Molecular events in the evolution of freeze resistant fish: type I AFP in Cunner (*Tautogolabrus adspersus*) and type III AFP in the rock gunnel

(Pholis gunnellus) and radiated shanny (Ulvaria subbifurcata)

By

© Rod S. Hobbs

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Abstract

Type I Antifreeze proteins (AFPs) have been fully characterized in two teleost Orders; Pleuronectiformes and Scorpaeniformes. Results from this study have confirmed that cunner [*Tautogolabrus adspersus* (Family Labridae)], a member of a third Order (Order Perciformes), also produce a type I AFP. This finding solidifies the hypothesis that type I AFPs are multiphyletic in origin and suggests that they represent remarkable examples of convergent evolution within three Orders of teleost fish. The cunner AFP was purified from blood plasma and found to belong to what is now known as classical type I AFP with their small size (mass = 4095.16 Da), alanine-richness (>57 mol%), high α -helicity (>99%) with the ability to undergo reversible thermal denaturation, 11-amino acid (ThrX₁₀) repeat regions within the primary structure, the capacity to impart a hexagonal bipyramidal shaping to ice crystals and the conservation of an ice binding site found in many of the other type I AFPs. cDNA library sequencing indicated that the protein is produced without a signal sequence yet the translated product of the AFP cDNA suggests that it codes for the AFP isolated from plasma.

Despite the fact that cunner produce AFPs, the AFP concentration within the blood is too low to provide whole body freeze protection. Southern and northern blot analyses carried out in this study establishes that the cunner AFP genes belong to a multigene family that is predominantly expressed in external epithelia (skin and gill filaments). These results support the hypothesis that the survival of cunner in icy waters is attributable in part to epithelial AFPs that help block ice propagation into their interior milieu. In contrast to the cunner, heterospecifics occupying the same habitat have greater freeze protection because they produce AFPs in the liver for export to the plasma as well as in external epithelia. Since the external epithelia would be the first tissue to come into contact with ice it is possible that one of the earliest steps involved in the evolution of freeze resistant fish could have been the expression of AFPs in tissues such as the skin. I suggest that this epithelial-dominant AFP expression represents a primitive stage in AFP evolution and propose that cunner began to inhabit "freeze-risk ecozones" more recently than heterospecifics.

Type III AFPs have been identified in a single suborder of fish (Zoarcoidei). In the eelpouts (family Zoarcoidae) and wolffishes (family Anarhichadidae), freeze protection is accomplished through the cooperation of two distinct groups of AFP isoforms: SP-Sephadex (SP) and QAE-Sephadex (QAE) binding types. Although members from most families within the suborder Zoarcoidei are known to produce type III AFP, the cooperative action between AFP isoforms has not been confirmed outside of these two families. Through sequence analysis of AFP cDNAs isolated in this study, I have confirmed the presence of both SP and QAE type III isoforms in two additional families within this suborder: Pholidae [rock gunnel (*Pholis gunnellus*)] and Stichaeidae [radiated shanny (*Ulvaria subbifurcata*)]. These results support the hypothesis that cooperativity between SP and QAE isoforms evolved prior to the divergence of families within the suborder Zoarcoidei.

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List of Abbreviations

AA	Amino acid
AFP	Antifreeze protein
AFGP	Antifreeze glycoprotein
AF(G)P	Antifreeze proteins and glycoproteins
AW	Atlantic wolffish
CD	Circular dichroism
CDS	Coding sequence
cDNA	Complementary DNA
CRD	Carbohydrate recognition domain
DNA	Deoxyribonucleic acid
FP	Freezing point
gDNA	Genomic DNA
HPLC	High performance liquid chromatography
kDa	Kilodalton
LGT	Lateral gene transfer
MALDI	Matrix assisted laser desorption ionization
MP	Melting point
mRNA	Messenger RNA
MS	Mass spectroscopy
Mya	Million years ago
nt	Nucleotide
ORF	Open reading frame
PCR	Polymerase chain reaction
QAE	Quaternized aminoethy
RLM-RACE	RNA ligase mediated rapid amplification of cDNA ends
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
S	Sedimentation coefficient
SP	Sulphopropyl
TH	Thermal hysteresis
Tm	Melting temperature

Chapter 1: Introduction

1.1 General Effects of Cold

Water is essential for the survival of all organisms. As the primary cellular solvent, water is required for the metabolic biochemical reactions necessary to sustain life. For these reactions to take place, however, cellular water must be available in its liquid form. While declines in temperature reduce the rates of these life sustaining biochemical reactions, reduction in temperature below the organism's freezing point can result in the formation of ice. Restriction of ice formation to extracellular spaces can lead to physiologically important osmotic changes in the concentrations of cellular ions. In fact, the sensitivity of an organism to extracellular freezing arises from this dehydrating effect, in addition to mechanical damage to biological components due to growing ice. At the cellular level both dehydration and extracellular freezing cause similar effects, and as a result elicit similar adaptive responses to limit tissue injury (Ring and Danks 1994, Convey 2000). In contrast, intracellular freezing generally results in cellular death as the expansion of water after freezing and the formation of ice crystals can damage cellular components (Mazur 1984).

Since ectotherms do not maintain internally generated heat, they remain at the temperature of the external environment. For the majority of the last 200 million years, the earth's climate was one of relatively warm temperatures (Fletcher *et al.* 2007, Retallack 2009). This provided ectotherms with an environment void of the risk of freezing. This changed abruptly about 30-45 Mya when environmental temperatures dropped and these organisms were threatened with the reality of sub-freezing environmental temperatures and ice for the first time (Coxall *et al.* 2005). Some organisms were unable to adapt to the changing conditions and many species became extinct (reviewed in Prothero 1994). What was catastrophic for some proved

fortuitous for others as niches left void by extinction provided opportunity to those that could withstand the harsh conditions. For instance, habitats rich in invertebrate food sources within freeze risk ecozones where competition was low provided motivation for invading species that could avoid freezing. In order to take advantage of these newly available resources, however, mechanisms had to evolve to allow ectotherms to tolerate these potentially lethal environments.

1.2 Strategies to Deal with Cold

The evolution of life in the presence of potentially lethal low temperatures and ice has resulted in two very different approaches to survival. Freeze tolerant organisms, including many insects, reptiles and amphibians have adapted to withstand the formation of ice in extracellular spaces. Many of these organisms can withstand the periodic freezing of a majority of their bodily water (Cai and Storey 1997). This is accomplished through a variety of means including controlled extracellular freezing and increased production of specific molecules that help reduce biological damage from ice and reduce osmotic stress (De Croos et al. 2004, Duman 2001, Storey and Storey 1992, Storey and Storey 1996). Extracellular freezing aided by ice nucleators, for instance, is induced at high sub-zero temperatures thereby minimizing the risk of fast uncontrolled freezing generally resulting from non-specific nucleation at low temperatures (Johnston 1990, Storey and Storey 1996). A decreased rate of ice formation not only limits the potential for cellular structural damage, but increases the time available for metabolic adjustments necessary for freeze survival. The allowance of extracellular freezing indirectly reduces the risk of intracellular freezing due to the associated osmotic movement of water out of the cell. Conversely, in some instances, excess water loss from the cell is prevented by the accumulation of low molecular weight polyols that maintain internal solute concentrations

(Zachariassen 1991) and prevent the cell from reaching a critical minimum cell volume (Storey and Storey 1996).

A seemingly less risky strategy is that of freeze avoidance, an approach employed by a variety of ectotherms, particularly fish where tolerance of freezing is not a viable response to cold. Marine fish from temperate and polar oceans can encounter seawater temperatures near -1.8 °C and survive by utilizing a variety of tactics. Many species of fish accumulate ions or small solutes such as Na⁺/Cl⁻ or glycerol to colligatively lower the freezing point of their plasma (Fletcher 1977, Lewis *et al.* 2004, Raymond 1992). Alternatively, some fish can live in a supercooled state at temperatures below their body freezing point by avoiding contact with ice and ice nucleators. However, ice contact in this supercooled state is lethal (Scholander *et al.* 1957). This means that fish living in marine habitats characterized by cold water temperatures and ice ("freeze risk ecozones") require alternative protection. This has been accomplished through the production of a specialized group proteins and glycoproteins.

1.3 AF(G)P Discovery and Mechanism of Action

The term "antifreeze" as it relates to freeze protection in fish was coined by a group of researchers in the 1950s. During an expedition to Labrador, Scholander and his colleagues observed abundant fish life despite the fact that the surrounding sea water temperature was approximately 1 °C below the freezing point of the fish's bodily fluids. They discovered that some of these fish could exist in a supercooled state, by residing at depths where contact with ice was unlikely, but that they immediately froze to death upon contact with ice. On the other hand, ice was not lethal to a second group of fish living under similar temperatures in shallow water (Scholander *et al.* 1957). An unknown "antifreeze" substance was deemed responsible for the

latter group's survival. Over the following decades, these "antifreezes" were identified as proteins and glycoproteins (DeVries and Wohlschlag 1969).

Antifreeze proteins (AFPs) and glycoproteins (AFGPs) (collectively termed AF(G)Ps) are up to 500 times more effective at lowering the freezing point of an aqueous solution than would be expected from their colligative properties alone. Unlike colligative solutes, AFPs do not significantly affect osmolality of the plasma (Fletcher *et al.* 1999) a critically important characteristic since most osmolytes become cytotoxic at concentrations required to afford freeze resistance. AFPs lower the freezing point of a solution by binding to specific planes of ice and inhibiting further growth, in a process called adsorption-inhibition (Fletcher *et al.* 2001, Raymond and DeVries 1977). During this process, the spacing of bound AFPs on ice makes further addition of water energetically unfavourable, effectively inhibiting the growth of an ice crystal (Fig 1-1). This creates a depression in the observable freezing point (non-equilibrium freezing point) of a solution and as a result a gap between the freezing point and melting point, termed thermal hysteresis (TH) (Wilson 1993).

1.4 AFP Classification

AFPs have now been described in a diverse assortment of organisms including plants (Griffith *et al.* 1992), insects (Graham *et al.* 1997), algae (Bayer-Giraldi *et al.* 2010), nematodes (Wharton *et al.* 2005) fungi and bacteria (Duman and Olsen 1993). Within teleost fish, AFPs are classified into five distinct groups according to their physical and biochemical properties.

Type I AFPs are the most comprehensively studied group of AFPs that were first identified and characterized in winter flounder [*Pseudopleuronectes americanus* (Duman and DeVries 1974)]. These are alanine-rich (~60 mol%), amphipathic α-helical proteins that range in

Figure 1-1. Mechanism by which AF(G)Ps cause FP depression (and TH), according to the theory of adsorption/inhibition [image modified from Desjardins (2013)]



size from about 3.3-17.8 kDa (Duman and DeVries 1976, Evans and Fletcher 2001, Gauthier *et al.* 2005, Low *et al.* 1998, Marshall *et al.* 2004). Since their initial discovery, type I AFPs have been described in a variety of related and unrelated fish species from three distinct teleost Orders: Pleuronectiformes (Family Pleuronectidae [seven species (Gauthier *et al.* 2005, Knight *et al.* 1991, Nabeta 2009, Scott *et al.* 1987, Scott *et al.* 1988a)]), Scorpaeniformes (Family Cottidae [four species (Chakrabartty *et al.* 1988, Hew *et al.* 1985, Low *et al.* 2001, Yang *et al.* 1988)], and Family Liparidae [two species (Evans and Fletcher 2001)]) and Perciformes [Family Labridae, two species (Evans and Fletcher 2004, Hobbs *et al.* 2011)].

Type II AFPs are large (ca. 11-24 kDa) cysteine rich molecules characterized by a β sheet secondary structure and the presence of five disulphide bridges. Type II AFPs have been characterized in sea raven [*Hemitripterus americanus*, Family Hemitripteridae, Order Scorpaeniformes (Ng *et al.* 1986, Slaughter *et al.* 1981)], longsnout poacher [*Brachyopsis rostratus*, Family Agonidae, Order Scorpaeniformes (Nishimiya *et al.* 2008)], the rainbow smelt and Japanese smelt [*Osmerus mordax* and *Hypomesus japonicus*, Family Osmeridae, Order Osmeriformes (Ewart *et al.* 1992, Yamashita *et al.* 2003)] and Atlantic herring [*Clupea harengus*; Family Clupeidae, Order Clupeiformes (Ewart and Fletcher 1993)]. These AFPs appear to have evolved from the carbohydrate recognition domain (CRD) of calcium-dependent (C-type) lectins (Ewart *et al.* 1992), traditionally utilized in the immune response by recognizing carbohydrate antigens on the surface of bacteria. Interestingly, differences have evolved as smelt and herring AFP contain the galactose-binding motif of galactose binding lectins and Ewart *et al.* (1998) identified this as the ice binding site in herring. This is not the case with the sea raven AFP (Loewen *et al.* 1998). In addition, herring and smelt AFP require Ca²⁺ for activity, as do C- type lectins (Ewart *et al.* 1992, Ewart and Fletcher 1993) while Ca^{2+} is not required for AFP activity in sea raven or longsnout poacher (Ng *et al.* 1986, Nishimiya *et al.* 2008).

Type III AFPs have been identified in members of five families of the Suborder Zoarcoidei (Order Perciformes) including the ocean pout [Macrozoarces americanus, Family Zoarcidae (Hew et al. 1988)], notched-fin eelpout [Zoarces elongates, Family Zoarcidae (Nishimiya et al. 2005)], European eelpout [Z. viviparous, Family Zoarcidae (Albers et al. 2007)], two Antarctic eelpouts [Austrolycicthys brachycephalum (Cheng and DeVries 1989) and Rhigophila dearborni (Schrag et al. 1987), Family Zoarcidae], Laval's eelpout [Lycodes] lavalaei, Family Zoarcidae (Shears et al. 1993)], Polar eelpout [L. polaris, Family Zoarcidae (Schrag et al. 1987)], radiated shanny [Ulvaria subbifurcata, Family Stichaeidae (Shears et al. 1993)], rock gunnel [Pholis gunnellus, Family Pholidae (Shears et al. 1993)], spotted wolffish [Anarhichasminor, FamilyAnarhichadidae (Cheng et al. 2006)] and Atlantic wolffish [Anarhichasminor, FamilyAnarhichadidae (Scott et al. 1988b)]. These small proteins (ca. 6.5 kDa) fold into a globular protein with one flat surface and exhibit no obvious bias for any particular amino acid (Sonnichsen et al. 1996). In ocean pout, at least 12 isoforms have been identified (Li et al. 1985) from which two immunologically distinct groups [SP Sephadex (SP) and QAE Sephadex (QAE) binding types] have been characterized (Hew et al. 1984, Hew et al. 1988).

Type IV AFP has only been described in longhorn sculpin (*Myoxocephalus octodecimspinosis*) as a 12.3 kDa, glutamine-rich protein that forms an antiparallel helix bundle (Deng *et al.* 1997). Recently, however, the validity of their inclusion with AFPs has been called into question as their affinity to ice appears to be coincidental (Gauthier *et al.* 2008).

The antifreeze glycoproteins (AFGPs) are found in Antarctic notothenioids (DeVries and Wohlschlag 1969) and northern cods (Hew *et al.* 1981). These O-glycosylated proteins range from 2-32 kDa and contain multiple Ala-Ala/Pro-Thr repeats with the disaccharide attached to each Thr residue (DeVries *et al.* 1971, Hew *et al.* 1981).

1.5 AF(G)P Physiology

AF(G)Ps are regulated in response to the environmental conditions faced by the fish with AF(G)Ps usually produced when and where there is a risk of freezing. As a result, fish inhabiting Arctic and Antarctic environments, where sea water temperatures are perpetually below the fish's freezing point, continually produce AF(G)Ps. In fact, AFGP synthesis in the Antarctic notothenioid *Pagothenia borchgrevinki* does not change with experimentally varied temperatures (O'Grady *et al.* 1982) indicating a likely genetic predisposition for continuous AFGP production. On the other hand, fish inhabiting north temperate environments follow a seasonal cycle of AF(G)P production with high AF(G)P levels in the winter and low or no AF(G)P produced in the summer. For instance, winter flounder and shorthorn sculpin produce AFPs in the fall prior to the threat of freezing and reach maximal levels when sea water temperatures are coldest (Fletcher *et al.* 1985a, Hew *et al.* 1980). Ocean pout, alternatively, produce AFPs year-round although winter levels are much higher than those observed in the summer (Fletcher *et al.* 1985b).

1.5.1 Liver-type AFP

In winter flounder, the liver was originally identified as the source of AFP production where they are translated as 82 residue preproproteins. The signal sequence is cleaved co-translationally during extracellular transport to yield a 61 residue proprotein which is finally processed in the blood to give a mature 37 residue AFP (Davies *et al.* 1982). The cycle of AFP production is regulated by the central nervous system in response to day length (Fletcher 1981).

During the summer when days are long, growth hormone (GH) is responsible for the inhibition of AFP mRNA transcription (Idler *et al.* 1989). As day length decreases in the fall GH production is reduced, thereby eliminating the negative regulation on AFP synthesis. Although water temperature appears to play no role in AFP mRNA production or termination, it does play a role in mRNA stability and accumulation as high water temperatures prevent AFP accumulation in the winter (Fletcher 1981). On the other hand, water temperature appears to be the sole factor responsible for AFGP regulation in Atlantic cod (Fletcher *et al.* 1987) where manipulating day length alone has no effect of AFGP production.

It has been demonstrated that fish skin provides an effective barrier to ice passage into the body, and that cell membranes intrinsically protect the cell from intracellular freezing at high freezing temperatures (Valerio *et al.* 1992). However, the gills and gut, with only a single layer of epithelial cells are prime locations for ice to enter circulation. Initially, the circulating AFPs originating from the liver were thought to be the sole means of extracellular freeze protection in fish, and that this was accomplished by binding to circulating ice crystals and preventing their growth. This seemed realistic since the freezing point of fish blood closely matched the whole fish freezing point (Fletcher *et al.* 1986, Fletcher *et al.* 2001). However, relatively recently, a novel group of AFPs were discovered in non-liver tissues in a number of fish. These were named "skin-type AFPs" as opposed to the classic "liver-type AFPs" originally described and are expressed in a variety of epithelial tissues, mainly skin and gill filaments (Gong *et al.* 1996, Schneppenheim and Theede 1982, Valerio *et al.* 1990).

1.5.2 Skin-type AFP

Skin-type AFPs were initially discovered in the European shorthorn sculpin (Schneppenheim and Theede 1982). However this finding along with a similar discovery in cunner (Valerio et al. 1990) was not fully appreciated until Gong et al. (1992) identified AFP mRNA in a variety of non-liver tissues in winter flounder and ocean pout. This was followed up by examining a winter flounder skin cDNA library for the presence of AFP genes, where a novel set was discovered (Gong *et al.* 1996). Interestingly, these skin-type AFPs lacked a signal or prosequence indicating a possible intracellular role. However, this hypothesis has yet to be substantiated as experiments by the same authors show retention of skin-type AFPs in the cytoplasm of gill epithelial cells, as well as in the interstitial spaces between skin cells (Murray et al. 2002a, Murray et al. 2002b). In addition, unlike the liver-type AFPs which exhibit extreme (500-700 fold) seasonal variation in their expression, skin-type AFPs only exhibit moderate 5-10 fold variation and are not regulated by growth hormone (Gong *et al.* 1995). Skin-type AFPs have now been characterized in a number of species (Evans and Fletcher 2004, Evans and Fletcher 2005, Gong et al. 1996, Low et al. 1998, Low et al. 2001,) illustrating that they may be ubiquitous among AFP producing fish. In fact, it has been hypothesized that a skin-type AFP is the ancestral AFP which gave rise to the liver-type AFP (Evans and Fletcher 2004, Low et al. 2002).

1.6 AF(G)P Evolution

Much effort has gone into elucidating the evolutionary origins of AF(G)Ps. A conundrum that has made this difficult is the fact that few clues are provided by the phylogenetic relationships among AF(G)P producing fish. For instance, species from closely related taxa can produce drastically different AF(G)Ps while distantly related species can produce virtually identical types (Fig 1-2). This has been explained in part by the fact that current families of teleost fish were already established (or in the process of being established) by the onset of **Figure 1-2.** AF(G)Ps distribution within AF(G)P-producing teleosts. The structures of the different AFPs are shown (that of the AFGP is not yet resolved). Image modified from Fletcher *et al.* (2001)



Cenozoic sea-level glaciations about 30-45 Mya (Deconto *et al.* 2008, Scott *et al.* 1986, Stickley *et al.* 2009, Zachos *et al.* 2001) resulting in their need to develop independent strategies for freeze protection. In addition, the multifaceted structure of ice provided multiple recognition sites for potential proteins to bind, allowing different protein molecules to achieve the same ice growth inhibiting effect (Davies *et al.* 2002). As a result, a protein with an affinity to any of these planes had the potential to evolve into an AF(G)P.

