MOLECULAR AND SEASONAL BASIS FOR FREEZE RESISTANCE IN WOLFFISH SPECIES (*Anarhichas lupus*, *A. minor*, AND THEIR HYBRIDS)

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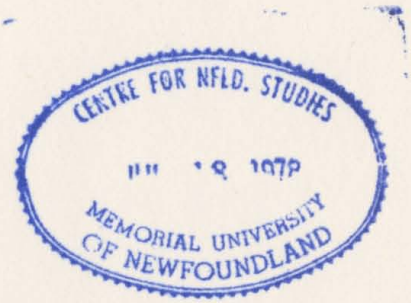
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MOLECULAR AND SEASONAL BASIS FOR FREEZE RESISTANCE IN WOLFFISH SPECIES (*Anarhichas lupus*, *A. minor*, and their hybrids)

By

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– ABSTRACT –

During winter, the coastal waters of Newfoundland can be considered a "freeze risk ecozone" for teleost fishes. The benthic Atlantic (*Anarhichas lupus* – AW) and spotted wolffish (*A. minor* – SW) reside at opposite ends of this ecozone, with the AW facing the greatest risk because of its shallower niche. To resist freezing, AW secretes five times the level of plasma antifreeze protein (AFP) activity than does SW.

The main basis for this difference in plasma AFP levels is gene dosage, as AW has approximately three times more AFP genes than SW. Perhaps as a result, AFP transcript levels in liver (the primary source of circulating AFPs) are several times higher in AW. One explanation for these gene and transcript dosage differences is the presence of tandemly arrayed AFP gene repeats in AW that make up two-thirds of its AFP gene pool. Such repeats are not present in SW. AW and SW diverged from a common ancestor at a time when the ebb and flow of northern glaciations would have resulted in the emergence of "freeze risk ecozones". The duplication/amplification of AFP genes in a subpopulation of ancestral wolffish would have facilitated the exploitation of this high risk habitat, resulting in the divergence and evolution of modern day AW and SW species.

Investigations on artificially produced AW/SW hybrids showed that all the AFP genes of SW are likely shared with AW, which supports recent gene amplification as an impetus for speciation. The high dynamism of the AW AFP locus (through high variability in dosage and organisation) was best visualized in the single haplotype

inherited by the hybrids. Their intermediate levels of plasma AFPs make these fish unfit for survival within the shallowest part of the "freeze risk ecozone".

While the total levels of AFP gene transcripts varied little from winter to summer in both species (especially in the liver), differential expression of members of the two subfamilies of type III AFPs, the SP- and QAE-type genes (appellation based on the ion-exchange Sephadex resins to which each type of isoform binds), was observed. While the expression of the QAE-type transcripts was ubiquitous (both temporally and spatially), that of the SP-type genes seemed to show some level of tissue specificity, which highlights the complex evolutionary history of these genes.

Co-authorship Statement

The author of the present thesis has made a major intellectual and practical contribution to all work reported therein, and is the principal author of the three research papers submitted therein in manuscript-format (Chapter 2 has been published in FEBS Journal; PubMed ID 22520964). The present author has been the main contributor in 1) the design and identification of the research proposal, 2) the practical aspects of the research, 3) the data analysis, and 4) the manuscript preparation.

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First, I would never have progressed this far without my family and husband's unconditional love and support. To you, I dedicate this thesis. **Merci d'avoir toujours été là pour moi, je vous aime!**

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Finally, a last thanks goes to the St. John's born and raised band "Hey Rosetta!", whose profound lyrics and great music accompanied my stay on the Rock (and a lot of my lab work).

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– LIST OF ABBREVIATIONS AND SYMBOLS –

aa, amino acid
AEP, Antarctic eelpout
AEBP, antifreeze enhancer-binding protein
AFGP, antifreeze glycoprotein
AF(G)P, antifreeze proteins & glycoproteins
AFP, antifreeze protein
AW, Atlantic wolffish
cDNA, complementary DNA
C/EBPα, CCAAT/enhancer binding protein
DIG, digoxigenin
DOW, deep ocean water
EAC, escape from adaptive conflict
ESU, evolutionary significant unit
F1, first generation
FH, freezing hysteresis
FP, freezing point
GDA, gene duplication/amplification
GH, growth hormone
GLM, general linear model
HC, heat capacity
IGF-1, insulin-like growth factor
INP, ice-nucleating proteins
IBS, ice-binding site
ka, kiloannum
LGT, lateral gene transfer
Ma, megaannum
MH, melting hysteresis
mRNA, messenger RNA
NaCl, sodium chloride
OP, ocean pout
QAE, quaternary aminoethyl
RE, restriction endonuclease
RFLP, restriction fragment length polymorphism
SAS, sialic acid synthase
SD, standard deviation
SHC, specific heat capacity
SP, sulfopropyl
SW, spotted wolffish
TH, thermal hysteresis
UTP, uracil triphosphate
UTR, untranslated region
VCH, volumetric heat capacity
wflAFP, winter flounder liver AFP
wfsAFP, winter flounder skin AFP
WGD, whole genome duplication

– CHAPTER 1 –

1.1 – Background of study

The challenge of subzero temperatures

Water exists in its aqueous form between 0°C and 100°C (at sea level). Since the appearance of life in the primal ocean, Earth has offered a climate where this temperature window could be satisfied, and evolution has worked around water as the universal solvent for the chemistry of life. However, important cooling events have punctuated Earth's climatic history. The onset of the most recent of these events, during the Cenozoic Era, brought forth the Antarctic glaciation at the Eocene-Oligocene boundary, ~ 34 Ma ago (Zachos *et al.*, 2001, 2008; DeConto *et al.*, 2008). The Earth then passed from a "Hot House" to an "Ice House" state that became firmly established by the mid-Miocene and still persists today (Katz *et al.*, 2008). The transition occurred quickly in geological time (Coxall *et al.*, 2005), and life at sea level found itself exposed to the threat of freezing for the first time after 200 Ma of relatively warm climate (Fletcher *et al.*, 2007; Retallack, 2009). Clades in all kingdoms encountered some level of extinction not only at the threshold of the Ice House, but also during the cooling preceding the glaciation (reviewed by Prothero, 1994; Thatje *et al.*, 2005). Indeed, lower temperatures lower the rate of biochemical processes, and total and uncontrolled freezing of an organism renders all water inaccessible to the cell's biochemistry. Moreover, because water expands while freezing, its solidification disrupts the tissues and mechanically damages the cell membranes, which results in cell leakage – and death – upon thawing. The establishment

of an Ice House on Earth thus could easily have been a death sentence for Life itself. Life, however, persisted and adapted.

Adaptations developed by homeothermic endotherms

First of all, freezing could be inherently avoided by those animals – birds and mammals – that had evolved the ability to metabolically generate and maintain high body temperatures independent of their thermal environment. Endothermy – which emerged independently in these two lineages during the Cretaceous, and possibly earlier in mammals (Ruben, 1995; Ruben & Jones, 2000; Hillenius & Ruben, 2004; Pörtner, 2004; Lovegrove, 2012) – was a convenient pre-requisite to a cooling climate: freezing could be avoided by metabolically keeping the body temperatures above the freezing point (FP) of the body fluids. As the global temperature progressively dropped during the Eocene, birds and mammals took over the niches left vacant by the retreating ectotherms, and eventually became the dominant terrestrial vertebrate clades in high latitude environments.

While endothermy allows animals to stay active in the cold, it is a highly costly adaptation, and the energy invested in keeping the body at a constant warm temperature in the face of heavy heat loss to the environment (homeothermy) must be maintained through constant food availability. Seasonal migration to warmer locations thus became a key to survival for those animals that cannot access enough resources during winter. The best known examples of animals using this strategy are found among birds, in which migratory behaviour is known to have evolved independently several times (Pullido,

2007), and in some instances, likely in response to climate cooling (see review by Salewski & Bruderer, 2007). In contrast, animals that are permanent residents of cold latitudes evolved resistance strategies such as better insulation against heat loss, rete mirabile to warm the blood going from the limbs to the heart, tissue eurythermy, relatively compact body shape, and higher metabolism (Scholander, 1955; Lovegrove, 2001; Pörtner, 2004). Among these animals, some actively forage while maintaining high metabolism and body temperatures, and rely on efficient body insulation while resting or sleeping. On the other hand, some birds will enter a torpid, hypothermic-hypometabolic state during the cold winter nights, and resume normal activity during the day, thus saving precious energy (Schleucher, 2004). This ability to switch from a homeothermic state to a more poikilothermic-like state on a daily basis is defined as heterothermy.

Some other animals take controlled hypothermia and hypometabolism a step further, by entering hibernation. This fascinating trait is thought to have evolved from the plesiomorphic ability of ancestral mammals to perform daily heterothermy (reviewed by Grigg *et al.*, 2004). In mammals, heterothermy likely led to the evolution of endothermy (Pörtner, 2004; Lovegrove, 2012), and is essentially lost in non-hibernating mammals. As winter closes in, hibernators accumulate food and/or fat reserves, select microenvironments (hibernacula) where the temperature will stay around or above 0°C, and allow their metabolism and body temperatures to lower significantly, in a controlled fashion. For instance, the core temperature of a hibernating Arctic ground squirrel will fall as low as -2 °C, while the ground temperature varies between -10 and -15 °C (Boyer & Barnes, 1999).

On another note – and interestingly – facultative endothermy exists in some plants and insects, while regional endothermy has been reported in reptiles and fish (Heinrich, 1974; McNab, 1983; Minorsky, 2003; Dickson & Graham, 2004). However, endothermy is not considered a tool against freezing in these organisms.

Adaptations developed by ectotherms

For those life forms that did not evolve endothermy, migration and hibernation are strategies in use. Many species of insects and fish will retreat to warmer regions at the approach of winter (Oberhauser & Peterson, 2003; Goddard & Fletcher, 2002). Others will enter dormancy in a hibernaculum where they are in no danger of freezing. This latter line of action is followed by some insects, including the mosquitoes (Becker *et al.*, 2010). Moreover, various amphibians and reptiles will bury in the soft sediment at the bottom of ice-covered ponds, lower their metabolism, and survive in anoxia in this thermally buffered environment (Boutilier *et al.*, 1997; Jackson, 2002). A shallow water marine fish, the cunner (*Tautogolabrus adspersus*), will hide among rocks or in crevices on the seabed, enter a torpid state, and avoid contact with suspended ice crystals while tolerating seawater temperatures near – 2 °C (Green & Farwell, 1971). All these animals, as do the endotherms, practice what is collectively called freeze avoidance strategies. For organisms that cannot select an environment or hibernaculum of stable, non-freezing temperature, two options remain: to tolerate or resist freezing.

Freezing tolerance is found among numerous life forms, including microbes, plants, invertebrates, amphibians, and reptiles (Kukal *et al.*, 1988; Storey & Storey, 1996;

Loomis & Zinser, 2001; Voituron *et al.*, 2002; Walker *et al.*, 2006). A fascinating and widely studied example of such freeze tolerance is that of the wood frog (*Rana silvatica*), which overwinters underneath the snow-covered leaf litter, and allows itself to freeze solid if temperatures fall below its equilibrium FP (reviewed by Storey & Storey, 2004). The freezing will initiate through skin contact with ice (Layne *et al.*, 1995), and will first spread within fluid spaces (*e.g.* the abdominal cavity, eye lens), then through the vascular spaces of the organs, drawing water out of the cells. Here, extracellular initiation of freezing is crucial. Indeed, as the highly organised intracellular environment is mechanically disrupted by the expanding ice, intracellular freezing leads to death in most cases (Sinclair & Renault, 2010). To avoid uncontrolled and harmful ice growth within its body, the wood frog utilizes ice-nucleating proteins (INPs). These INPs are of two sources: some are secreted in the frog's plasma (Wolanczyk *et al.*, 1990; Storey *et al.*, 1992), while others originate from INP-producing bacteria colonising the skin and gut of the animal (Lee *et al.*, 1995). By lowering the amount of undercooling of its body fluids, the INPs grant the wood frog a controlled and progressive freezing. In the absence of ice nucleators (small ice crystals or impurities), undercooling occurs when the temperature of a given fluid is lowered below its FP. An undercooled fluid will ultimately freeze sponteanously several degrees below its FP, at a point called the undercooling point.

INPs were shown to reduce the amount of undercooling, promoting freezing nearer the FP of a given fluid. For freeze-tolerant organisms, this is of crucial importance: the higher the degree of undercooling, the faster the ice growth as nucleation initiates, and the higher the potential for damage to the cell membranes. INPs thus promote a more

controlled, gradual ice growth to occur, which lowers chances of lethal punctures or shearing to the cellular membranes. As ice slowly propagates, the frog's cells will get progressively dehydrated by osmosis. By becoming highly concentrated in salt and other osmolytes (including the cryoprotectant glucose), the inner cell environment will have a lower FP that will prevent it from freezing at the temperatures the organism endures. The animal will survive in this condition until the spring, when it thaws and resume activity. While some details of this mechanism change among species, one combination remains crucial: the presence of both cryoprotectants and ice nucleators, which together promote controlled extracellular ice growth.

Finally, a strategy shared by a staggering number of ectotherms allows survival at subzero temperatures: freeze resistance. This is accomplished by a decrease of the organismal FP below that of the surrounding environment. The most obvious advantage of this strategy is the maintenance of activity in the cold polar environments, and during winter in the temperate regions of the globe. Still, some freeze resistant species overwinter in an inactive state. The spruce budworm is one such example (reviewed by Tyshenko *et al.*, 1997). These insects spend the winter hidden in cocoons at the tip of conifer branches where they can withstand temperatures of $\leq$ -30°C. They decrease the FP of their hemolymph using different cryoprotectants, such as glycerol, which can be accumulated at high concentrations before reaching cytotoxic levels (glycerol is considered an osmotically compatible solute, as opposed to NaCl; Watanabe *et al.*, 2003), as well as unique molecules, called antifreeze proteins (AFPs) (Hew *et al.*, 1983; Qin *et al.*, 2007). AFPs inhibit ice growth by lowering the FP of their hosts to or preferentially,

past the coldest temperature they face in their environment, and can be found across five kingdoms of life (according to Cavalier-Smith, 2004), within an extensive list of freeze resistant ectothermic organisms ranging from bacteria (Gilbert *et al.*, 2004; Raymond *et al.*, 2008), fungi (Hoshino *et al.*, 2003) and microalgae (Janech *et al.*, 2006), to vascular plants (Griffith & Yaish, 2004), nematodes (Wharton *et al.*, 2005), Collembola (Graham & Davies, 2005), arachnids (Duman *et al.*, 2004), insects (see above), crustaceans (Kiko, 2010), and fishes (reviewed in Fletcher *et al.*, 2001; Goddard & Fletcher, 2002). The rich diversity of these proteins between and even within these groups suggests that AFPs evolved independently several times in response to the shared threat of freezing faced by life on Earth during the Cenozoic glaciations.

Interestingly, the synthesis of AFPs is not limited to freeze resistant organisms. For instance, a freeze tolerant insect has recently been found to produce antifreeze agents (Walters *et al.*, 2009). The role of this agent within the freeze tolerance strategy of this animal is unknown, but may prevent the recrystallization of ice during freeze-thaw episodes (see later sections). In freeze tolerant plants, the presence of weak AFPs exist as a mechanism to favor the formation of ice at relatively high temperatures, which allows for a slower more controlled ice growth (they can act as INPs), and the inhibition of recrystallization during freeze-thaw cycles (Griffith & Yaish, 2004). Finally, an extreme case of freeze-resistance was recently discovered in an Alaskan population of the red flat bark beetle (*Cucujus clavipes*) which can survive temperatures down to -100 °C (Sformo *et al.*, 2010). These insects do so in a vitrified state that is accomplished through extensive dehydration and elevation of glycerol and AFP concentrations. Here, the AFPs

not only lower the FP of these insects, but also mask potential ice nucleators. When the hemolymph of the beetle reaches the supercooling point (~ -40 °C), the animal freezes instantly. In some cases, as the supercooling point dropped below 58 °C (which can be encountered in the insect's harsh winter environment), the occurrence of vitrification was demonstrated through the use of differential scanning calorimetry (Sformo *et al.*, 2010). In this instance, no ice crystals formed: the water instead adopted a glass-like state (amorphous ice) without the expansion characteristic of normal ice formation. Mechanical cell damage was thus avoided.

AFPs and INPs are part of a functional group of proteins recently grouped under the general appellation "ice-binding proteins" (IBP) (Janech *et al.*, 2006). As their name indicates, all IBPs share a high affinity to the molecular structure of water in its solid phase, to which they bind. Today, the AFPs are the most widely studied of the IBPs, and were the first to be discovered by DeVries & Wohlschlag (1969) in the plasma of a freeze-resistant Antarctic teleost fish, the bald rockcod (*Trematomus/Pagothenia borchgrevinki*, family *Nototheniidae*).

The discovery of AFPs in freeze resistant teleost fishes

The road to the understanding of organismal freeze resistance opened in the early 1950s, when Dr. R. H. Backus asked Dr. P. F. Scholander the following question:

"When arctic fishes swim about in ice water at -1.7 to -1.8°C, why don't they freeze? Do they have twice as high an osmotic concentration as ordinary fishes […]?" (Scholander *et al.*, 1957)

This prompted the latter to go to Labrador. After many experiments on fishes retrieved from the frigid waters of Hebron Fjord – and measurements of their blood FPs – the scientist gave what he believed then to be an easy answer to what appeared to be an "attractively simple problem":

"The shallow water fish moving about in the ice in the winter double their osmoconcentration at that time, *i.e.*, until it almost matches the sea water, and are thereby protected against freezing." (Scholander *et al.*, 1957)

These fish did have FPs nearing that of seawater, but Dr. Scholander was mistaken in the interpretation of the underlying cause. This is because his field osmometer didn't offer the accuracy needed to detect a feature nowadays considered diagnostic of the presence of AFPs: freezing hysteresis (FH) – *i.e.* the discrepancy between the FP and the melting point (MP) of a solution. Although he did notice a slight FH (~0.1°C) in the plasma of his fishes, the value was so low (and only 0.05°C above the difference measured in his control) that it was not given further thought. In fact, we now know that these measurements were underestimated. However, one thing was certain to this researcher and his colleagues: whatever was responsible for the lower FPs of the shallow water Labradorean fishes, it was not NaCl (Scholander *et al.*, 1957). The fishes were "somewhat protected", and the molecule(s) responsible were still to be discovered.

A few years later, Scholander and his colleges went back to Hebron Fjord, in order to identify the osmolyte(s) responsible for the high winter FPs of these fishes. The species investigated then included the shorthorn sculpin (*Myoxocephalus scorpius*) and

the Greenland cod (*Gadus ogac*). They measured the plasma concentrations of a vast selection of osmolytes, but their compiled contribution (converted in °C of FP depression) still did not match the absolute FPs measured with their osmometer (Gordon *et al.*, 1962). They postulated that an obscure "antifreeze substance" may be responsible for this gap in their values. Interestingly, the amino acid present at the highest concentration in the shorthorn sculpin plasma was alanine (Gordon *et al.*, 1962). Nearly two decades later, a high-alanine content AFP would be discovered by Hew *et al.* (1980) in the plasma of that very species.

During the late 1960s, Arthur L. DeVries – then a doctoral student under the supervision of Dr. Donald Wohlschlag – found that several species of Antarctic notothenioid fish had lower blood FPs than expected for teleosts, which are normally hyposmotic to their environment. He successfully isolated the osmolyte responsible for what he initially interpreted as being an isosmotic state with seawater, and deduced that it was a set of carbohydrate-containing proteins (DeVries & Wohlschlag, 1969). The heavily glycosylated proteins were purified and characterised (DeVries *et al.*, 1970; Komatsu et al., 1970), and their sequence and structure eventually solved (DeVries *et al.*, 1971; Shier *et al.*, 1972). This family of antifreeze agents became known as "antifreeze glycoproteins" (AFGPs).

AF(G)Ps grant freeze resistance through thermal hysteresis

Had these early investigators used a vapour point osmometer instead of a freezing point osmometer, the discovery of AFGPs and AFPs might have taken much longer. This

is because these fascinating proteins do not act in the same way as other solutes to lower the FP of a solution. In chemistry, the FP is a colligative property of a solution, *i.e.*, a property that undergoes changes with the addition of solutes, and that will solely depend on the amount of solutes added (number of molecules), not on their identity (size or mass. For instance, 1 M sucrose (342 g/l) and 1 M NaCl (58.44 g/l) will lower the FP of a solution to the same extent. Colligative FP depression occurs because the molecules of solute disturb the order and availability of the water molecules to a growing crystal. Hence, energy needs to be removed from the system for this entropy to decrease and for ice to form. As the FP decreases, so does the MP, as the two are at equilibrium. Thus, in a system that follows colligative laws, the FP is termed "equilibrium FP".

