA digested with the restriction enzyme HindIII. These labelled fragments were able to withstand stringent washing conditions indicating that their sequences match that of the probe perfectly or nearly so. It is important to note that the Maxi probe was prepared using a cDNA segment corresponding to a sequence region of the mature Maxi protein not found in the smaller liver AFPs.

The intensity of the hybridization signal in the control lane containing the equivalent of a single copy of Maxi per genome (single copy loading control) allows evaluation of the number of copies of the Maxi gene present in the winter flounder genome. The less intense bands observed on the genomic blot appear to represent single copies of the Maxi AFP gene (Figure 4. 1). The more intense bands, on the other hand, may represent genomic restriction fragments containing more than one copy of Maxi I AFP genes or alternatively two different genomic restriction fragments of similar size each containing one or more antifreeze protein genes. It was not surprising that the Maxi genes are found in lower numbers than the smaller AFPs. As noted in the

Introduction, this modest concentration is sufficient to have a substantial freezing point depression effect because the Maxi AFP is so active (Graham et al. 2008c, Marshall et al. 2005). When comparing the hybridization patterns of DNA from individual fish of the same geographic location, there are differences among their hybridization patterns, indicating genetic polymorphism among individuals sampled. It can be speculated that there are at least five copies of Maxi in the genome of the winter flounder, but some winter flounder have higher or lower numbers of copies. In particular, one fish from Passamaquoddy Bay that showed not a single copy of the gene. It can be argued that this can be due to poor quality DNA but this was not the case as all gDNA was tested by gel electrophoresis. Furthermore, the same result was obtained by qPCR studies done on the same fish (see Chapter 3 of this thesis). Antifreeze activity analysis showed that a couple of fish that did not have the Maxi AFP gene did have the equivalent antifreeze activity, suggesting that there might be other isoforms of the AFP Maxi gene different from the one used in the analysis of genes by qPCR and Southern blotting.

Since most AFPs belong to multi-gene families, these results clearly show that a multi-gene family encodes the Maxi AFPs; however, the arrangement of these copies in the genome cannot be established on the basis of Southern blotting alone. For this, it would be necessary to clone and sequence the larger hybridized fragments or screen a genomic library to identify Maxi clones and perform additional restriction map analysis of each isolated clone. Given that other winter flounder AFP studies involving Southern blots have shown that the short liver-expressed AFPs (e.g. wflAFP-6 and wflAFP-8) are arranged in tandem repeats, based on the location and intensity of the bands (Scott et al. 1985) we can then argue that the similar banding pattern observed in our southern blot analysis suggest that the Maxi genes are not tandemly arranged in the genome but rather linked and irregularly spaced. This is not surprising considering that skin AFPs as well as wflAFP-9 AFP genes are also linked but irregularly spaced in the genome of the

winter flounder (Davies 1992, Davies and Gauthier 1992, Gong et al. 1996). Similarly, Southern blot studies from the smooth flounder (*Liopsetta putnami*) and American plaice (*Hippoglossoides platessoides*) have shown that they only present linked but irregularly spaced genes; and other relatives such as the yellowtail flounder (*Limanda ferruginea*) also have a multi-gene family of AFPs that are linked but irregularly spaced (Scott et al. 1988). This suggests that AFP gene families have evolved in a similar manner.

Winter flounder and yellowtail flounder live within the same geographical range (Leim and Scott 1966), but the yellowtail flounder lives offshore at depths ranging from 60 to 100 m (Johnson et al. 1999) while the winter flounder lives inshore at depths of 25 m in average (Pereira et al. 1999). So the winter flounder is an obligate AFP producer due to its exposure to ice in shallow water while the yellowtail flounder is rarely exposed to ice nucleators (Fletcher et al. 1985a, Scott et al. 1988). Recently it was discovered that the American plaice produces the hyperactive form, but not the smaller liver AFP type. So, it has been suggested that this newly discovered Maxi AFP gene could be the ancestor gene that gave rise to the smaller liver and skin isoforms (Gauthier et al. 2005, Graham et al. 2008c). The Maxi AFP may therefore be a very important AFP that not only helps winter flounder adapt to the colder temperatures inshore, but may also be a critical gene that gave rise to the smaller AFPs or tissue specific AFPs in other species that also evolved to cope with their specific environments.

Chapter 5

Discussion

AFPs are biologically important to fish because they contribute to the freeze resistance necessary to survive in the cold. Additionally, freeze resistance is dependent on the type of AFP, protein concentration and on the specific interaction of AFPs with ice crystals. Furthermore, protein concentration is dependent on AFP gene copy number. So, determining the number of AFP genes in a fish can predict the fish freeze resistance.

Measuring AFP gene copy number has always been done by Southern blot hybridization (Hayes et al. 1991). This technique is time consuming and requires tens of microgram quantities of high quality DNA and even though it can be accurate in analyzing low gene copy numbers and gene organization, it's not practical when processing many samples or high-copy genes. So, an alternative to Southern blot was necessary to study AFP gene copy numbers.

We designed a quantitative PCR assay for the quantification of AFP genes. We implemented a standard curve method that allowed the analysis of several samples from all target locations at once. For this, we created sets of primers that only produced specific products corresponding to each specific target AFP gene. This was verified by cloning and sequencing multiple clones from each qPCR product. Determining the absolute copy number is of utmost importance in studies of gene dosage. So, for calibration of our data, we used circular plasmid DNA with an insert of the target AFP gene as an internal standard, and obtained the final copy numbers on a genome basis. Calibration using plasmid DNA as the internal control has been used successfully in qPCR (Whelan et al. 2003, Wilhelm et al. 2003). For our method, we were required to calculate the amount of DNA corresponding to one haploid genome with precision. This

step is necessary because the genome size of an organism describes the DNA content in picograms and it represents the number of base pairs present.