Despite the aforementioned handicap, research has presented compelling cases for the evolutionary origin of a number of AF(G)Ps. The most convincing of these is the trypsinogenlike gene origin for AFGPs in Antarctic Notothenioids. In this case, not only has sequence analyses comparing the AFGP to a pancreatic trypsinogen gene implied a direct evolutionary link (Chen *et al.* 1997), but a transcriptionally active chimeric trypsinogen-AFGP gene has been isolated (Cheng and Chen 1999). This all but confirms the evolutionary pathway between protease and AFGP genes by a process of neofunctionalization after gene duplication.

Unfortunately, determining the evolutionary history of AF(G)Ps is not always straight forward, as indicated by the controversial hypothesis presented to explain the evolution of very similar type II AFPs in the genetically unrelated species sea raven, smelt and herring (Fig 1-2). One thing that appears likely is that all type II AFPs evolved from c-type lectins, specifically their carbohydrate recognition domain (Ewart and Fletcher 1993, Ewart *et al.* 1998). The controversy lies in whether convergent evolution (Fletcher *et al.* 2001), direct descent (Liu *et al.* 2007) or the recently hypothesized lateral gene transfer (LGT) (Graham *et al.* 2012) was the cause for such genetically dissimilar species producing the nearly identical type II AFPs. Current support lies with LGT due to the shortcomings of a convergent evolution hypothesis, mainly that non-coding DNA regions of type II genes share striking sequence similarity between the

different species (Graham *et al.* 2008), a detail not expected from convergent evolution, but supportive of LGT. Since non-coding DNA would not be under selective pressure (as with the coding DNA) one would expect significant differences in non-coding DNA sequences between the species in question (especially smelt and herring) due to the large genetic distance between these groups. In addition, the fact that no type II AFP homologues have been found at the same locus in any other species sheds significant doubt on the possibility of descent from a common ancestor as this would have required multiple occasions of gene loss.

A similar case of an AF(G)P evolving from a polysaccharide-binding protein is found with the type III AFPs. In this case, however, the pathway resembles that of the AFGPtrypsinogen previously described where gene duplication followed by neofunctionalization resulted in the generation of a protein with novel function. Type III AFPs show homology with sialic acid synthase (SAS) (Baardsnes and Davies 2001), an enzyme involved with sialic acid metabolism. The SAS-B protein was shown to have rudimentary TH activity in addition to SAS activity when expressed *in vitro*, satisfying one requirement of the escape from adaptive control (EAC) theory (Deng *et al.* 2010).

In the above cases, the impetus for AF(G)P evolution seems to have been a whole genome duplication event that occurred in an early teleost ancestor (Hoegg *et al.* 2004, Volff 2005). Generation of a duplicated set of genes would provide the raw materials required for AF(G)P evolution. The presence of a second gene would allow mutational modification of the duplicate without affecting the original, thereby freeing the latter to possibly develop a novel function such as ice affinity.

The selective advantage afforded to fish that expressed AF(G)Ps led to the amplification of AF(G)P genes during Cenozoic cooling. This has been demonstrated through genomic

(Southern blot) analyses on numerous AF(G)P producing species. In fact, the presence of AF(G)P multigene families appears to be the rule with the extent of amplification directly related to the physical environmental conditions faced by the fish, with the one exception being the smelt (Graham *et al.* 2012). For instance, winter flounder [~80 AFP gene copies (Gong *et al.* 1996, Scott *et al.* 1985)], and wolffish [~85 gene copies (Scott *et al.* 1988b)] inhabit the shallow, ice-laden waters of the north Atlantic ocean. Alternatively, a deeper dwelling cousin of the winter flounder, the yellowtail flounder only has about 10-12 AFP gene copies in its genome (Scott *et al.* 1988a), while the counterpart to the Atlantic wolffish (AW), the spotted wolffish has only a third the number of AFP genes as AW (Desjardins *et al.* 2012), because the risk of ice contact and sub-zero water temperatures is lower than that of their shallow water counterparts. This effect of environment on AF(G)P gene amplification is directly evident in ocean pout where population differences in AFP copy number have been observed (Hew *et al.* 1988).

1.7 Experimental Animals

Cunners (*Tautogolabrus adspersus*, Walbaum, 1792) belong to the Order Perciformes (family Labridae) whose members are typically found in tropical and subtropical areas of the Atlantic, Pacific and Indian Oceans. Cunners, however, are abundant in the more northerly waters of the Atlantic Ocean as far north as Newfoundland, Canada (Scott and Scott, 1988). These fish generally reside in shallow coastal waters throughout the year and as a result, encounter sub-freezing water temperatures and ice during the winter. During this time, cunners are known to become torpid and cease feeding as a behavioural strategy for freeze avoidance (Green and Farwell, 1971).

Radiated shannies (*Ulvaria subbifurcata*, Storer, 1839) (Order Perciformes, family Stichaeidae) and rock gunnels (*Pholis gunnellus*, Linnaeus, 1758) (Order Perciformes, family

Pholidae) belong to closely related families of small, eel-like fish that inhabit shallow, inshore waters of the Pacific and Atlantic Oceans. In the Atlantic Ocean, both can be found as far north as Newfoundland, Canada (Scott and Scott, 1988).

1.8 Thesis Objectives

The aim of this study was to examine the AFPs, their genes, genomic organization and expression in type I and type III AFP producing representatives from two Suborders, Labroidei and Zoarcoidei, within the Order Perciformes (Fig 1-2). The Labrids that inhabit the north east coast of North America are the only Perciform group known to produce type I AFPs. The cunner *Tautogolabrus adspersus* which resides along coastal Newfoundland is the northernmost representative of this group. Previous preliminary studies of this species established that cunner produce a type I AFP. The present study was designed to fully characterize this AFP and its gene(s) to determine how closely its sequence, structure and tissue specific expression resembled those observed for the type I AFPs found in the right-eyed flounders (Order Pleuronectiformes), and the sculpins and liparids (Order Scorpaeniformes).

Type III AFPs have been identified in five of the nine families in the Suborder Zoarcoidei (Order Perciformes): Zoarcidae, Cryptacanthodidae, Anarhichadidae, Pholidae and Stichaeidae. The remaining four have not yet been examined. Genomic Southern analyses of members from four of the five identified families (Cryptacanthodidae excluded) have established that in each case the type III AFPs are encoded by multigene families. In addition detailed structural studies of the AFPs in members of two of these families [Zoarcidae (eelpouts) and Anarhichadidae (wolffish)] revealed that in each case they are present as mixtures of SP-Sephadex (SP) and QAE-Sephadex (QAE) binding isoforms. Although both isoforms can bind to ice the SP isoform has little ability to prevent ice growth. However, the SP isoform can block ice growth if small

quantities of the QAE isoform are present in the same solution. Therefore it is evident that both isoforms act cooperatively to protect eelpouts and wolffishes from freezing. The question to be resolved is whether cooperativity of the SP and QAE isoforms is confined to the eelpout and wolffish families or is it common to all AFP producing families within the Suborder Zoarcoidei? The present study was carried out to answer this question in part by determining whether SP and QAE AFP genes are expressed in the radiated shanny (Family Stichaeidae) and rock gunnel (Family Pholidae).

1.9 References

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Chapter 2: Isolation and characterization of type I antifreeze proteins from cunner, *Tautogolabrus adspersus*, Order Perciformes

Preface

This chapter consists of a version of a manuscript published in the journal FEBS J [**Hobbs** RS, Shears MA, Graham LA, Davies PL,Fletcher GL (2011) Isolation and characterization of type I antifreeze proteins from cunner, *Tautogolabrus adspersus*, order Perciformes.FEBS J. 278(19):3699-710]. The experimental research and data analyses were performed by RSH. The manuscript was written by RSH with editorial input from MAS, LAG, PLD and GLF.

Abstract

Antifreeze proteins (AFPs) are produced by many species of teleost fish that inhabit potentially lethal ice-laden seawater and afford them protection from freezing. To date type I AFPs have been fully characterized in two teleost Orders; Pleuronectiformes and Scorpaeniformes. In this study, we report the isolation and complete characterization of a type I AFP present in fish from a third Order: cunner (*Tautogolabrus adspersus*); Order Perciformes (Family Labridae). This protein was purified from blood plasma and found to belong to what is now known as classical type I AFP with their small size (mass = 4095.16 Da), alanine-richness (>57 mol%), high α -helicity (>99%) with the ability to undergo reversible thermal denaturation, 11-amino acid (Thr X_{10}) repeat regions within the primary structure, the capacity to impart a hexagonal bipyramidal shaping to ice crystals and the conservation of an ice binding site found in many of the other type I AFPs. Partial *de novo* sequencing of the plasma AFP accounted for approximately half of the peptide mass. Sequencing of a combined liver and skin cDNA library indicated that the protein is produced without a signal sequence. In addition the translated product of the AFP cDNA suggests that it codes for the AFP isolated from plasma. These results further solidify the hypothesis that type I AFPs are multiphyletic in origin and suggest that they represent remarkable examples of convergent evolution within three Orders of teleost fish.

2.1 Introduction

Many species of teleost fish that inhabit potentially ice-laden seawater during the winter produce antifreeze proteins/glycoproteins for protection against freezing (Fletcher *et al.* 1986). The antifreeze proteins (AFPs) are produced by diverse fish species and are classified into four distinct categories (types I-IV) based on their physical characteristics. It should be noted, however, that the type IV AFP found in longhorn sculpin plasma does not appear to function as an AFP and its affinity for ice might be coincidental (Gauthier *et al.* 2008). Despite their structural differences, all AFPs work non-colligatively by depressing the freezing point of an aqueous solution via an adsorption-inhibition mechanism (Raymond and DeVries 1977). Reviews on structure/function characteristics, diversity, physiology and molecular genetics of fish AFPs are available (Davies *et al.* 2002, Ewart *et al.* 1999, Fletcher *et al.* 2001, Harding *et al.* 1999).

Of the four classes of AFPs, the type I AFPs originally discovered in winter flounder (*Pseudopleuronectes americanus*) (Duman and DeVries 1974) have been the most extensively studied. These proteins are alanine-rich, amphipathic α -helical proteins. They have a helix periodicity of three turns every 11 residues rather than the more typical five turns every 18 residues (Sicheri and Yang 1995). This periodicity is reflected in the sequences, which contain 11-amino acid repeats (ThrX₁₀) where X is frequently alanine (Low *et al.* 2001). Since their initial discovery these type I AFPs have been characterized and sequenced in members of two distinct teleost Orders: Pleuronectiformes (Family Pleuronectidae [seven species (Gauthier *et al.* 2005, Knight *et al.* 1991, Nabeta 2009, Scott *et al.* 1987, Scott *et al.* 1988, Hew *et al.* 1985, Low

et al. 2001, Yang *et al.* 1988)], and Family Cyclopteridae [two species (Evans and Fletcher 2001)]).

Cunners (*Tautogolabrus adspersus*) belong to the Order Perciformes (family Labridae), marine fishes typically found in tropical and subtropical areas (Scott and Scott 1988). Unlike most members of this family, cunners are prevalent in the more northerly waters of the Atlantic Ocean as far north as Newfoundland, Canada. During winter, they become torpid and remain in shallow waters where contact with sub-freezing temperatures and ice makes them prime candidates for AFP production. AFP activity has been reported in cunner skin extracts (Valerio *et al.* 1990, Valerio *et al.* 1992) and more recently, a type I AFP was purified and partially characterized from cunner skin homogenates (Evans and Fletcher 2004). Low levels of AFP activity have also been detected in cunner blood plasma during winter suggesting the presence of circulating type I AFP.

The purpose of this study was to purify, characterize and sequence a circulating plasma AFP from cunner and, with the use of the amino acid sequence, isolate and sequence the AFP cDNA corresponding to the mRNA responsible for its production. The results indicate that the sequence and structure of the cunner AFP bears a remarkable resemblance to what are regarded as the small classical type I AFP isotypes found in the Cottids and Pleuronectids. This discovery provides further insight into the evolutionary origins of type I AFPs and the structural features required for helix stability and their ability to bind ice.

2.2 Materials and Methods

2.2.1 Sample collection

Fifty cunner individuals were collected by Ocean Sciences Centre (OSC) divers near Logy Bay, Newfoundland and Labrador, Canada in the winter of 2008 and maintained at the

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OSC in 250 l aquaria supplied with flowing seawater (32-33‰ salinity) under seasonally normal photoperiod and water temperature. Water temperature ranged from 15 °C in August to -1.8 °C during April. One group of fish were blood sampled (0.5 ml) at monthly intervals in order to determine whether there was a seasonal cycle in plasma AFP activity. Other fish were exsanguinated in March to obtain enough plasma for AFP purification. Fish were anesthetized using MS-222 and bled from a caudal blood vessel using syringes equipped with 20 gauge needles. The freshly drawn blood was aliquoted into heparin-containing BD Vacutainers[®], mixed briefly, and centrifuged at 5000 x g for 10 min. Following centrifugation, blood plasma was removed, placed in plastic centrifuge tubes and stored in a -70 °C freezer prior to analysis.

All measures were taken to minimize pain and discomfort during animal experiments. Guidelines followed were those of the Canadian Council on Animal Care (CCAC).

2.2.2 Isolation and purification of plasma AFP

Plasma samples collected during March were pooled (8-12 ml) and applied to a Sephadex G-75 gel-permeation column (2.5 x 90 cm) maintained at 4 °C and eluted with 0.1 M NH₄HCO₃. Individual fractions (4.5 ml) were lyophilized and resuspended in 100 μ l of 0.1 M NH₄HCO₃ and tested for AFP activity. AFP-active fractions were pooled and applied to a second Sephadex G-75 gel-permeation column (1.5 x 90 cm). Fractions were again lyophilized and resuspended in 100 μ l of 0.1 M NH₄HCO₃ to test for AFP activity. AFP-active fractions were pooled, lyophilized and resuspended in 500 μ l of 0.1 M NH₄HCO₃. This partially purified AFP was further purified by reversed-phase HPLC using a Phenomenex Jupiter 5 μ m C18 column (250 x 4.6 mm, 300 Å). A gradient of 30 - 60% acetonitrile, 0.1% trifluoroacetic acid (TFA) (Solvent A) and 0.1% TFA (Solvent B) was used at a flow rate of 1 ml/min. Individual peaks were collected, lyophilized and resuspended in 50 μ l 0.1 M NH₄HCO₃ for AFP activity measurements

to identify the peak containing AFP. The corresponding fractions from several purification runs were then pooled, lyophilized, resuspended in 30 μ l water and desalted with ZipTip[®] pipette tips (Millipore, Etobicoke, ON) as per manufacturer's instructions. The AFP concentration was determined by amino acid analysis and serial dilutions were prepared in water to generate a TH activity curve. This HPLC purified AFP sample was separated on 14% polyacrylamide gels in a Tris-Tricine buffer system (Schagger and von Jagow 1987) and stained with 0.3 M CuCl₂ (Lee *et al.* 1987).

2.2.3 Measurement of antifreeze activity

Thermal hysteresis (TH) was measured using a Clifton Nanolitre Osmometer (Clifton Technical Physics, Hartford, NT), following the procedure of Evans *et al.* (2007). TH is defined as the difference between the melting point and non-equilibrium freezing point of a solution (in °C) and is used as a measure of antifreeze activity. TH measurements were carried out on HPLC purified AFP as outlined in the previous paragraph. TH of G-75 purified AFP and plasma samples was measured without further processing. All measurements were made in triplicate. Video recordings were taken of ice crystals during some of the analyses allowing individual frames to be converted into photographs.

2.2.4 AFP characterization

Amino acid analysis and matrix-assisted laser desorption ionization (MALDI) mass spectrometry (MS) was performed at the Advanced Protein Technology Centre, Hospital for Sick Children, Toronto, ON, Canada. Briefly, samples for amino acid analysis were dried in a vacuum centrifugal concentrator and subjected to vapor phase hydrolysis by 6N HCL, 1% phenol at 110 °C for 24 h under pre-purified nitrogen atmosphere. Following hydrolysis, excess HCl was removed by vacuum and hydrolysates derivatized with phenylisothiocyanate (PITC) at room

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temperature. Derivatized amino acids were analyzed using the Waters Pico-Tag system. For MALDI-MS, and MALDI-MS/MS, HPLC-purified AFP was analyzed in 0.1 M NH₄HCO₃ on the Applied Biosystems/MDS Sciex API QSTAR XL Pulsar MALDI QTOF as previously described (Wood *et al.* 2008). Resulting scans were provided as .wiff files and analyzed using Analyst[®] software from Applied Biosystems/MDS Sciex.

2.2.5 Secondary structure of plasma AFP

Circular dichroism (CD) spectra were recorded on a 40 μ g/ml AFP (in 0.1 M NH₄HCO₃) solution using a Jasco-810 spectropolarimeter with a water-jacketed 2 mm quartz cuvette. The temperature of the cuvette was maintained using a CTC-345 circulating water bath. Buffer was scanned to establish a baseline CD spectrum which was subtracted from subsequent scans of the protein solution. Spectra were obtained from 195 to 250 nm, with a 0.1 nm step, 100 nm/min scanning speed and 0.5 s response time. CD spectra were obtained starting at 1 °C, then at 5 °C intervals from 5 to 70 °C at a heating rate of 0.25 deg/min with eight accumulations per temperature to obtain a melting curve for the protein. Following the melting experiment the protein sample was incubated at 4 °C overnight and re-scanned at 1 °C to determine the extent of refolding. The mean residue ellipticity at a given wavelength (θ)_n was expressed in Deg·cm²·dmol⁻¹ (Chen *et al.* 1974) and the percentage of helicity was calculated from the mean residue ellipticity at 222 nm as previously described (Chen *et al.* 1974, Low *et al.* 2001).

Helical wheel diagrams were constructed using pepwheel from EMBOSS (*http://emboss.bioinformatics.nl/cgi-bin/emboss/pepwheel*) with the number of steps set to 11 and the number of turns set to three (Baardsnes *et al.* 1999).

2.2.6 cDNA library synthesis and 454 sequencing

Total RNA was isolated from cunner skin and liver tissues collected during the winter using TRIzol[®] reagent as per manufacturer's instructions (Invitrogen Life Technologies, Burlington, ON). Approximately 160 µg skin RNA and 240 µg liver RNA were pooled and enriched for messenger RNA (mRNA) using an Oligotex direct mRNA mini kit (Qiagen Inc., Chatsworth, Ca). Duplicate cDNA libraries were created following the Roche cDNA rapid library preparation method for 454 sequencing using the cDNA synthesis system and primer random as per manufacturer's instructions (Roche Applied Science, Laval, QC). These cDNA libraries were stored on dry ice and shipped to the McGill University and Genome Quebec Innovation Centre for sequencing with Roche GS-FLX Titanium technology. The resulting cDNA sequences were computationally translated into all six reading frames and scanned for the presence of a portion of the cunner AFP which was obtained from MS/MS sequencing.

2.3 Results

2.3.1 Purification and thermal hysteresis (TH) activity of AFP from cunner plasma

Plasma AFP activity showed a distinct seasonal cycle (Fig 2-1) with measurable levels of TH first appearing in November, reaching peak values (0.16 ± 0.02 °C) during March and gradually declining to zero by August. The peak in TH correlates with, and contributes to, the seasonal increase in plasma freezing point depression.

When blood plasma was fractionated by size-exclusion chromatography, a single peak of AFP activity, and associated bipyrimidal ice crystal morphology (Fig 2-2a, inset), was observed in fractions 62-76 (Fig 2-2a). The partially purified protein was further resolved on HPLC and a single peak exhibiting AFP activity was identified at approximately 21 min, 42% solvent A (Fig

Figure 2-1. Seasonal cycle of AFP activity in cunner blood plasma. Two cunners were blood sampled and analyzed for AFP activity at monthly intervals. All values are plotted as means \pm standard deviation



2-2b). Since this peak was closely preceded by a neighbouring inactive peak, it was re-applied to the HPLC column which resulted in isolation of a single, distinct AFP active peak (Fig 2-2b, inset). This peak was collected and analyzed by SDS-PAGE. Coomassie blue and Amido black do not stain type I AFP from winter flounder (Fourney *et al.* 1983) so the gel was stained using copper, which produced a negative stain (photographed against a black background). HPLC-purified cunner AFP migrated as a single band at a rate slightly slower than winter flounder AFP (mass ~ 3.3 kDa) but faster than the 10 kDa standard indicating that the molecular mass was approximately 4 - 5 kDa (Fig 2-2c). Further analysis by MALDI MS determined that the AFP consisted of two species with monoisotopic masses of 4095.16 and 4153.20 Da, which are identical save a minor post translational modification as explained later (Fig 2-3). Based on these results, the cunner AFP had been purified to near homogeneity, since it eluted as a single peak on HPLC and migrated as a single band on SDS-PAGE.