In contrast, the FP of a solution that contains AF(G)Ps will not be at equilibrium with its MP (non-equilibrium FP). This is because these proteins work non-colligatively, *i.e.* they cause FP depression in a way that would not be predicted by their concentration alone. For instance, while the glycopeptides from Antarctic were found to be as effective as NaCl in depressing the FP of water when compared on a mass basis (DeVries & Wohlschlag, 1969), the AFGP proved to be 200 to 300 times more efficient when compared to the salt on a molal basis (DeVries *et al.*, 1970). Such non-colligative action of AF(G)Ps is a clear advantage for an organism, as most osmolytes would become cytotoxic at the concentrations necessary to provide freezing resistance. The mechanism by which AF(G)Ps lower the FP of solutions is accomplished by the binding of the proteins to the surface of nascent ice crystals, which halts their growth. Unrestricted ice

growth, *i.e.* freezing, will only occur once the non-equilibrium FP is reached. This explains why FH is characteristic of aqueous solutions that contain AF(G)Ps (Fig. 1.1).

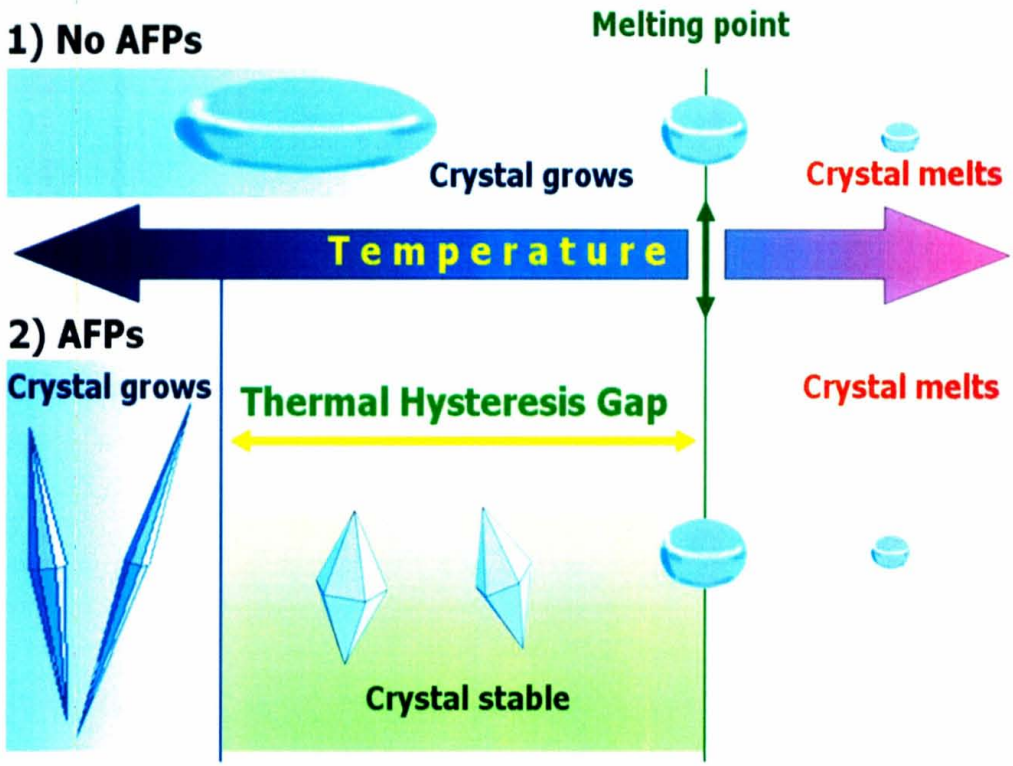

Fig. 1.1. Thermal hysteresis and behaviour of ice crystals in presence (2) and absence (1) of AF(G)Ps. Image graciously provided by Dr. Peter Davies.

The measurement of FH was first conducted by DeVries (1971) on a pure fraction of AFGPs from the serum of an Antarctic notothenioid fish. He was the first to propose that the glycoproteins lower the FP of fish plasma by adsorption onto the surface of ice crystals. This led to the development of the theory of "adsorption/inhibition" as an

explanation for the non-colligative action of the AF(G)Ps on FP (Raymond & DeVries, 1977). A schematic of the FP depression mechanism can be visualised below (Fig. 1.2).

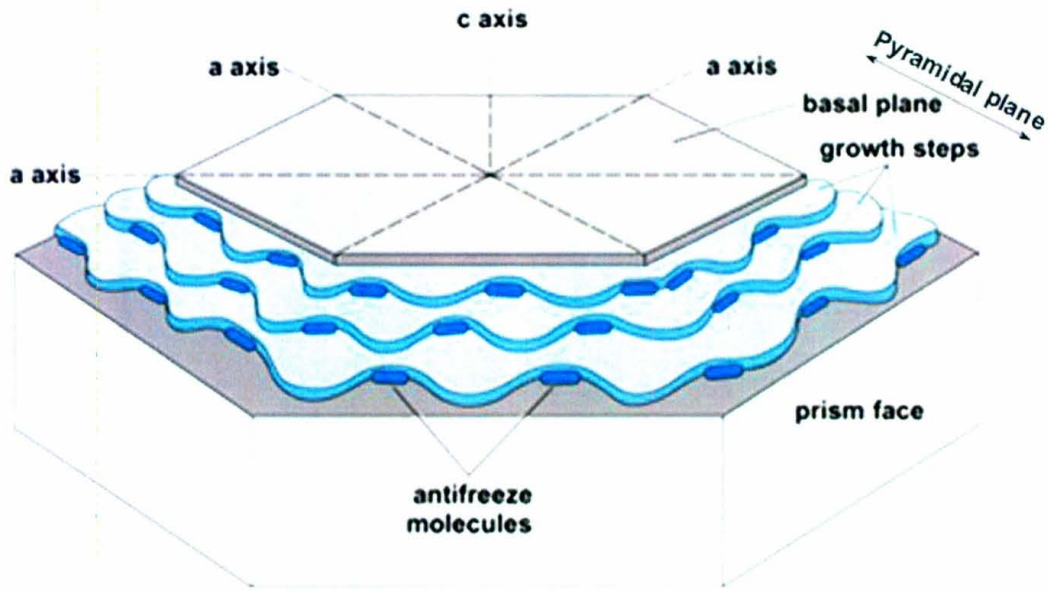

Fig. 1.2. Mechanism by which AF(G)Ps cause FP depression (and TH), according to the theory of adsorption/inhibition (image modified from: Arthur L. DeVries, "Antifreeze (biology)," in AccessScience, ©McGraw-Hill Companies, 2008, http://www.accessscience.com).

If the temperature of a solution containing ice crystals with adsorbed AF(G)Ps is lowered below the equilibrium FP (which is equal to the MP, hence its name), curved ice fronts begin to form between adjacent adsorbed AF(G)Ps. As ice normally forms in a highly organised fashion, growth of these fronts will halt at a certain level of curvature (Kelvin effect). The extent to which the FP will be depressed will be a function of the concentration of AF(G)Ps. The closer together are the bound proteins at the crystal's

surface, the faster the curvature of the intercalated ice fronts attains a threshold whereby growth ceases. Hence, in the presence of AF(G)Ps, growth will only resume once lower temperatures are reached. At that point, new water molecules with lower kinetic energy will be able to bind to the crystal, despite doing so into a less ordered and angular fashion. The temperature at which this occurs corresponds to the non-equilibrium FP. An ice crystal then appears to burst out of control along its c-axis (Fig. 1.2), as the curved ice fronts finally resume growth and fuse over the embedded AFPs. The AF(G)Ps are then buried with a speed being proportional to the extent to which the fluid was undercooled (*i.e.* cooled below the equilibrium FP).

If the AF(G)Ps halt ice growth because they adsorb to the surface of an ice crystal, could they prevent melting from occuring? Knight & DeVries (1989) were the first to obtain qualitative evidence that this is indeed the case. They observed irregular faceting forming within a hole that they had drilled in ice and filled with an AFP-containing solution. This observation strongly suggested that the proteins had bound to the walls of the hole and would not allow melting to occur uniformly, as it did in the control experiment (conducted with pure water). Their work offered qualitative evidence that the MP can be affected by adsorbed AF(G)Ps, and that some level of melting hysteresis (MH) will occur in AF(G)P-containing solutions (akin to the FH that was by then already routinely measured). The topic was then virtually forgotten for about two decades, until Celik *et al.* (2010) resumed investigation and found quantitative evidence of melting hysteresis MH in the presence of AFPs. Using a Clifton nanoliter osmometer (a device which allows the user to visualise the behaviour of microscopic ice crystals in controlled

thermal conditions), they froze a solution containing AFPs, and slowly melted it until a single tiny crystal remained. The highest temperature at which this crystal would stop shrinking and remain stable was determined as the MP of the solution. They then gradually lowered the sample's temperature down through its FH window to a point where the crystal burst and the sample re-froze. Finally, they increased the temperature until the sample started to melt again. Interestingly, at the melting temperature determined above, all but the original ice crystal had melted. This is because the AF(G)Ps bound to that crystal before the sample was re-frozen, and were now preventing bound water molecules from leaving the ice fraction for the liquid fraction. The sample's temperature needed a further increase in temperature in order to finally melt the crystal. Even in the presence of relatively high concentrations of AFPs (< 10 mg/ml), the resulting MH was small (at most, a few tenths of a degree) when compared to what the FH would have been. This explains why it could not easily be detected before, when the Clifton nanoliter osmometer was used with a coarser temperature control. The study of Celik *et al.* (2010) not only gave irrefutable proof of the existence of MH, it also consolidated the theory of adsorption/inhibition (along with studies from Wilson *et al.*, 1993; Pertaya *et al.*, 2007) according to which AF(G)Ps bind irreversibly to ice (Raymond & DeVries, 1977).

The amount of thermal hysteresis (TH = FH + MH) measured in an aqueous sample has been shown to be proportional to the concentration of AF(G)Ps in solution (DeVries, 1971; Celik *et al.*, 2010), according to a non-linear relationship which can be determined by building a standard curve of TH/[AFP] for any new AF(G)P tested (Davies

& Hew, 1990) (see Fig. 1.3). In contrast, other solutes (like NaCl or sugars), follow a linear relationship with temperature (DeVries *et al.*, 1970).

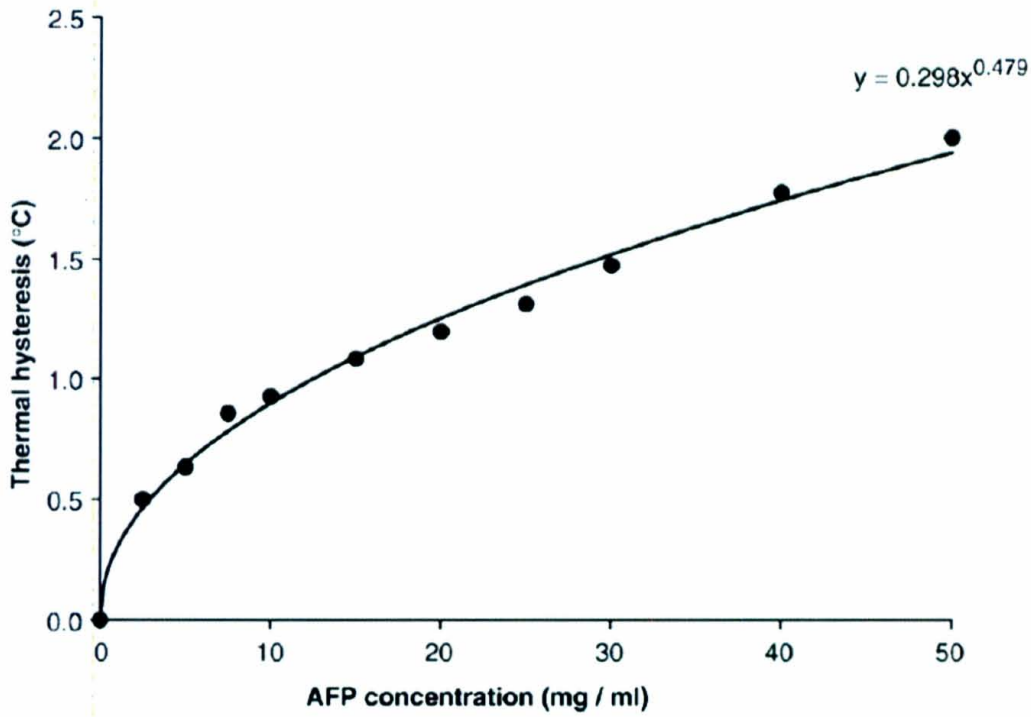

Fig. 1.3. Standard curve of TH/[AFP] from Desjardins *et al.* (2006), using type III AFPs from ocean pout (*Macrozoarces americanus*) resuspended in ammonium bicarbonate buffer (0.01 M).

For a given [AF(G)P], a threshold (plateau) will be approached where the FP/MP of the solution cannot be decreased (FP) or increased (MP) significantly further from the equilibrium FP/MP. The location of this threshold will depend on the type of AFP investigated, and on the presence of other osmolytes in solution, some of which have been

shown to enhance the activity of AF(G)Ps (Duman, 2001; Jin, 2003; Evans *et al.*, 2007; Amornwittawat *et al.*, 2009; Gong *et al.*, 2010). The magnitude of the TH of a sample is also inversely proportional to the size of the crystal and the rate of cooling used during measurement (Takamichi *et al.*, 2007). *In vivo*, AF(G)Ps will bind to nascent ice crystals that may be too small to be observed through a microscope. As a result, the non-equilibrium FP and MP of an AF(G)P-containing aqueous solution is likely always underestimated when measured in the laboratory.

Other properties of AF(G)Ps

While the achievement of freeze-resistance in living organisms by the FP depression of their physiological fluids is a key feature of the AF(G)Ps, these proteins display other unique and important properties. One of those is the inhibition of ice recrystallization (Knight *et al.*, 1984). Ice recrystallization happens when the temperature of a frozen sample fluctuates slightly below its MP. This can be visualised and assessed experimentally using a "splat assay". Here, a bit of sample is splattered upon a plate of frozen metal and incubated for hours at low subzero temperatures (Knight *et al.*, 1988). Under such thermal conditions, the tiny crystals thus generated will remain unchanged in the presence of even trace amounts of AF(G)P, while those within a sample devoid of antifreeze will gradually increase in size, and decrease in numbers (as the water of the smallest crystals is redristributed to the biggest ones). The outcome of ice recrystallisation can be experienced in everyday life, as coarse ice crystals in an ice cream quart or other frozen food item that has spent too much time in the grocery's freezer (which are self-

defrosting regularly). To solve such palatability issues, a frozen dessert-producing company (Breyers, Unilever) actually patented the use of a fish AFP in some of their products (U.S. Patent # 20050129810, Fig. 1.4). The AFP (which they re-labelled as "ice-structuring protein" to enhance public perception and avoid confusion with ethylene glycol, the car antifreeze) is expressed by and extracted from cultures of genetically-modified yeast (*Saccharomyces cerevisiae*) that contains an AFP gene from the teleost ocean pout (*Macrozoarces americanus*), found in the shallow waters of the North Atlantic Ocean. The cost of including AF(G)Ps into frozen products is negligible, because AF(G)Ps perform ice recrystallization inhibition at very low concentrations (10^{-6} mg/ml, Knight *et al.*, 1984). A team from the University of Guelph is currently working on AF(G)Ps applications in the frozen foods industry; they are interested in AFPs from winter wheat grass, *Triticum aestivum* (Kontogiorgos *et al.*, 2007).

Ingredients

Light Ice Cream: Skim Milk, Sugar, Corn Syrup, Cocoa (Processed With Alkali), Milkfat, Whey, Maltodextrin*, Propylene Glycol Monoesters, Cellulose Gel, Carob Bean Gum, Mono And Diglycerides, Cellulose Gum, Guar Gum, Carrageenan, Ice Structuring Protein, Vitamin A Palmitate. Milk Chocolatey Coating: Sugar, Vegetable Oil (Coconut, Soybean), Milk, Cocoa Butter, Chocolate Liquor, Whey Powder, Soy Lecithin, Natural Vanilla Flavor. Fudge Swirl: Sugar, Water, Corn Syrup, Chocolate Liquor, Cocoa Processed With Alkali, Modified Tapioca Starch, Nonfat Dry Milk, Salt, Potassium Sorbate (Used To Protect Quality). Dark Chocolate Chips: Sugar, Chocolate Liquor, Cocoa Butter, Soy Lecithin. * Ingredient Not In Regular Ice Cream

Fig. 1.4. A fish AFP is part of the ingredients listed in a frozen dessert produced by Breyers (Unilever). Source: http://www.breyers.com/products/Smooth-and-Dreamy-Bars-and-Sandwiches/Triple-Chocolate-Chip-Bars.aspx

In nature, ice recrystallization can be a life-threatening issue, both during cycles of extreme freeze-thaw (Walker *et al.*, 2006), and when slight variations around the non-equilibrium FP and MP occur. For instance, some AFGP-producing notothenioid fishes that live in the perennially icy waters of the Antarctic are known to harbour ice crystals within their spleen (Praebel *et al.*, 2009). Further investigation revealed that ice crystals bound with AFGPs are selectively absorbed by phagocytes in the spleen tissue, where they are stored (Evans *et al.*, 2010). To reach this conclusion, the researchers used nanospheres coated with fluorescently-labelled AFGPs that they injected in the fish's bloodstream. It is yet unverified if these Antarctic fishes can ever remove their internal ice, but it is theoretically possible. Indeed, these fish can encounter local temperature variations that span their TH window in nature. This may occur when performing vertical movements through the water column during summer towards the warmer surface layers (Hunt *et al.*, 2003). That is where the danger arises. Were it not for AF(G)Ps conferring anti-recrystallisation properties, a fish moving to warmer waters at temperatures between its non-equilibrium FP and MP (TH gap) could suffer damage to its tissues as some of its splenic ice crystals would grow bigger. Fortunately, the ice grains are completely neutralised in the presence of AF(G)Ps. Recrystallisation inhibition is possible because, as mentioned above, AF(G)Ps bind irreversibly to ice.

Yet another property of AF(G)Ps appears to be cell membrane protection at low temperatures (Rubinsky *et al.*, 1990; reviewed by Wang, 2000 and Tomczak *et al.*, 2002). At hypothermic temperatures, the membranes of non-acclimated cells will transiently leak as they pass from a liquid-crystalline state to a gel-like state (reviewed in Tomczak *et al.*,

2002). During the phase transition, both states are thought to co-exist, which may actually cause the leakage through packing defects (Clerc & Thompson, 1995). Rubinsky *et al.* (1990, 1991, 1992) were the first to measure an effect of AF(G)Ps on cell survival in cold, hypothermic temperatures. They chilled pig oocytes in the presence of AFGPs extracted from notothenioid blood and did not observe the level of damage that would usually occur at these temperatures. Instead, at a concentration of 1 mg/ml, 80% survival was observed among the cells. As an explanation, they proposed that the AF(G)Ps prevent mammalian cell leakage by blocking the ion channels, which they viewed as the cellular weak link. Because a later study by Hays *et al.* (1996) showed that AF(G)Ps preserve the integrity of liposomes (artificially prepared vesicles whose membrane do not contain proteins, therefore ion channels), it was speculated that AF(G)Ps would instead improve the *interaction* between the membrane proteins and phospholipids, as the phase transition takes place (Tomczak & Crowe, 2002). The AF(G)Ps may do so by inserting themselves into the membrane, or binding to its surface , depending of the nature of the AF(G)P studied (reviewed in Inglis *et al.*, 2006). These observations are exciting because they imply that the AF(G)Ps could be used for cold storage of human cells and organs (Lee *et al.*, 1992; Tablin *et al.*, 1996; Amir *et al.*, 2004a, 2004b), which would extend their "shelf life" between donor and patient, although certain issues need to be resolved first (see Inglis *et al.*, 2005). This membrane stabilization property also implies that in nature, AF(G)Ps might be useful to organisms facing high subzero (non-freezing) temperatures, through a mechanism yet to be fully elucidated.