Since variation in the size of the winter flounder genome has not been studied, we calculated the winter flounder genome size in all locations targeted; this was done successfully by flow cytometry. Flow cytometry is one of the most common methods used today to analyze genome size and it is based on the analysis of DNA content of thousands of individual nuclei (Lamatsch et al. 2000). Our analysis of winter flounder genome size did not show significant differences among locations. This was important in order to allow accurate qPCR analysis. Additionally, the genome size results we obtained were very similar to those obtained by bulk fluorometric assay (Hinegardner and Rosen, 1972) and Feulgen image analysis densitometry (Hardie and Hebert, 2003). In general, the qPCR method was able to detect single copies of AFP genes. Any well characterized genomic DNA could potentially be measured with the added advantage of requiring very small quantities of DNA.

The analysis of AFP genes by qPCR showed that fish from northerly locations such as Newfoundland had more copies of AFPs genes in general compared to fish from southerly locations such as Georges Bank. This is in accordance with what was found by Southern blot previously (Hayes et al. 1991). Since water depth and latitude are factors influencing freeze resistance, it is possible that Georges Bank fish living in deeper water, had weaker selection pressure for freeze resistance and hence less copy numbers of AFP genes, while winter flounder that reside northerly in Newfoundland are obligate producers of AFPs due to the risk of encountering ice.

The concentration of AFPs in the blood reflects the extent of the freezing conditions that fish like winter flounder encounter. In winter flounder, the production of high levels of AFP in the blood is necessary to survive freezing conditions. Since higher production of a protein can be achieved by generating multiple copies of the genes, it is

not surprising winter flounder evolved a multi-gene family of AFPs genes, a strategy that allows them to produce higher levels of AFPs. A similar example of adaptation is the gene amplification of esterase genes that emerged by natural selection in mosquitos of the species *Culex pipiens* (*Guillemaud et al. 1997*), multiple copies of the genes enhance the production of the metabolic enzymes resulting in resistance to organophosphates. The enzymes work by quickly metabolizing the insecticide to non-toxic products allowing the mosquitos to survive in very toxic environments.

The combined numbers of wflAFP-6 and wflAFP-8 in winter flounder from Newfoundland were previously reported at 30-40 copies per diploid genome by Southern blotting (Hayes et al. 1991, Scott et al. 1985, Scott et al. 1988). These genes were quantified together due to their sequence similarity (Davies 1992). Our qPCR method was able to quantify each gene separately with precision showing that there were on average 33 copies of wflAFP-6 and 8 copies of wflAFP-8 per haploid genome in samples from Newfoundland. The copy number we determined is higher than the reported copy number for these genes. We attribute this to the limited sensitivity of southern blotting. The higher number of wflAFP-6 genes relative to wflAFP-8 genes is in accordance with studies showing that the most common AFP in fish serum is wflAFP-6 (Fourney et al. 1984).

There were also differences in copy numbers within locations, especially copy numbers of wflAFP-6, some fish had very few copies while others had hundreds. Gene copy number variation is not uncommon, in gene duplication by unequal crossing over in tandem repeats, some gene copies that are in tandem can be clustered together and oriented in the same direction. During unequal crossing over individual copies can be removed from (or added to) via recombination or replication errors, leading to alleles with different numbers of repeats (Graham 1995, Magadum et al. 2013, Zhang 2003).

There was less variation in the copy numbers of the hyperactive Maxi within locations, however it was very surprising that there were very few copies of this gene. This may be explained by the fact that Maxi is a bigger protein and more active than the others AFPs, so that it increases freeze resistance even when present at low copy numbers (Marshall et al. 2004). The analysis of antifreeze activity and gene copy numbers revealed that some fish had high antifreeze activity even with low copy number of AFPs genes, this raised the possibility that Maxi is encoded by a multigene family with other members not represented in the qPCR assay. To answer this question we implemented Southern blot analysis of Maxi and found that although the specific gene copy numbers could not be determined, the band pattern and organization of Maxi indicated a multi-gene family. More work by qPCR would need to be done to investigate Maxi gene copy numbers in winter flounder. For now, knowing that Maxi is composed of a multi-gene family means that there might be other isoforms contributing to the winter flounder freeze resistance.

Through this work, a standardized method that could be implemented in a laboratory setting was developed. Sensitive qPCR based dosage assays for the genes encoding the four major serum AFPs (wflAFP-6, wflAFP-8, wflAFP-9 and Maxi) were developed. These assays were then applied to measure gene dosage in flounder from populations spanning a wide geographic range along the eastern coast of North America. This method can be used to study more deeply the variability of AFP genes in different populations of winter flounder, but also can be used to analyze fish stocks for aquaculture. Winter flounder is a promising species for aquaculture because is naturally tolerant to the low water temperatures in the region due to its AFPs. Also, winter flounder is well-adapted to culture practices and grow relatively fast (Litvak 1999). Fish stocks could be screened by the qPCR method we employed, not only to identify fish broodstock that are best fitted on a geographical basis, but also to potentially confirm the

cold fitness of the fish intended for an aquaculture project. Our results suggest that the qPCR assay suite developed in this project would provide suitable data for selecting source locations for the most freeze-resistant fish. Flounder with the highest dosages of one type of AFP or a combination of AFPs should be the best equipped to avoid freezing and potentially be used in coastal cold-water aquaculture.

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