2.3.2 Amino acid analysis and tandem MS/MS sequencing

Amino acid analysis of the cunner AFP (Table 2-1) revealed an abundance of alanine residues (57 mol%) which is only slightly lower than that seen in winter flounder type I AFP (Gong *et al.* 1996). Glutamic acid + glutamine (9.2 mol%) and lysine (9.5 mol%) were the next most abundant residues, followed by threonine (6.2 mol%). The concentration of the sample was calculated at 0.04 mg/ml. Notably absent from the analysis were the aromatic residues Phe, Trp and Tyr which explains the AFP's lack of absorbance at 260-280 nm.

Partial *de novo* sequencing of MS/MS spectra generated the sequence SAPQ<u>LAAEATAAAALKAAEATK</u>, which accounted for approximately half of the expected peptide mass. The underlined portion of this peptide was used as a query sequence to scan **Figure 2-2.** Purification of AFP from cunner plasma. (A) Fractionation of cunner plasma on a G-75 Sephadex size-exclusion column. Cunner blood plasma (~9 ml) was applied to the column and fractions exhibiting TH activity (62-76) as indicated by the dashed line were pooled for repurification on G-75. The figure shows the results of the first round of gel-filtration. AFP active fractions were pooled for further purification on reversed-phase HPLC. Inset: ice crystal formed in the presence of size-fractionated cunner plasma AFP. (B) G-75 purified AFP (~200 µg) was separated on a Phenomenex Jupiter 5 µm C18 column (250 x 4.6 mm, 300Å) with a flow rate of 1 ml/min and a 30-60% acetonitrile, 0.1% trifluoroacetic acid gradient. The single peak that exhibited TH activity (indicated by the arrow) was re-applied to the column (inset). (C) SDS-PAGE separation of cunner HPLC-purified AFP on a 14% polyacrylamide gel in Tris-Tricine buffer system. Lane 1, Bio-Rad Precision Plus ProteinTM dual color standard (10 µl), lane 2, cytochrome c standard (mass = 12,500 Da, 5µg), lane 3, *Pseudopleuronectes americanus* AFP (mass~ 3,300 Da, 10 µg) (DeLuca *et al.* 1998), lane 4, HPLC-purified cunner AFP (mass = 4,095 Da, 3 µg)



Figure 2-3. Mass spectra of purified cunner type I AFP. [M+H]⁺ peak at 4095.16 represents the mature AFP while monoisotopic peak at 4153.20 represents an intermediate form in the post-translational processing of the cunner AFP



Amino acid	Mol%	Observed number*	Expected number [#]
Asx	1.9	0.9	1
Glx	9.2	4.1	4
Ser	3.9	1.8	2
Gly	2.8	1.3	2
Thr	6.2	2.8	3
Ala	57.2	25.7	25
Pro	2.5	1.1	1
Met	2.0	0.9	1
Leu	4.5	2.0	2
Lys	9.5	4.3	6
Total	99.7	44.9	47

Table 2-1. Amino acid analysis of cunner AFP

* Based on mol% from amino acid analysis and a mature protein length of 45 residues

[#]From *in silico* translation of the cDNA sequence [including the two C-terminal residues (GK)

that are removed post-translationally]

computationally translated protein sequences obtained from 454 sequencing of a cunner cDNA library.

2.3.3 Antifreeze activity and ice crystal morphology

The TH activity of cunner AFP was measured over a range of concentrations from 0 - 10 mg/ml (Fig 2-4). TH activity of the cunner AFP was 0.82 °C at 10 mg/ml, which is higher than both the winter flounder HPLC-6 and yellowtail flounder AFPs, but lower than the winter flounder AFP9 variant. Cunner AFP was particularly effective at low concentrations, where its TH was comparable to that of the winter flounder AFP9 variant (Chao *et al.* 1996). The reason for the intermediate activity of cunner AFP between that of winter flounder HPLC-6 and the more highly active AFP9 is not immediately obvious from the sequence. The ice-binding surfaces of cunner (presumed to begin after the Pro residue) and HPLC -6 are very similar, unlike AFP9, which contains one extra 11 amino-acid repeat that extends the ice-binding surface. However, the cunner protein is clearly intermediate in length (45 vs. 37 and 51 residues after post-translational processing) and increased protein size, irrespective of an increase in the ice-binding footprint, can increase TH (DeLuca *et al.* 1998).

The ice crystal morphology observed in the presence of cunner AFP is similar to that observed with other type I AFPs (Evans and Fletcher 2001, Kuiper *et al.* 2002), in that the crystal grows into a hexagonal bipyramid upon slight undercooling (Fig 2-4, inset).

2.3.4 Secondary structure analysis of cunner AFP

The CD spectra of a cunner AFP sample at low temperature resembled that of other alanine-rich α -helical proteins (Chao *et al.* 1996, Deng and Laursen 1998, Evans and Fletcher 2001, Gauthier *et al.* 2005, Wallimann *et al.* 2003) with well-defined minima near 208 and 222 nm (Fig 2-5a). At 1 °C, the peptide is nearly fully α -helical (99.8%) and it appears almost fully

Figure 2-4. Thermal hysteresis (TH) activity curve for cunner AFP. The plot of TH as a function of cunner AFP concentration (mean ± standard deviation) is compared against those for winter flounder AFP9, winter flounder HPLC6 and yellowtail flounder AFP [modified from Chao *et al.* (1996)]. Inset: ice crystal in the presence of 10 mg/ml cunner plasma AFP



denatured by 65 °C as this curve and that at 70 °C overlap, and the helical content is calculated to be around 25%. The change in helicity with temperature is gradual and starts occurring immediately upon heating from 1 °C to 5 °C with an estimated midpoint of thermal denaturation (Tm) of ~ 30 °C (Fig 2-5b). This denaturation is largely reversible as the protein regained 91% of its original helical content upon cooling back to 1 °C (data not shown).

2.3.5 cDNA library sequencing and analysis

A 1/8 GS-FLX Titanium sequencing reaction generated over 82,000 cDNA sequence reads with a median read length of 233 bp. Expressed sequence tags (ESTs) were computationally translated in all six reading frames and scanned for the query sequence LAAEATAAAALKAAEATK resulting in 112 positive matches (~0.14%). Alignment of the positive ESTs generated a consensus sequence which includes a complete ORF, including putative in-frame start and stop codons (Fig 2-6). This ORF codes for an alanine-rich protein of 47 residues in length, with a mass of 4239.24 Da and contains three 11-amino-acid repeats (ThrX₁₀) which are characteristic of the majority of type I AFPs (Fig 2-7). The protein is produced without an obvious signal sequence (Genbank accession no. **JF937681.2**).

2.4 Discussion

The results of this study clearly demonstrate that cunner produce type I AFPs and secrete them into the circulatory system on a seasonal basis with the highest levels occurring during the winter months. These cunner proteins can be classified as classical type I AFPs due to their small size, high alanine content, three 11-amino acid (ThrX₁₀) repeat regions within the primary structure, α -helical secondary structure that can undergo reversible thermal denaturation, and the capacity to impart a hexagonal bipyramidal shaping to ice crystals. **Figure 2-5**. Thermal denaturation of cunner AFP determined by circular dichroism (CD). **(A)** CD spectra of cunner AFP taken over the range 1-70 °C. **(B)** Mean residue ellipticity of cunner AFP at 222 nm during thermal denaturation from 1-70 °C. The midpoint (Tm) was calculated from the mean residue ellipticity at 50% helix



Figure 2-6. Nucleotide sequence (Genbank accession no. **JF937681.2**) and primary translation product of cunner AFP cDNA. The ORF is capitalized, whereas the 5'- and 3'-UTRs are in lower case letters. In-frame start and stop codons are underlined. The arrow indicates the presumed site of post translational processing leading to enzymatic conversion of Ala-Gly-Lys to Ala-NH₂. A single 11-amino-acid repeat (ThrX₁₀) is underlined in bold

1	gat	tt	tgt	gga	tca	aag	ttc	aata	agt	tct	ctc	tct	aca	aca	aaA	TGG	ATTO	2
1]	MI 1	DS	5
51	AGO	GAA	AAA	GTG	GCAG	CCT	CAG	CTT	GCT	GCT	GAA	GCT.	ACT	GCT	GCA	GCT	GCA	2
4	0	3 3	К	S	A	Р	Q	L	A	A	Е	A	T	A	A	A	A	
101	TTI	AAA	GCT	GCI	'GA/	AGC	TAC	CAA	AGC	TGC.	AGC	TAA	AGC	TGC	AGC	TGA	AGC	ľ
20	L	K	A	A	E	A	_ т	K	A	A	A	K	A	A	A	Е	A	
151	AC	[GC]	TAA	AGC	TGC	CAG	CTG	CAG	CTG	CTG	GAA	AAT	AGC	tgc	tgg	age	tgea	£
37	т	A	K	7	1	A J	A J	A Z	A .	A	G	К						
										î								
201	ggg	gga.	agc	cgc	etge	ctt	cgt	cct	ttg	ggċ	caa	cta	aaa	tgg	ctg	ctg	gc	

251 ccagggatttt

Figure 2-7. Alignment of known type I AFP protein sequences based on primary structural characteristics. The amino acid sequence alignment was made by ClustalW (*http://www.ebi.ac.uk/Tools/msa/clustalw2/*). Residues conserved throughout all sequences indicated by *. Residues differing from the majority are highlighted grey. Threonine (T) residues at the beginning of the 11-amino-acid repeats (ThrX₁₀) are highlighted green. Abbreviations used: Wfl-HPLC6, winter flounder liver HPLC 6 (Davies *et al.* 1982); AP-AFP, Alaskan plaice plasma AFP (Knight *et al.* 1991); Wfl-AFP9, winter flounder liver AFP9 (Chao *et al.* 1996); YT-AFP, yellowtail flounder AFP (Scott *et al.* 1987); WfsAFP2, winter flounder skin AFP 2 (Gong *et al.* 1996); SS-3, shorthorn sculpin plasma AFP 3 (Hew *et al.* 1985); AS-1, Arctic sculpin plasma AFP 1 (Yang *et al.* 1988); GS-5, grubby sculpin plasma AFP 5 (Chakrabartty *et al.* 1988); SS-8, shorthorn sculpin plasma AFP 8 (Baardsnes *et al.* 2001); AS-3, Arctic sculpin plasma AFP 3 (Yang *et al.* 1988)

Wfl-HPLC6 AP-AFP Wfl-AFP9 YT-AFP	D <mark>T</mark> ASDAAAAAALTAANAKAAAEL	TAANAAAAAAATAR TARDAAAAAAATAAAAR TAANAAAAAAATAAAARG TAAAAAEAAAATAAAARG	37 40 52 48
cunner	MDSGKSAPQLAAEA <mark>T</mark> AAAALKAAEA	<mark>T</mark> KAAAKAAAEA <mark>T</mark> AKAAAAAAGK	47
WfaleDo	мод од и д д д д <mark>и</mark> д д д и д д д д д д д		20
WISAFP2		I NAAAAAAAAA I - NAGAAR	22
SS-3	MNAPARAAAK <mark>T</mark> AADALAAAKK	TAADAAAAAAAA	33
AS-1	MDAPARAAAK <mark>T</mark> AADALAAANK	TAADAAAAAAAA	33
GS-5	MDAPAIAAAK <mark>T</mark> AADALAAAKK	TAADAAAAAAKP	33
Lss-AFP	MDAPAKAAAK <mark>T</mark> AADAKAAAAK	<mark>T</mark> AADALAAANK <mark>T</mark> AAAAKAAAK-	42
GS-8	MDGETPAOKAARLAAAAAALAAK	TAADAAAKAAA TAAAAA	40
SS-8	MDGETPAOKAARLAAAAAALAAK	TAADAAAKAAATAAAAASA	42
AS-3	MDGETPAQKAARKAAAAAAAAAA	TAADAAAAAA	32
	* ** * *	* * *	

The type I AFPs discovered to date fall into three categories: plasma-type AFPs produced in the liver and exported into circulation through a traditional signal sequence mediated pathway; skin-type AFPs lacking signal sequences and thought to act at both intracellular and extracellular locations; and the hyperactive AFPs. The plasma and skin-type AFPs from winter flounder are small (3.3 to 4.5 kDa) while the hyperactive AFP is much larger (34 kDa) (Davies *et al.* 1982, Gong *et al.* 1996, Graham *et al.* 2008). However, all contain 11-residue (ThrX₁₀) repeat sequences that appear more regularly than those in type I AFPs from sculpins (Baardsnes *et al.* 2001, Chakrabartty *et al.* 1988, Yang *et al.* 1988).

An alignment of the cunner AFP sequence with the other known type I sequences illustrates the similarity between these proteins (43-64% identity, Fig 2-7). This similarity is further demonstrated by the remarkable conservation of the putative ice binding face elucidated by Baardsnes *et al.* (1999) for the type I AFPs (Fig 2-8).

It is apparent from the results of this study that the theoretical mass of the cunner AFP (4239.24 Da) as deduced from the cDNA sequence differed from the mass of the circulating plasma AFP as determined by mass spectrometry (4095.16 Da). It is reasonable to think that this discrepancy is due to post-translational modifications of the AFP similar to those reported for the winter flounder HPLC-6 and -8 AFP (Hew *et al.* 1986). In the case of the cunner AFP it is likely that the C-terminal lysine was cleaved post-translationally followed by removal of the C-terminal glycine during amidation of the penultimate alanine residue (Chao *et al.* 1996, Hew *et al.* 1986) (Fig 2-6). This is supported by the amino acid analysis where both glycine and lysine are underrepresented in the purified protein compared to the *in silico* predicted protein sequence

Figure 2-8. Helical wheel representations of type I AFPs. Basic residues are in blue, acidic residues are in red and threonine (T) residues commencing 11-amino-acid repeats are in green. The ice binding face, as proposed by Baardsnes *et al.* (1999) is indicated by a solid black line. Wfl-HPLC6, winter flounder liver HPLC-6 (Davies *et al.* 1982); GS-5, grubby sculpin plasma AFP 5 (Chakrabartty *et al.* 1988); Lss-AFP, longhorn sculpin skin AFP (Low *et al.* 2001); SS-3, shorthorn sculpin plasma AFP 3 (Hew *et al.* 1985); Wfs-AFP2, winter flounder skin AFP 2 (Gong *et al.* 1996)



(Table 2-1). Amidation of the C-terminus eliminates a helix destabilizing negative charge and appears to be crucial for formation of a helix cap structure (Sicheri and Yang 1995) and proper AFP function. A chemically synthesized winter flounder AFP (HPLC-6) lacking this C-terminal amide group displayed significantly reduced TH activity compared to its amidated counterpart (Patel and Graether 2010, Tong *et al.* 2000). Similar modifications have been observed in other proteins with the removal of multiple C-terminal residues followed by α -amidation of the remaining glycine residue (Stevens *et al.* 2009, Yoo Joon *et al.* 1982). In fact, over 50% of all bioactive proteins (i.e. those capable of interacting with or affecting living cells and/or tissues) are modified via this α -amidation process at their C-terminal end (Fisher and Scheller 1988, Siegel *et al.* 1999).

A second post-translational modification that appears likely is an N-terminal blockage of the cunner AFP since multiple attempts at Edman sequencing failed to produce sequence information (data not shown). N-terminal acetylation is the most widespread modification observed in Eukaryotes (Polevoda and Sherman 2000) and without the amino group, the residue cannot react with phenylisothiocyanate in the Edman reaction. This modification is observed in both the shorthorn sculpin type I AFP (SS-8) and winter flounder skin-type I AFP (sAFP1), which have N-terminal sequences similar to the cunner AFP, beginning with Met-Asp (Baardsnes *et al.* 2001, Gong *et al.* 1996). Acetylation appears necessary for full AFP activity as a recombinant shorthorn sculpin AFP (SS-3) produced without the N-terminal acetylation was devoid of AFP activity (Fairley *et al.* 2002). However, after treatment of the recombinant SS-3 with acetic anhydride, AFP activity was recovered to near that of AFP purified from plasma.

Additional evidence for these modifications comes from MS analysis on the purified protein where a peak with monoisotopic mass of 4153.20 is observed in addition to the $[M+H]^+$

peak at 4095.16 (Fig 2-3). This matches the predicted mass of the cunner AFP with N-terminal acetylation and removal of the C-terminal lysine (4153.16), which is an intermediate form in the proposed C-terminal processing of the cunner AFP. Taken together, the N-terminal acetylation and the C-terminal lysine removal followed by glycine amidation would produce a protein with a predicted monoisotopic mass of 4095.15 Da for the singly charged cunner AFP, which is virtually identical to the observed mass of 4095.16 Da.

The AFP isolated from cunner blood plasma in the present study does not appear to be the same as that identified in the skin by Evans and Fletcher (2004). A preliminary study by these authors identified AFPs from skin extracts that were much larger (~ 7000 Da) than the circulating AFPs and contained amino acids (Arg, Val, Ile and Phe) which were not detected in the plasma AFP. In addition the skin AFP only contained 1% Glx as compared to over 9% in the plasma AFP. This could indicate that there are other AFP isoforms or variants present in skin.

At the present time the tissue from which the AFP mRNA originated is unknown. In winter flounder, serum isoforms are synthesized primarily by the liver as preproproteins containing both a signal peptide and a cleaved N-terminal region (Davies *et al.* 1982, Hew *et al.* 1986). However, the cunner transcript does not encode a signal sequence typically required for external secretion of proteins. Similar type I AFPs lacking signal sequences have been found in winter flounder skin (Gong *et al.* 1996, Murray *et al.* 2002a, Murray *et al.* 2002b, Murray *et al.* 2003), and a skin origin for a plasma type I AFP has been suggested for snailfish (Evans and Fletcher 2005a, Evans and Fletcher 2005b). A non-liver origin for plasma antifreeze glycoproteins (AFGPs), which also lacks signal peptides, has been suggested where the AFGPs are made in the pancreas, secreted into the digestive tract and absorbed into the blood (Cheng *et al.* 2006).

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CD measurements indicate an α -helix content of over 99% for cunner AFP based on its predicted size of 45 amino acids (Table 2-1), which is close to values obtained for other type I AFPs (Chao *et al.* 1996, Evans and Fletcher 2001, Gauthier *et al.* 2005, Marshall *et al.* 2005). Similar to the type I AFP variant from winter flounder (Chao *et al.* 1996) cunner AFP exhibited a reversible thermal denaturation in the range of 1 - 70 °C, with a loss of less than 10% of the original helicity when the AFP solution was cooled back to 1 °C. The estimated Tm of ~30 °C is comparable to the winter flounder HPLC 6 (~28 °C) (Wen and Laursen 1993).

The seasonal cycle in cunner plasma AFP levels is similar to that observed in other type I AFP-producing fish, with peak levels occurring in the winter when sea water temperatures are the coldest, and undetectable amounts in the late summer/early fall when sea water temperatures are warmest (Hew and Yip 1976) (Fig 2-1). However, even during months of peak AFP levels the increase in freeze protection afforded by the AFP (0.16 °C) over that conferred by the colligative properties of the plasma was minimal and only lowered the freezing point of the blood plasma to ~ -0.95 °C. Since sea water temperatures decline to approximately -1.8 °C during winter this would result in nearly a full degree difference between the fish's freezing temperature and the temperature of the surrounding sea water. This situation would put cunner at considerable risk of freezing should they come into contact with ice.

The low levels of AFP activity in cunner plasma cannot be attributed to a weakly active protein because the TH curves show that the activity of cunner AFP is greater than that of the major circulating AFP in winter flounder (HPLC 6) [Fig 2-4, Evans and Fletcher (2001)]. This suggests that the low levels of plasma AFP activity are attributable to low AFP concentrations of approximately 0.2 mg/ml (Fig 2-4). Winter flounder, in contrast to the cunner, have plasma AFP levels exceeding 10 mg/ml during winter.

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Gene dosage appears to play an important role in plasma AFP abundance, with fish at greater risk of freezing requiring multiple gene copies in order to produce sufficient AFP for freeze protection. For example, AFP is abundant in winter flounder and ocean pout (*Macrozoarces americanus*) during winter, and these species are estimated to have 40 (Scott *et al.* 1985) and 150 (Hew *et al.* 1988) AFP gene copies, respectively. In fact, Hew *et al.* (1988) found a direct correlation between the potential risk of exposure to ice at subzero temperatures, plasma AFP levels and AFP gene copy number in two geographically distant populations of ocean pout. Similarly, Duncker *et al.* (1999) observed a direct relationship between AFP copy number and AFP activity in transgenic *Drosophila melanogaster*. Studies using Southern blotting procedures might reveal whether or not the low levels of plasma AFP could be attributable to low AFP gene copy number.