The storage of reproductive cells and embryos at subzero temperatures in the presence of AFPs may represent an additional path, and encouraging results have been obtained (Rubinsky *et al.*, 1992; Arav *et al.*, 1993; Robles *et al.*, 2005; Le François *et al.*, 2008). Experiments are currently under way in Dr. Fletcher's lab to see if a line of cells from the non-AF(G)P-producing Chinook salmon (*Oncorhynchus tshawytscha*) exposed to cold/freezing temperatures will show increased survival in the presence of AFPs. This role in the prevention of cell leakage during exposure to cold temperatures also attracted the attention of the cosmetic industry, with LIFTLAB recently commercialising a line of facial creams containing a fish AFP among its ingredients (http://theliftlab.com/v1/pages/SHOP.html). The company currently holds patents that grant them the right to use AF(G)Ps from fish and insects in their products. They renamed the AF(G)Ps "cell protection protein", in an attempt to emphasis their claim that the customer's skin would be protected against cold damage.

Interestingly, another potential application of AF(G)Ps involves a result that is the polar opposite to those just mentioned: cell destruction. This was inspired by another unique property of the AF(G)Ps: ice-shaping. As illustrated in Fig. 1.2, bound AF(G)Ps will cause an ice crystal to develop an hexagonal shape by binding on its prism face (as opposed to ice with no AF(G)Ps, which will expand as a rounded disk). Because the proteins can also bind to the pyramidal plane of the growing crystal, but cannot do so on its basal plane, the ice will elongate along the c-axis (Fig. 1.2). This eventually stabilises the crystal as a hexagonal bipyramid between the equilibrium and non-equilibrium FPs of the fluid (see image on Fig. 1.1). Once the non-equilibrium FP is reached (which should

not happen in the host organism), growth will resume in an uncontrolled fashion, with needle-like projections sprouting from the tips of the bipyramid along the c-axis. The higher the [AF(G)P], the greater the undercooling, and the more dramatic this phenomena will be. Obviously, if prompted to occur *in vivo*, the explosive growth of such needle-like crystals would potentially puncture and/or shear any cell membranes in their paths. This cell-killing consequence of the ice-shaping properties of AF(G)Ps has been noticed and tested by medical research teams in an attempt to improve the success rate of cryosurgery. Used on small and localised tumors, cryosurgery is performed by locally freezing cancerous cells with liquid nitrogen circulated through a "cryoprobe". When highly concentrated AFP solutions were injected into the tumors prior to freezing, resulting cell death percentages were found to be significantly higher in mice (Pham *et al.*, 1999) and rats (Muldrew *et al.*, 2001).

This destructive effect of ice helps us to understand why organisms that tolerate freezing (such as *Rana pipiens*) vitally need to control how and where ice grows within their bodies, by the secretion of INPs. This is also why freeze-resistant animals – such as some teleostean fishes and the spruce budworm – must not undercool past their non-equilibrium FP. As a result, organismal freeze resistance will be finely tuned to the needs of the organism. For instance, the spruce budworm will produce enough glycerol and AFPs to keep its body fluids liquid down to -30 °C and slightly colder, while marine teleosts only need to protect themselves from freezing down to ~ -2 °C, the FP of seawater. As water is a more thermally buffered medium than is air, it does not freeze down to great depths at low atmospheric temperatures.

The threat of freezing in the marine environment: the case of the teleost fishes

Unlike terrestrial organisms, aquatic organisms are not typically exposed to temperatures lower than the FP of water, freeze-resistant or freeze-tolerant invertebrates inhabiting the intertidal zone are an exception (Aarset, 1982; Murphy, 1983). Indeed, even in bitterly cold atmospheric conditions, no substantial body of water will freeze to the bottom. Fresh water reaches its maximum density at 4 °C, and the deepest regions of big lakes will stay at this temperature year-round. In the oceans, water (at 35 ppt salinity) is at its densest as it reaches its FP (~ -2.0 °C), while the temperature of the deep ocean water (DOW) remains stable between 0 and 2 °C (Lear *et al.*, 2000). Thus, animals that live deeper in the water column are protected against freezing by their very location.

The FP and other colligative properties of seawater will obviously vary according to its salt contents. The high thermal buffering capacity of seawater can be best understood when comparing its physical properties to those of air (see Table 1.1). At 0 °C and sea level atmospheric pressure, 35 ppt seawater has a specific heat capacity (SHC) that is four-fold that of air and a density that is ~ 800 times higher. On a volumetric basis, this means that ~ 3200 times more heat would need to be applied to a given volume of seawater, in order to elevate its temperature by the same amount as the equivalent volume of air. Scaled up to the total mass they occupy on Earth, the heat capacity (HC) of the oceans would be ~ 1000 times higher than that of the whole atmosphere. The seawater would then have to absorb roughly 1000 times more heat than the latter, before its global temperature increases a mere 1 °K or 1 °C (Table 1.1). Of course, these are rough estimates, but they allow a general appreciation of the formidable thermal buffering

power of water, and explain why the surface temperature of great bodies of water can decrease enough to cause the formation of an ice cover, while the temperature of the underlying waters will remain quite stable.

Medium	SCH (kJ/kg·K)	Density (kg/m^3)	VCH (kJ/m^3·K)	Total mass (kg)	Total HC (kJ/kg·K)
Seawater	3.99	1028.10	4102.12	1.4×10^{21}	5.6×10^{21}
Air	1.00	1.29	1.29	5.2×10^{18}	5.2×10^{18}

Table 1.1. Specific heat capacity (SHC), density, volumetric heat capacity (VHC), total mass, and estimated total heat capacity (total HC) of seawater and air (to a salinity of 35 ppt).

Freeze-intolerant marine plants and invertebrates, which are isosmotic to seawater (Scholander *et al.*, 1957), are in no danger of freezing if they encounter ice within the water, as long as they are not exposed to the colder air. In the case of marine fishes, the ancient groups Chondrichthyes, Coelacanth, and Agnatha are isosmotic (or slightly hyperosmotic) to seawater. The first two groups accumulate diverse osmolytes – principally urea and trimethylamine oxide – tot that end, while the latter maintains a fluid composition highly similar (almost isoionic) to that of seawater (Griffith, 1981, 1987). To maintain isosmoticity with seawater is how, for example, the Greenland shark can swim underneath the Arctic and North Atlantic sea ice in search of prey (Skomal & Benz, 2004) without any need for AF(G)Ps. The marine teleost fishes, however, are different in that they universally maintain a hyposmotic state comparatively to their environment (Griffith, 1981; Evans, 2008). This feature is likely a remnant of a freshwater emergence

(reviewed in Finn & Kristoffersen, 2007). These fish are thus in danger of freezing if they contact ice at temperatures below their FP. In comparison, fresh water teleosts are hyperosmotic to their environment (Evans, 2008). As such, and unlike their marine counterparts, they are not threatened by freezing and do not need AF(G)Ps.

From polar to temperate latitudes, seawater will freeze nearer the coast, where the proximity to colder land and lower heat input from buffering deeper waters will favour its surface freezing. A coastal freeze-risk ecozone can thus be found seasonally in these regions, where the risk of freezing will decrease with depth. Without adaptation, marine teleosts could freeze if they contact ice crystals in the water column. As ice has a lower density than liquid water, it has positive buoyancy, and will normally stay near the surface (see exceptions below). Thus, freeze-susceptible fishes will normally be found in deeper waters, where they survive below their FP in an undercooled state (Scholander *et al.*, 1957). Conversely, marine teleosts living in shallower surface and coastal waters are expected to face, at least seasonally, the threat of freezing. Consequently, most fish that stay in the shallowest part of the "freeze risk ecozone" are fully protected by AF(G)Ps.

However, ice can occasionally occur at greater depths, which renders difficult a clearly defined depth limit to the "freeze risk ecozone". For instance, if a loose slushy ice cover exists at the surface, high wind-driven turbulence can drive surface frazil ice to a depth of several metres (Svensson & Omstedt, 1998). Ice crystals brought down by water column convective overturning, or when icebergs scrape the seafloor can occur as well (reviewed in Goddard & Fletcher, 2002). Moreover, ice can be found tethered to the bottom (anchor ice reaches depths of 30 m in Antarctica, but lesser depths in the Arctic;

Dayton *et al.*, 1969; Reimnitz *et al.*, 1987). Finally, it can form underneath a deep coastal glacial ice sheet (*e.g.* ice platelets were collected at a depth of 250 m near the Filcher Ice Shelf in Antarctica, Dieckmann, 1986), or can reach down following denser brine sinking towards the seafloor from forming surface ice ("brinicles"; Martin, 1974; see also: http://www.bbc.co.uk/nature/15835017). As the harshest conditions occur in the Antarctic (deeper anchor ice and ice formation down to several hundreds of meters), fish that live in these waters often produce high AF(G)P levels, even when found at great depths (DeVries & Cheng, 2005). However, all deeper dwelling temperate North Atlantic fish investigated for TH in their plasma (*e.g.* the American plaice, *Hippoglossoides platessoides*, and the spotted wolffish, *Anarhichas minor*) were found to have levels insufficient to grant freeze resistance (Goddard & Fletcher, 2002; Desjardins *et al.*, 2006). Exceptional meteorogical conditions or icebergs (scouring the sea floor) moving ice crystals into deeper water notwithstanding, fish living in temperate waters deeper than 30 m theoretically escape the threat of freezing. Thus, the here-proposed "freeze risk ecozone" concept (coined by Dr. Garth L. Fletcher) comprises depths where these phenomena can occur.

In general, teleost fish plasma or serum osmolarities translate to an equilibrium FP that ranges between -0.6 and -0.8 °C (Holmes & Donaldson, 1969). Some polar fishes have evolved slightly higher serum osmolarities than their temperate counterparts (Gordon *et al.*, 1962; O'Grady & DeVries, 1982), with resulting equilibrium FPs dropping as low as -1.2 °C in some species (Dobbs & DeVries, 1975). However, the colligative contribution of the osmolytes was never sufficient to bring their plasma

equilibrium FP down to that of their surroundings. Some temperate teleosts also show seasonal fluctuation of their plasma osmolyte concentrations, with higher values reached in winter (Fletcher, 1977, Lewis *et al.*, 2004; Desjardins *et al.*, 2006). For instance, the rainbow smelt (*Osmerus mordax*), found in the northern Atlantic, synthesises high plasma levels of relatively inert glycerol, which contributes ~ 0.5 °C of FP depression in the winter (Lewis *et al.*, 2004). Nevertheless, these fish – as well as other temperate and polar species – still need to lower their FP further in order to resist freezing. This is where the AF(G)Ps enter the equation.

Attaining freeze resistance by evolving an isosmotic state with seawater would have warranted a whole re-adaptation of organismal, physiological and biochemical processes in marine teleosts. Thus, to evolve AF(G)Ps in order to lower the FP non-colligatively to (or below) that of seawater, appears a more facile solution, as it does not affect the ionic and osmotic balance of the fish. Perhaps for this reason, the evolution of AF(G)P in marine teleosts came out as the universal response to the threat of freezing, with these proteins emerging independently in a beautiful example of convergent evolution (Scott *et al.*, 1986; Fletcher *et al.*, 2001; Goddard & Fletcher, 2002).

Teleost fishes as a model for the study of AFP evolution

Teleost fishes emerged relatively recently, perhaps during the middle or late Triassic, 220 to 200 Ma ago (Nelson, 2006). Their ancestor, shared by other ray-finned fishes, likely dwelled within warm climatic conditions (Prochnow *et al.*, 2006). It is thus pretty safe to postulate that the teleostean fishes only faced a serious threat of freezing

since the Cenozoic. Indeed, the Earth was mostly within a climate "Hot House" before that era, since as far back as ~200 Ma ago (Early Jurassic) (Fletcher *et al.*, 2007; Retallack, 2009; Price & Nunn, 2010; Dera *et al.*, 2011; Jenkyns *et al.*, 2011). This makes the study of AF(G)P evolution easier within this group than – for instance – in insects, which are an incommensurably diverse and ancient clade. Indeed, the first fossil evidence of an insect dates back to the early Devonian, some ~ 400 Ma ago (Engel & Grimaldi, 2004), which means that members of this clade were exposed to one other major glacial event before the Cenozoic Era (Scheffler *et al.*, 2003).

Teleosts are the most speciose and diversified vertebrate clade on Earth. Nelson's description gives a proper measure of that statement:

"About 26 840 extant species, about 96% of all extant fishes, placed in 40 orders, 448 families, and 4278 genera" (Nelson, 2006).

Recent evidence from the field of molecular biology points to a whole genome duplication (WGD) event as a trigger to this explosive radiation (Hoegg *et al.*, 2004; Volff, 2005). The main argument behind this is that a greater genetic template was made available to evolution. The important evolutive role of gene duplication was brought under the spotlight by Ohno (1970), who proposed that after a duplication event, selective pressures that preserve the integrity of a given gene sequence are relaxed on one of the copies, which then becomes free to accumulate random mutations. From there, three main paths await for such a duplicate: 1) to be conserved as it is, or 2) to accumulate mutations

that will progressively erode its sequence (turning it into a pseudogene) or 3) enable it to evolve into a different functional entity (Wagner, 1998; Zhang, 2003; Innan & Kondrashow, 2010). For the latter option, an evolving duplicate can serendipitously develop a completely novel function (neofunctionalisation) or simply improve a pre-existing, possibly competing one (subfunctionalisation).

To possess at least two copies of each gene obviously allowed the evolution of an array of new phenotypes in the rapidly diversifying teleosts. With time, a lot of the supplemental copies that were not selected for were eventually lost. The most dramatic case known to science of a post-duplication genome contraction in a fish can be seen in the puffer fish *Tetraodon nigroviridis*, for which the genome has been sequenced (Jaillon *et al.*, 2004). Following detailed analysis, it was concluded that only 15 % of the gene duplicates were conserved in that species (Brunet *et al.*, 2006). Such genome contraction (eventually leading to secondary diploidization) was observed in most teleost species investigated (de Peer *et al.*, 2009). Nevertheless, the WGD event that occurred at the base of the teleost lineage likely helped these fish to achieve their remarkable biodiversity. Teleost fishes dominate virtually all ecological niches in the oceans, from the great depths to the shallows, from the tropics to the poles, where sea ice eventually became prevalent.

Possessing some genes in duplicate (or more) may have offered a stepping stone for the evolution of AF(G)P in marine teleosts. At the onset of the first undisputed Cenozoic sea-level glaciations, at the Eocene-Oligocene boundary some 34 Ma ago (Zachos *et al.*, 2001, 2008; DeConto *et al.*, 2008), the teleostean marine shore fauna already bore a striking resemblance to modern assemblages (Greenwood *et al.*, 1966),

which means that contemporary teleost suborders and families were already – or about to be – established (Scott *et al.*, 1986). This situation likely explains the high diversity of AF(G)Ps evolved by fish belonging to distinct clades (Fig. 1.5). Indeed, it is now agreed that the several extant AF(G)P-bearing fish groups evolved their AF(G)Ps independently, in front of the same and relatively recent threat of freezing (Scott *et al.*, 1986; Chen *et al.*, 1997a; Fletcher *et al.*, 2001). Today, these AF(G)Ps are classified as type I, II, III AFPs and AFGPs based on their distinct structures and composition (Davies & Hew, 1990), and the elucidation of their evolutionary origins is the focus of ongoing research (see following section).

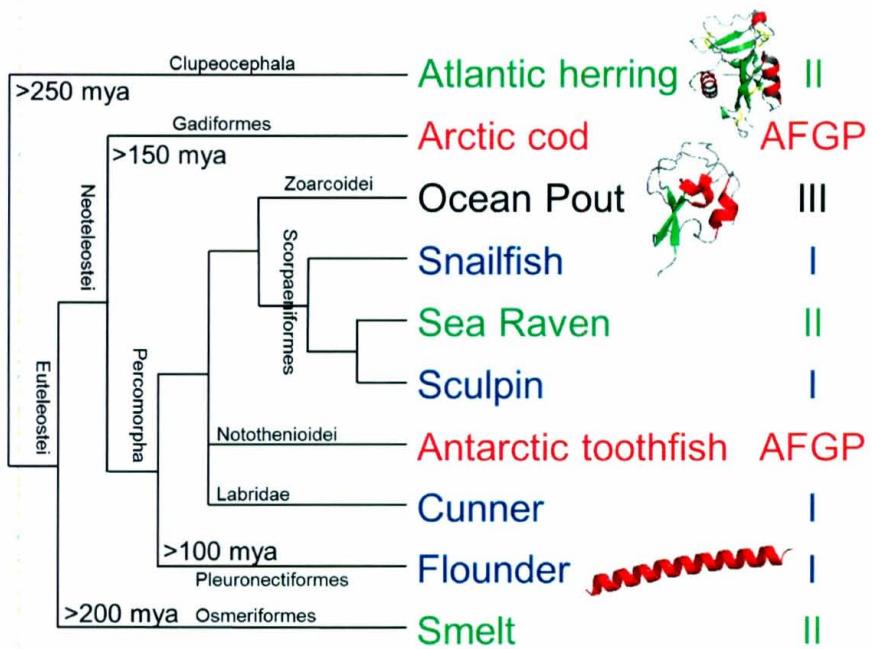

Fig 1.5. AF(G)Ps distribution within AF(G)P-producing teleosts. The structures of the different AFPs are shown (that of the AFGP is not yet resolved), and the approximate times of divergence of the older fish clades are indicated. This image is used with the permission of Gauthier *et al.* (2011), who used the phylogenetic tree built by Miya *et al.* (2003), and the divergence times proposed by Azuma *et al.* (2008).

Origin and emergence of the fish AF(G)Ps

The WGD event that occurred in the early teleost ancestor may have generated part of the raw materials needed to rapidly evolve a response to the new risk of freezing death. To deduce the origin of the AF(G)P genes and their products first involves the comparison of their sequences to other nucleotide and amino acid sequences available in the databases.

The first AF(G)P for which an evolutionary origin has been investigated is the type II AFP. These AFPs have been found and characterised in the Atlantic herring (*Clupea harengus*; family Clupeidae, order Clupeiformes) (Ewart & Fletcher, 1990; Ewart & Fletcher, 1993), the rainbow smelt and Japanese smelt (*Osmerus mordax* & *Hypomesus japonicus*, family Osmeridae, order Osmeriformes) (Ewart & Fletcher, 1990; Ewart *et al.*, 1992; Yamashita *et al.*, 2003), the sea raven (*Hemitripterus americanus*, family Hemitripteridae, order Scorpaeniformes) (Slaughter *et al.*, 1981; Ng & Hew, 1992), and the longsnout poacher (*Brachyopsis rostratus*, family Agonidae, order Scorpaeniformes) (Nishimiya *et al.*, 2008). The presence of five disulfide bridges is the major distinguishing feature of these 14 to 24 kDa globular proteins (see Davies & Sykes, 1997). Although very similar in structure, a major difference exists: the proteins from the sea raven and longsnout poacher (order Scorpaeniformes) do not need calcium for activity, while the ones from herring and smelt do (Ewart *et al.*, 1992; Nishimiya *et al.*, 2008). This raised the possibility of an evolutionary origin from distinct precursor genes (Loewen *et al.*, 1998). However, the present consensus is that all type II AFPs evolved from a Ca^{2+}-dependant C-type lectin gene (Ewart *et al.*, 1992, Ewart & Fletcher, 1993;

Liu *et al.*, 2007; Graham *et al.*, 2008a). C-lectins are sugar-binding proteins whose role lies in mechanisms of recognition between cells and proteins. The type II AFP is obviously derived from their carbohydrate-binding site (Ewart *et al.*, 1998). However, the genetic background for the evolutive mechanisms involved in the emergence of these AFPs is still lacking. For now, a simplified and likely explanation is that a C-type lectin progenitor gene was duplicated, with one copy eventually diverging into the type II AFP precursor gene (Cheng & DeVries, 2002).

When comparing the four different type II AFPs together, another question comes to mind: are these AFPs related, and if so, how? On that question, no consensus has been reached as of yet. A first suggestion was that the Ca^{2+}-dependent and -independent AFPs are the result of convergent evolution (Fletcher *et al.*, 2001), with the duplication and divergence of the original C-lectin gene occurring independently within the three teleost orders mentioned above. This offered a first tentative explanation for the presence of very similar AFPs among these distantly related fish groups (see Fig. 1.5). A recent study by Liu *et al.* (2007) suggested an alternative hypothesis: an initial Ca^{2+}-dependent C-lectin gene duplication happened in the common ancestor to all these fish groups, with one of the copies eventually giving rise (by subfunctionalisation) to a type II AFP. This AFP would have then evolved differently within the descendent fish clades, with a more recent loss of their last Ca^{2+}-binding site, causing the AFP from the Scorpaeniformes to become Ca^{2+}-independent. However, when looking at the great genetic distance separating these fishes, and the absence of AFP II genes in many clades that emerged after the Clupeiformes (see Fig. 1.5), the scenario of widespread gene loss appears quite unlikely,

although it obviously occurred in some cases (*e.g.*, *Tetraodon nigroviridis*; Liu *et al.*, 2007). This is especially true as there seems to be no selective pressure acting towards the loss of unneeded genetic material in eukaryotes (Davies, pers. com.).

While it is very likely that the AFPs from the sea raven and the longsnout poacher share a common ancestor from an older Scorpaeniformes fish (~ 67% identity between their amino acid sequences), common ancestry by descent appears somewhat far-fetched when comparing the AFPs of the herring and the smelts (~ 84% identity between their amino acid sequences). Indeed, these species are quite far apart on the evolutionary tree (see Fig. 1.5). As mentioned above, it seems incredible that the type II AFPs would have been kept in the herring, but lost in the entire Eutelostei subdivision, except for the smelts and a few Scorpaeniformes. Emergence in a common ancestor to the herring and these other fishes (dating back to > 250 Ma ago, Azuma *et al.*, 2008) would also have had to predate the onset of the Cenozoic glaciations by more than 200 Ma. This is counterintuitive, considering that the recognised stimulus for AFP evolution is the presence of sea ice (Scott *et al.*, 1986).

This latter reasoning brings forth again the afore-mentioned scenario of an independent emergence of these proteins by convergent evolution (Fletcher *et al.*, 2001). However, if convergent evolution can potentially produce two nearly identical groups of isoforms independently by acting on the coding sequence of a single gene (or genes; see next case), one would expect to see important differences in the non-coding regions (5' and 3' UTRs, and introns), which would not be subjected to selection. Thus, important sequence divergence should be present among these sequences, especially considering the

great genetic distance separating, for instance, the herring and the smelts. Recent comparison of the AFP II DNA sequences from these two species (on their shared lengths) revealed high identities between the exons (~ 88-97%), but (surprisingly) slightly higher identities between their introns (~ 88-99%) (Graham *et al.*, 2008a). This intriguing feature inspired yet another scenario by the latter authors: the type II AFPs may have spread across unrelated fish taxa through lateral gene transfer (LGT). That hypothesis is currently being explored by Dr. P.L. Davies' team at Queen's University (Kingston, ON).

The second AF(G)P for which the evolutionary origin was investigated – and eventually resolved – is the AFGP found in fishes of the suborder Notothenioidei. Chen *et al.* (1997a) obtained the sequence of a complete gene and compared it to the sequences available in the databases. Their best hit was a trypsinogen-like serine protease gene (cDNA) from a flatfish. Intriguingly, the two gene sequences appeared completely unrelated; the only homologies (> 70%) were found in small portions of the sequences corresponding to the C- and N-terminals ends of the proteins, and within the DNA flanking the gene at its 3′ end. Chen and colleges (1997a) then retrieved and sequenced a trypsinogen-like serine protease gene from a notothenioid fish (*Dissostichus mawsoni*) and again, compared that sequence to that of the AFGP gene. This time, more resemblance was found. These genes shared high identity between their first exon (96%), which codes for a signal peptide, and the shared part of the adjacent intron (93%). The next region of high identity was observed between the sixth exon of the protease gene and the 3′ end of the AFGP gene. In between, the sequences were completely unrelated.

In lieu of the four other exons found in the protease gene, the AFGP gene has an extensive second exon. This exon codes for a highly repetitive AFGP polyprotein, containing several AFGPs linked to each other by a conserved three-residue sequence (Leucine-*Asparagine*-Phenylalanine or Leucine-*Isoleucine*-Phenylalaline), with individual AFGP proteins being released following post-translational removal of that linker sequence (Hsiao *et al.*, 1990). Each AFGP is essentially a repetition of a single monomer (threonine-alanine-alanine), that bears a disaccharide on its threonine (Thr) hydroxyl group (DeVries, 1988); this in turn makes them heavily glycolysated, hence their name. Their repetitive structure and other factors related to their expression render them difficult to work with (Brown & Sönnichsen, 2002); as a result, their tertiary structure remains unresolved.

Interestingly, this repeated three-residue motif within each AFGP, which corresponds to the nucleotide sequence "aca-gcg-gca", was found as a single copy at the junction between the first intron and the second exon in the notothenioid protease gene (Chen *et al.*, 1997a). To explain the evolution of an AFGP gene from the latter, these authors proposed that the original protease gene first underwent duplication. Eventually, one copy would have had its short "aca-gcg-gca" sequence amplified extensively, while its second to fifth exons would have been deleted (along with the associated introns). Shortly after the publication of this research, the discovery of an active chimeric (hybrid) gene that contains a partially amplified "aca-gcg-gca", nested within the complete sequence of the protease gene (Cheng & Chen, 1999) validated these authors' hypothesis.

The emergence of the nothothenioid AFGP has been hypothesised by Cheng *et al.* (2003) to coincide with the species explosion of Antarctic representatives of the suborder Notothenioidei. These fish likely initiated their radiation an estimated 24 Ma ago (Matshiner *et al.*, 2011), in response to the intensification of the sea level Antarctic glaciations at the Oligocene-Miocene transition (Naish *et al.*, 2001). The rapidly diversifying notothenioid fishes gradually spread to all niches available within this frigid environment, perhaps starting by invading the shallow water habitats where massive local extinctions may have occurred (Eastman, 2005). From there, the notothenioids gradually replaced fish in other habitats as well, becoming one of the most impressive fish flock known to biology (Eastman & McCune, 2000), along with the cychlids from the great East African lakes (Verheyen *et al.*, 2003). They are today the dominant teleost clade in Antarctica waters, representing ~55% of the species richness (Clarke & Johnston, 1996). This case illustrates a classical case of neofunctionalisation, as the original trypsinogen-like protease gene, which would likely code for a digestive enzyme, did not have any precursor antifreeze activity to improve upon following duplication.

Amazingly, AFGPs have been found in various species of northern cod (Hew *et al.*, 1981; Chen *et al.*, 1997b). Despite being nearly identical to those of the notothenioids, the AFGPs evolved by gadoids were hypothesised to have a distinct evolutionary origin (Chen *et al.*, 1997b). Three main facts support this assumption (reviewed by Cheng & DeVries, 2002). First of all, the signal sequence of the gene coding for the AFGP in the cods is different from that of the notothenioid's gene, and no homologous sequence could be found while browsing the databases. Second, the short repeated motif "Thr-Ala-Ala"

that forms the backbone of both AFGPs is coded by distinct codons between the two fish groups, and the amino acid sequence that acts as a spacer in the cod AFGP is completely different (O'Grady & DeVries, 1982; Chen *et al.*, 1997b). Finally, these fish belong to different superorders (cods: Paracanthopterygii; notothenioids: Acanthopterygii) and are thought to have evolved in geographic isolation (Northern and Southern hemisphere origin for the cods and notothenioids, respectively). This considerable genetic distance (Fig. 1.5) renders an independent origin of the AFGP genes by convergent evolution a more plausible scenario than their inheritance from a common ancestor (followed by subsequent loss in all other families of their respective suborders) (Chen *et al.*, 1997b). Indeed, as discussed before in the case of the type II AFPs, the latter scenario would imply that the acquisition of a functional AFGP had to occur well before the initiation of the Cenozoic glaciations. For now, the origin of the cod AFGP remains a complete mystery, and may represent another example of *de novo* gene evolution (neofunctionalisation) in front of a new environmental stress.

Another class of AF(G)Ps, generally grouped as type I AFPs, have a still unresolved evolutionary origin. These alanine-rich, alpha-helix structured proteins also form the most heterogeneously widespread AF(G)P group (Fig. 1.5), having been reported in fishes from four families (Pleuronectidae: righteye flounders (Fourney *et al.*, 1984; Scott *et al.*, 1985); Cottidae: sculpins (Hew *et al.*, 1980; Low *et al.*, 1998); Cyclopteridea: snailfishes (Evans & Fletcher, 2001; Evans & Fletcher, 2005); and Labridae: the cunner, *Tautogolabrus adspersus* (Evans & Fletcher, 2004; Hobbs *et al.*, 2011)). These families can in turn be divided among three orders: the Pleuronectiformes

(Pleuronectidae), Scorpaeniformes (Cottidae, Liparidae, and Cyclopteridae), and Perciforms (Labridae), which all belong to the superorder Acanthopterygii (Nelson, 2006). The most studied type I AFPs originate from the winter flounder, and all share a repetitive unit of 11 residues (Lin & Gross, 1981): Threonine-X_2-(Aspartic acid or Asparagine)-X_7, where "X" is most often Ala (Scott *et al.*, 1987). These AFPs are highly similar in their primary sequence among the three suborders, with half of the remaining AFPs also having 11 repeat units of the Alanine (Ala) residue (except for the sculpin and snailfish AFPs).

A common ancestry for these sequences among fish from three suborders thus appears quite unlikely, and Gauthier *et al.*'s (2011) analysis strongly argues for a repeated independent evolution of the type I AFP within the Acanthopterygii by convergence, in response to the relatively recent onset of the Cenozoic sea-level glaciations in well-established fish superorders (Scott *et al.*, 1986). The evolutionary origin of each of these genes may then involve distinct precursor genes. The best attempt to find such an ancestral precursor was made by Evans & Fletcher (2005), who studied sequences isolated from a cDNA library constructed from snailfish liver mRNA. Their goal was originally to find the nucleotide sequence coding for this species' type I AFP. Upon sequencing of three of their positive clones (that were hybridised with a snailfish AFP I cDNA probe), these authors first came across two sequences of an egg shell protein, and one sequence of type II keratin. When aligned, significant homologies between portions of the coding sequence of these latter cDNAs and the AFP sequence were found. These

researchers thus proposed that the AFPs from the snailfish may have evolved by co-option of one of these genes.

In contrast to the other AF(G)Ps, the type III AFPs have been exclusively found in representatives of a single suborder: the Zoarcoidei (order Perciformes). Species from five families (out of the nine) have been investigated for the presence of the type III AFPs and tested positive for the gene (Davies *et al.*, 1988; Shears *et al.*, 1993). These species are members of the families Zoarcidae (eelpouts), Pholidea (gunnels), Stichaeidae (pricklebacks), Cryptacanthodidae (wrymouths), and Anarhichadidae (wolffishes). Following searches within the available databases, Baardsness & Davies (2001) hypothesised that the evolutionary origin of these small (~ 7 kDa) globular proteins was a sialic acid synthase (SAS) gene. SAS is an intracellular enzyme that catalyzes synthesis of sialic acids from N-acetylmannosamine or Man-NAc-6-phosphate and phosphoenolpyruvate. The SAS gene comprises six exons, with the last one corresponding to the sugar-binding domain of the enzyme. The enzyme functions as a homodimer, where the C-terminal of one molecule seals the active site of the other. By so doing it also provides residues that interact with the bound substrate (Gunawan *et al.*, 2005). Baardsness & Davies (2001)'s analysis revealed that the mature short AFP III sequence shared significant identity with that last exon. A decade later, Deng *et al.* (2010) sequenced the AFP locus of an Antarctic zoarcid fish (*Lycodichthys dearboni*) and found a SAS gene (called SAS-B) flanking an extensive tandem array of AFP genes. On top of its sugar-binding domain, other regions of homology the SAS-B gene shared with the AFP gene were its 5′ UTR and the beginning of its first exon. The afore-mentioned

authors discovered that SAS-B had in fact been translocated to this locus from its original SAS locus, following a duplication event, as the original SAS genes (A and B) are located on a different chromosome from the AFP genes. Thus, a likely hypothesis for the evolution of a type III AFP from a SAS-B gene would be a duplication and translocation of a SAS-B gene, followed by the loss of exons # 1 to # 5 (except for the first part of this first exon) within one of the duplicates, leaving the small sugar-binding domain (exon # 6) bound to its putative signal sequence (first part of exon 1) by the remaining intron # 5 ready to evolve into a fully functional systemic AFP.

Interestingly, Deng and colleagues (2010) reported that the SAS B protein, once expressed *in vitro* and purified, exhibited weak TH (up to 0.015 ˚C) at a concentration of 2 mg/ml. At an identical concentration, the AFP III obtained from that same fish showed substantial TH (up to 0.67 ˚C). The reported expression of trace activity by this functional SAS gene brought the authors to conclude that the evolution of the type III AFP illustrated well the concept of "escape from adaptive conflict" (EAC). The EAC model (derived from the subfunctionalisation model) states that a precursor gene with an emergent function (besides its primary function) could see this new function selected for and improved to a certain degree prior to gene duplication (Hughes, 1994; Piatigorsky & Wistow, 1991). Further evolution of the nascent function would be halted by the attainment of conflict with the gene's main function. At that point, duplication would be required to allow further specialisation, by freeing one copy for the evolution of its alternative function. By comparison, the gene subfunctionalisation model stipulates that evolution of the new underlying function will only initiate *after* duplication.

In conclusion, despite highly different evolutionary origins, and the resulting diverse structures, the fish AF(G)Ps all display TH through their ability to bind to ice crystals and halt their growth (Davies & Hew, 1990; Fletcher *et al.*, 2001; Davies *et al.*, 2002). A minute ice crystal presents different planes on which an AF(G)P can bind (see Fig. 1.2): the prism, basal and numerous pyramidal planes, which differ by the spacing between the oxygen atoms of the bound water molecules. Such differential spacing obviously allowed for the diversity of AF(G)P observed today, where different AF(G)P types will bind to different planes of an ice crystal. For instance, winter flounder (*Pseudopleuronectes americanus*) type I AFPs will bind to a pyramidal plane of ice (Knight *et al.*, 1991), while type III from ocean pout (*Macrozoarces americanus*) will bind to both pyramidal and primary prism faces (Antson *et al.*, 2001) thanks to a compound ice-binding site (Garnham *et al.*, 2010). This compound site is fully functional for the so-called QAE type III AFP isoforms, while the isoforms of the other known type (dubbed SP type), have a deficient prism plane ice-binding site (appellation based on the ion-exchange Sephadex resins to which each type of isoform binds; see Chapter 2 for further details). Most other AF(G)Ps also bind to the prism and pyramidal planes, which generates a stabilised hexagonal bypiramidal crystal. That crystal will burst along its c-axis if the temperature is lowered beyond the non-equilibrium FP afforded by the AF(G)Ps. Binding to the basal plane of ice seems to be a feat only accomplished by a few AFPs (known as hyperactive AFPs), and is considered to be the basis of their potency (Scotter *et al.*, 2006). The only fish AF(G)Ps likely able to bind to the basal plane is the winter flounder hyperactive AFP (Graham *et al.*, 2008b), discovered by Marshall *et al.* (2004).

The search for the AF(G)Ps' respective binding sites has been the object of numerous studies, and following the determination of their tertiary structures (by NMR and/or X-ray crystallography), diverse mutant forms of these proteins (especially types I and III AFPs) have been generated and tested for TH, in order to determine the residues that are important for binding. A few models to explain how AF(G)Ps bind to ice have been proposed. The first such model proposed that binding happens through hydrogen bonding at AF(G)P surfaces that are complementary to the ice lattice (Raymond & DeVries, 1977; Chou, 1992; Jia *et al.*, 1996). This model by itself has a few caveats; for example, it fails to explain why free water would not be preferred to ice for binding (Davies *et al.*, 2002). Other studies hypothesised that the binding was mostly performed through hydrophobic effects, where bound water molecules would be released from the ice-binding site upon AF(G)P attachment to the crystal's surface (Chao *et al.*, 1997). However, results from molecular dynamic simulations suggests that a relatively hydrophobic antifreeze protein face could organise and keep water molecules within a lattice near its surface (as clathrates, or ice-like waters), which would in turn help the AFP to bind to ice, through hydrogen bonding (Nutt & Smith, 2008). The recent discovery of bound waters onto a crystallised AFP supports such a scenario, that in turn lead to the proposition of the "anchored clathrate" model for AF(G)P binding, which would theoretically be applicable to all AF(G)P types (Garnham *et al.*, 2011). If proven true, such a mechanism would be the basis for the convergent evolution of all AF(G)Ps.

The first steps into the evolution of fish AF(G)P multigene families

Almost all AF(G)P-producing fishes that have had their AF(G)P gene locus studied were found to have their AF(G)P genes organised as multigene families (Hew *et al.*, 1995; Chen *et al.*, 1997b; Fletcher *et al.*, 2001, Cheng *et al.*, 2003), the exception being the smelt that very likely inherited one type II AFP gene copy from the herring through LGT (Graham *et al.*, 2008a; Graham , com. pers). As AF(G)Ps obviously did not show the activity they do today when they first emerged, the development of multigene families of pro-AF(G)P genes must have been an essential corner stone for the development of freeze resistance in full strength icy seawater. An interesting (yet unanswered) question is: how did fish initially survive in ice laden environments without a fully developed AF(G)P arsenal (*i.e.* only armed with one or low copy numbers of nascent, proto-AF(G)P genes that translated into AF(G)Ps that showed only low to marginal activity)? As there is no such thing as freezing "just a little" (Scholander *et al.*, 1957), how did fish succeed in filling the ≤ 1.0 °C gap between their equilibrium FP and that of seawater rapidly enough not to go extinct in face of the cooling climate?

A tentative resolution of this apparent paradox is to postulate that emergence of the first AFP genes occurred in an environment where the FP of seawater was near the equilibrium (colligative) FP of the fish. Marine environments of low salinities do occur, and are most frequently encountered in shallow coastal waters where heavy rain, river discharges and important levels of ice melting take place. For example, extensive areas of brackish waters can be encountered along the Arctic coast, particularly in Northern Russia, thanks to the freshwater input of major river systems and summer ice melt

(AMAP, 1998; Fig. 1.6). Important estuaries also exist at lower latitudes. Fish may have evolved a more efficient freeze resistance strategy as they moved into saltier icy waters.

Interestingly, many of the most efficient AF(G)P-producing species encountered today show some degree of euryhalinity/haloplasticity and can – or could potentially – survive in brackish waters. Examples include representatives of the Zoarcoidei, Scorpaeniformes, Clupeidae, Osmeridae (which are mostly anadromous), Gadidae, Labridae, and especially the Pleuronectidae (Novikow *et al.*, 2000; Methven *et al.*, 2001; Le François *et al.*, 2004; Magnussen *et al.*, 2008). For instance, the starry flounder (*Platichthys stellatus*), a marine fish of the family Pleuronectidae, was reported > 100 meters upstream in the Columbia River (reviewed in Orcott, 1950).

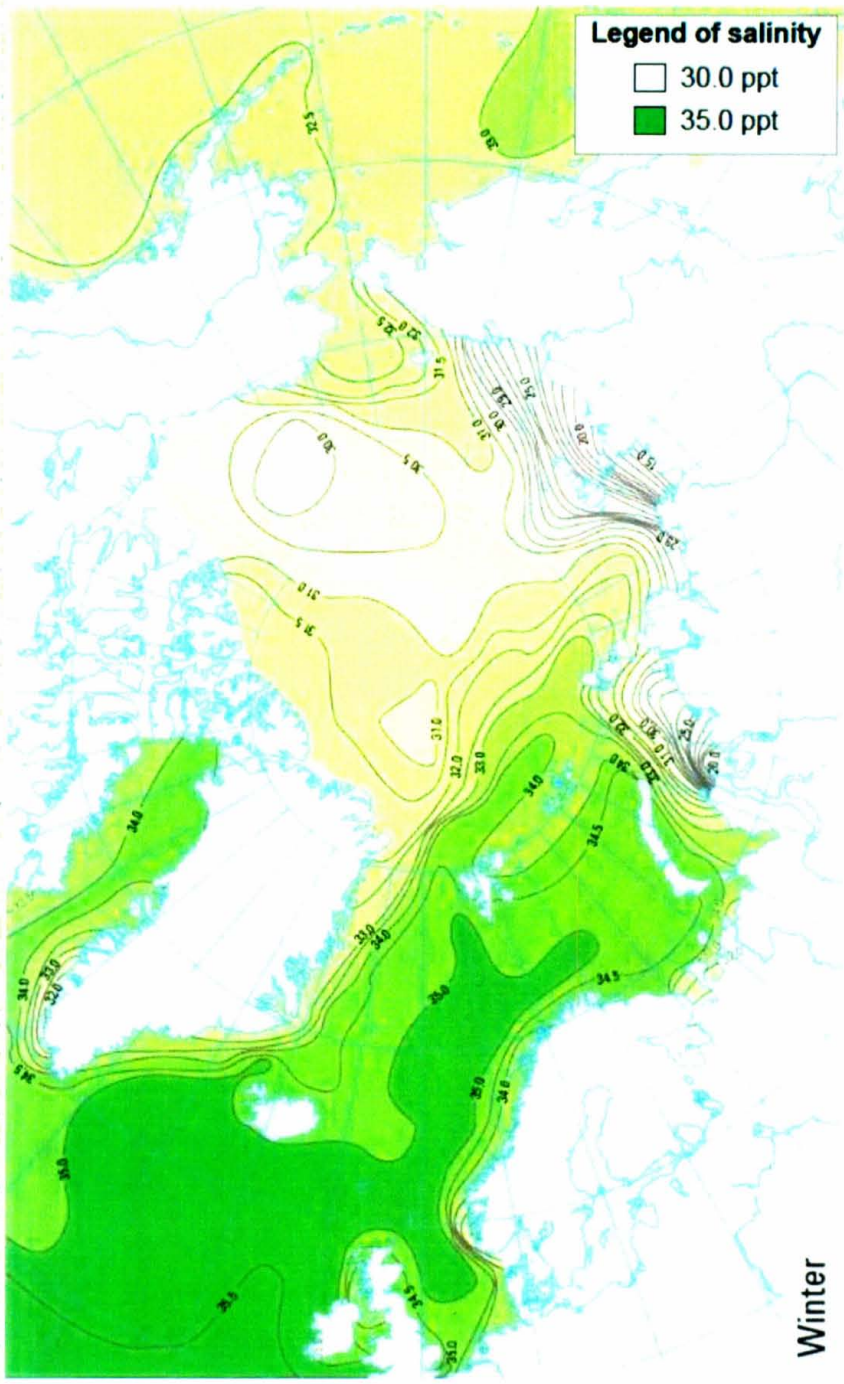

Fig. 1.6. Winter salinities (ppt) of the surface waters of the Arctic Ocean, and Northern Pacific and Atlantic oceans (modified from AMAP, 1998).