During the winter, water temperatures along the north east coast of Newfoundland generally decline to -1 °C to -1.8 °C for three to four months (Jan-April). Cunners are the only fish occupying the shallow and potentially ice-laden waters of this habitat with levels of plasma AFP that are not sufficient to protect them from freezing. All of the other species studied to date have high plasma levels of type I, type II, type III AFP or AFGP that increase their freeze resistance to safe levels [type I: winter flounder (*P. americanus*) (Marshall *et al.* 2004), shorthorn sculpin (*M. scorpius*) (Hew *et al.* 1980), Atlantic snailfish (*L. atlanticus*) (Evans and Fletcher 2001); type II: smelt (*Osmerus mordax*) (Lewis *et al.* 2004); type III: rock gunnel (*Pholis gunnellus*), and radiated shanny (Ulvaria subbifurcata) (Shears *et al.* 1993); AFGP: juvenile Atlantic cod (*Gadus morhua*) (Goddard *et al.* 1992)]. Cunners appear to overcome the lack of sufficient plasma AFP by entering somewhat protected microhabitats such as rock crevices, ceasing to feed and going into metabolic torpor thereby reducing the potential for ice

contact (Green and Farwell 1971, Lewis and Driedzic 2007). The discovery of AFP in cunner skin extracts leads one to think that perhaps they provide additional protection by helping to block ice propagation into the fish. This has been shown for winter flounder skin (Valerio *et al.* 1992). However this strategy is not foolproof because ice can be abundant in such waters and during storms ice crystals enter the refugia and occasionally result in mass mortalities (Green 1974).

The inadequately low levels of plasma AFP in cunner, a species that is at the northernmost point of its geographical range, suggest that, in contrast to heterospecifics, this species began to exploit subzero and at times ice-laden habitats in relatively recent times. Therefore it may be that there has been insufficient time for the process of natural selection to result in a more effective AFP production system, usually through gene amplification and in some cases with the genes in extensive tandem repeats (Hew *et al.* 1988, Scott *et al.* 1985).
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Chapter 3: Epithelial dominant expression of antifreeze proteins in cunner suggests recent entry into a high freeze-risk ecozone.

Preface

This chapter consists of a version of a manuscript published in the journal Comp Biochem Physiol A Mol Integr Physiol. [**Hobbs** RS, Fletcher GL (2012) Epithelial dominant expression of antifreeze proteins in cunner suggests recent entry into a high freeze-risk ecozone. Comp Biochem Physiol A Mol Integr Physiol. 164(1):111-8]. The experimental research and data analyses were performed by RSH. The manuscript was written by RSH with editorial input from GLF.

Abstract

Most marine teleost fishes residing in a high freeze-risk ecozone, such as the coastal waters of Newfoundland during winter, avoid freezing by secreting high concentrations of antifreeze proteins (AFPs) into their blood plasma where they can bind to and prevent the growth of ice that enters the fish. Cunner (Tautogolabrus adspersus), which overwinter in such shallow waters are the only known exception. Although this species does produce type I AFPs, the plasma levels are too low to be of value as a freeze protectant. Southern and northern blot analyses carried out in this study establishes that the cunner AFP genes belong to a multigene family that is predominantly expressed in external epithelia (skin and gill filaments). These results support the hypothesis that the survival of cunner in icy waters is attributable in part to epithelial AFPs that help block ice propagation into their interior milieu. In contrast to the cunner, heterospecifics occupying the same habitat have greater freeze protection because they produce AFPs in the liver for export to the plasma as well as in external epithelia. Since the external epithelia would be the first tissue to come into contact with ice it is possible that one of the earliest steps involved in the evolution of freeze resistant fish could have been the expression of AFPs in tissues such as the skin. We suggest that this epithelial-dominant AFP expression represents a primitive stage in AFP evolution and propose that cunner began to inhabit "freezerisk ecozones" more recently than heterospecifics.

3.1 Introduction

Most marine teleosts are unable to inhabit "freeze-risk ecozones" (subzero ice laden waters) characteristic of polar and sub-polar oceans because the temperature of the water (-1.9 °C) can be a full degree lower than the freezing point of their body fluids (-0.7 to -0.9 °C). A number of teleosts survive in this environment by producing antifreeze proteins (AFPs) or glycoproteins (AFGPs) that bind to the surface of ice crystals that may form within their body fluids, and thereby inhibit their growth. Without the protective effects of AFPs, undercooled fish freeze and die on contact with ice (Fletcher *et al.* 1986, Scholander *et al.* 1957).

Three physiologically functional types of AFPs (types I-III) as well as the AFGPs have been described in a variety of fish taxa (Davies *et al.* 2002, Ewart *et al.* 1999, Fletcher *et al.* 2001, Gauthier *et al.* 2008). A fourth AFP (type IV) originally identified in longhorn sculpin is no longer considered to function as an antifreeze (Deng *et al.* 1997, Gauthier *et al.* 2008). Although diverse in primary sequence and secondary structure, all AFPs and AFGPs lower the non-equilibrium freezing point of aqueous solutions non-colligatively by binding to specific planes of seed ice, modifying its shape and restricting further growth by an adsorption-inhibition mechanism (Davies *et al.* 2002, Jia and Davies 2002, Knight *et al.* 1991, Raymond and DeVries 1977).

Type I AFPs, the most extensively studied AFPs, are alanine-rich, amphipathic α-helical proteins first described in righteye flounders (Duman and DeVries 1974, Low *et al.* 2001, Scott *et al.* 1987, Sicheri and Yang 1995). These AFPs have been fully characterized in four teleost families from three distinct Orders: Order Pleuronectiformes [Family Pleuronectidae (Gauthier *et al.* 2005, Knight *et al.* 1991, Nabeta 2009, Scott *et al.* 1987, Scott *et al.* 1988)], Order Scorpaeniformes [Family Cottidae (Chakrabartty *et al.* 1988, Hew *et al.* 1985, Low *et al.*

2001,Yang *et al.* 1988) and Family Cyclopteridae (Evans and Fletcher 2001)] and Order Perciformes [Family Labridae (Hobbs *et al.* 2011, Evans and Fletcher 2004)]. The remarkable similarity in primary sequence and tertiary structure of the AFPs from these three Orders of teleosts suggests that they are the result of convergent evolution (Hobbs *et al.* 2011).

Cunner (*Tautogolabrus adspersus*) belongs to a Family (Labridae, Order Perciformes) of marine fishes typically found in tropical and subtropical areas (Scott and Scott 1988). However this species is distributed along the Atlantic coast of North America from the temperate waters of Chesapeake Bay to the north coast of Newfoundland Canada, where freezing water temperatures and ice during the winter cause a significant threat to their survival (Green 1974). Although AFPs present in cunner are believed to play a role in their ability to survive such harsh conditions (Evans and Fletcher 2004, Valerio *et al.* 1990) a recent study has demonstrated that the plasma levels are too low to significantly improve their freeze resistance (Hobbs *et al.* 2011). Thus the physiological significance of AFPs in cunner plasma remains in question.

Previous research has shown that cunner skin, in contrast to blood plasma, has relatively high levels of AFP activity leading to the suggestion that the AFPs could serve to help prevent ice propagation across external epithelial tissues (Valerio *et al.* 1990). Such a protective effect has been demonstrated for winter flounder where AFPs have been shown to help prevent ice propagation across the skin (Valerio *et al.* 1992a). This skin protective hypothesis is supported at the molecular level by the discovery of a novel subclass of type I AFP genes (skin-type) that are predominantly expressed in epithelial tissues of winter flounder (*Pseudopleuronectes americanus*) and in two species of sculpin, shorthorn (*Myoxocephalus scorpius*) and longhorn (*M. octodecemspinosus*).

Our recent sequencing of a cunner cDNA library indicates that the cunner AFP gene codes for a protein that lacks an obvious secretary signal sequence; a characteristic that is shared by all of the skin-type I AFPs. However since the library used to isolate the cunner AFP cDNA was derived from a mixture of liver and skin mRNA the tissue source of the AFP mRNA is unknown.

The present study establishes that cunner AFPs belong to a multigene family that is predominantly expressed by the external epithelia. In addition, the cunner's closest relative (*Tautoga onitis*) on the western Atlantic and a resident of more southerly waters also possessed a multigene family of type I AFP genes. It is suggested that cunner began to inhabit "freeze risk ecozones" more recently than heterospecifics.

3.2 Materials and Methods

3.2.1 Sample Collection

Cunners were collected by Ocean Sciences Centre (OSC) divers near Logy Bay, Newfoundland and Labrador, Canada in the winter of 2008 and maintained at the OSC in 250 1 aquaria supplied with flowing seawater (32-33‰ salinity) under seasonally normal photoperiod and water temperatures. Water temperatures ranged from 15.1 °C in August to -1.8 °C during April. Fish were euthanized with an MS-222 overdose and the tissues were removed immediately, frozen in liquid nitrogen and stored at -70 °C until analysis.

Tautogs (*Tautoga onitis*) were collected by otter trawl near Menemsha Bight, Martha's Vineyard, MA, USA and maintained at the Marine Biological Laboratory, Woods Hole, MA, USA in a 5000 gal aquarium supplied with running seawater. Fish were euthanized with MS-222 overdose and liver samples were excised, cut into ~1 mm thick pieces and treated with DMSO/salt DNA preservation solution (20% DMSO, 0.25 M sodium EDTA, saturated NaCl, pH 7.5) as previously described (Seutin *et al.* 1991).

3.2.2 RNA Ligase-Mediated Rapid Amplification of cDNA Ends (RLM-RACE)

Total RNA was isolated from cunner tissue samples using TRIzol[®] reagent (Invitrogen) as per manufacturer's instructions. GeneRacerTM cDNA was generated from total RNA by ligation of the GeneRacerTM RNA oligo to full length mRNA followed by reverse transcription with the GeneRacerTM poly dT primer and superscript III reverse transcriptase as per manufacturer's instructions (Invitrogen). GeneRacerTM cDNA was used as the template for primary PCR with GeneRacerTM 3'/Cunner F1 or GeneRacerTM 5'/Cunner R1 primers (Fig 3-1). This was followed by nested PCR using the primary PCR product diluted 1/20 in water as the template with GeneRacerTM 3' nested/Cunner F2 or GeneRacerTM 5' nested/Cunner R2 primers to amplify both the 5' and 3' ends of the cunner AFP gene. The nucleotide sequences of the primers used (in 5' - 3' direction) were:

Cunner F1: GAT TCA GGA AAA AGT GCA CCT CAG CTT

Cunner R1: CTG GGC CAG CAG CCA TTT TAG TTG G

Cunner F2: CAC CTC AGC TTG CTG CTG AAG CTA C

Cunner R2: TTA GTT GGC CCA AAG GAC GAA GCA GC

PF1: GTG GAT CAA GTT CAA TAG TTC TC

PR1: TGT ACC CGT AGG TAA AAG ATG TG

PCR reactions were performed in a Mastercycler[®] gradient thermal cycler (Eppendorf Scientific). Amplification was achieved in a 25 μ l reaction containing one unit of AccuprimeTM GC Rich DNA polymerase (Invitrogen), 1 x AccuPrimeTM GC-rich buffer B [60 mM Tris-HCl (pH 9.2), 1.5 mM MgSO₄, four dNTPs (200 μ M each of dATP, dGTP, dCTP and dTTP), 30

Figure 3-1. Nucleotide sequence of the complete *T. adspersus* liver/skin cDNA generated by RLM-RACE illustrating primer annealing sites. The ORF is capitalized, whereas the 5'- and 3'- UTRs are in lower case letters. In frame start and stop codons are in bold. Primers PF1, Cunner F1, Cunner R1 and PR1 are underlined while Cunner F2 and Cunner R2 are italicized

CTGC
CTGC
ГGAAG
agctg
ctggc
ttgaa
ttagc
accta
aaa

mM NaCl with proprietary thermostable AccuPrimeTM proteins and enhancers], template cDNA (100 ng for primary PCR and 2 μ l of the diluted primary PCR product for nested PCR) and 0.2 μ M of both 5' and 3' primers. The conditions for PCR were as follows: initial denaturation at 95 °C for three min followed by 30 cycles of denaturation at 95 °C for 30 s, primer annealing at 58 °C for 30 s and elongation at 72 °C for 90 s. A final step at 72 °C for 10 min ensured complete elongation of products. PCR products were separated on 1.2% agarose/TBE (45 mM Tris-borate, 1 mM EDTA) gels containing 0.5 μ g/ml ethidium bromide for nucleic acid visualization.

3.2.3 Examination of Tissue Specific Expression of the Cunner AFP Gene

3.2.3.1 RNA Probe Production

3.2.3.1.1 Bacterial Preparation

Cunner AFP cDNA was amplified with PCR using primers PF1/PR1 and a cunner GeneRacerTM skin/liver cDNA pool as the template. Cycling conditions were as follows: initial denaturation at 95 °C for three min followed by 30 cycles of denaturation at 95 °C for 30 s, primer annealing at 54 °C for 30 s and elongation at 72 °C for 90 s. A final step at 72 °C for 10 min ensured complete elongation of products. The resulting PCR product was separated on a 1.2% agarose/TBE gels containing 0.5 μ g/ml ethidium bromide and extracted using the QIAquick Gel Extraction Kit (Qiagen) as per manufacturer's instructions. The purified cDNA was ligated into the pGEM[®]-T easy vector and transformed into *E. coli* bacteria [DH5 α (Invitrogen)] according to the pGEM[®]-T easy vector system protocol (Promega).

3.2.3.1.2 Plasmid Purification

Bacteria were grown in Luria-Bertani (LB) medium (10 g/L bacto-tryptone, 5 g/L bactoyeast extract, 10 g/L NaCl, pH 7.0) which was supplemented with ampicillin (100 μ g/ml) based on the resistance gene encoded by the vector transformed into the cells. The cultures were grown overnight with shaking at 37 °C. Aliquots of the culture were transferred to a 1.5 ml microcentrifuge tube and the cells were pelleted by centrifugation in a tabletop microcentrifuge at 10,000 x g for 30 s. Plasmid DNA was purified using the QIAprep Mini Prep Kit (Qiagen) according to the manufacturer's protocol and eluted in 50 μ l of 10 mM Tris-HCl (pH 8.5). The resulting DNA was analyzed using a diagnostic restriction enzyme (RE) digest as well as Sanger sequencing. For an RE diagnostic digest, 4 μ l of DNA was digested with 1 μ l EcoRI for 1.5 h at 37 °C and analyzed by agarose gel electrophoresis.

Plasmid DNA was linearized at a restriction site downstream of the cloned insert according to standard RE protocol (Invitrogen). Specifically, 10 µg of plasmid DNA was cut using 10 µl of the appropriate RE and 10 µl of the appropriate RE buffer by incubating at 37 °C for 1.5 h. The linearized plasmid was then purified using the QIAquick Gel Extraction Kit (Qiagen) as per manufacturer's protocol. The concentration of the purified linearized plasmid DNA was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific).

3.2.3.1.3 DIG-RNA Labeling

The linearized plasmid was used to generate labeled RNA for use as an antisense probe for northern blot analyses in an *in vitro* transcription reaction with digoxigenin-11-UTP (DIG) according to manufacturer's protocol (DIG Northern Starter Kit, Roche Applied Science). A plasmid containing an 1111 bp region amplified from a chicken tubulin gene (Genbank accession no. **V00389**) was used to create the DIG labeled internal control probe in the same manner as previously described.

3.2.3.2 Northern Blot Analysis

Formaldehyde agarose gel electrophoresis was performed as per methods outlined in DIG Northern Starter Kit (Roche Applied Science) with slight modifications. RNA was separated on a 1.2% formaldehyde agarose gel prepared with 1 x MOPS buffer (20 mM MOPS, 5 mM NaAc, 2 mM EDTA, pH 7.0) and 0.22 M formaldehyde. Five µg total RNA was used for each tissue in a final volume of five µl. 15 µl of freshly prepared loading buffer (50% formamide, 6% formaldehyde, 1 x MOPS, 10% glycerol, 0.05% bromophenol blue, 6 µg ethidium bromide) (3:1 ratio with RNA) was added to each sample, mixed and heated at 70 °C for 10 min and briefly chilled on ice. RNA was electrophoretically separated at 130 V in 1 x MOPS buffer, visualized under UV light and photographed using the MultiImage[™] light cabinet and AlphaImager[™] 1220 Documentation and Analysis System (Alpha Innotech Corporation).

RNA was transferred to a positively charged nylon membrane (Roche Applied Science) using the VacuGene[™] XL vacuum blotting system (Amersham biosciences) with 10 x SSC buffer (0.3 M NaCl; 30 mM sodium citrate, pH 7.0) as per manufacturer's protocol. After the transfer period, the RNA was cross-linked to the membrane using a UV Stratalinker[®] 2400 (Stratagene). Pre-hybridization, hybridization and immunological detection conditions were as per methods outlined in DIG Northern Starter Kit (Roche Applied Science). After immunological detection, the membrane was exposed to BioMax Light chemiluminescent film (Kodak) for 20 min and processed with GBX Developer/Fixer (Kodak) as instructed by the manufacturer.

3.2.3.3 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

GeneRacerTM cDNA was used as the template for RT- PCR experiments investigating tissue specific expression of the cunner AFP gene. Reactions were performed in a Mastercycler[®] gradient thermal cycler (Eppendorf Scientific). Amplification was achieved in a 25 µl reaction containing one unit of AccuPrimeTM GC-rich DNA polymerase (Invitrogen), 1 x AccuPrimeTM GC-rich buffer B [60 mM Tris-HCl (pH 9.2), 1.5 mM MgSO₄, four dNTPs (200 µM each of dATP, dGTP, dCTP and dTTP), 30 mM NaCl with proprietary thermostable AccuPrimeTM proteins and enhancers], 100 ng template cDNA and 0.2 µM of both Cunner F1 and Cunner R1 primers. Initially, cDNA samples were analyzed for the presence of the housekeeping gene βactin to ensure the quality of cDNA (Hobbs and Fletcher 2008). After the quality of cDNA samples was verified, amplification of cunner AFP cDNA was achieved as follows: initial denaturation at 95 °C for three min followed by 30 cycles of denaturation at 95 °C for 30 s, primer annealing at 59 °C for 30 s and elongation at 72 °C for 90 s. A final step at 72 °C for 10 min ensured complete elongation of products. PCR products were separated on 1.2% agarose/TBE (45 mM Tris-borate, 1 mM EDTA) gels containing 0.5 µg/ml ethidium bromide.

3.2.4 AFP Gene Variability in Wrasses

3.2.4.1 DNA Probe Production

A Southern probe was generated using the PCR DIG Probe Synthesis Kit (Roche) according to manufacturer's instructions. A plasmid containing the PCR product obtained with primers PF1/PR1 (Fig 3-1) and a cunner skin/liver cDNA pool (see section 3.2.3.1.1) was used as the template for the PCR labeling reaction with primers PF1/PR1. Cycling conditions were as follows: initial denaturation at 95 °C for three min followed by 30 cycles of denaturation at

95 °C for 30 s, primer annealing at 52 °C for 30 s and elongation at 72 °C for 60 s. A final step at 72 °C for 10 min ensured complete elongation of products.

3.2.4.2 Southern Blot Analysis

Genomic DNA (gDNA) was extracted from liver samples from ocean pout, cunner and tautog (*T. onitis*), a closely related genus in the Family Labridae, using a MasterPureTM complete DNA purification kit (Epicentre Biotechnologies) according to manufacturer's instructions. DNA quality (purity and size) was assessed via spectrophotometric absorbance (260/280 ratio) using a NanoDrop 2000 spectrophotometer (Thermo Scientific) and direct visualization on ethidium bromide stained agarose gels. DNA aliquots were digested with either SacI, EcoRI or BamHI using the restriction digestion protocol suggested by the manufacturer (New England Biolabs). Following digestion, DNA was precipitated and purified using ethanol precipitation. Specifically, 1/10 volume of 3M sodium acetate (pH 5.2) was added to each digest and mixed by inversion. Next, two times the digest's volume of ethanol was added, mixed by inversion and held on ice for five minutes. After centrifugation at 10,000 x g for 30 min, the supernatant was removed. The DNA pellet was washed twice with 70% ethanol, dried briefly and resuspended in TE buffer (pH 8.0). 10 µg of each digest was separated on ethidium bromide stained 0.8% agarose gel at low voltage (50-60 V). Included on the gel were cunner AFP cDNA standards for use in gene copy number estimation. The DNA in the gel was depurinated (250 mM HCl) for 15 min, followed by denaturation (0.5 M NaOH) for 2 x 15 min and neutralization (0.5 M Tris-HCl, pH 7.5; 1.5 M NaCl) for 2 x 15 min. DNA was transferred to a positively charged nylon membrane (Roche Applied Science) using the VacuGene[™] XL vacuum blotting system (Amersham biosciences) with 20 x SSC (0.6 M NaCl; 60 mM sodium citrate, pH 7.0) as per manufacturer's instructions. After the transfer period, the DNA was cross-linked to the

membrane via UV light using a UV Stratalinker[®] 2400 (Stratagene). Pre-hybridization, hybridization and immunological detection conditions were as per methods outlined in DIG Northern Starter Kit (Roche Applied Science). After immunological detection, the membrane was exposed to BioMax Light chemiluminescent film (Kodak) for 10-20 min and processed with GBX Developer/Fixer (Kodak) as instructed by the manufacturer.