The above-mentioned fish taxa are mostly found in the Northern hemisphere. In comparison, the salinity of the water is higher and more stable in Antarctica, as no significant seasonal melting of the ice shelves or freshwater input from rivers occurs around the polar continent (O'Grady & DeVries, 1982). The endemic and fully AFGP-fortified Antarctic notothenioid fishes, thought to have originated *in situ* (Eastman & McCune, 2000), may thus have evolved and strengthened their antifreeze defense by other means. One possible scenario (which may also apply to some Northern hemisphere species) is that they did so in deeper waters, during the progressive cooling of Antarctica (Deng *et al.*, 2010). As the temperature slowly cooled below their non-colligative FP, fish with low amounts of a nascent AF(G)Ps dwelling in waters 0.1 ˚C below their equilibrium FP would theoretically be fully protected, assuming that their AF(G)Ps show enough activity to lower their FP of that 0.1 ˚C. Moreover, if ice does intrude at depths, the crystals would become thermally unstable when brought down through warmer waters (Deng *et al.*, 2010). The oppressive effect of hydrostatic pressure would also make it increasingly difficult for water to stay in its solid phase, as the FP of seawater linearly decreases with depth (~ 0.0076 °C/10 m) (Fujino *et al.*, 1974). Therefore, these intruding crystals would be quite small, and as a consequence, easier to neutralise with low concentrations of a nascent AF(G)P. This is because TH granted by any AF(G)P is inversely proportional to crystal size (Takamichi *et al.*, 2007). As water continues to cool down gradually over a scale covering millions of years, the freeze resistance of a given species could have progressively improved to the point where it could dwell safely in subzero icy surface waters.

The gradual improvement of freeze resistance in fish, be it in brackish or deeper warm water environments, is supported by the finding that the precursor genes in the Zoarcoids (see previous section) only showed modest activity (Deng *et al.*, 2010). Moreover, the ancestral chimeric protease/AFGP gene of the Nothotheinioids shows transcriptional activity, which indicate that these genes are functional (Cheng & Chen, 1999). The resulting proto-AFGP, although not tested for activity, most likely expresses some levels of TH, due to the significant amount of Thr-Ala/Pro-Ala repeats (basis for antifreeze activity) coded by the gene. Finally, it is noteworthy that a fifth type of AF(G)P, originally called type IV AFP, was characterised by Deng *et al.* (1997, 1998). It was found in the plasma of longhorn sculpin (*Myoxocephalus octodecemspinosus*), a fish of the order Scorpaeniformes that synthesises a type I AFP in its skin (Low *et al.*, 2001). In this case, Deng *et al.* (1997) proposed that the precursor gene of the new type IV AFP was one encoding an apolipoprotein, although evolutionary separation from this alleged precursor is not clearly apparent (Gauthier *et al.*, 2008). Then classified as a new AFP, this protein's status was recently revised. Indeed, despite having the potential to develop antifreeze activity (it expresses traces of TH at high concentrations), this protein apparently was not selected for this role, and thus never evolved into a functional AFP (Gauthier *et al.*, 2008). It is possible that, throughout the vast proteome of any given fish, numerous other proteins happen to exhibit traces of TH. These in turn may have the potential to develop antifreeze activity, given the right conditions.

The TH of any proto-AF(G)P, as low as it likely was, could have meant the difference between life and death during a slight undercooling in a fish's environment.

From there, the first step into getting freeze protection in saltier/colder waters would have been to somehow compensate for the initial low activity. An "easy" way to achieve this is to increase the amount of circulating AF(G)P, through the evolution of higher gene dosage. In at least two verified (and two suspected) cases, the evolution of an AF(G)P followed the duplication of an ancestral gene, with modifications accumulating on one of the copies through point mutations, indels, and other mechanisms involved in gene creation, such as atypical splicing and retrotransposition (Ewart *et al.*, 1998; Evans & Fletcher, 2005; Cheng & Chen, 1999; Babushok *et al.*, 2007; Deng *et al.*, 2010). The emergent proto-AF(G)P gene could eventually be duplicated.

In eukaryotic genomes, newly duplicated genes are most often closely located, as direct or inverted repeats. They generally appear as a consequence of mistakes during DNA replication or repair mechanisms, or by meiotic unequal crossing-over, as a result of a non-allelic homologous recombination event between repetitive flanking sequences (Andersson & Hughes, 2009; Hastings *et al.*, 2009). If Darwinian selection favours higher expression of the phenotype, preservation of both copies of a newly duplicated gene will be promoted (Kondrashov & Kondrashov, 2006; Conant & Wolfe, 2008). The resulting doubled expression levels represent a convenient shortcut to the longer trial-and-error mutational process of evolving higher transcription rates (by mutating the promoter region), or improving the original function (by mutating the coding region) on a single gene. More importantly however, a gene tandem becomes the template for higher scale gene amplification, given that positive selection remains poised towards increased

expression of that particular phenotype. The result is the generation of a gene tandem array.

Once an initial AF(G)P gene duplication has spread within a fish population over generations, further gene amplification most often occurs by meiotic unequal crossing-over (Hastings *et al.*, 2009). Another amplification mechanism, called rolling circle replication, can create copies of a gene within extrachromosomal circular DNA, initially excised from its original locus (Cohen & Lavi, 2009). This replication mode can produce several copies of the circular sequence at once, independently of the amount of starting material, and the resulting amplified DNA can eventually be re-integrated at the locus of origin through homologous recombination, or elsewhere in the genome (Cohen & Lavi, 2009). Given that natural selection continues to favour an increased number of AF(G)P gene copies over time, more beneficial crossing-over events (and/or rolling circle replication events) will be accumulated over generations. In the end, the increased gene dosage will lead to a concomitant improvement of the freezing resistance potential of the fish through higher synthesis and levels of circulating AF(G)P. Additionally (and as mentioned above), evolution can also act to modify the phenotype itself, by selecting for individuals bearing new beneficial point mutations within those amplified genes.

One or several such gene duplication-amplification (GDA) events will eventually lead to the creation of multigene families. Examples of well-known and ubiquitous gene families include the ribosomal proteins genes (Hatanaka & Galetti, 2004), and the histone genes (Hentschel & Birnstiel, 1981). Interestingly, they also include the C-lectins (Ewart *et al.*, 2001), the trypsinogen- (or trypsin-) like serine proteases (Barrett & Rawlings,

1995), and the sialic acid synthases (at least two members in fish; Deng *et al.*, 2010), which – as discussed in the previous section – supplied members to the evolutionnary processes that lead to the emergence of AF(G)Ps in teleost fishes.

Mechanisms shaping the diversity of AF(G)P gene multifamilies and role of gene dosage in fish speciation

GDA is in fact a ubiquitous mechanism of rapid adaptation to new environmental challenges (Hastings, 2007; Andersson & Hugues, 2009). However, to say that GDA alone has shaped the diversity observed today within all known AF(G)P multigene families would be erroneous. In fact, these gene families have also experienced episodes of contraction/erosion, which may have taken place during interglacials, when selective pressures for the expression of high levels of circulating AF(G)Ps were relaxed. The present ice-age has seen several glacial events (52 in 2.8 Ma), interrupted by interglacial periods (Gibbard & Cohen, 2008). Concurrently, selective pressures for freeze resistance may have gone through as many cycles of intensification/relaxation. During the glacials, sea ice would have been present at lower latitudes, while interglacials would have been characterised by ice formation occurring only at relatively high latitudes, such as we observe today. As glaciers retreated, some populations of AF(G)P-producing fishes would have moved into the newly re-exposed shallow water habitats, thereby remaining in contact with ice. Populations of others freeze resistant fish, however, may have not changed their geographical distribution, and adapted to the warming conditions as a result. In the absence of sea ice, part of their AF(G)P genes would have been deleted by successive crossing over events (and/or inactivated by mutations), as selection would

have stopped to encourage the maintenance of freeze resistance. After several generations, the resulting offspring would survive with lower AF(G)P gene copy numbers, and form an AF(G)P-deficient population. Given enough time, populations exhibiting differences in their freeze resistance capabilities could undergo speciation.

An example of species that have lost most of their AF(G)P genes due to the absence of Darwinian selection for freeze resistance are the temperate notothenioid fishes derived from the Antarctic-evolved family Nototheniidae. These fish are found in the waters of New Zealand (*Notothenia angustata* and *Notothenia microlepidota*) and South America (*Paranotothenia magellanica*), where ice does not form. They are thought to have moved Northwards following the expansion of the austral glacial fronts over the Southern Ocean (likely during the Late Miocene, ~ 11 Ma ago; Bargelloni *et al.*, 2000; Cheng *et al.*, 2003). These fish have only two to four copies of the AFGP gene, some of which are pseudogenes.

Confronted with the next glaciation, populations/species of secondarily freeze-susceptible fish may be extirpated or go extinct, or regain high AF(G)P gene dosage through the amplification mechanisms described in the previous section. They also could retreat to deeper and/or warmer waters, in order to avoid a threat that they can no longer handle. Several deeper dwelling fish species that belong to clades known to produce AF(G)Ps still possess some of these genes, and express low to negligible TH in their plasma. These include, for instance, several righteye flounder species (Scott *et al.*, 1988b; Goddard and Fletcher, 2002). Another example is the spotted wolffish (*Anarhichas minor*). A member of the suborder Zoarcoidei (type III AFP-producing fishes), this

benthic fish shows low to trace amounts of plasma TH (Desjardins *et al.*, 2006). In comparison, its sister species, the shallower dwelling Atlantic wolffish (*Anarhichas lupus*), produces high plasma levels of the type III AFPs (Desjardins *et al.*, 2006; 2007). As they are closely related, and both occupy distinct thermal ecozones along a depth gradient (one with the risk of freezing, the other without), these bottom-dwelling fishes offer a good opportunity to study the genetic bases for the evolution of differential freeze resistance capacities, and the potential role such differences play in speciation (see objective of study and chapter 2 of this thesis).

Although an obvious way to regain antifreeze protection in the face of a recurrent threat of freezing is to re-amplify part of an AF(G)P multigene family within a fish population, another possible mechanism could involve the acquisition of genes from another, better protected species. The afore-mentioned LGT hypothesis, according to which an AFP gene from herring would have brought freeze resistance to the smelt, is such an example (Graham *et al.*, 2008a; Graham, com. pers.). In the case of closely related taxa however, a more conventional way to increase one species' AF(G)P gene arsenal could be through natural hybridisation with a fully protected species.

Natural hybridisation is now widely recognized as a source of evolutionary novelty (Dowling & Secor, 1997; Arnold, 1992, 1997; Barton, 2001; Mallet, 2005). If the fitness of a hybrid is superior or equal to that of at least one of its parental species, hybridisation represents a potential shortcut for the restoration of freeze resistance in a population of marine teleosts. If the hybrids are fertile and hybridisation does occur frequently enough, the evolution of functional antifreeze protection could happen within

two scenarios: the creation of a distinct "hybrid species" or the introgression of AF(G)P genes from the hybrids back into a population of the unprotected species (Baack & Rieseberg, 2007; Mallet, 2007). In both cases, as generations pass, a stable AF(G)P locus may develop, after both alleles of most genes become stabilised within the population, which leads to an increased gene dosage, and eventually speciation.

Fish are well known for their propensity to hybridise in nature, especially within freshwater habitats, obviously due to their more constrained physical environment (Hubbs, 1955). For instance, introgressive hybridisation between the rapidly evolving cichlid fishes of Lake Malawi (Africa) generated several new species (Genner & Turner, 2012). Indeed, an ancient hybridization event between a shallow-water rocky habitat "Mbuna" species and a fish belongin to a "Shallow-Benthic" soft-sediment feeder group resulted in the evolution and radiation of a third group of species (the so-called Deep-Benthic types), which species adapted to low light habitats. Thus, although the genomic DNA of these later fish was more closely related to that of the shallow benthic feeders, analysis of their mitochondrial DNA revealed that it was inherited from the Mbuna species (Genner & Turner, 2012).

Within the marine realm, Hubbs (1955) reported that most cases of natural hybridisation occur among flatfish species. These fish often share spawning grounds and readily hybridise not only across species, but also across genera. This indicates the existence of relatively weak pre-and post-zygotic barriers to reproduction among these fishes. For example, when artificial fertilisation is practised on flatfishes (for aquaculture purposes), viable hybrids can even be successfully obtained by crossing species belonging

to different families (*e.g. Paralichthys olivaceus* and *Kareius bicoloratus*; You *et al.*, 2009). This indicates a lack of gametic isolation, and apparent absence of post-zygotic barrier as the offspring develops and survives. While pre-zygotic barriers result from temporal, habitat, behavioural, mechanical and gametic isolation, post-zygotic barriers correspond to inviability of the hybrids, their sterility, and low fitness in succeeding generations, which is termed hybrid breakdown (Ellisson & Burton, 2008). The strength of these barriers thus dictates the success of hybridization.

The creation of hybrids has gained in popularity in the field of aquaculture, as the F1-hybrids often exhibit what is called "hybrid vigour" (positive heterosis), and sterility (Bartley *et al.*, 2001), which together results in higher growth rates and lower production costs. It can also allow the creation of animals that combine the best characteristics of each parental species. The generation of such high efficiency stocks motivated several breeding initiatives in aquaculture research & development. For instance viable hybrids of the Atlantic and spotted wolffishes were recently created in Québec (Gaudreault *et al.*, 2009), in order to obtain a fish that displays both the high growth rate of the spotted wolffish in cold water (Moksness, 1994), and the freeze resistance of the Atlantic wolffish (Desjardins *et al.*, 2006; 2007). The choice of these species was motivated by their overall high potential for cold water farming (Le François *et al.*, 2002). During growth trials however, the hybrids only displayed growth performances comparable to those of the spotted wolffish (Gaudreault *et al.*, 2009), and thus no apparent hybrid vigour, although more tests are needed. These fish would be expected to produce plasma AFP at an intermediary level to that of their parental species, assuming that this

phenotype is inherited in a Mendelian fashion. In this context, these fish offer a great opportunity to study the impact of hybridisation on freeze resistance and its genetic components (see objective of study and chapter 3 of the present thesis).

Factors controlling the expression of multigene-based freeze resistance

Fish AF(G)P multigene families can comprise anywhere from two members (in the temperate notothenioid *Notothenia angustata*, suborder Notothenioidei; Cheng *et al.*, 2003) to over a hundred (~150 members in the ocean pout, *Macozoarces americanus*, suborder Zoarcoidei; Hew *et al.*, 1988). In all cases, multiple isoforms (distinct sequences) have been found within a given AF(G)P family, which differ in size and composition/structure (hence in potency). Such isoforms may even show different patterns of expression (in timing and site).

The species in which the physiology and molecular biology of multigene-based antifreeze protection has been most thoroughly studied is the winter flounder (*Pseudopleuronectes americanus*, family Pleuronectidae) (reviewed in Fletcher *et al.*, 2001; Miao *et al.*, 2002), a small flatfish that inhabit the shallow coastal waters of the Northwestern Atlantic (Scott & Scott, 1988). This species produces three main families of type I AFPs isoforms: the so-called liver AFPs (wflAFPs; Davies *et al.*, 1982), skin AFPs (wfsAFPs; Gong *et al.*, 1996), and the thermally labile hyperactive AFPs (Marshall *et al.*, 2004; 2005). In general, the onset of AFP mRNA transcription in winter flounder is associated with the gradual shortening of day length during the fall, while cold temperatures are needed for the accumulation/retention of the AFPs in the plasma

(Fletcher, 1981; Vaisius *et al.*, 1989). Following several experiments spanning a period of a decade (reviewed in Fletcher *et al.*, 1989), it was concluded that the production of growth hormone (GH) by the pituitary during the summer (but not during the winter) inhibits AFP synthesis in the liver of winter flounder through an unknown physiological chain reaction. They proposed a model where the hypothalamus inhibits the production of GH by the pituitary, in response to the shortening of day length. From there, the transcription of the AFP genes proceed. Following their secretion from the liver, the pre-AFPs signal sequences are cleaved off, and the resulting mature AFPs are distributed throughout the organism.

A decade later, Dr. Hew's team added elements to this model. Their research focused on the molecular interactions between the wflAFP genes and some transcription factors occurring within the hepatocytes. They observed that the intron of the wflAFP gene featured an enhancer element (which they named "element B") that binds a liver-enriched transcription factor, called CCAAT/enhancer binding protein (C/EBPα) (Chan *et al.*, 1997; Miao *et al.*, 1998a). This transcription factor was responsible for the liver-specific transcription of the wflAFP genes, and may act in conjunction with a second factor, which was dubbed antifreeze enhancer-binding protein (AEBP) (Chan *et al.*, 1997). Miao *et al.* (2002) proposed that the insulin-like growth factor (IGF-1) could act as an intermediary between GH and wflAFP gene transcription within the hepatocytes. According to their scenario, the absence of circulating GH in the fall would halt the hepatic synthesis of IGF-1. The absence of this factor would in turn activate (or increase

the concentrations) of the above-mentioned transcription factors. However, experimental evidence for such a link is not available as yet.

While the wflAFPs are expressed exclusively in the liver (Gong *et al.*, 1996; Hew *et al.*, 1999), the wfsAFPs seem to be ubiquitously expressed in the fish, with higher expression in the peripheral tissues such as the gill filaments and skin (Gong *et al.*, 1996). Their synthesis also seems less responsive to seasonality than in the wflAFPs (Gong *et al.*, 1995). The different expression patterns of the wflAFP and wfsAFP isoforms appears primarily to result from a variation within the short intronic sequence of their genes. In the wflAFP genes, this sequence (ataatgtttcatcagcactt), corresponds to the afore-mentionned "element B" (Chan *et al.*, 1997) that unlocks the transcription of wflAFP mRNAs exclusively in the liver (Miao *et al.*, 1998a). In the wfsAFP gene, the presence of an additional "TA" (ataatgttt**ta**catcagcactt) within that same intronic sequence (known in this case as element "S") prevents proper binding of the C/EBPα (but not that of the AEBP) to the DNA (Miao *et al.*, 1998b). Consequently, the expression levels of the wfsAFPs are reduced and ubiquitous. Interestingly, another difference between the wflAFP and wfsAFP isoforms is that the latter lack a secretory signal sequence (Gong *et al.*, 1996). Consequently, these AFPs remain intracellular and/or very localy distributed within the skin's interstitial space (Murray *et al.*, 2000; 2002). Obviously, more research is needed in order to fully understand the transcriptional control of this gene, as a supplemental 241bp intronic fragment, exclusive to the wfsAFPs, is also suspected to play a role in the regulation of expression (Miao *et al.*, 1998b). The ubiquitous nature of

the wfsAFPs motivated some authors to hypothesise that they are precursors to the wflAFPs (Gong *et al.*, 1996; Low *et al.*, 2002).