3.2.4.3. Polymerase Chain Reaction

Cunner gDNA was used as the template in polymerase chain reaction (PCR) experiments for the purpose of sequencing AFP genes. PCR reactions were performed in a Mastercycler[®] gradient thermal cycler (Eppendorf Scientific). Amplification was achieved in a 25 µl reaction containing one unit of AccuPrimeTM GC-rich DNA polymerase (Invitrogen), 1 x AccuPrimeTM GC-rich buffer B [60 mM Tris-HCl (pH 9.2), 1.5 mM MgSO₄, four dNTPs (200 µM each of dATP, dGTP, dCTP and dTTP), 30 mM NaCl with proprietary thermostable AccuPrimeTM proteins and enhancers], 100 ng genomic DNA and 0.2 µM of both PF1 and PR1 primers. The amplification conditions were as follows: initial denaturation at 95 °C for three min followed by 30 cycles of denaturation at 95 °C for 30 s, primer annealing at 52 °C for 30 s and elongation at 72 °C for 90 s. A final step at 72 °C for 10 min ensured complete elongation of products. PCR products were separated on 1.2% agarose/TBE (45 mM Tris-borate, 1 mM EDTA) gels containing 0.5 µg/ml ethidium bromide.

3.2.5 cDNA Cloning and Sequencing

PCR products of interest were extracted using a QIAquick Gel Extraction Kit (Qiagen) as per manufacturer's instructions. Pure PCR products were ligated into the pGEM[®]-T easy vector (Promega) and transformed into *E. coli* DH5 α cells as per manufacturer's instructions. 50 µl aliquots of transformed bacteria were spread on pre-warmed, X-gal in dimethylformamide (DMF) treated (40 μ l of 40 mg/ml solution/plate) LB agar [10 g/L bacto-tryptone, five g/L bactoyeast extract, 10 g/L NaCl (pH 7.0), 15 g/L bacto-agar] plates supplemented with ampicillin (100 μ g/ml) and incubated overnight at 37 °C. Individual bacterial colonies containing inserts were patch plated for short-term storage. DNA from individual bacterial colonies was purified using a Qiagen Plasmid Mini Kit (Qiagen). Purified plasmid DNA was sequenced at Memorial University of Newfoundland's (MUN) CREAIT network on the 3730xl capillary DNA analyzer (Applied Biosystems) using pUC plasmid primers M13F and M13R. Sequencing results were provided as .abi files from which text sequences and chromatograms were viewed using Sequence Scanner version 1 (Applied Biosystems) to confirm accuracy of the provided text (Phred cut-off = 40). DNA sequences were analyzed and aligned using Vector NTI (Invitrogen) and the clustalW alignment option (AllignX).

3.3 Results

3.3.1 Identification of a Complete Cunner AFP cDNA by RLM-RACE

PCR amplification of a GeneRacer^{$^{\text{M}}$} cDNA pool in conjunction with AFP-specific and GeneRacer^{$^{\text{M}}$} primers generated PCR products that were purified and sequenced. These sequences were aligned and used to generate the complete (contiguous) cDNA with at least three-fold coverage at every base of the sequence (Fig 3-1). The resulting cunner AFP cDNA is identical to that previously described (Hobbs *et al.* 2011) with the addition of two bp at the 5' end (5' UTR) and 187 bp at the 3' end (3' UTR) to give a complete cDNA of 448 bp in length (Fig 3-1) including a potential polyadenylation signal near the 3' end of the cDNA (Genbank accession no. **JF937681.2**).

3.3.2 Tissue Specific Expression of a Cunner AFP Gene

AFP mRNA expression analyses using northern blotting and RT-PCR procedures were carried out on two individuals. Since the results obtained for both individuals were identical, only one of them was chosen for illustration (Fig 3-2 & 3-3).

Northern blot analyses revealed the presence of high levels of type I AFP mRNA in skin and gill tissue, moderate levels in spleen and low levels in intestinal and stomach tissues (Fig 3-2). No AFP mRNA was detected in liver and heart. Two major hybridization signals for cunner AFP mRNA were observed in all positive tissues [~500 and ~700 nucleotides (nt)], the smaller of which corresponds well with the RACE determined cDNA sequence (Fig 3-1) plus a typical poly (A) tail (Brawerman 1981, Schiavi *et al.* 1994, Wahle 1995). A larger, less intense signal (~1400 nt) was also observed in skin, gill and spleen samples. The intense signals observed in skin and gills were obtained as a result of extended exposure times required to detect low signals observed in intestine and stomach. No AFP mRNA was observed in the negative control ocean pout (*Macrozoarces americanus*) liver RNA sample. Control tubulin mRNA was detected in all tissues examined.

RT-PCR analysis identified the presence of cunner AFP mRNA (207 bp product) in all tissues tested. However the amplicon obtained for liver mRNA was barely detectable and could be the result of contamination by a few cells from a contiguous tissue during dissection (Fig 3-3). β -actin mRNA (675 bp product) was detected in all tissues including the AFP negative control (ocean pout liver). No cunner type I AFP mRNA was detected in the ocean pout liver sample and neither cunner AFP mRNA nor β -actin products were detected in the PCR negative control (water).

Figure 3-2. Northern blot analysis of cunner total RNA from various tissues as seen on a denaturing agarose gel. Total RNA (5 μ g) was separated on a formaldehyde agarose gel prior to blotting onto a positively charged nylon membrane. Top panel probed with a cunner AFP RNA probe. Middle panel probed with a chicken tubulin probe (internal control). Bottom panel is total RNA following electrophoresis viewed under UV light. Ocean pout liver RNA (opL) was used as a negative control. Abbreviations used: nt; nucleotides, L; liver, Sk; skin, I; intestine, St; stomach, G; gill, H; heart, Sp; spleen



Figure 3-3. RT-PCR analysis of cunner total RNA from various tissues as seen on a 1.2% agarose gel. Primers were designed from cunner cDNA to generate a 207 bp product. Internal control primers were designed from the Atlantic salmon β -actin gene sequence to generate a 675 bp product. Ocean pout liver cDNA (opL) was used as an AFP negative control while water was used as a PCR negative control. Abbreviations used: M; 1 Kb Plus DNA ladder (Invitrogen), L; liver, Sk; skin, I; intestine, St; stomach, G; gill, H; heart, Sp; spleen, W; water



3.3.3 Seasonal Expression of a Cunner AFP Gene

Northern blot analyses indicated that the level of expression of type I AFPs in skin varied with season (Fig 3-4). The hybridization signal was strongest in January (winter), somewhat weaker in October (fall) and April (spring) and very weak in August (summer). The two major hybridization signals were observed (~500 and ~700 nt) in all samples, while the larger, less intense signal (~1400 nt) was present in the fall, winter and spring samples, but absent in the summer sample.

3.3.4 Cunner and Tautog AFP Genes are Part of Multi-gene Families

The presence and abundance of AFP genes in the genomes of cunner and *T. onitis* was examined using genomic Southern blot analyses. Two individual fish per species were examined and the results for both were identical. The results from a single fish from each species are presented in Fig 3-5. Hybridization of a cunner AFP DNA probe to RE digested gDNA produced at least nine hybridization signals in cunner ranging in size from about one kB to approximately 23 kB and two corresponding signals in *T. onitis* ranging in size from about 4.4 kB to approximately 10 kB. No hybridization signals were observed in ocean pout gDNA (Fig 3-5a). Multiple hybridization signals were observed in all samples probed with a chicken tubulin DNA probe (Fig 3-5b).

Sequencing of PCR products obtained from cunner gDNA (cgDNA) and cunner AFP specific primers PF1 and PF2 identified the presence of two genes differing by the number of introns present (Fig 3-6). CgDNA1 is 494 bp in length while CgDNA2 is 686 bp. Both genes contain the same intron from positions 64-158 while CgDNA2 has an additional intron from positions 374-565 (Fig 3-6). CgDNA1 has a single silent nucleotide substitution relative to the cDNA sequence (Genbank accession no. **JF937681.2**) at position 208 where cytosine replaces

Figure 3-4. Seasonal northern blot analysis of cunner total RNA from skin tissues as seen on a denaturing agarose gel. Total RNA ($2 \mu g$) was separated on a formaldehyde agarose gel prior to blotting onto a positively charged nylon membrane. Top panel probed with a cunner AFP RNA probe. Bottom panel is total RNA following electrophoresis viewed under UV light. Abbreviations used: nt; nucleotides



Figure 3-5. Southern blot analyses of cunner, tautog and ocean pout gDNA. DNAs (10μg) were digested with either SacI (S), EcoRI (E) or BamHI (B)and separated on a 0.8% agarose gel. (a) was probed with a cunner AFP DNA probe while (b) was probed with a chicken tubulin probe (Genbank accession no. **V00389**). Inset of blot (a): hybridization intensity corresponding to one, 10 and 25 AFP copies/genome to aid in cunner AFP copy number determination. DNA Molecular Weight Marker II, DIG-Labeled (Roche) is shown to the left of both blots. The origin of the gel is indicated by horizontal arrow



Figure 3-6. Nucleotide sequences of AFP genes identified from cunner gDNA (CgDNA1 and CgDNA2) relative to cunner cDNA (CcDNA; Genbank accession no. **JF937681.2**). Identical nucleotides relative to cunner cDNA represented by dots while base substitutions are indicated. The coding sequence is capitalized (highlighted green). Intervening sequences (highlighted blue) and untranslated regions (highlighted pink) are in lowercase lettering while the location of introns not present in a sequence represented by hyphens. The locations of primer annealing sites are underlined

CcDNA:	cagattttgtggatcaagttcaatagttctctctctacaacaaa <mark>ATGGATTCAGGA</mark>	56
CgDNA 1:		
CYDNA 2.		
	AAAA	112
	\cdots gtgagtgtctttatttatatgaaggtatatgtgatcacttttcatgcctctt	
	····gtgagtgtctttatttatatgaaggtatatgtgatcacttttcatgcctctt	
	<mark>@TGCACCTCAGCT</mark>	168
	atatqaqatqqatcactqctqtttcttctctcqcccattqtaq	100
	atatgagatggatcactgctgtttcttctctcgcccattgtag······	
	TGCTGCTGAAGCTACTGCTGCAGCTGCACTTAAAGCTGCTGAAGCTACCAAAGCTG	224
	CAGCTAAAGCTGCAGCTGAAGCTACTGCTAAAGCTGCAGCTGCAGCTGCTGGAAAA	280
	TAGctqctqqaqctqcaqqqqaaqccqctqcttcqtcctttqqqccaactaaaatq	336
	••••••••••••••••••••••••••••••••••••••	
		202
		392
	······ <mark>ttccaagactttaaagatt</mark>	
		448
		504
		560
	${\tt atgaaataacctgtaccagagtctgcattacaagacttcattaagagtaacaaaag}$	
		616
		010
	<pre>taatggggg</pre>	
	tgcaagcatacatgagttagcatgctattttctatgtacttaaaatcatgactttg	672
	•••••	
	${\tt cacatcttttacctacgggtaca}$ aataaagtaacctgaaagtttgaaactgtcaaa	728
	•••••	
	aaaaaa 735	

thymine, while CgDNA2 has two substitutions in the 3' UTR at positions 329 where thymine replaces cytosine and 603 where guanine replaces adenine. The *in silico* deduced amino acid sequence for both genomic clones is identical to that previously identified from cunner cDNA (Hobbs *et al.* 2011).

3.4 Discussion

The results of this study demonstrate that the type I AFP genes in cunner belong to a multigene family that are predominantly expressed in gill and skin tissues with little or no AFP transcripts in the liver. This result together with our recent discovery that the cunner AFP gene codes for an AFP that has no secretory signal indicates that it can be classified as a skin-type I AFP that is almost identical in size and structure to the skin-type I AFPs first discovered in winter flounder skin (Gong *et al.* 1996), and later in longhorn and shorthorn sculpins respectively (Low *et al.* 1998, Low *et al.* 2001).

The significance of AFP expression in skin to the welfare of fish residing in polar and subpolar marine waters is embodied within the "skin protection hypothesis" which suggests that AFPs in skin functions to protect the epithelial cells directly by preventing nascent ice crystal formation and growth within the interstitial space, while at the same time blocking ice propagation across the epithelial layer and into circulating body fluids [for details see Fletcher and Davies (2012)].

Previous research has shown that the external epithelia of fish residing in ice laden sea water is an excellent barrier to ice propagation; the effectiveness of which can be enhanced when the skin is fortified with AFPs to the point where propagation temperatures can be well below the freezing point of sea water (Cziko *et al.* 2006, Turner *et al.* 1985, Valerio *et al.* 1992a, Valerio *et al.* 1992b). In the case of the cunner this barrier could effectively prevent ice crystals from

entering the circulatory system where there are negligible levels of plasma AFPs. This may be a major reason why cunner avoid freezing during winter (Hobbs *et al.* 2011, Valerio *et al.* 1990).

The results from northern analyses of cunner tissues support the "skin protection hypothesis" in that the tissues most likely to come into contact with or contain ice crystals (Praebel *et al.* 2009) express high levels of AFP mRNA (Fig 3-2). Moreover tissue AFP mRNA levels were correlated with the likelihood of ice contact with the highest transcript levels in the skin and gill filaments which are constantly in direct contact with the external sea water, and lower levels in stomach and intestine where ice contact would be limited to periods of sea water ingestion. Cunner enter into a state of metabolic torpor during winter when feeding ceases and drinking rates along with ice ingestion would be minimal (Green and Farwell 1971, Lewis and Driedzic 2007).

An apparent anomaly to the relationship between AFP mRNA levels and the risk of ice contact is the level of expression in the spleen. Significant levels of AFP gene or transgene expression driven by AFP promoters has also been observed in splenic tissue of winter flounder, ocean pout and transgenic Atlantic salmon (Fletcher and Davies 2012, Gong *et al.* 1992, Gong *et al.* 1996, Hobbs and Fletcher 2008) raising questions as to its functional significance. The answer may lie with observations reported in a recent study showing that ice crystals were frequently found in the spleens of the Antarctic notothenioid fish *Pagothenia borchgrevinki* to the exclusion of all other internal tissues (Praebel *et al.* 2009). This was taken one step further when Evans *et al.* (2011) demonstrated that phagocytic cells in the spleen actively engulf AFGP-labeled ice-mimicking nanoparticles and transport them to the interior of the spleen. This evidence, along with reports of circulating anti-ice IgGs (Verdier *et al.* 1996) leaves no doubt that ice crystals can form within or gain entry into fish and remain there for long periods of time.

Since cunner plasma has very low AFP levels and thus limited ability to prevent ice crystal growth it may be that plasma AFPs are solely used to label ice for splenic uptake, where a localized source of AFPs can prevent growth of stored ice crystals until seasonal warming.

All of the skin-type I AFPs lack the secretory signal sequence usually required for extracellular transport suggesting that they remain and function intracellularly. However since the amino acid sequence of the circulating AFPs in cunner is identical to that encoded by the AFP gene it seems evident that the protein must be able to exit from the cellular compartment (Hobbs et al. 2011). Direct evidence for this comes from an immunohistological study of winter flounder skin revealing that skin-type AFPs were localized in the interstitial space in close association with the cell membrane and absent from the cytoplasm (Fletcher and Davies 2012, Murray et al. 2003). Further evidence for the presence of skin-type AFPs in the extracellular compartment comes from a study of antifreeze activity (thermal hysteresis, TH) in cunner skin. This study showed that there was a gradient of AFP activity ranging from the highest levels in the cellular epidermis (avg. 0.6 °C), lower levels in the acellular dermis (avg. 0.14 °C) and undetectable levels in the plasma (Valerio et al. 1990). Since the individual fish in this study had no measurable levels of AFPs in the plasma the dermal AFPs must have been produced by the epidermal cells. Alternative pathways for protein export that circumvent the typical endoplasmic reticulum-Golgi apparatus route have been described (Menon and Hughes 1999, Mignatti et al. 1992).

Hybridization of a cunner AFP cDNA probe to restriction enzyme digested cunner DNA resulted in at least nine individual bands, indicating that the cDNA probe bound, and remained bound after stringency washes, to DNA sequences within these restriction fragments. In the event that each band represents a single gene, we can estimate that there are approximately 10

AFP genes within the cunner genome. At this time, it is unknown how many of these genes are functional as well as how they are organized in the cunner genome. Similarly, interpretation of the tautog (*T.onitis*) Southern results are guarded as we can only safely say that there are DNA sequences within the tautog genome that share identity with the cunner AFP gene. Whether or not these are homologous will require identification of the DNA sequences in question.

It is well accepted that the principal physiological function of AFPs is to prevent internal ice growth in fish. Therefore it is reasonable to assume that their first appearance within a taxon would have occurred when one or more members of this group were exposed to, or invaded what can be called "freeze-risk ecozones" that are characterized by water temperatures below the colligative freezing points of their body fluids, and the presence of ice. The first opportunity for modern day teleosts to enter such ecozones would have been the initiation of sea level Cenozoic glaciation some 30-45 Mya when seawater temperatures would have declined to their freezing points (Deconto et al. 2008, Stickley et al. 2009, Zachos et al. 2001). These conditions which have persisted at the high latitudes to the present day created freeze-risk ecozones that were inhabited by an abundance of invertebrate prey that could only be exploited by fish that were freeze resistant. This environment is considered to be the impetus for the evolution of the four known AF(G)P types in unrelated fish taxa (Scott *et al.* 1986) and subsequent taxa diversification. An excellent example of the latter is the relationship between antifreeze proteins and the evolution of Antarctic notothenioids published recently by Near et al. (2012). A second example points to the role of AFP gene duplication/amplification with regards to the the divergence and evolution of modern-day Atlantic and spotted wolffish species within the past million years (Desjardins et al. 2012).
Although considerable progress has been made with respect to identifying the ancestral precursor proteins/genes to modern day AF(G)P (Baardsnes and Davies 2001, Chen *et al.* 1997, Deng *et al.* 2010, Evans and Fletcher 2005a, Ewart *et al.* 1998, Graham *et al.* 2012) we have only rudimentary knowledge of some of the intermediate steps involved in the evolution of fully freeze resistant fish capable of residing in ice laden sea water. We know less about the time scale over which these events took place.

Since the external epithelia would be the first tissue to come into contact with ice it is possible that one of the earliest steps involved in the evolution of freeze resistant fish could have been the expression of AFPs in tissues such as the skin. Initially weakly active ice binding proteins could have served to protect the epithelial tissues from damaging effects of ice formation within the interstitial space when the fish first encountered ice. These early ice binding proteins would have provided the fish with a selective advantage over conspecifics and set the stage for the evolution of effective AFPs and the emergence of new species.

The earliest example supporting this hypothesis came from the discovery of a skin-type type I AFP gene family in winter flounder that is distinctly different from that of the multi- gene family responsible for the AFPs produced by the liver for export to the circulatory system. Since members of this skin-type AFP gene family lacked signal and pro-sequences they appeared to be more primitive than those encoded by the liver type AFP genes (Gong *et al.* 1996): the implication being that the liver type evolved from the skin-type AFPs. Further support for this hypothesis comes from the work of Evans and Fletcher (2005b) on snailfish where all of the fish examined expressed the skin-type AFP mRNA in the skin whereas less than half of them showed expression in the liver. This phenomenon has all of the appearances of a snapshot of "evolution

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in action" where the transition of AFP expression in skin only, to both liver and skin, is incomplete.

T. adspersus is the only species overwintering in shallow and frequently ice laden waters of Newfoundland that relies solely on AFP expression in skin without the assistance of sufficient levels of plasma AFPs (Hobbs *et al.* 2011). Could it be that this species has not had sufficient time to evolve a more effective expression system that would ensure the secretion of high levels of AFPs into the plasma and extracellular space? Could it be that the cunner entered its current icy habitat more recently than heterospecifics that produce both plasma and skin AFPs?