The third known variant of winter flounder AFP, discovered more recently by Marshall *et al.* (2004) was dubbed "hyperactive" AFP. Its discovery resolved a consistent discrepancy between the measured non-equilibrium plasma FPs of the fish and that of their environment (Scott *et al.*, 1988a). This big (~ 16,7 kDa) mainly alpha-helical protein form homodimers (33,4 kDa) and shows activity 10 to 100 times higher than that of the other winter flounder AFP isoforms (Marshall *et al.*, 2005). Interestingly, and unlike other fish AF(G)Ps, is suspected to be able to bind to the basal plane of an ice crystal (Graham *et al.*, 2008b), hence its hyperactivity (Scotter *et al.*, 2006). Such an AFP was found to be the only freeze protectant circulating in American plaice (*Hippoglossoides platessoides*) by Gauthier *et al.* (2005).

From the example of the winter flounder, it appears likely that the control of gene expression within AF(G)P multigene families is a complex process in general. Other than the establishment of a causal link between environmental factors and the timing of AF(G)P appearance in the blood (Fletcher *et al.*, 1985; 1987; Lewis *et al.*, 2004; Desjardins *et al.*, 2006), or the observation of presence/absence of AF(G)P mRNAs in different fish tissues (Gong *et al.*, 1992), not much is known about how AF(G)P expression is regulated in other fish species. In the case of the Atlantic cod (*Gadus morhua*), temperature is known to be the main factor regulating expression of the AFGPs (Fletcher *et al.*, 1987). In type III AFP-producing fishes, photoperiod may be the main trigger, and the temperature the secondary modulator, as these fish seem unable to stop

producing AFP during summer although the levels are significantly lower (Fletcher *et al.*, 1985; Desjardins *et al.*, 2006; 2007; Enevoldsen *et al.*, 2003). The presence of two main classes of type III isoforms (QAE- and SP-types) in the ocean pout (and potentially other species belonging to the suborder Zoarcoidei) justifies the exploration of differential expression among classes of paralogs within a multigene family. Here again, the Atlantic and spotted wolffishes are interesting candidates for such an investigation (see objective of study and chapter 4 of this thesis).

1.2 – Objectives of study

The present study was initiated with the general objective of investigating the molecular bases for interspecific differences and seasonal variation in AFP production in wolffish species. It is known from a previous study that the Atlantic wolffish (AW) produces more plasma AFPs than does the spotted wolffish (SW) (Desjardins *et al.*, 2006), and that the levels of TH in both species show a strong seasonal component, with the highest values reached in winter. The result of this work can be divided into three chapters, presented in manuscript form within the present thesis:

Chapter 2: Antifreeze protein gene amplification facilitated niche exploitation and speciation in wolffish

Evidence from the literature consistently links levels of circulating AF(G)Ps in fish to the degree of environmental severity in term of risk of freezing (Fletcher *et al.*, 1985; Goddard & Fletcher, 2002; Desjardins *et al.*, 2006; Bilyk & DeVries, 2010). Higher

plasma TH has in turn been correlated with higher AF(G)P gene dosage (Scott *et al.*, 1988a; Hew *et al.*, 1988; Cheng *et al.*, 2003). Thus fish that inhabit the shallower waters of the freeze risk ecozone, such as the AW, would be expected to possess more copies of their AF(G)P genes than do species that dwell into deeper waters of the same ecozone, such as the SW. In order to verify if AFP gene dosage may be involved in the interspecific difference in plasma TH previously measured between these two species (Desjardins *et al.*, 2006), Southern blots were performed on wolffish DNA. Levels of AFP transcripts in the liver and other tissues were also compared using Northern blotting, and AFP transcripts (as cDNA) were sequenced and analysed, to verify that the AFP genes from both species are functional. Indeed, some species with lower AF(G)P expression were found to have AF(G)P pseudogenes (Cheng *et al.*, 2003). The results are discussed within the context of climatic change characteristic of the present ice age.

Chapter 3: Effect of interspecific hybridization on freeze resistance capacity in wolffish and its evolutionary significance

The recent generation of AW and SW hybrids from captive experimental aquaculture broodstock offered a great opportunity to investigate how the AFP genes are inherited in members of the family Anarhichadidae, as well as the outcome with respect to freeze resistance. Gene dosage and organisation were compared among the hybrids and their parental species by Southern blotting. Expression of the genes was investigated by Northern blotting. Thus we could account for an effect other than that of a variation in gene copy number impacting AFP mRNA transcription levels. Finally, the levels of

plasma AFP (as TH) were compared among all fish, in order to evaluate the freeze resistance capacity of the hybrids. The results are discussed with an emphasis on hybrid fitness at different points of the glacial-interglacial cycle, *i.e.* when the intensity of selective pressures for the conservation of high AF(G)P gene dosage may change in temperate fish of the freeze-risk ecozone.

Chapter 4: Temporal and spatial expression of type III AFP genes in wolffish species

Contrarily to the notothenioids from Antarctica, which produce high levels of AFGP year-round, temperate species, including AW and SW (Desjardins *et al.*, 2006) show seasonal fluctuations of their plasma AF(G)P levels, with high TH in winter and low or no antifreeze activity during the summer. In wolffish, it is not yet known how transcript levels are responding to environmental cues. Here, Northern blotting was used to see if the AFP mRNA levels reflect the levels of TH measured in the plasma of the AW and SW during winter and summer. Furthermore, as it is known that different isoforms can be subjected to differential control within a given species (Gong *et al.*, 1995), RT-PCR analyses using primers specific to SP- and QAE-type AFP genes were run on cDNA samples from different tissues sampled in summer and winter, in order to see if the two main AFP gene subfamilies are expressed differently within (and between) species, and between seasons. The results were discussed in relation to evolution of paralogs within a mutigene family.

– CHAPTER 2 –

"Antifreeze protein gene amplification facilitated niche exploitation and speciation in wolffish"

2.1 – Introduction

Changing environmental conditions are an important driving force of natural selection, and the mechanisms behind adaptive responses to past climate change can sometimes be deduced by examining extant organisms. Some 34 Ma ago, the onset of the Cenozoic glaciations challenged life on Earth with subzero temperatures (Zachos *et al.*, 2001; DeConto *et al.*, 2008), likely for the first time after a ~ 200 Ma period of relative warm climate (Fletcher *et al.*, 2008; Retallack, 2009). Eventually, glaciations reached sea-level and marine life inhabiting shallow waters, which became ice-laden, had to face the threat of freezing.

Unlike marine invertebrates, which are generally isosmotic with seawater, most fish are hyposmotic and can freeze at a higher temperature than the freezing point (FP) of seawater (~ -1.9 °C). Therefore, in the absence of agents that inhibit freezing, undercooled fish exposed to sea ice rapidly freeze and die (Scholander *et al.*, 1957). Many northern species survive exposure to subzero temperatures below their FP by residing in deeper waters, where they can avoid or reduce the probability of ice contact. Consequently, only species that have evolved a resistance to freezing are able to colonize and exploit the shallower water niches within a "freeze risk ecozone", where the threat of ice contact at subzero temperatures follows a depth and latitudinal gradient.

The most studied freeze resistance mechanism in teleost fish is the production of antifreeze proteins (AFPs). Evidence indicates that the structurally distinct types of AFPs and antifreeze glycoproteins (AFGPs) known to science arose independently in a number of diverse fish species (Scott *et al.*, 1986; Chen *et al.*, 1997a,b; Loewen *et al.*, 1998; Fletcher *et al.*, 2001; Baardness & Davies, 2001; Cheng & Detrich, 2007; Deng *et al.*, 2010; Hobbs *et al.*, 2011). These proteins act by non-colligatively lowering the the FP of physiological fluids below their equilibrium FP by binding to, and halting the growth of nascent ice crystals through an adsorption-inhibition mechanism (Raymond & DeVries, 1977; Wilson *et al.*, 1993; Pertaya *et al.*, 2007; Celik *et al.*, 2010). The thermal hysteresis (TH) that results is a measure of antifreeze activity (Kao *et al.*, 1986), and the temperature a which the plasma of AFP-bearing fish will freeze is termed the non-equilibrium FP.

Adaptation to environmental change can occur by the progressive accumulation of point mutations, which change the sequence and/or expression levels of a gene in a favourable way. However, this process can be very slow in the face of acute environmental stress. In contrast, gene duplication/amplification (GDA) is a more facile and rapid solution to such stress. GDA can foster adaptive evolution in two main ways. First, it can increase expression levels to bolster a useful phenotype (Hurles, 2004). For instance, the physiological challenges imposed by the frigid conditions encountered in the Antarctic marine environment likely led to the duplication of at least 118 different genes in the notothenioid fishes (Chen *et al.*, 2008). Second, it provides extra copies that may be less constrained by selection. These copies can then accrue point mutations and other genetic modifications (Ohno, 1970) which may lead to improved or altered function or

the creation of a new phenotype, through gene sub- or neofunctionalisation (Hurles, 2004). Adaptive changes brought about by GDA have repeatedly been observed in the laboratory and in the field to promote resistance against different types of stressors in an array of unicellular and multicellular organisms (Hastings, 2007). In AFP-producing fishes, GDA appears to be responsible for the emergence and diversification of the diverse AFP multigene families in polar and temperate marine fishes (Hew *et al.*, 1988; Scott *et al.*, 1988a,b; Hayes *et al.*, 1989; Chen *et al.*, 1997a,b; Cheng *et al.*, 2003; Graham *et al.*, 2008a; Deng *et al.*, 2010; Hobbs *et al.*, 2011; Nicodemus-Johnson *et al.*, 2011). Eventually, GDA events within a population could lead to the emergence of new species if the stress is maintained long enough to act as a substrate for natural selection.

The Atlantic wolffish (*Anarhichas lupus* – AW) and the spotted wolffish (*Anarhichas minor* – SW) are two bottom-dwelling species of the family Anarhichadidae (suborder Zoarcoidei) inhabiting the waters of the North Atlantic (Barsukov, 1959; Scott & Scott, 1988). The AW can be found in shallow areas (Keats *et al.*, 1985) during periods when sea ice occurs (Côté, 1989), *i.e.* in the "freeze risk ecozone", whereas the SW is usually found at depths greater than 100 m (Kulka *et al.*, 2004) where the potential for ice contact would be minimal. Both species are known to possess and express AFP genes (Scott *et al.*, 1988b; Shears *et al.*, 1993; Desjardins *et al.*, 2006; Cheng *et al.*, 2006). However, only the AW seems to have sufficient levels of AFP in its plasma to resist freezing in ice-laden seawater (Desjardins *et al.*, 2006). Previous studies have noted that fish that have a higher probability of encountering ice (according to latitude, depth of habitat, and salinity) generally have increased levels of plasma AFPs (Hew *et al.*, 1988;

Scott *et al.*, 1988a; Hayes *et al.*, 1991; Wöhrmann, 1997; Goddard & Fletcher, 2002; Nabeta, 2009; Bilyk & DeVries, 2010). The one known exception is the cunner (*Tautogolabrus adspersus*) (Hobbs *et al.*, 2011).

Wolffishes, and other members of the suborder Zoarcoidei, produce type III AFPs (Wang *et al.*, 1995a,b; Hew *et al.*, 1988; Scott *et al.*, 1988b; Shears *et al.*, 1993; Nishimiya *et al.*, 2005). Interestingly, type III AFP has evolved from the small C-terminal domain of the enzyme sialic acid synthase (SAS) (Baardsnes & Davies, 2001; Deng *et al.*, 2010), which is essential for sugar-substrate manipulation (Gunawan *et al.*, 2005; Reaves *et al.*, 2008). It is postulated that an initial duplication in a hypothetical ancestral zoarcoid generated a copy of the SAS gene that evolved towards improvement of initially weak antifreeze activity (Deng *et al.*, 2010). At some point, the portion of the SAS gene encoding the C-terminal domain became linked to an exon encoding a signal peptide. Further amplification would have facilitated high expression levels of an initially weak AFP, while mutations accumulated to optimize both expression levels and antifreeze activity. This is a clear example of adaptive GDA.

All type III AFP-producing fish species examined so far possess multiple AFP gene copies and/or isoforms (Hew *et al.*, 1988; Scott *et al.*, 1988b; Shears *et al.*, 1993; Nishimiya *et al.*, 2005; Deng *et al.*, 2010). These small, globular ~ 7 kDa AFPs can be roughly subdivided into two groups (SP or QAE), based on their ability to bind to either SP or QAE chromatography resin (Hew *et al.*, 1984). While there is > 75% identity among QAE isoforms and > 90% among SP isoforms, lower identity (~ 55%) is observed between the two groups (Hew *et al.*, 1988). To date, SP and QAE isoforms have been

found in AW and SW, respectively (Scott *et al.*, 1988b; Cheng *et al.*, 2006), as well as in fish of a related family, the Zoarcidae (Hew *et al.*, 1984, 1988; Nishimiya *et al.*, 2005). Some of the differences in amino acid sequence between QAE and SP isoforms translate into structural differences within their compound ice-binding site (Garnham *et al.*, 2010), such that the SP isoforms show greatly reduced affinity for the primary prism plane. Indeed, SP isoforms tested were unable to completely halt ice growth unless a small amount (1%) of the fully active QAE form was also present, which implies cooperative action (Nishimiya *et al.*, 2005; Takamichi *et al.*, 2009). This case illustrates the level of complexity that rapid adaptation to a new challenge can attain through adaptive GDA under the influence of strong positive selective pressures.

The initiation and development of adaptive GDA is more difficult to witness in complex organisms such as vertebrates, as opposed to microbes or lower multicellular life forms, whose rapid generation times allow for complete laboratory studies. In higher animals, evidence for GDA can often only be observed as "snap-shots" from the natural environment, and deduced through genomic sequence and/or gene dosage comparisons among populations experiencing different environmental conditions. As AW and SW are closely related (sister species) (Johnstone *et al.*, 2007; McCusker & Bentzen, 2010) yet inhabit different depth ranges along the sea-floor habitat, they provide a valuable opportunity to explore the effect of adaptive GDA and differential Darwinian selection on the type III AFP multigene family. In the present study, we compared the gene copy number, nucleotide sequences, and mRNA expression levels of the AFPs in a variety of tissues of these two species. The data clearly indicate that the higher freeze resistance

capacity in the more shallow-dwelling AW, as compared with the deeper dwelling SW, is strongly correlated with gene dosage. This agrees well with the role of GDA in enabling fish to adapt rapidly to the threat of freezing, brought on by the onset of glaciation events.

2.2 – Material and Methods

Animals, experimental conditions and tissue sampling

Atlantic wolffish (AW) were reared from two fertilized egg masses collected in Conception Bay (NL, Canada) in 2000, and were part of the same stock as the fish used in an earlier study (Desjardins *et al.*, 2007). Spotted wolffish (SW) were obtained from crosses between wild fish that had been collected in 2003 from the northern part of the Gulf of St. Lawrence on Beaugé Bank (QC, Canada). Both collection sites lie near the 50th parallel, where water temperatures are heavily influenced by the cold Labrador Current (deYoung & Sanderson, 1995; Colbourne, 2004; Galbraith *et al.*, 2010). All fish were hatched and reared at the Centre Aquacole Marin de Grande-Rivière (MAPAQ, QC, Canada) before being transferred to the Institut Maurice-Lamontagne (Mont-Joli, QC, Canada) where they were reared at 9 °C. They were air-lifted from Mont-Joli to the Ocean Sciences Centre (Memorial University of Newfoundland, St. John's, NL, Canada) during October 2006, where they were maintained in separate tanks (2 m x 2 m x 0.3 m) at seasonally ambient temperature and photoperiod (Fletcher, 1977), and fed formulated food *ad libitum* (Ewos, Marine).

Blood and tissue sampling took place in early February, during a time when plasma AFP activity levels typically are at their peak (Desjardins *et al.*, 2006; 2007). Fish

were held in a smaller water tank to which a lethal dose of anesthetic (benzocaine $_{final}$[50 mg/L]) was added to the water. After 10 min, mensurations were taken, blood was sampled from a caudal blood vessel and treated as described by (Desjardins *et al.*, 2006), and the plasma stored in a -70 ˚C freezer. Some aliquots of whole blood were also frozen in liquid nitrogen. The anaesthetized fish were given a sharp blow on the head, to ensure that they were dead, prior to dissection. Tissues (liver, skin, gill filaments, stomach, intestine, heart, white muscle, kidney, head kidney, spleen, and brain) were removed from the fish as rapidly as possible, immediately frozen in liquid nitrogen, and stored at -70 ºC before use.

The guidelines of the Canadian Council on Animal Care were followed during transport and care of experimental animals. All measures were taken to keep pain and discomfort of the fish to a minimum during the blood/tissue samplings.

Thermal hysteresis measurements

Wolffish plasma antifreeze activity was measured as thermal hysteresis (TH, in ºC), which is defined as the difference between plasma melting and freezing temperatures in the presence of ice. Measurements were performed using a Clifton Nanolitre Osmometer (Clifton Technical Physics, Hartford, NY, USA) and by following the procedure of (Evans *et al.*, 2007). The temperatures at which a small crystal will start to melt or grow correspond to the melting point and non-equilibrium FP of the fish's plasma, respectively.

Southern blot analysis

High molecular weight genomic DNA was extracted from frozen liver tissue using the method of (Blin & Stafford, 1976) with modifications (Scott *et al.*, 1985). DNA aliquots (10 µg) were then extensively digested with REs *Eco*RI, *Bam*HI, *Sac*I or *Ase*I (New England BioLabs, Pickering, ON, Canada). Restriction digests were electrophoresed overnight on 0.8% agarose gels (30 V; 1.5 V/cm), and blotted (Southern, 1975) onto (+) Zeta-Probe GT membranes (Bio-Rad, Richmond, CA, U.S.A.) using alkaline capillary transfer accordingly to manufacturer's instructions. All following steps were performed as per Graham *et al.* (2008a). The blots were reprobed with β-tubulin cDNA from chicken (Accession no. **V00389**; from 326 bp to 1423bp) as a loading control.

A first-strand cDNA pool was generated from OP liver mRNA that was reverse transcribed using SuperScript II™ RT and an oligo$(dT)_{20}$ primer (Invitrogen Canada Inc.) as per the manufacturer's protocol. PCR products were generated using primer set 5′ #2 and 3′ #1 (see PCR conditions below, and Table 2.1), subcloned into a pCR®2.1-TOPO® plasmid vector (Invitrogen Canada Inc.) using the TOPO® TA Cloning® Kit (Invitrogen Canada Inc.), and then transformed into Subcloning Efficiency™ DH5α™ Competent Cells (Invitrogen Canada Inc.) as per manufacturer's instructions. Isolated colonies were inoculated into LB growth medium and plasmid DNA was purified using a Qiaprep Spin Mini Prep Kit™ (Qiagen Inc., Mississauga, ON, Canada). Several clones were sequenced (three-fold coverage) at the McGill University and Genome Québec Innovation Centre (Montréal, QC, Canada). A PCR fragment was amplified as above from a clone containing a 385 bp insert corresponding to bases 368 to 453 and 628 to 926 of the

genomic sequence OP3A from OP (Accession no. **J03923**). This fragment was labeled by the random priming method (Invitrogen Canada Inc., Burlington, ON, Canada) in the presence of alpha^{32}P-dCTP. The resulting probe is expected to hybridize to both QAE and SP-type AFP sequences.

Northern blot analysis

Frozen tissue samples from two individuals of each species were homogenized in TRIzol® reagent (Invitrogen Canada Inc., Burlington, ON, Canada), and total RNA isolated according to the manufacturer's instructions. All samples were treated with amplification grade DNase. For each tissue analyzed, 1 μg of total RNA was separated on a denaturing 1.2% agarose gel containing 0.67% formaldehyde. The RNA was then blotted onto a positively charged nylon membrane (Roche Diagnostics, Laval, QC, Canada) using a VacuGene XL Vacuum Blotting System® (Amersham Biosciences, Piscataway, NJ, USA), and cross-linked with UV light.

The cloned 385 bp fragment used above for probing Southern blots was subcloned into the pGEM®-T Easy Vector (Promega Corp., Madison, WI, USA), which has both SP6 and T7 DNA polymerase recognition sites. A non-radioactive DIG-11-dUTP labeled AFP RNA probe was generated by *in vitro* transcription of this construct using the DIG Northern Starter Kit® (Roche Diagnostics Canada).

Pre-hybridization, hybridization, washing and detection procedures were performed according to, and using the reagents recommended by, the protocol provided with the DIG Northern Starter Kit®. Following incubation with CDP-Star®, the

chemiluminescent signals generated by the hybridized probe were recorded digitally by exposure in a light cabinet (G:BOXiChemi), using the image acquisition software GeneSnap 7.02 from Syngene (Frederick, MD, USA). To ensure the quality of RNA, the blots were stripped and reprobed with a DIG-11-dUTP labeled chicken β-tubulin RNA probe (see above section). Atlantic salmon (*Salmo salar*) liver total RNA was used as a negative control.

Cloning of AFP genomic and cDNA sequences

Genomic AFP sequences

AW and SW AFP genes were amplified from genomic DNA by PCR using the primers 5′ #1 and 3′ #1 (see PCR conditions, Table 2.1, and Fig. 2.1). These primers were designed from known sequences (Accession nos. **J03923**, **J03924**, and **M22125**) in order to amplify the complete open reading frame (ORF) of both SP and QAE type III AFP genes. Products were ligated into pCR®2.1-TOPO®, transformed, screened and sequenced as above. At least three-fold coverage was obtained for all sequences. Sequence processing, analysis and comparison were carried out using the CLC Sequence Viewer 6.5.1 software (CLC bio, Katrinebjerg, Denmark).

AFP cDNA sequences

First-strand cDNA was generated from DNase-treated AW and SW total RNA, as described above. One tenth of each RT reaction was then included in PCR reactions using either the primer pair 5′ #2 and 3′ #1 or 5′ #1 and 3′ QAE (see PCR conditions and Table

2.1). Primer 3′ QAE was designed from the genomic sequence AWG1, obtained during this study (Accession no. **JQ040521**). Products were resolved on a 1.25% agarose gel and visualized with ethidium bromide. Individual bands were excised, purified using a QIAquick Gel Extraction Kit™ (Qiagen Inc.), then cloned and sequenced as described above.

PCR conditions

All PCR reactions were performed using an Eppendorf Mastercycler® (Eppendorf Canada, Mississauga, ON, Canada) in the presence of Taq DNA polymerase (Invitrogen Canada Inc.), and sequence-specific primers (Table 2.1). The PCR conditions were as follows:

1) Primer set 5′#1/3′#1 and 5′#2/3′#1: the initial denaturation step was carried out at 95 °C for 2 min, followed by 30 cycles of 95 °C (30 s), 60 °C (30 s), and 72 °C (60 s), ending with a final 72 °C elongation step of 10 min.

2) Primer set 5′#1/3′QAE: conditions were identical to those above with the exception of the annealing temperature, which was 65 °C.

Note: upon manuscript publication, nucleotide sequence data will be available in the GenBank database under the accession nos. AWG1-4, JQ040521, JQ040515, JQ040516, JQ040517; AWE1-3, JQ040522, JQ040523, JQ040524; SWG1-3, JQ040518, JQ040519, JQ040520.

Table 2.1. PCR primers used to amplify AFP sequences from AW and SW and their annealing position on reference sequences. See Fig. 2.1 for the location of the annealing sites of the first three primers, which were designed to amplify both SP and QAE sequences, relative to an SP-type AFP sequence. Primer 3′ QAE is specific for some QAE sequences and was designed based on the sequence of AWG1 after it was cloned using 5′ #1 and 3′ #1.

Primer name	Sequence (5′– 3′)	Ref. seq. Accession nos.	Position
5′ #1	gttaagtcctcccacatactg	**M22125**	270-290
3′ #1	tccggacagacttgggtttgtg	**M22125**	945-924
5′ #2	tctcagccacagccatgaagtc	**M22125**	387-408
3′ QAE	ctacgcatacgttttcaccatc	**JQ040521**	586-565

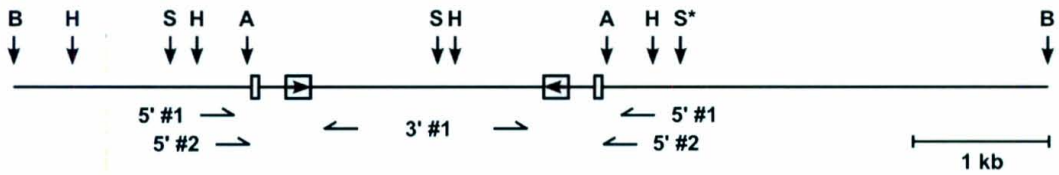

Fig. 2.1. Map of the tail-to-tail inverted AFP gene duplicates from the *Bam*HI clone isolated by (Scott *et al.*, 1988b) from which two *Hin*dIII subclones were sequenced, each bearing one AFP gene (Accession no. **M22125**). The first gene, AW1.9 (referred in the present work as AWir) is in a sense orientation, while the second gene, AW1.5, is in inverted orientation. The sequences of both genes are identical, and their coding sequences are shown as grey blocks, with arrows inside indicating the direction of transcription. Restriction sites for the various REs (B, *Bam*HI; H, *Hin*dIII; S, *Sac*I(*Sst*I); A, *Ase*I) are indicated above the line. The third *Sac*I (*Sst*I) site is polymorphic (indicated by an asterisk), and there are actually three cleavage sites for *Ase*I within a 21 bp region (positions 362-382 and 3033-3013, detail not shown) upstream of each gene. The annealing sites for primer pair 5′ #1 and 3′ #1, and primer 5′ #2 are indicated by arrows underneath the sequence (see Table 2.1 for their precise position on the source sequence). Note: the primers are not to scale.

2.3 – Results

Higher plasma TH activity in the AW compared to the SW is related to gene dosage differences

Representative individuals of the two wolffish species, sampled in February, were tested for their plasma thermal hysteresis (TH) and plasma non-equilibrium freezing point (FP) values (Table 2.2). The two SW individuals had an average TH value of 0.19 °C, which, in combination with colligative solutes, resulted in a FP ~ 1 °C higher than the FP of seawater. In contrast, the two AW individuals had TH values of ~ 1 °C for non-equilibrium FPs of -1.50 °C and -1.85 °C. At this time, the temperature of the ambient seawater in the tanks where the wolffishes were held was still above zero (1.80 °C).

Table 2.2. Mensurations, plasma non-equilibrium freezing points (FP) and thermal hysteresis (TH) values for AW and SW individuals 1 and 2.

	AW					SW			
Fish #	Mass (g)	Length (cm)	FP (°C)	TH (°C)	Fish #	Mass (g)	Length (cm)	FP (°C)	TH (°C)
1	1614	57.8	-1.50	0.82	1	483	36.6	-0.90	0.24
2	1500	56.5	-1.85	1.19	2	946	40.5	-0.82	0.13

There is some uncertainty as to how *in vitro* TH measurements translate into protection from freezing in the wild. For technical reasons and to facilitate comparisons between samples, TH measurements are typically done with a standard cooling rate that is more rapid than would be encountered in the wild, and a large (50 μm) seed ice crystal is

used to start the measurements, both of which tend to lower the absolute TH value (Takamichi *et al.*, 2007). Nevertheless, it is clear that the TH values in the AW are four to five-fold higher than in the SW, using this protocol. As the relationship between TH and [AFP] is hyperbolic rather than linear (Davies & Hew, 1990), the resulting interspecific difference in circulating AFP concentrations is even greater than the TH values suggest.

The above-mentioned results prompted us to examine AFP gene dosage among wolffishes. Southern blot analyses showed that type III AFP genes were present in both species, but that there were many more copies of the genes in AW than in SW (Fig. 2.2). When the DNAs were digested with the restriction endonuclease (RE) *Eco*RI, the differences in the hybridization patterns detected with an AFP probe (AFP) were quite marked. The AW blot showed seven or eight positive fragments ranging from 3 kb to well over 20 kb (Fig. 2.2A). The intensity of the signal for some of the larger fragments suggested the presence of many co-migrating gene copies. In contrast, only four or five positive fragments were observed on the SW blot. These fragments did not comigrate with any AW fragments nor were they particularly intense. The *Bam*HI digests present a quantitatively similar picture (Fig. 2.2A). However, in these digests, the strongest signal, which is indicative of many gene copies, is now found at around 8 kb in AW. In the *Sac*I digests, the average size of the hybridizing bands was lower, and 15 to 17 bands of varying intensity were observed in AW. In comparison, only seven bands were observed for SW. These latter signals were of a more uniform intensity, consistent with the presence of only one or two copies of the type III AFP gene per band.

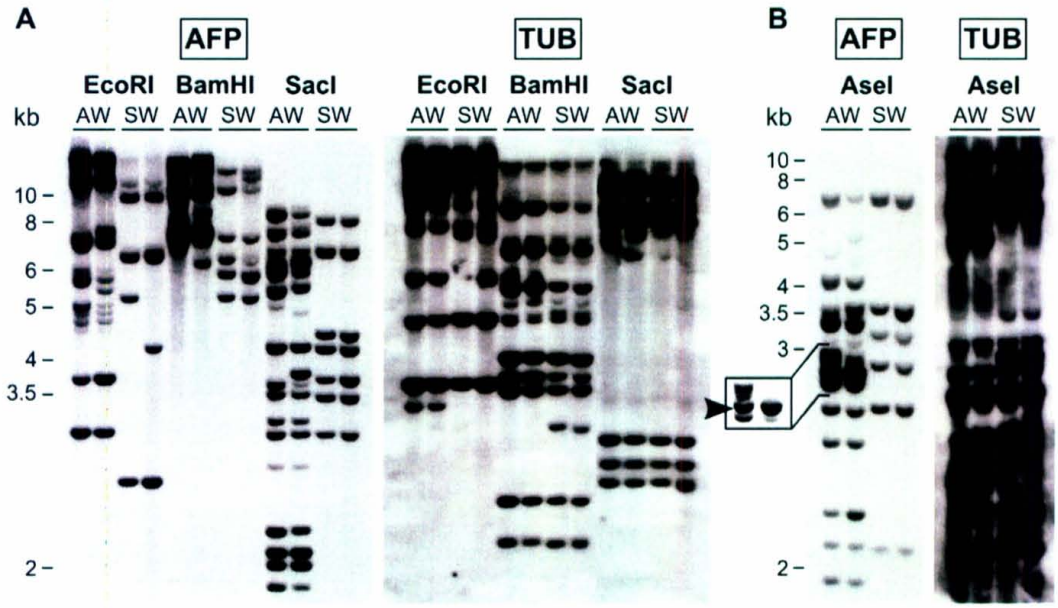

Fig. 2.2. Genomic Southern blots for Atlantic wolffish (AW) and spotted wolffish (SW) individuals 1 and 2 probed for AFP genes. The genomic DNA was digested with REs *Eco*RI, *Bam*HI, *Sac*I (A) and *Ase*I (B). Each blot was probed with a portion of an ocean pout QAE-type AFP gene (AFP) then reprobed with chicken β-tubulin cDNA (TUB) as a loading control. The positions of the DNA size markers are indicated on the left. Individuals 1 and 2 are displayed in their numerical order. A section of the *Ase*I blot corresponding to the signal in the vicinity of 2.6 kb from a shorter exposure is shown on Fig.2.1B, and the putative location of the 2.6 kb band corresponding to the inverted SP-type gene duplicates sequenced by Scott *et al.* (1988b) is indicated by an arrow.

To validate that similar quantities of genomic DNA were originally loaded on the gel, the blot was stripped and reprobed with β-tubulin cDNA (TUB), which is also encoded as a multigene family (Fig. 2.2A). The similar signal intensity for these genes between AW and SW for all three digests indicates that comparable amounts of DNA were loaded in each lane. The banding patterns for TUB were very similar between

species, with only a few restriction fragment length polymorphisms (RFLPs), such as the absence of the 3.3 kb band in the *Eco*RI digest of SW DNA. This is consistent with recent speciation in which there has not been sufficient time for variation to emerge in this particular gene family. In contrast, very few of the AFP gene bands had the same size between the two wolffish species, which suggests that the AFP gene locus has undergone dramatic changes since divergence from a common ancestor was initiated in these wolffishes. A comparison of the overall signal intensity suggests that there are ~ 3 times as many AFP genes in AW than in SW, which is consistent with the higher TH values seen in AW plasma.

Differences in AFP gene copy numbers within a tandem array containing inverted repeats may account for most of the gene dosage differences between AW and SW.

The main difference between the Southern blots of the two species was the presence of intense bands in the AW blot with no equivalent in the SW blot. These strong signals were evident at ~ 8 kb in the *Bam*HI lanes (Fig. 2.2A). This is consistent with previous restriction mapping and partial sequencing done by Scott *et al.* (1988b), which indicated that the genome of AW contains a tandem array of many copies of a repeated unit, which in turn contains two AFP genes in an inverted orientation (Fig. 2.1). These units, flanked by *Bam*HI sites, do not contain any *Eco*RI sites. Consequently, the high molecular weight signals detected in the *Eco*RI lanes likely correspond to DNA fragments containing multiple copies of this iterated unit. The lack of these intense bands in SW suggests that the tandem repeats are missing (or present at only one or a few copies). The *Sac*I digests support this hypothesis (Fig. 2.2A). Indeed, these digests in SW lack the intense signals

found in the vicinity of 2 kb and 6 kb in AW. In the latter species, *Sac*I (*Sst*I) cuts on either side of each AFP gene within the inverted repeats, generating fragments of ~ 2 kb. As one of the restriction sites is polymorphic, ~ 6 kb fragments are generated that contain ~ 4 kb of intergenic sequence (Fig. 2.1) (Scott *et al.*, 1988b). Therefore, these intense AW signals likely correspond to fragments containing a single gene that originate from the tandem array of inverted 8-kb duplicates visible in the *Bam*HI digests.

A smaller proportion of the AFP genes previously mapped from AW also show evidence that they were originally tandemly arrayed. In this locus, however, the genes are irregularly spaced and the inverted repeat unit is absent (Scott *et al.*, 1988b). Since there is a *Sac*I (*Sst*I) site between most of these genes, it is likely that most of the lower-intensity bands visible on the *Sac*I blot originate from that minor gene component and contain but a single copy of the AFP gene (Fig. 2.2A).

Analysis of two tail-to-tail AW genes previously cloned and sequenced by Scott *et al.* (1988b) indicated that *Ase*I cuts 22 bp upstream of the initiation codon, in the highly conserved portion of the 5′ UTR of each gene in the inverted repeat (Fig. 2.1). This would generate fragments of ~ 2.6 kb in length containing the two tail-to-tail genes within each repeat. An intense hybridization signal that accounted for roughly two thirds of the total hybridization was observed at this position for both AW specimens. Only a single, low intensity band was observed in the same region for SW, again suggesting that this unit was not amplified within this species. An expansion of this region showed that there were some differences between the two AW individuals (Fig. 2.2B, detail). Additional differences between individuals of the same species (more so in AW) were observed in most of the digests, suggesting that the AFP loci are polymorphic.

Total liver AFP gene transcript levels are consistent with gene dosage differences

The presence and levels of AFP mRNA transcripts from a number of tissues were determined by Northern blot analyses of both species (Fig. 2.3). AFP gene expression in AW was found to be at its highest in the liver (Fig. 2.3A,B, lane Li), which is the main secretory organ for most plasma proteins in fish (Fletcher *et al.*, 2001). Liver expression was much lower in SW (Fig. 2.3C-D, lane Li) and this was confirmed when the two species were compared on the same blot (Fig. 2.3E, lanes Li). The peripheral tissues (skin, gill filaments, stomach, and intestine) had significant levels of AFP transcripts in both species (Fig. 2.3, lanes Sk, Gi, St, and In, respectively). Very low levels were detected in some of the other tissues, such as the kidney, but solely in AW (Fig. 2.3A,B). The only notable difference between individuals of one species (SW) was in the levels of AFP transcript observed in the intestine. However, this could be a result of sampling differences, as only a small portion from the middle region of each intestine was used. Finally, AFPs were not expressed in blood cells, so the transcripts detected in the various tissues were endogenous and did not arise from the blood. Overall, tissue expression of AFP gene transcripts is higher in the AW than in the SW, particularly in the liver. This is consistent with – although perhaps not exclusively due to – the higher AFP gene dosage observed in the former species (Fig. 2.2).

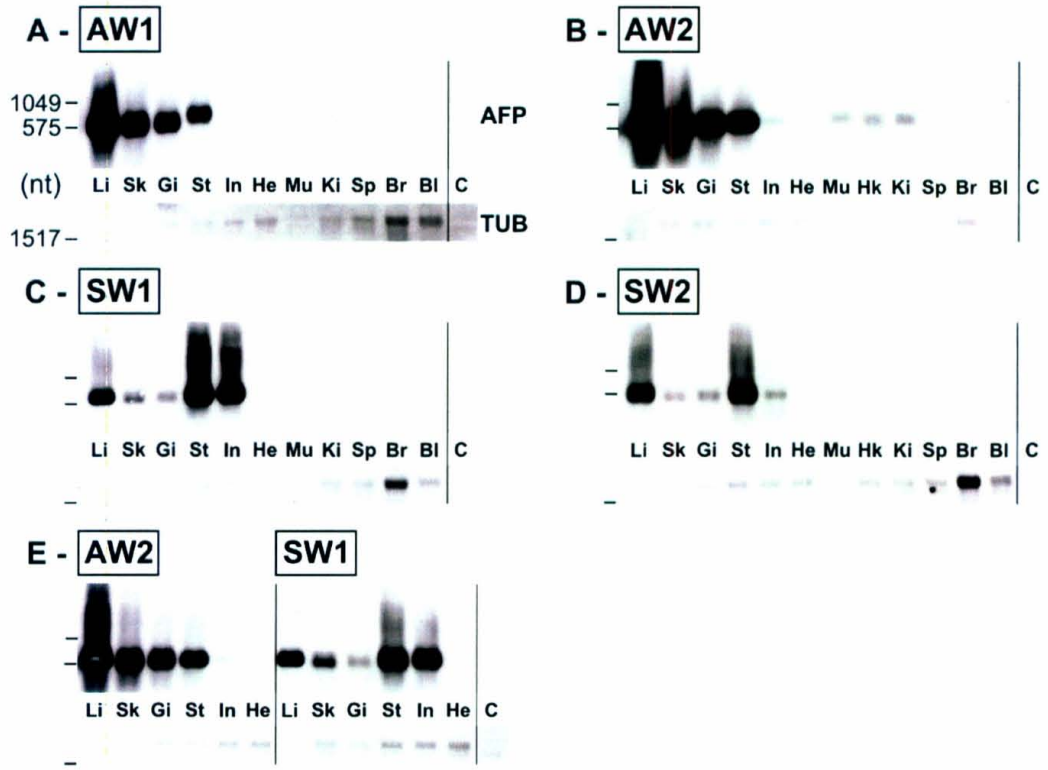

Fig. 2.3. Northern blots of Atlantic wolffish (AW) and spotted wolffish (SW) probed for AFP transcripts. Two AW and two SW individuals were included in the analyses. Blots (A) and (B) correspond to AW individuals 1 and 2, respectively, while the blots (C) and (D) correspond to SW individuals 1 and 2. One fish of each species (individuals AW2 and SW1) were also compared on the same blot (E). The tissues analyzed were liver (Li), skin (Sk), gill filaments (Gi), stomach (St), intestine (In), heart (He), white muscle (Mu), kidney (Ki), head kidney (Hk), spleen (Sp), brain (Br), blood (Bl). Atlantic salmon liver mRNA was included as a negative control (C). Each blot was reprobed with β-tubulin (TUB) mRNA as a loading control. The positions of the RNA size markers (nt) are indicated on the left.

Each blot was reprobed with a β-tubulin mRNA probe to confirm RNA integrity and loading. This control house-keeping gene transcript was detected in all tissues but was particularly abundant in the brain, which is known to be rich in tubulin (Lewis *et al.*, 1985; Parker & Detrich, 1998). The signal appeared faint in AW liver, which may indicate that the relative proportion of this transcript in this fixed amount of mRNA (1 μg) was reduced due to the high concentration of the AFP transcript (Hobbs *et al.*, 2008).

AW and SW AFPs are similar to those of other type III producing fishes

A number of cDNA and genomic clones were obtained by PCR amplification from both wolffish species. The deduced amino acid (aa) sequences are aligned in Fig. 2.4A,B. A total of nine unique sequences were obtained from AW; six SP isoforms and three QAE isoforms. The three unique sequences obtained from SW encode SP isoforms, and two encode QAE isoforms previously reported by Cheng *et al.* (2006).