Available evidence indicates that the Atlantic Labrids have a temperate western Atlantic origin with possible tropical roots (Hanel *et al.* 2002). In contrast, it is generally accepted that all of the other northern species that produce type I AFPs [Pleuronectidae, Cottoidei (Cottidae and Cyclopteridae)], arose in the north Pacific (personal communication, Joseph S. Nelson; Munroe 2005). If this is the case then of all of the groups of fish possessing type I AFPs the Labrids are the only ones that moved into ice laden waters from the Atlantic. It appears likely that the north Pacific species exploiting food resources along the edge of the polar ice would have evolved AFP freeze protection prior to extending their range through the Arctic and into the north Atlantic during interglacial periods. Therefore species with north Pacific beginnings may have developed AFP freeze protection before the labrids entered ice laden marine waters.

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Chapter 4: Type III antifreeze protein SP and QAE isoform cooperativity evolved prior to suborder Zoarcoidei divergence.

Abstract

The production of antifreeze proteins (AFPs) is a prerequisite for most species of teleost fish inhabiting the "freeze risk ecozones" of the north Atlantic ocean as a means to prevent potentially lethal internal ice growth. Type III AFPs have been identified in a single suborder of these fish (Zoarcoidei). In the eelpouts (family Zoarcoidae) and wolffishes (family Anarhichadidae), freeze protection is accomplished through the cooperation of two distinct groups of AFP isoforms: SP-Sephadex (SP) and QAE-Sephadex (QAE) binding types. Although members from most families within the suborder Zoarcoidei are known to produce type III AFP, the cooperative action between AFP isoforms has not been confirmed outside of these two families. Through sequence analysis of AFP cDNAs isolated in this study, we have confirmed the presence of both SP and QAE type III isoforms in two additional families within this suborder: Pholidae [rock gunnel (*Pholis gunnellus*)] and Stichaeidae [radiated shanny (*Ulvaria subbifurcata*)]. These results support the hypothesis that cooperativity between SP and QAE isoforms evolved prior to the divergence of families within the suborder Zoarcoidei.

4.1 Introduction

Organisms that inhabit sub-freezing ice-laden environments require a means to prevent uncontrolled growth of internal ice (Duman and Olsen 1993, Fletcher *et al.* 2001). Most marine teleosts that inhabit such "freeze risk ecozones" produce antifreeze proteins (AFPs) or glycoproteins (AFGPs) as a protective mechanism against this potentially lethal *in vivo* growth of ice (Fletcher *et al.* 1986). These unique molecules protect the organism by binding directly to specific surfaces of ice crystals via an adsorption-inhibition mechanism, thus preventing further growth and ultimately death of the fish (Celik *et al.* 2010, Jia and Davies 2002, Knight *et al.* 1991, Raymond and DeVries 1977, Wilson 1993). To date, three physiologically functional groups of AFPs (types I-III) as well as the AFGPs have been described in a variety of fish taxa (Davies *et al.* 2002, Ewart *et al.* 1999, Fletcher *et al.* 2001, Gauthier *et al.* 2008).

The type III AFPs and their genes are restricted in distribution to the suborder Zoarcoidei. They were first isolated and characterized from ocean pout (*Macrozoarces americanus*, family Zoarcidae) (Hew *et al.* 1988) and have since been found to be present in four additional Zoarcoidei families: Anarhichadidae, [spotted wolffish (*Anarhichas minor*) and Atlantic wolffish (*A. lupus*)]; Pholidae [rock gunnel (*Pholis gunnellus*)]; Stichaeidae [radiated shanny (*Ulvaria subbifurcata*)] and Cryptacanthodidae [wrymouth (*Cryptacanthodes macullatus*)] (Davies *et al.* 1988, Desjardins *et al.* 2012, Scott *et al.* 1988, Shears *et al.* 1993).

Genomic Southern blotting analysis of four of the five aforementioned families, wrymouth being the exception, has established in all cases that type III AFP are encoded by multigene families (Hew *et al.* 1988, Scott *et al.* 1988, Shears *et al.* 1993). However structural studies of these ~ 7 kDa globular proteins have only been conducted on members of two of them, Zoarcidae (eelpouts) and Anarhichadidae (wolffishes), where they have been shown to be present as mixtures of SP-Sephadex (SP) and QAE-Sephadex (QAE) binding isoforms whose sequences are approximately 55% identical (Hew *et al.* 1988). Both of these isoforms have the capacity to bind to ice, however the SP isoform, in contrast to the QAE, has little or no ability to prevent ice crystal growth. This incapacity appears to be due to structural differences between the ice-binding surfaces of the two isoforms (Garnham *et al.* 2010, Jia *et al.* 1996, Nishimiya *et al.* 2005, Takamichi *et al.* 2009, Yang *et al.* 1998). However, full ice growth inhibitory activity can be imparted upon SP isoforms by the addition of minute amounts of a QAE isoform (Nishimiya *et al.* 2005, Takamichi *et al.* 2009). Therefore, from a physiological viewpoint it is evident that the SP and QAE isoforms act cooperatively to protect eelpouts and wolffishes from freezing.

Radiated shannys and rock gunnels inhabit shallow inshore waters where they are frequently exposed to subzero temperatures and ice during the winter months (Scott and Scott 1988). Both of these species are known to produce high plasma levels of type III AFP that are encoded by multigene families (Shears *et al.* 1993). However since no nucleotide or protein sequencing has been reported for these species we do not know whether they, in common with the eelpouts and wolffishes, possess SP and QAE isoforms.

In the present study we examine the nucleotide sequences and mRNA expression levels of AFP genes in the radiated shanny and rock gunnel. The results indicate that these species possess both SP and QAE type III AFP genes and strongly supports the hypothesis that the cooperative interaction between the QAE and SP isoforms to protect these species from freezing evolved prior the divergence of the suborder Zoarcoidei into at least four families: Zoarcidae, Anarhichadidae, Pholidae, and Stichaeidae.

4.2 Materials and Methods

4.2.1 Sample Collection

Radiated shannys and rock gunnels were collect by Ocean Sciences Centre (OSC) divers near Logy Bay, Newfoundland and Labrador, Canada and maintained at the OSC in 250 l aquaria supplied with flowing sea water (32-33‰ salinity) at seasonally ambient water temperature and photoperiod (Fletcher 1977). Fish were euthanized via an MS-222 overdose and tissues were removed and immediately flash frozen in liquid nitrogen or submerged in TRIzol[®] reagent for processing. Frozen tissue samples were stored in a -70 °C freezer prior to analysis.

4.2.2 RNA Ligase Mediated Rapid Amplification of cDNA Ends (RLM-RACE)

Total RNA was isolated from radiated shanny and rock gunnel tissue samples using TRIzol[®] reagent (Invitrogen) as per manufacturer's instructions. A GeneRacer[™] RNA oligonucleotide was ligated to radiated shanny and rock gunnel full length mRNA to allow for subsequent 5' RLM-RACE experiments. All RNA samples were reverse transcribed using Superscript III reverse transcriptase and the GeneRacer[™] poly dT primer (Invitrogen) according to the manufacturer's protocol. cDNA generated from radiated shanny and rock gunnel liver and skin mRNA was used as a 3' RACE PCR template with GeneRacer[™] 3' primer and an ocean pout type III AFP specific 5' primer (OPRTF, Table 4-1). PCR reactions were performed in a Mastercycler[®] gradient thermo cycler (Eppendorf Scientific). Amplification was achieved in a 50 µl reaction containing two units of Taq DNA polymerase (Invitrogen), 1 x PCR buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl], 1.5 mM MgCl₂, four dNTPs [200 µM each of dATP, dGTP, dCTP and dTTP (Invirtogen)], 100 ng template cDNA and 0.2 µM of both 5' and 3' primers. Touchdown PCR conditions utilized for 3' RACE were as follows: initial denaturation at 94 °C for three min followed by 13 cycles of denaturation at 94 °C for 45 s, primer annealing at 67 °C

Drimon	Sequence
rimer	Sequence
GeneRacer 3'	5'-GCTGTCAACGATACGCTACGTAACG-3'
OPRTF	5'-CGG TTT GCT TTT CGT CCT CCT-3'
GeneRacer 5'	5'-CGACTGGAGCACGAGGACACTGA-3'
S&G R1	5'-GGA AGA AAC TCC TTG GCA CCC TCA GA-3'
GeneRacer 5' nested	5'-GGACACTGACATGGACTGAAGGAGTA-3'
OAE R1	5'-CAG ACT GAC TAG TCG GGG AAT GTC CT-3'
C	
SP R1	5'-CTG GYC CTT AGC CAC TRK CTG GTT CAC-3'

5'-CAC TTC ATT CTC CGC TAA TTA ATT AAT T-3'

5'-GTG CCA AGG AGT TTC TTC CCA A -3'

5'-CCA TCA AAT CTC AAC ATA GTC TCC-3'

S&G 5' RACE 2

Probe F1

Probe R1

Table 4-1. Primers utilized in PCR experiments. Primers are written in $5' \rightarrow 3'$ orientation

decreasing to 55 °C for 30 s and elongation at 72 °C for 30 s, ending with 19 cycles of 94 °C for 45 s, 55 °C for 30 s and 72 °C for 30 s. A final step at 72 °C for four min ensured complete elongation of products.

Nested touchdown PCR was utilized for 5' RACE on radiated shanny and rock gunnel cDNA. In the primary reaction GeneRacerTM cDNA from liver and skin was pooled and used as the template with GeneRacerTM 5' primer and S&G R1 primer (Table 4-1). Primary touchdown PCR conditions utilized were as follows: initial denaturation at 94 °C for three min followed by 11 cycles of denaturation at 94 °C for 30 s, primer annealing at 72 °C decreasing to 62 °C for 30 s and elongation at 72 °C for 60 s, ending with 20 cycles of 94 °C for 30 s, 62 °C for 30 s and 72 °C for 60 s. A final step at 72 °C for 10 min ensured complete elongation of products. The PCR products generated in the primary reaction were diluted 20 x with water and used as template for secondary PCR with GeneRacerTM 5' nested primer and either QAE R1 or SP R1 (Table 4-1). Secondary touchdown PCR conditions utilized were as follows: initial denaturation at 94 °C for 30 s, primer annealing at 72 °C for 30 s, 65 °C for 30 s and 72 °C for 90 s, ending with 25 cycles of 94 °C for 30 s, 65 °C for 30 s and 72 °C for 90 s. A final step at 72 °C for 10 min ensured complete elongation of products.

The sequence information gathered from sequencing of 5' RACE PCR products (see Section 4.2.3) was used to design sense primers near the 5'end of the radiated shanny and rock gunnel AFP genes. These primers were utilized in a second 3' RACE experiment to obtain as much of the gene sequence as possible in a single experiment. GeneRacerTM liver and skin cDNAs were pooled and used as the template for touchdown PCR with primers S&G 5' RACE 2 and GeneRacerTM 3' primer (Table 4-1) with the same cycling conditions as 5' RACE. PCR

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products were separated on 1% agarose/TBE (45 mM Tris-borate, 1 mM EDTA) gels containing 0.5 µg/ml ethidium bromide for nucleic acid visualization.

4.2.3 cDNA Cloning and Sequencing

PCR products were gel extracted using a QIAquick gel extraction kit (Qiagen) as per manufacturer's instructions. Pure PCR products were ligated into the pGEM-T easy vector (Promega) and transformed into E. coli DH5a cells as per manufacturer's instructions. 50 µl aliquots of transformed bacteria were spread on pre-warmed, X-gal in dimethylformamide (DMF) treated (40 µl of 40 mg/ml solution/plate) LB agar [10 g/L bacto-tryptone, five g/L bactoyeast extract, 10 g/L NaCl (pH 7.0), 15 g/L bacto-agar] plates supplemented with ampicillin (100 µg/ml) and incubated overnight at 37 °C. Individual colonies containing inserts were patch plated for short-term storage. DNA from individual bacterial colonies was purified using a Qiagen plasmid mini kit (Qiagen). Purified plasmid DNA was sequenced at the Memorial University of Newfoundland (MUN) CREAIT network on a 3730xl capillary DNA analyzer (Applied Biosystems) using pUC plasmid primers M13F and M13R. Sequencing results were provided as .abi files from which text sequences and chromatograms were proof read using Sequence Scanner version 1 (Applied Biosystems) to confirm accuracy of the provided text. DNA sequences were analyzed and aligned using Vector NTI (Invitrogen) and the clustalW alignment option (AlignX) to generate complete contigs.

4.2.4 Northern Blot Analyses

4.2.4.1 RNA Probe Production

4.2.4.1.1 Plasmid Purification

A northern blot RNA probe was generated from cDNA obtained from PCR using primers ProbeF1/ProbeR1 (Table 4-1) with a plasmid containing a rock gunnel skin 3' RACE PCR product as template. The PCR product was gel purified, ligated into the pGEM®-T easy vector system and transformed into *E. coli* bacteria [DH5α (Invitrogen)] as previously described.

Bacteria were grown in Luria-Bertani (LB) medium (10 g/L bacto-tryptone; 5 g/L bactoyeast extract; 10 g/L NaCl, pH 7.0) which was supplemented with ampicillin (100 μ g/ml) based on the resistance gene encoded by the vector contained within the cells. The cultures were grown overnight with shaking at 37 °C. A 1.5 ml aliquot of the culture was transferred to a 1.5 ml microcentrifuge tube and the cells were pelleted by centrifugation in a tabletop microcentrifuge at 10,000 x g for 30 s. Plasmid DNA was purified using the QIAprep Mini Prep Kit (Qiagen) according to the manufacturer's protocol and eluted in 50 μ l of 10 mM Tris-HCl (pH 8.5). DNA was analyzed using a diagnostic restriction enzyme (RE) digest as well as Sanger sequencing. For RE diagnostic digests, 4 μ l of DNA was digested with 1 μ l EcoRI for 1.5 hours at 37 °C, and analyzed by agarose gel electrophoresis.

Plasmid DNA was linearized at a restriction site downstream of the cloned insert according to standard RE protocol (Invitrogen). Specifically, 10 µg of plasmid DNA was cut using 10 µl of RE and 10 µl of the appropriate RE buffer by incubating at 37 °C for 1.5 hours. The linearized plasmid was then purified using the QIAquick Gel Extraction Kit (Qiagen) as per manufacturer's protocol. The concentration of the purified linearized plasmid DNA was analyzed using the NanoDrop 2000 spectrophotometer (Thermo Scientific).

4.2.4.1.2 DIG-RNA Labeling

The linearized plasmid was used to create labeled RNA for use as a probe for northern blot analyses in an *in vitro* transcription reaction with digoxigenin-11-UTP (DIG) according to

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manufacturer's protocol (DIG Northern Starter Kit, Roche Applied Science). A plasmid containing a 1111 bp region amplified from a chicken tubulin gene (Genbank accession no.**V00389**) was used to create the DIG labeled internal control probe in the same manner as previously described.

4.2.4.2 Northern analysis

Formaldehyde agarose gel electrophoresis was performed as per methods outlined in the DIG Northern Starter Kit (Roche Applied Science) with slight modifications. RNA was separated on a 1.2% formaldehyde agarose gel prepared with 1 x MOPS buffer (20mM MOPS; 5mM NaAc; 2mM EDTA; pH 7.0) and 0.22 M formaldehyde.Two µg total RNA was used for each tissue in a final volume of five µl. 15µl of freshly prepared loading buffer (250 µl of 100% formamide; 83 µl of 37% formaldehyde solution; 50 µl 10 x MOPS; 50 µl 100% glycerol; 10 µl 2.5% bromophenol blue; 57 µl water; 6 µg ethidium bromide) (3:1 ratio with RNA) was added to each sample, mixed and heated at 70 °C for 10 min and briefly chilled on ice. RNA was electrophoretically separated at 130 V in 1 x MOPS buffer, visualized under UV light and photographed using the MultiImageTM light cabinet and AlphaImagerTM1220 Documentation and Analysis System (Alpha Innotech Corporation).

RNA was transferred to a positively charged nylon membrane (Roche Applied Science) using the VacuGene[™] XL Vacuum blotting system (Amersham biosciences) with 10 x SSC buffer (0.3 M NaCl; 30 mM sodium citrate, pH 7.0) as per manufacturer's protocol. After the transfer period, RNA was cross-linked to the membrane via UV light using a UV Stratalinker[®] 2400 (Stratagene). Pre-hybridization, hybridization and immunological detection conditions were as per methods outlined in DIG Northern Starter Kit (Roche Applied Science). After immunological detection, the membrane was exposed to BioMax Light chemiluminescent film

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(Kodak) for 30-60 min and processed with GBX Developer/Fixer (Kodak) as instructed by the manufacturer.

4.3 Results

4.3.1 SP and QAE Type III AFP genes in radiated shanny and rock gunnel

Preliminary PCR reactions were performed on radiated shanny and rock gunnel GeneRacerTM cDNA using a GeneRacerTM 3' primer and a 5' primer designed from an ocean pout type III AFP gene (Hobbs and Fletcher 2008). These reactions generated products that were sequenced and subsequently identified as SP and QAE isoforms of type III AFP genes. The sequence data obtained was then used to design PCR primers specific to regions of radiated shanny and rock gunnel AFP genes that differed markedly between the SP and QAE AFP isoforms which would be used in conjunction with the GeneRacer[™] 5' primer in 5' RACE. The 5' sequence data obtained from 5' RACE was used to design a final primer located as near to the 5' end of the AFP CDS as possible. This primer was then used in conjunction with SP or QAE specific 3' primers to amplify nearly full length SP and QAE cDNAs. Alignment of these sequences generated contiguous sequences representing SP and QAE type III isoforms from both radiated shanny and rock gunnel with at least three-fold coverage at each base (Figs 4-1 to 4-4) (Genbank Accession numbers: radiated shanny QAE JX178756.1, radiated shanny SP JX178755.1, rock gunnel QAE JX178758.1, rock gunnel SP JX178757.1). Rock gunnel QAE AFP cDNA is 550 base pairs (bp) in length and comprised of a 77 bp 5' untranslated region (UTR), 200 bp 3' UTR and 273 bp CDS that codes for a 90 amino acid (aa) translated gene product (Fig 4-1). Rock gunnel SP AFP cDNA is 542 bp in length and comprised of a 77 bp 5' UTR, 198 bp 3' UTR and 267 bp CDS that codes for an 88 amino acid (aa) translated gene product (Fig 4-2). Radiated shanny QAE AFP cDNA is 550 base pairs (bp) in length and

Figure 4-1. Rock gunnel QAE AFP cDNA sequence. The peptide coding sequence is written in capital letters while untranslated regions are in lowercase. The deduced amino acids are displayed above their corresponding codons and in bold. The start (ATG) codon is underlined while the stop codon (TAA) is identified with *. A putative polyadenylation signal (aataaa) is italicized and underlined. Genbank Accession no. **JX178758.1**

agaagtetea getacagett teactteatt etcegetaat taattaatta 50 G 8 М Ν S A Ι L Т ttaattaatt aagtctcagc cacagctATG AATTCAGCTA TTTTAACTGG 100 ьь C V D N м т S Α 24 F L F v G S TTTCCTTTTC GTCCTCCTTT GTGTCGACAA CATGACTTCA GCCGGCTCGG 150 GKSV VAN QLI Р I N т Α 41 L т GTAAATCCGT GGTGGCCAAC CAGCTGATCC CCATAAATAC TGCCCTGACT 200 LVA R Ι м MKAE PMG I P A E 58 CGGATCATGA TGAAGGCGGA GTTGGTCGCC CCAATGGGCA TCCCCGCCGA 250 D I P R ьv S LOV N R Α v P M 74 GGACATTCCC CGACTAGTCA GTCTGCAAGT CAACAGGGCA GTGCCGATGG 300 GТ т L М ΡD М V K т ү Ρ Α Κ Q * 90 GCACAACCCT CATGCCAGAC ATGGTGAAAA CGTACCAACC AGCGAAGTAA 350 ttctgagggt gccaaggagt ttcttcccaa aaccagaaga agaaatgccc 400 cctctcacaa tcaaccttgt ttttgtcaga aacccaagtc tgtccggatg 450 ttaactgaac atgtcaaaac ctgtggagac tatgttgaga tttgatggtc 500 tgaaaagata aagcatataa ataaaatttt gcccaaaaaa aaaaaaaaa 550

Figure 4-2. Rock Gunnel SP AFP gene sequence. The peptide coding sequence is written in capital letters while untranslated regions are in lowercase. The deduced amino acids are displayed above their corresponding codons and in bold. The start (ATG) codon is underlined while the stop codon (TAG) is identified with *. A putative polyadenylation signal (aataaa) is italicized and underlined. Genbank Accession no. **JX178757.1**

agaagtetea getacagett teactteatt etcegetaat taattaatta 50 S A G 8 М Ν Ι L Т ttaattaatt aagtctcagc cacaactATG AATTCAGCTA TTTTAACTGG 100 FLF VLL CVDN MTS AGS 24 TTTCCTTTTC GTCCTCCTTT GTGTCGACAA CATGACTTCA GCCGGCTCGT 150 I P M SVVA SQL NTAL т ΡA 41 CCGTGGTGGC CAGCCAGCTG ATCCCCATGA ATACTGCCCT GACTCCGGCG 200 ммк G V V V SPA GIP FAEM58 ATGATGAAGG GGGTTGTGGT CAGCCCAGCA GGCATCCCGT TCGCAGAGAT 250 SRI VGK Q V N Q I V A K D Q 74 GTCCAGAATA GTGGGAAAGC AAGTGAACCA GATAGTGGCT AAGGACCAAA 300 Q P A K TLMP S мv КТҮ * 88 CCCTCATGCC AAGCATGGTG AAAACGTACC AACCAGCGAA GTAGttctga 350 gggtgccaag gagtttcttc ccaaaaccag aagaagaaat gccccctctc 400 acaatcaacc ttgtttttgt cagaaaccca agtctgcccg gatgttaact 450 gaacatgtca aaacctgtgg agactatgtt gagatttgat ggtctgaaaa 500 542 gataaagcat ata*aataaa*a ttttgcccaa aaaaaaaaaa aa

Figure 4-3. Radiated shanny SP AFP gene sequence. The peptide coding sequence is written in capital letters while untranslated regions are in lowercase. The deduced amino acids are displayed above their corresponding codons and in bold. The start (ATG) codon is underlined while the stop codon (TAG) is identified with *. A putative polyadenylation signal (aataaa) is italicized and underlined. Genbank Accession no. **JX178755.1**

agaagtetea getacagett teactteatt eteegetaat taattaatta 50 S A ΙL T G 8 М Ν ttaattaatt aagtctcagc cacaaccATG AATTCAGCTA TTTTAACTGG 100 FLF VLL C V N M S S A 24 A Q S TTTTCTTTTC GTCCTCCTTT GTGTCAACAT GAGTTCAGCC GCCCAGTCCG 150 V V A T QLI Ρ I N TALT PAM 41 TGGTGGCCAC TCAGCTGATC CCCATAAATA CTGCCCTGAC TCCGGCAATG 200 PF MKG MDVN PSG I Т EKS58 ATGAAGGGGA TGGATGTCAA CCCAAGCGGC ATCCCGTTCA CGGAGAAGTC 250 V N Q P V V K G 74 Т L v GKQ 0 т CACACTAGTG GGAAAGCAAG TGAACCAGCC AGTGGTTAAG GGCCAGACCC 300 LMRN MVK Ρ 82 * TCATGCGAAA TATGGTGAAA CCATAGgcaa agaagttgtt ttgagggtgc 350 caaggagttt cttcccaaaa ccaaaagaag aaatgtcccc tctcacaatt 400 aatcttgttt ttgtcacaaa cccaaatctg tccggatgtt aactgaacat 450 gtcaaaacct gtggagacta tgttgagatt tgatgttctg aaaagataaa 500 541 gcctata*aat aaa*aggttgc ccaaaaaaaa aaaaaaaaa a

Figure 4-4. Radiated shanny QAE AFP gene sequence. The peptide coding sequence is written in capital letters while untranslated regions are in lowercase. The deduced amino acids are displayed above their corresponding codons and in bold. The start (ATG) codon is underlined while the stop codon (TAG) is identified with *. A putative polyadenylation signal (aataaa) is italicized and underlined. Genbank Accession no. **JX178756.1** agaagtetea getacageet teactteatt eteegataat taattaatta 50 S V G 8 М Ν Ι F Т ttaattaatt aagtctcagc cacaactATG AATTCAGTTA TTTTTACTGG 100 LVF CVDN M S Α 24 VLL S Α S TTTGGTCTTC GTCCTCCTTT GTGTCGACAA CATGAGTTCA GCCGCCTCGG 150 GQSV VAN QL Ι Р I N т Α L т 41 GTCAGTCCGT GGTGGCCAAC CAGCTGATCC CCATCAATAC TGCCCTGACT 200 MRAE V V S I P A E 58 LVM Ρ ЬG CTGGTGATGA TGAGGGCGGA GGTGGTCTCC CCATTGGGCA TCCCCGCCGA 250 DIP R ьv SLQV N R A V PL 74 GGACATTCCC CGACTAGTCA GTCTGCAAGT GAACAGGGCA GTGCCGCTGG 300 GΤ T L т A E M V K G Y S Ρ Α ĸ * 90 GCACAACCCT CACGGCAGAG ATGGTGAAAG GGTACAGCCC GGCTAAGTAG 350 ttcttgagggt gccaaggagt ttcttcccaa aaccaacaga agaaatgccc 400 cctctcacaa ttaaccttgt ttttgtcaca aacccaagtc tgtccggatg 450 ttaactgaac atgtcaaaac ctgtggagac tatgatgaga tttgatgttc 500 tgaaaagata aagcctatga ataaagtttt gcccaaaaaa aaaaaaaaa 550 comprised of a 77 bp 5' UTR, 200 bp 3' UTR and 273 bp CDS that codes for a 90 amino acid (aa) translated gene product (Fig 4-3). Radiated shanny SP AFP cDNA is 541 bp in length and comprised of a 77 bp 5' UTR, 215 bp 3' UTR and 249 bp CDS that codes for an 82 aa translated gene product (Fig 4-4).

4.3.2 QAE and SP Genes Predate Suborder Zoarcoidei Divergence

A multiple sequence alignment of radiated shanny and rock gunnel QAE and SP cDNAs illustrates the high sequence identity between all four sequences (85-92%) (Fig 4-5). This can be further investigated by comparing 5' UTRs (96-99% identity), 3' UTRs (94-99% identity) or CDSs (71-87% identity) independently. Comparison of cDNA sequences with other family members of the suborder Zoarcoidei, Atlantic wolffish (*Anarhichas lupus*, family Anarhichadidae), ocean pout (*Macrozoarces americanus*) and Antarctic eelpout (*Lycodichthys dearborni*, family Zoarcidae) revealed 78-91% identity among all sequences (Fig 4-6). This is due to 70-89% sequence identity among CDSs, 70-99% among 5' UTRs , and 85-99% among 3' UTRs (data not shown). This high sequence identity of type III AFP cDNA among different families is depicted by the nearly continuous straight line in dot matrices (Fig 4-7).

Alignment of the *in silico* translated type III AFP gene products from the radiated shanny and rock gunnel with those of other members of the suborder Zoarcoidei, family Zoarcidae [ocean pout, notched-fin eel pout (*Zoarces elongates*)], and family Anarhichadidae (Atlantic wolffish) illustrates a high amino acid sequence identity among the QAE (70-86%) (Fig 4-8A) and SP (70-88%) (Fig 4-8B) isoforms. **Figure 4-5.** Multiple sequence alignment of radiated shanny (*Ulvaria subbifurcata*) and rock gunnel (*Pholis gunnellus*) AFP cDNA sequences. The nucleotide alignment was constructed using ClustalW (*http://www.ebi.ac.uk/Tools/msa/clustalw2/*). Residues conserved throughout all sequences indicated by * below. Residues differing from the majority are highlighted grey

shannyQAE gunnelQAE gunnelSP shannySP	AGAAGTCTCAGCTACAGCCTTCACTTCATTCTCCGATAATTAAT	60 60 60 60
shannyQAE gunnelQAE gunnelSP shannySP	AAGTCTCAGCCACAACT ATG AATTCAGTTATTTTTACTGGTTTGGTCTTCGTCCTCCTTT AAGTCTCAGCCACAGCT ATG AATTCAGCTATTTTAACTGGTTTCCTTTTCGTCCTCCTTT AAGTCTCAGCCACAACT ATG AATTCAGCTATTTTAACTGGTTTCCTTTTCGTCCTCCTTT AAGTCTCAGCCACAACC ATG AATTCAGCTATTTTAACTGGTTT T CTTTTCGTCCTCCTTT ***********************	120 120 120 120
shannyQAE gunnelQAE gunnelSP shannySP	GTGTCGACAACATGAGTTCAGCCGCCTCGGGTCAGTCCGTGGTGGCCAACCAGCTGATCC GTGTCGACAACATGACTTCAGCCGGCTCGGGTAAATCCGTGGTGGCCAACCAGCTGATCC GTGTCGACAACATGACTTCAGCCGGCTCGTCCGTGGTGGCCAGCCAGCTGATCC GTGTCAACATGAGTTCAGCCGCCCAGTCCGTGGTGGCCACTCAGCTGATCC ***** ******* ******* * * **********	180 180 174 171
shannyQAE gunnelQAE gunnelSP shannySP	CCATCAATACTGCCCTGACTCTGGTGATGATGAGGGCGGAGGTGGTCTCCCCCATTGGGCA CCATAAATACTGCCCTGACTCGGATCATGATGAAGGCGGAGTTGGTCGCCCCAATGGGCA CCATGAATACTGCCCTGACTCCGGCGATGATGAAGGGGGTTGTGGTCAGCCCAGCAGGCA CCATAAATACTGCCCTGACTCCGGCAATGATGAAGGGGATGGAT	240 240 234 231
shannyQAE gunnelQAE gunnelSP shannySP	TCCCCGCCGAGGACATTCCCCGACTAGTCAGTCTGCAAGTGAACAGGGCAGTGCCGCTGG TCCCCGCCGAGGACATTCCCCGACTAGTCAGTCTGCAAGTCAACAGGGCAGTGCCGATGG TCCCGTTCGCAGAGATGTCCAGAATAGTGGGAAAGCAAGTGAACCAGATAGTGGCTAAGG TCCCGTTCACGGAGAAGTCCACACTAGTGGGAAAGCAAGTGAACCAGCCAG	300 300 294 291
shannyQAE gunnelQAE gunnelSP shannySP	GCACAACCCTCACGGCAGAGATGGTGAAAGGGTACAGCCCGGCTAAG TAG TTCTGAGGGT GCACAACCCTCATGCCAGACATGGTGAAAACGTACCAACCA	360 360 354 348
shannyQAE gunnelQAE gunnelSP shannySP	GCCAAGGAGTTTCTTCCCAAAACCAACAGAAGAAATGCCCCCTCTCACAATTAACCTTGT GCCAAGGAGTTTCTTCCCAAAACCAGAAGAAGAAATGCCCCCCTCTCACAATCAACCTTGT GCCAAGGAGTTTCTTCCCAAAACCAGAAGAAGAAATGCCCCCCTCTCACAATCAACCTTGT GCCAAGGAGTTTCTTCCCCAAAACCAAAAGAAGAAATGTCCCCCTCTCACAATTAATCTTGT *********************************	420 420 414 408
shannyQAE gunnelQAE gunnelSP shannySP	TTTTGTCACAAACCCAAGTCTGTCCGGATGTTAACTGAACATGTCAAAACCTGTGGAGAC TTTTGTCAGAAACCCAAGTCTGTCCGGATGTTAACTGAACATGTCAAAACCTGTGGAGAC TTTTGTCAGAAACCCAAGTCTGCCCGGATGTTAACTGAACATGTCAAAACCTGTGGAGAC TTTTGTCACAAACCCAAATCTGTCCGGATGTTAACTGAACATGTCAAAACCTGTGGAGAC	480 480 474 468
shannyQAE gunnelQAE gunnelSP shannySP	TATGATGAGATTTGATGTTCTGAAAAGATAAAGCCTATGAATAAAGTTTTGCCCAAAAAA TATGTTGAGATTTGATGGTCTGAAAAGATAAAGCATATAAATAA	540 540 534 528
shannyQAE gunnelQAE gunnelSP shannySP	AAAAAAAAA 550 AAAAAAAAA 550 AAAAAAAA 542 AAAAAAAAAAAAAA 541	

Figure 4-6. Multiple sequence alignment of AFP cDNA sequences from various members of Zoarcidae. The nucleotide alignment was constructed using ClustalW

(*http://www.ebi.ac.uk/Tools/msa/clustalw2/*). Residues conserved throughout all sequences indicated by * below. Residues differing from the majority are highlighted grey. Start and stop codons are underlined and in bold. Sequences terminate at poly (A) signal. OP5A, Ocean pout (*Macrozoarces americanus*) (Hew *et al.* 1988); AW, Atlantic wolffish (*Anarhichas lupus*) (Scott *et al.* 1988); OP3A, Ocean pout (*Macrozoarces americanus*) (Hew *et al.* 1988); AE, Antarctic eelpout (*Lycodichthys dearborni*) (Cheng *et al.* 2006)

Shanny (QAE)	-AGAAGTCTCAGCTACAGCCTTCACTTCATTCTCCGATAATTAATTAATTAT	51
Gunnel (QAE)	-AGAAGTCTCAGCTACAGCTTTCACTTCATTCTCCGCTAATTAATTAATTAT	51
Shanny (SP)	-AGAAGTCTCAGCTACAGCTTTCACTTCATTCTCCGCTAATTAATTAATTAT	51
Gunnel (SP)	-AGAAGTCTCAGCTACAGCTTTCACTTCATTCTCCGCTAATTAATTAATTAT	51
OP5A (SP)	-AGAAGTCTCAGCTACAGCTTTCACTTCGATCTCCGATAATTAAT	59
AW (SP)		51
OP3A (OAE)	- <u>Δ</u> GA Δ <u>G</u> TCTCAGCTACAGCTTTCACCTTCCCCG	51
$\Delta E (\Delta E)$		50
AL (QAL)	* * ** * ***** ******* ******* ********	50
Chapper (OAE)		105
Cuppel (OAE)		105
Champie (CD)		105
Shanny (SP)		105
Gunnel (SP)		105
OP5A (SP)	TAATTAATTAAGTCTCAGCCACAGCC ATG AAGTCCGTTATTTTAACCGGTTTGC	113
AW (SP)	TAATTAATTAAGTCTCAGCCACAGCC ATG AAGTCAGCTATTTTTAACCGGTTTTGC	105
OP3A (QAE)	TAATTAATTAAGTCTCAGCCACAGCC ATG AAGTCAGTCATTTTAACTGGTTTGC	105
AE (QAE)	TAATTAATTAAGTCTCAGCCACAGCCACAGCCACAGCCATGAAGTCAGTTGTTTTAACTGGTTTGC	110

Shanny (QAE)	TCTTCGTCCTCCTTTGTGTCGACAACATGAGTTCAGCCGCCTCGGGTCAGTCCGTGGTGG	165
Gunnel (QAE)	TTTTCGTCCTCCTTTGTGTCGACAACATGACTTCAGCCGGCTCGGGTAAATCCGTGGTGG	165
Shanny (SP)	TTTTCGTCCTCCTTTGTGTCAACATGAGTTCAGCCG <mark>C</mark> CCAGTCCGTGGTGG	156
Gunnel (SP)	TTTTCGTCCTCCTTTGTGTCGACAACATGACTTCAGCCGGCTCGTCCGTGGTGG	159
OP5A (SP)	TTTTCGTCCTCCTTTGTGTCGACCACATGACAGCCAGCCAGTCCGTGGTGG	164
AW (SP)	TTTTCGTCCTCCTTTGTGTCGACCACCTGAGTTCAGCCAGC	159
OP3A (OAE)	TTTTCGTCCTCCTTTGTGTCGACCACATGAGTTCAGCCAGC	159
AE (OAE)	TGTTCGTCCTCCTTTGTGTCGACCACATGAGTTCAGCCAACAAGGCGTCCGTGGTGG	167
(= /	* *************************************	
Shanny (OAE)	ССААССАССТСАТССАТСААТАСТССССТСАСТСАСТСА	225
Cuppel (OAE)		225
Shappy (SD)		225
Currel (CD)		210
		219
OPSA (SP)		224
AW (SP)		219
OP3A (QAE)	CCACCCAGCTGATCCCCCATAAATACTGCCCTGACTCTGGTGATGATGACGACACGGGTTA	219
AE (QAE)	CCAACCAGCTGATCCCCCATAAATACTGCCCTGACTCTGATAATGATGAAGGCGGAGGTGG	227
	*** ***********************************	
Shanny (QAE)	TCTCCCCATTGGGCATCCCCGCCGAGGACATTCCCCCGACTAGTCAGTC	285
Gunnel (QAE)	TCGCCCCAATGGGCATCCCCGCCGAGGACATTCCCCCGACTAGTCAGTC	285
Shanny (SP)	TCAACCCAAGCGGCATCCCGTTCACGGAGAAGTCCACACTAGTGGGAAAGCAAGTGAACC	276
Gunnel (SP)	TCAGCCCAGCAGGCATCCCGTTCGCAGAGATGTCCAGAATAGTGGGAAAGCAAGTGAACC	279
OP5A (SP)	CCAACCCAATAGGCATCCCGTTCGCAGAGATGTCCCAAATAGTGGGGGAAGCAAGTGAACA	284
AW (SP)	TCAACCCAGCGGGCATCCCGTTCGCGGAGATGTCCCAAATCGTGGGAAAGCAAGTGAACA	279
OP3A (QAE)	TCTACCCAACGGGCATCCCCGCCGAGGACATTCCCCCGATTAGTCTCAATGCAAGTGAACC	279
AE (QAE)	TCACCCCAATGGGCATCCCCGCCGAGGACATTCCCCGAATAATCGGAATGCAAGTGAACA	287
	* **** ******* * ** * ** * * * * * * * *	
Shanny (OAE)	GGGCAGTGCCGCTGGGCACAACCCTCACGGCAGAGATGGTGAAAGGGTACAGCCCGG	342
Quild,		
Gunnel (QAE)	GGGCAGTGCCGATGGGCACAACCCTCATGCCAGACATGGTGAAAACGTACCAACCA	342
Gunnel (QAE) Shanny (SP)	GGGCAGTGCCGATGGGCACAACCCTCATGCCAGACATGGTGAAAACGTACCAACCA	342 330
Gunnel (QAE) Shanny (SP) Gunnel (SP)	GGGCAGTGCCGATGGGCACAACCCTCATGCCAGACATGGTGAAAACGTACCAACCA	342 330 336
Gunnel (QAE) Shanny (SP) Gunnel (SP) OP5A (SP)	GGGCAGTGCCGATGGGCACAACCCTCATGCCAGACATGGTGAAAACGTACCAACCA	342 330 336 341
Gunnel (QAE) Shanny (SP) Gunnel (SP) OP5A (SP) AW (SP)	GGGCAGTGCCGATGGGCACAACCCTCATGCCAGACATGGTGAAAACGTACCAACCA	342 330 336 341 336
Gunnel (QAE) Shanny (SP) Gunnel (SP) OP5A (SP) AW (SP) OP3A (OAE)	GGGCAGTGCCGATGGGCACAACCCTCATGCCAGACATGGTGAAAACGTACCAACCA	342 330 336 341 336 339
Gunnel (QAE) Shanny (SP) Gunnel (SP) OP5A (SP) AW (SP) OP3A (QAE) AE (OAE)	GGGCAGTGCCGATGGGCACAACCCTCATGCCAGACATGGTGAAAACGTACCAACCA	342 330 336 341 336 339 338

Shanny (QAE)	CTAAG TAG TTCTGAGGGTGCCAAGGAGTTTCTTCCCAAAACCAACAGAAGAAATGCCCCC402
Gunnel (OAE)	CGAAGTAATTCTGAGGGTGCCAAGGAGTTTCTTCCCAAAACCAGAAGAAGAAATGCCCCC402
Shanny (SP)	AGAAGTTGTTTTGAGGGTGCCAAGGAGTTTCTTCCCAAAACCAAAAGAAGAAGAATGTCCCC390
Gunnel (SP)	CGAAG TAG TTCTGAGGGTGCCAAGGAGTTTCTTCCCAAAACCAGAAGAAGAAATGCCCCC396
OP5A (SP)	GAAAG TAG TTCTGAGGGTGCCAAGGAGCTTCTTCCCAAAACCAAAAGAAGAAGAATGCCCCC401
AW (SP)	CAAAG TAG TTCTGAGGGTGCCAAGGAGTTTCTACCCAAAACCAAAAGAAGAAGAAATGCCCCC396
OP3A (OAE)	CGCCGAAGAAC TGA AGGTGCCAAGGAGTTTCTTCCCCAAAACCAAAAGAAGAAGAAGAAGCCTCC 399
AE (OAE)	AGAAG TAG TTCTGAGCGTGCCAAGGAGTTTCTTCCCAAAACCAAAAGAAAG
(<u>g</u>)	* *** *********************************
Shanny (OAE)	TCTCACAATTAACCTTGTTTTTGTCACAAACCCAAGTCTGTCCGGATGTTAACTGAACAT 462
Gunnel (OAE)	TCTCACAATCAACCTTGTTTTTGTCAGAAACCCAAGTCTGTCCGGATGTTAACTGAACAT 462
Shanny (SP)	TCTCACAATTAATCTTGTTTTTGTCACAAACCCAAATCTGTCCGGATGTTAACTGAACAT 450
Gunnel (SP)	TCTCACAATCAACCTTGTTTTTGTCAGAAACCCAAGTCTGCCCGGATGTTAACTGAACAT 456
OP5A (SP)	TCTCACAATTAACCCTGTTTTTGTCACAAACCCAAGTCTGTTAACTGAACAT 453
AW (SP)	TCTCACAATTAACCTTGTTTTTGTCACAAACCCAAGTCTGTCCGGATGTTAACTGAACAT 456
OP3A (OAE)	TCTCACAATTAACCTTGTTTTTGTCACAAACCCAAGTCTGTCCGTATGTTAACTGAACAT 459
AE (OAE)	TCTCACAATTAACCTTGTTTTTGTCACAAACCCAAGTCTGTCCGTATGTCAACTGAACAT 458
	******* ** * **************************
Shanny (OAE)	GTCAAAACCTGTGGAGACTATGATGAGATTTGATGTTCTGAAAAGATAAAGCCTATGAAT 522
Gunnel (OAE)	GTCAAAACCTGTGGAGACTATGTTGAGATTTGATGGTCTGAAAAGATAAAGCATATAAAT 522
Shanny (SP)	GTCAAAACCTGTGGAGACTATGTTGAGATTTGAGATGTTCTGAAAAGATAAAGCCTATAAAT 510
Gunnel (SP)	GTCAAAACCTGTGGAGACTATGTTGAGATTTGATGGTCTGAAAAGATAAAGCATATAAAT 516
OP5A (SP)	GTCAAAACCTGTGGAGACT-GTTGAGATTTGATGTTCTGAAAAGATAAAGCCTATAAAAT 512
AW (SP)	GTCAAAACCTGTGGAGACTATGTTGAGATTTGATGTTCTGAAAAGATAAAGCATATCAAT 516
OP3A (OAE)	GTCAAAACCTGTGGAGACTATGTTGAGATTTGAGATGTTCTGAAAAGATAAATCATTTAAAT 519
AE (OAE)	GTCAAAACCTGTGGAGACTGTTGAGATTTGATGTTCTGAAAAGATAAAGCCTATAT 514
(<u>g</u>)	***************************************
Shanny (OAE)	AAA 525
Gunnel (OAE)	AAA 525
Shanny (SP)	AAA 513
Gunnel (SP)	AAA 519
OP5A (SP)	AAA 515
AW (SP)	AAA 519
OP3A (OAE)	AAA 522
AE (OAE)	AAA 517
\ X++ /	***

Figure 4-7. Dot matrix comparisons of selected cDNA sequences encoding the type III AFPs. Actual sequences are presented in Fig 4-6. A line indicates a match of 10 out of 13 bases. Matricies generated at *http://www.vivo.colostate.edu/molkit/dnadot*



Figure 4-8. Multiple sequence alignment of known type III AFP sequences based on primary structural characteristics. The amino acid alignment was constructed using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Residues conserved throughout all sequences are indicated by * below. Residues differing from the majority are highlighted grey. Putative icebinding residues are highlighted in blue (pyramidal plane binding) and green (primary prism plane binding) according to Garnham et al. (2010). A) Alignment of QAE-type III isoforms. Radiated shanny (U. subbifurcata) and rock gunnel (P. gunnellus) QAE amino acid sequences obtained from in silico translation of QAE cDNA sequences obtained from the current study. AWE2, Atlantic wolffish (Anarhichas lupus) (Desjardins et al. 2012); OP HPLC 12, Ocean pout (*M. americanus*) (Hew *et al.* 1988); nfeAFP10, Notched-fin eel pout (*Zoarces elongates*) (Nishimiya et al. 2005). B) Alignment of SP-type III isoforms. Radiated shanny (U. subbifurcata) and rock gunnel (P. gunnellus) SP amino acid sequences obtained from in silico translation of SP cDNA sequences obtained from the current study. AWE3, Atlantic wolffish (A. lupus) (Desjardins et al. 2012); OP HPLC 9, Ocean pout (M. americanus) (Hew et al. 1988); nfeAFP6, notched-fin eel pout (Z. elongates) (Nishimiya et al. 2005)

A	Shanny QAE: Gunnel QAE: AWE2: OP HPLC 12: nfeAFP10:	ASGQ-SVVAN GSGK-SVVAN NQASVVAT NQASVVAN NQASVVAT ****	QLIPINTAL QLIPINTAL QLIPINTAL QLIPINTAL QLIPINTAL ********	I LVMMRAEVVS I RIMMKAELVA I LVMMTTAVVT I LVMMRSEVVT I LVMMKAKVAT * **	PLGIPAEDIP PMGIPAEDIP PTGIPAEDIP PVGIPAEDIP PMGIPAEDIP * ******	RLVSLQVNRA 49 RLVSLQVNRA 49 RLVSMQVNRA 48 RLVSMQVNRA 48 RIVSMQVNRA 48 * ** *** *
	Shanny QAE: Gunnel QAE: AWE2: OP HPLC 12: nfeAFP10:	VPLGTTLT VPMGTTLM VPMGTTLM VPLGTTLM VACGTTLM * ****	AE MVKGYSI PD MVKTYQI PD MVKFYCI PD MVKGYPI PG MVKTYTI *** *	PAK 68 PAK 68 JCAP 68 PA 66 PVK 67		
В	Shanny SP: Gunnel SP: AWE3: OP HPLC 9: nfeAFP6:	AQSVVATQLI GSSVVASQLI SQSVVATQLI SQSVVATQLI GESVVATQLI **** ***	PINTALTPAM PMNTALTPAM PINTALTPIM PMNTALTPAM PINTALTPAM * ******	MKGMDVNPSG MKGVVVSPAG MKGQVVNPAG MEGKVTNPIG MEGKVTNPSG * * * * *	IPFTEKSTLV IPFAEMSRIV IPFAEMSQIV IPFAEMSQIV IPFAEMSQIV *** * * * **	SKQVNQPVVK 50 SKQVNQIVAK 50 SKQVNRPVAK 50 SKQVNRIVAK 50 SKQVNTPVAK 50 ***** * *
	Shanny SP: Gunnel SP: AWE3: OP HPLC 9: nfeAFP6:	GQTLMRNMVK DQTLMPSMVK GQTLMPNMVK GQTLMPGMVK *** ***	P 61 IYQPAK 66 IYRAAK 66 IYAA 64 IYVPAK 66			

4.3.3 Expression of type III AFP genes in radiated shanny and rock gunnel

AFP mRNA expression analyses using northern blotting procedures were carried out on two individuals. The results obtained for both individuals of both species were slightly different and are displayed separately (Fig 4-9 and 4-10).

Northern blot analyses of rock gunnel type III AFP gene expression revealed the presence of very high levels of AFP mRNA in liver, high levels in skin and gill tissue and very low levels in intestine, muscle, and heart of both individuals. The primary difference between the two individuals was observed in the stomach where moderate to high levels of AFP mRNA were observed in one individual (Fig 4-9a) while very low levels were observed in another (Fig 4-9b). Finally, low levels of AFP mRNA were observed in spleen from a single individual (Fig 4-9b). No AFP mRNA was detected in the negative control cunner skin mRNA. A single hybridization signal was observed in all positive tissues [~600 nucleotides (nt)], which corresponds well with the RACE determined cDNA sequences (Fig 4-1 to 4-4) plus a typical poly (A) tail (Brawerman 1981, Schiavi *et al.* 1994, Wahle 1995).

Northern blot analyses of radiated shanny type III AFP expression were slightly more variable compared to those of the rock gunnel. In one individual, high AFP mRNA levels were observed in liver, moderate levels in skin and stomach, low levels in gill and very low to undetectable levels in intestine, muscle and kidney (Fig 4-10a). In the second individual, very high AFP mRNA levels were observed in liver, high levels in skin, gill and stomach, moderate levels in muscle, low levels in heart (single sample only, Fig 4-10b) and very low to undetectable levels in intestine and kidney.

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Figure 4-9. Northern blot analysis of rock gunnel (*P. gunnellus*) total RNA from various tissues as seen on a denaturing agarose gel. Total RNA ($2 \mu g$) from two individuals (A and B) was separated on a formaldehyde agarose gel prior to blotting onto a positively charged nylon membrane. Top panel probed with a type III AFP RNA probe (see section 4.2.4.1.1). Middle panel was probed with a chicken tubulin RNA probe (internal control). Bottom panel is RNA following electrophoresis viewed under UV light. Cunner skin RNA (cSK) was used as a negative control. Abbreviations used: nt; nucleotides, S; sedimentation factor, L; liver, Sk; skin, G; gill, I; intestine, St; stomach, M; muscle, H; heart, Sp; spleen





Figure 4-10. Northern blot analysis of radiated shanny (*U. subbifurcata*) total RNA from various tissues as seen on a denaturing agarose gel. Total RNA (2 μg) from two individuals (A and B) was separated on a formaldehyde agarose gel prior to blotting onto a positively charged nylon membrane. Top panel was probed with a type III AFP RNA probe (see section 4.2.4.1.1). Middle panel was probed with a chicken tubulin RNA probe (internal control). Bottom panel is RNA following electrophoresis viewed under UV light. Cunner skin RNA (cSK) was used as a negative control. Abbreviations used: nt; nucleotides, S; sedimentation factor, L; liver, Sk; skin, G; gill, I; intestine, St; stomach, M; muscle, H; heart, K; kidney





4.4 Discussion

Five of the nine families of the suborder Zoarcoidei have tested positive for the presence of type III AFP genes and/or type III AFP gene expression products (Davies *et al.* 1988, Hew *et al.* 1988, Shears *et al.* 1993). These include the wrymouths (family Cryptacanthodidae), wolffishes (family Anarhichadidae), eel pouts (family Zoarcidae), gunnels (family Pholidae) and pricklebacks (family Stichaeidae), all of which inhabit freeze risk ecozones of the north Atlantic ocean. Of these five families, SP and QAE isoforms of type III AFPs have been purified and characterized from two: family Zoarcidae (Hew *et al.* 1988, Nishimiya *et al.* 2005, Shears *et al.* 1993) and family Anarhichadidae (Desjardins *et al.* 2012).

The results of this study clearly demonstrate that the rock gunnel (family Pholidae) and radiated shanny (family Stichaeidae) express both SP and QAE type III AFP isoforms as a means of freeze protection. This conclusion is based on the similarity in cDNA and deduced peptide sequence compared to those of other type III AFP producing members of the suborder Zoarcoidei. As a result, we now know that all of the families within the suborder Zoarcoidei that are known to have their AFP encoded by multigene families [Zoarcidae (Hew *et al.* 1988), Anarhichadidae (Desjardins *et al.* 2012, Scott *et al.* 1988), Pholidae and Stichaeidae (Shears *et al.* 1993)] express a mixture of both SP and QAE type III isoforms [Figs 1-4, (Desjardins *et al.* 2012, Hew *et al.* 1988)]. Alignment of radiated shanny and rock gunnel AFP peptide sequences with other Zoarcid members illustrated the similarity in primary structure, particularly within SP and QAE groups (Fig 4-8). In particular, the putative ice binding sites (Garnham *et al.* 2010) are highly conserved within QAE and SP groups in the representatives examined.

With SP isoforms the lack of thermal hysteresis (TH) is thought to be due to ineffective primary prism plane binding. The functional pyrimidal plane binding area, however, is

responsible for the SP isoforms ice shaping ability. Although the Ile to Met substitution identified at position 12 of the gunnel SP isoform does lie within this binding site, it is a conservative substitution not likely to affect protein folding and functionality (Robson and Suzuki 1976). Incidentally, this identical substitution has been observed in isoforms from several species [Fig 4-8; Desjardins *et al.* (2012)].

In QAE isoforms, two substitutions were observed in the rock gunnel AFP, both in the primary prism plane binding site. The Val to Ile substitution at position 22 is highly conservative and not likely to affect protein folding and therefore functionality (Robson and Suzuki 1976). The Leu to Arg substitution at position 21, however, is less conservative.

The overall conservation among residues within the ice binding sites is indicative of functionality of the radiated shanny and rock gunnel AFPs identified in this study.

Alignment of Zoarcid AFP cDNA sequences, including those identified from radiated shanny and rock gunnel in the current study also illustrates the remarkable similarity within this group (Fig 4-6). This is highlighted by a nearly continuous straight line in representative dot matricies (Fig 4-7). Particularly interesting is the similarity between non-coding regions of the type III AFP sequences. Although these regions often house functional domains important for post-transcriptional gene expression and thus subject to stronger selective constraints, the high degree of similarity observed cannot be explained by this alone. Within non-functional regions, random mutations would not be constrained by selection; as a result, non-homologous genes would be expected to be varied in these regions due to random mutation alone. The remarkable conservation observed in this study undoubtedly represents recent descent from a common ancestor. In addition, the identification of SP and QAE isoforms in most species of type III AFP producing fish in this suborder strongly indicates that the multigene family responsible for their

production had already evolved prior to family level divergence. The time frame for such events are not fully understood, however recent fossil evidence suggest that by ~11-12 Mya, the Pholids had already diverged from the other Zoarcid families (Nazarkin 2002). The evolution of this multigene family therefore predates this time period. Recent evidence points towards the development of northern sea ice ~ 30-45 Mya (Deconto *et al.* 2008, Stickley *et al.* 2009). The presence of homologous AFP in Zoarcid families point toward divergence of these families while encountering freeze risk ecozones during this time.

Tissue specific mRNA expression patterns observed in this study are similar to those seen in other Zoarcid family members including ocean pout (Hew *et al.* 1988, Hobbs and Fletcher 2008), and Atlantic and spotted wolffish (Desjardins *et al.* 2012). In all of these studies, expression of type III AFP mRNA has been observed in multiple body tissues, with high levels noted in the liver. This is consistent with the liver typically functioning as the main secretory organ for plasma proteins in fish (Fletcher *et al.* 2001).

It has recently been demonstrated that although SP type III AFPs do indeed have affinity to ice, and can impart hexagonal shaping when bound to ice crystals, they cannot prevent ice crystal growth due to structural differences in the ice binding site corresponding to the primary prism plane of ice (Garnham *et al.* 2010). It has even been suggested that these SP AFPs should be reclassified as "ice growth modifiers" (Harding *et al.* 1999, Takamichi *et al.* 2009). The limitation of SP isoforms, however, is overcome by the addition of minor quantities of a "fully active" QAE isoform (Nishimiya *et al.* 2005, Takamichi *et al.* 2009) indicating that cooperation between SP and QAE isoforms is a requirement for freeze protection in eelpouts and wolffishes. Specifically, it has been suggested that binding of the "active" QAE isoform is initially required in order to slow the growth speed of seed ice thereby allowing the adsorption of "less active,"

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more abundant SP isoform and ultimate halting of ice growth (Nishimiya *et al.* 2005). Support for this theory comes from a recent report where an SP/QAE mixture containing only 0.001 mM QAE produced a higher TH compared to a 0.05 mM QAE alone (Takamichi *et al.* 2009). Similar instances of isoform cooperation have been reported (Osuga *et al.* 1978, Schrag and DeVries 1983, Wang and Duman 2005).

The identification of SP and QAE isoforms in radiated shanny and rock gunnel, along with the observation that these are undoubtedly homologous with previously identified type III AFPs found in the Zoarcoidei, provide strong support for the hypothesis that cooperation between SP and QAE isoforms as a freeze protection strategy evolved prior to the divergence of the suborder Zoarcoidei into at least four families: Zoarcoidae, Anarhichadidae, Pholidae, and Stichaeidae.

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Chapter 5: Summary

5.1 Review of thesis objectives

The primary objective of this work was to examine AFPs and their genes from specific members of the family Labridae and suborder Zoarcidae within the Order Perciformes. Two specific questions were addressed in order to meet this objective:

1. Is cunner type I AFP similar to those of right-eye flounders, sculpins or snailfish?

2. Did cooperativity between SP and QAE type III AFP isoforms develop prior to the divergence of Zoarcid families?

To answer the first question, my goal was to isolate, purify and fully characterize an AFP from cunner using standard biochemical techniques. This preliminary information would then be used to identify and examine the nucleotide and amino acid sequences corresponding to the AFP by utilizing molecular cloning techniques. It was my intention to use the information obtained to elaborate and help clarify the function and evolution of type I AFPs within these groups.

To answer the second question, my intent was to identify SP and QAE type III AFP cDNA sequences from members of two unanalyzed families within the suborder Zoarcoidei using molecular cloning techniques. This information would then be compared with corresponding sequences from other Zoarcoidei family members to elucidate the evolutionary relationship.

This thesis has described my success at answering both of these questions.

5.2 Isolation and characterization of type I AFP in cunner

The impetus for this study came from the observation that cunner blood plasma exhibits TH during the winter, indicating the presence of AFPs. Like many AFP producing teleosts, an annual cycle of AFP production was initially identified where the highest levels of TH (in blood plasma) are observed in the winter when water temperatures are the coldest, and no TH is observed in the summer. It was interesting to observe that even at peak AFP production, whole body freeze protection could not be provided by AFP alone. As discussed in the thesis, the cunner relies on behavioural adaptations in conjunction with AFP production to minimize the risk of freezing.

In order to characterize the AFP responsible for the TH observed in cunner blood, the protein first needed to be isolated from the fish. I was successful in utilizing chromatographic techniques (size exclusion and HPLC) to isolate and purify an AFP from cunner blood plasma. Confirmation of type I classification was obtained through CD and amino acid analyses which identified high alanine content and α -helicity characteristic of type I AFPs. The activity of the purified AFP, as well as the structure of ice crystals formed in its presence was similar to that of type I AFPs isolated from various flounder species. MS analysis and partial MS/MS sequencing completed characterization by providing the peptide mass and partial amino acid sequence. In addition, protein sequencing identified repetitive elements in the AFP previously identified as characteristic of type I AFPs.

5.3 Expression pattern of type I AFP in cunner

The identification of the complete cDNA responsible for AFP production resulted from a combination of *de novo* sequencing of a mixed liver/skin cDNA library and RACE/PCR techniques. This sequence computationally translated a protein identical to that obtained from MS/MS sequencing of the purified AFP, with the addition of complete 5' and 3' ends (including UTRs), thereby validating the initial result.

Once the cDNA coding for the cunner AFP was available my intent was to examine gene expression patterns (tissue-specific and seasonal) as well as genomic organization using

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molecular techniques. Results from a seasonal northern analysis confirmed the previously identified seasonal cycle of AFP production. Tissue specific northern and RT-PCR analyses, however, identified a lack of expression in liver; the typical source for blood bound AFP production in teleosts. Instead, significant AFP mRNA was observed in epithelial tissues, namely skin and gill, expressed from what appears to be a multigene AFP family. This, in conjunction with the fact that the protein is produced without an obvious signal sequence, resulted in our "skin-type AFP" classification of the cunner AFP. To our knowledge, the cunner is the only overwintering fish off the coast of Newfoundland that relies solely on AFP expression in the skin without the assistance of high AFP levels in the plasma. As a result of these findings, in addition to the cunner's geographic origin, we reason that the cunner has not yet had the time needed to evolve the live-type AFP genes relied upon by most teleost fish for freeze protection and therefore represent an intermediate step in the evolution of freeze resistant fish.

5.4 Identification of SP and QAE genes in Pholidae and Stichaeidae

It was previously known that eelpouts (family Zoarcidae) and wolffishes (family Anarhichadidae) rely on the cooperation between SP and QAE type III AFP isoforms to protect them from freezing during the winter. We also knew that all of the other families from the suborder Zaorcoidei produce type III AFPs. However, because of a lack of data, it was unknown whether the SP/QAE cooperativity found in the eelpouts and wolffishes was derived within these groups or if it evolved earlier during the speciation of Zoarcoidei families from a common ancestor. This research aimed to answer this question by isolating (if present) SP and QAE genes from radiated shannies and rock gunnels and comparing their nucleotide sequences with those of the eelpouts and wolffishes. SP and QAE type III AFP genes were successfully isolated from radiated shannies and rock gunnels using a combination of RACE and RT-PCR with PCR primers specific to distinct regions of known SP or QAE genes. Sequence analyses of the isolated genes as well as their deduced peptides illustrated high sequence identity indicative of descent from an ancestor common to the Zoarcoidei families.

Type III AFP gene expression patterns observed from radiated shanny and rock gunnel tissue mRNAs were similar to those of other type III AFP producing fish with nearly ubiquitous expression across tissues, and the highest levels observed in liver.