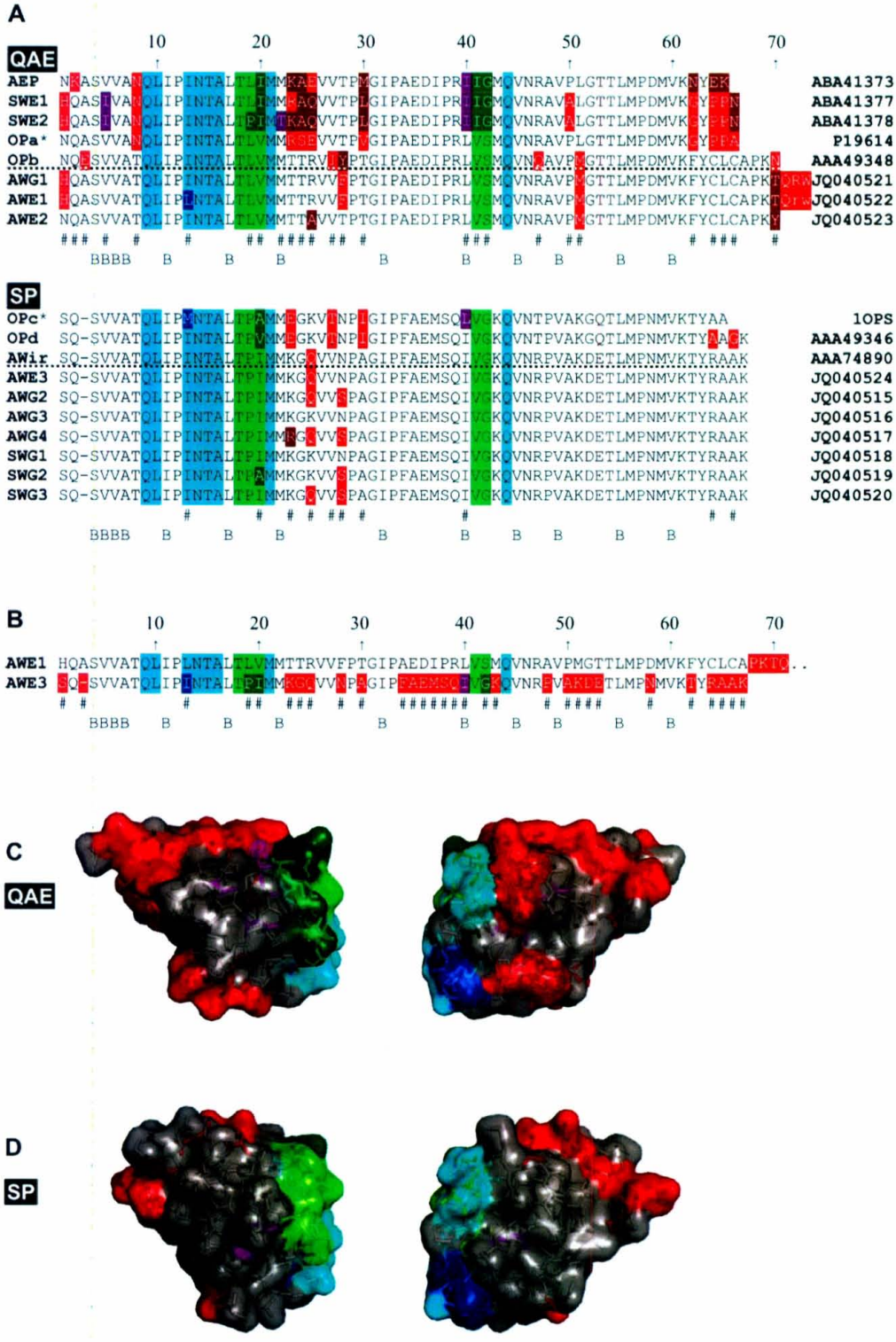
A
QAE
AEP
SWE1
SWE2
OPa*
OPb
AWG1
AWE1
AWE2
ABA41373
ABA41377
ABA41378
P19614
AAA49348
JQ040521
JQ040522
JQ040523
SP
OPc*
OPd
AWir
AWE3
AWG2
AWG3
AWG4
SWG1
SWG2
SWG3
1OPS
AAA49346
AAA74890
JQ040524
JQ040515
JQ040516
JQ040517
JQ040518
JQ040519
JQ040520
B
AWE1
AWE3
C
QAE
D
SP

Fig. 2.4. Comparison of the deduced type III AFPs from Atlantic wolffish (AW) and spotted wolffish (SW) with those from ocean pout (OP) and Antarctic eelpout (AEP) (suborder Zoarcoidei). (A) Alignments of QAE and SP isoforms. Three AW and two SW QAE isoforms are aligned in the top block with two OP sequences; OPa (HPLC-12, for which the structure is known, albeit with the C-terminus changed from YPPA to YAA) and OPb (OP3A, which is most similar to the AW isoforms); and an AEP sequence (RD2, which is most similar to the SW isoforms). Note that the "E" in third position in OPb is not present in the GenBank file but more recent sequencing of cDNAs (including the sequence we used as a probe in this study) strongly suggests that the actual splice acceptor site is three bases upstream of the one originally predicted. SP isoforms from AW and SW are aligned in the bottom block with AWir (AW1.9, encoded by one of the genes within a repeat with an inverted duplication), and two OP sequences; OPc (HPLC-3, for which a structure is known) and OPd (OP5A, which is the most similar to the wolffish isoforms). GenBank, SwissProt or PDB database accession nos. are given at the end of the sequences, and the literature references for each can be found in the text (section 4). Sequences below the dashed line in each block were obtained in this study. Putative ice-binding residues are highlighted in cyan (pyramidal plane binding) and green (primary prism plane binding), according to Garnham *et al.* (2010). Polymorphisms are highlighted in red (with additional differences in dark red), darker blue or green if on the ice-binding surface, or purple if in the core. Only the mature AFP sequences are shown as the signal peptide (MKSAILTGLLFVLLCVDHMSSA) was identical for all the wolffish sequences obtained in this study. The letters G and E in the names of the wolffish sequences indicate whether they were amplified from (G)enomic DNA or (E)xpressed cDNA templates, respectively. The mRNAs were obtained from liver (SWE1, AWE1, AWE3), skin (AWE2) and pancreas (SWE2) (Cheng *et al.*, 2006; this study). Polymorphic sites are indicated below the alignments with "#", and core residues with side-chains that are entirely buried or that show only limited surface exposure in recessed areas are indicated by "B". Asterisks indicate the two sequences with X-ray structures that are shown in (C) and (D). Note: lowercase letters in AWE1 indicate residues that are encoded by the end of the primer used to amplify this sequence. (B) Alignment of representative AW QAE and SP AFPs colored as in (A). Note that only one core residue is altered. (C) Structure of QAE-type OPa (HPLC-12) with residues colored according to their highlighting in (A), with non-highlighted residues in grey. The image on the right was rotated 180° about the Y-axis. The site of the single conservative substitution on the basal plane ice-binding face (I to L in AWE1, position 13) is in dark blue. Visible on the more variable pyramidal plane ice-binding face are the polymorphisms at sites 19, 20, 41 and 42 (in dark green). (D) Structure of SP-type OPc (HPLC-3) with coloring and rotation as above. The single conservative substitution in the core (I to L) is in purple. The I to M substitution on the basal plane ice-binding face is in dark blue. The single polymorphism on the pyramidal plane ice-binding face (position 20) is indicated in dark green. This image was aligned to the QAE isoform, so that the orientation is the same.

The three newly cloned QAE isoforms from AW were compared to the two published QAE sequences from SW (Cheng *et al.*, 2006) and to three more isoforms obtained from other species of the suborder Zoarcoidei (Fig. 2.4A, top). The three sequences we retrieved from AW are over 93% identical (gaps excluded), and share 80-85% identity with the ocean pout (*Macrozoarces americanus*; OP) isoform OPa (HPLC 12) (Hew *et al.*, 1988) for which an X-ray crystal structure has been determined (Jia *et al.*, 1996). However, they show even higher similarity to OPb (OP3A) (Hew et al., 1988), with identities of 90-91%. The two SW isoforms, which are 95% identical, show the highest identity (83-85%) to the AEP (RD2) isoform from Antarctic eelpout (AEP; *Lycodichthys dearborni*) (Wang *et al.*, 1995a). In contrast, the identity between the AW and SW sequences is lower, at just over 70%. This is not unexpected as QAE isoforms are quite variable, especially near the C terminus. They can even be quite different within a species, as indicated by the ocean pout sequences OPa and OPb, which are only 79% identical. Therefore, the lack of higher identities between the QAE isoforms of SW and AW may be a result of sampling bias, as only five sequences are now known.

It is possible to compare both the coding (276 shared bp) and non-coding sequences of OPb and AWG1 as they were cloned from genomic DNA. These two AFPs are 91% identical at the protein level but their coding sequences are 96% identical (data not shown). A significant percentage (67%) of these mutations causes missense changes and the 174 bp long intronic sequences are 96% identical as well. This suggests that the protein sequence may be under selection for mutational improvements while that same selection maintains the amplicon numbers within the AFP gene locus (Bergthorsson *et al.*, 2007). The portions of the 5′ and 3′ UTRs interior to the primer annealing sites are also

very well conserved with 97% and 99% identity, over 110 and 73 bp respectively. Additionally, the 3′ UTR includes an indel of 31 bp.

The SP-type AFP isoforms from both species were compared to AWir (AW1.9), one of the two AFP genes found in the inverted repeat from AW that was cloned and sequenced by (Scott *et al.*, 1988b) (Fig. 2.4A, bottom). Interestingly, AWE3, which was deduced from a liver cDNA sequence, was an exact match to AWir. When all wolffish sequences are compared, there are only four polymorphic sites visible, and the sequences show at most three polymorphisms (> 96% identity). In addition, some of the sequences are identical between the two species (AWG2/SWG3 and AWG3/SWG1). Two OP isoforms are shown for comparison; OPc (OP5A) (Hew *et al.*, 1988) for which an X-ray crystal structure has been determined (Yang *et al.*, 1998), and OPd (HPLC3) (Hew *et al.*, 1988), which shows the highest overall identity (83 to 86%) of all published zoarcoid sequences to the wolffish genes. Note that OPc is based upon a post-translationally modified protein sequence and that cleavage can occur at both termini, which is thought not to affect activity (Hew *et al.*, 1988; Li *et al.*, 1985).

The high similarity between the SP isoforms of the wolffishes extends to their DNA sequences, including the intronic and partial 5′ and 3′ UTR sequences (data not shown). For instance, AWG3/SWG1 are also identical at the nucleotide level, while AWG2/SWG3 differ only at one position in the 181 bp intron and two positions within the 79 bp of the 3′ UTR. Pairwise comparisons between all isoforms of both species indicate that both coding and non-coding regions are over 96% identical.

In wolffishes, the variability among the QAE sequences is higher than among the SP isoforms. This was also observed within other representatives of the Zoarcoidei, the

OP (Hew *et al.*, 1988), and the notched-fin eelpout (*Zoarces elongatus*) (Nishimiya *et al.*, 2005). However, the greatest differences are seen when QAE and SP isoforms are compared. For example, two representative sequences from AW (Fig. 2.4B) share only 56% identity. The best conserved residues are those that are buried and make up the hydrophobic core of the protein. The ice-binding residues are fairly well conserved while there is a great deal of variability in surface residues that are not on the ice-binding surfaces.

AW and SW AFPs are likely functional

To assess the potential effects of sequence polymorphisms on TH activity, the aa variations in the AFP sequences (Fig. 2.4A) were mapped onto the crystal structures of representative QAE (OPa) and SP (OPc) isoforms in figures 2.4C and 2.4D, respectively. There are 26 polymorphic sites in the QAE isoforms shown in Fig. 2.4A, but the last four are found at the highly variable C terminus that was modified for crystallization (Jia *et al.*, 1996; Yang *et al.*, 1998), so they are not considered further. Only three (20%) of the thirteen core residues are polymorphic (Fig. 2.4A&C, in purple), and two of these substitutions are so conservative (V to I at position 5, and L to I at position 40) that they are unlikely to significantly affect the protein fold. The substitution of the smaller T residue for M at position 22 could potentially alter the packing of the core to some degree, but is unlikely to totally disrupt the structure. It may be partially compensated for by the spatially adjacent substitution of the slightly larger I at position 5. In contrast, the surface exposed residues that are not thought to be involved in ice binding are far more

polymorphic (Fig. 2.4A&C, in red and dark red) than the core residues, as there are 14 differences at 37 sites (38% polymorphic). These substitutions are generally less conservative than those observed in the core.

The same general pattern is observed in the polymorphisms found in the SP isoforms. For instance, there is only one core residue polymorphism; the same highly conservative I to L substitution at position 40 as observed in the QAE isoforms (Fig. 2.4A&D, in purple). In fact, this substitution is the only one observed in the core when a SP and QAE isoform from AW are compared (Fig. 2.4B). There are two differences at the C-terminus of the SP isoforms, and five differences among their surface residues located between the putative ice binding sites. Here again, these substitutions tend to be less conservative.

The QAE isoforms have a total of five polymorphisms (Fig. 2.4A&C, in dark green and dark blue) within the 13 residues that are thought to be involved in ice binding. The I to L substitution found at position 13 in AWE1 is not found in any other isoform in the database but is so conservative that it is unlikely to drastically reduce antifreeze activity. All of the other polymorphisms are located among residues that belong to the surface postulated to bind to the prism plane of ice (in green) (Garnham *et al.*, 2010). The V to I substitution at position 41 in the SW sequence has also been observed in AEP (Fig. 2.4A), whereas the other three polymorphisms at positions 19, 20 and 42 are predominant in the SP isoforms from wolffish.

SP isoforms have been shown to have ice-shaping activity but lack thermal hysteresis in the absence of QAE isoforms (Nishimiya *et al.*, 2005; Takamichi *et al.*, 2009). This had been attributed to a lack of prism plane binding (Garnham *et al.*, 2010).

Substitutions at position 20, (shown in dark green, Fig. 2.4A&D) lie on the non-functional prism plane binding site of SP isoforms and should not influence ice-binding activity. The I to M substitution at position 13 (in dark blue) does lie on the functional pyramidal plane binding site (cyan), but is an extremely conservative substitution and has been seen in numerous isoforms from several species (Fig. 2.4A&D and data not shown).

Taken together, the sequences shown in Fig. 2.4A exemplify the remarkable diversity that exists within the AFP gene pool of wolffish (and the AFP III multigene family in general), which is a signature of a highly dynamic locus. The isoforms can be categorized as SP- or QAE-types based on their overall sequence similarities and it is likely that their ice-binding properties would closely correspond to those seen for the QAE and SP isoforms in other species. Thus, none of the polymorphisms that were observed in SW appear strongly deleterious or sufficiently different from those found in other type III AFPs to suggest that they would lead to a drastic loss of activity relative to the isoforms found in AW. Therefore, it is unlikely that point mutations contribute to the reduced freeze-resistance of SW.

2.4 – Discussion

Differences in AFP gene dosage correlate with environment requirements

Following the analysis of a number of genomic and expressed AFP sequences and comparison with sequences in the databases (Fig. 2.4), it was found that Atlantic (AW) and the spotted (SW) wolffish each possess and express both SP and QAE isoforms (Cheng *et al.*, 2006; this study). A combination of these two isoforms is the basis for type

III AFP-mediated freeze resistance (Nishimiya *et al.*, 2005; Takamichi *et al.*, 2009). Moreover, their AFPs appear to be functional, and are likely to be under selection for improved function and maintenance of high dosage in AW. As there are ~ three times more AFP gene copies in AW than in SW (Fig. 2.2), it would appear that gene dosage is largely responsible for the higher plasma TH levels observed in AW. This is in accordance with the differences in risk of freezing faced by the two species, as a result of the depth of their respective habitats. AW are found in the shallow waters of Conception Bay Newfoundland during spring where they can be exposed to sub-zero temperatures in the presence of ice, whereas SW remain in deep water (Keats *et al.*, 1985; Kulka *et al.*, 2004).

According to a modeling study by Svensson & Omsted (1998), small pieces of ice can be driven down to depths of 20 m and more under intense storm conditions. The presence of suspended ice particles, which can nucleate freezing, poses a real threat for fish that are undercooled and lack adequate protection. Indeed, winter kills of fish due to freezing are not uncommon (Green, 1974; Hoag, 2003; Fletcher & Davies, 2012). Considering this, it is likely that SW would freeze to death if exposed to ice, as its average plasma non-equilibrium FP (-0.86 ˚C) is well above that of the surface waters of Conception Bay in April (-1.82 to -1.83 ºC) (deYoung & Sanderson, 1995). AW from Conception Bay were previously shown to have an average non-equilibrium FP of -1.71 (SE ± 0.15 ˚C) during the early spring (Desjardins *et al.*, 2007), which corresponds well with the values obtained in the present study (average of -1.68 ˚C). Although this is slightly higher than the actual freezing temperature of seawater, these values are determined using isolated plasma in artificial conditions (as outlined earlier), which are very likely to underestimate the antifreeze protection conferred to the fish in their natural

environment (Takamichi *et al.*, 2007). Moreover, the fish's freeze resistance is likely further improved by the presence of AFPs in epithelial tissues such as skin, gills and stomach (Fig. 2.3). In fact, the skin itself has been shown to act as an efficient barrier against ice propagation (Valerio *et al.*, 1992).

A positive correlation between the probability of a fish encountering ice and AFP gene dosage is a recurrent observation and has been found to be the main cause for intra- and interspecific differences in levels of circulating AFPs (Hew *et al.*, 1988; Scott *et al.*, 1988a; Hayes *et al.*, 1991; Cheng *et al.*, 2003; Nabeta, 2009; Bilyk *et al.*, 2010). For example, OP from Newfoundland waters were found to have ~ 150 copies of type III AFP genes while those from Southern New Brunswick (where sea ice is less prevalent) had about one fourth this number of genes (Hew *et al.*, 1988). The levels of plasma TH measured in these fish (Fletcher *et al.*, 1985) were consistent with their respective gene dosage. A similar study compared four species of righteye flounders (family Pleuronectidae) that synthesise type I AFPs (Scott *et al.*, 1988a). These fish inhabit inner-shelf waters off the Newfoundland coast. Southern blot analyses revealed that the two species found in the shallowest areas, the winter flounder (*Pseudopleuronectes americanus*) and the smooth flounder (*Pleuronectes putnami*), had the highest AFP gene copy numbers. This was mirrored by plasma high TH levels during winter, which translated into non-equilibrium FPs of -1.7 °C (winter flounder) and -1.8 °C (smooth flounder) (Goddard & Fletcher, 2002). In contrast, the two other species investigated, the yellowtail flounder (*Limanda ferruginea*) and the American plaice (*Hippoglossoides platessoides*), had lower non-equilibrium FPs of -1.1°C (Goddard & Fletcher, 2002). These species are found at depths where they are less likely to encounter ice.

Recent amplification of AFP genes may have led to speciation in wolffishes

AW has been shown to have approximately two thirds of its AFP genes organized as an array of ~ 8 kb direct tandem repeats that each contains two SP genes in inverted orientation, with the remaining genes clustered and irregularly spaced (Scott *et al.*, 1988b). Our Southern blots are consistent with this result (Fig. 2.2A) and the *Ase*I digests allowed us to visualize the tandemly repeated gene copies as a cluster of three to four intense bands of ~ 2.6 kb (Fig. 2.1 & 2.2B). The slight size differences observed among these bands is likely due to variability among the intergenic sequences of these duplicates (see restriction maps in Scott *et al.* (1988b)). In contrast, intensely hybridizing fragments of this size, or any size for that matter, are not present in SW. We estimate that SW has roughly a third of the AFP genes of AW, and that the primary difference between the two species is that these tandem inverted repeats of SP-type genes were not amplified in SW. AW shows much higher levels of AFP mRNA in the liver (Fig. 2.3), suggesting that these tandem genes are largely responsible for the level of transcript observed in that tissue. As the liver is the primary source of circulating AFPs (Fletcher *et al.*, 2001), this amplified gene array could be responsible for the higher plasma TH levels measured in the AW.

Extensively amplified genes are a hallmark of rapid adaptation (Reams & Neidle, 2004; Fondon III & Garner, 2004; Hastings, 2007). Recent GDA is typified by evenly-spaced tandem arrays of virtually identical genes. Over time, these tend to change into clusters of linked but irregularly spaced genes that are more variable in sequence (Graham, 1995). Tandem arrays are highly unstable, as they are hotspots for unequal crossing over during meiotic homologous recombination events (Despons *et al.*, 2011):

consequently, they may vary within a species. This appears to be the case in AW as the lengths of the tandem duplications vary more in one individual than in the other (Fig. 2.2B, detail). While tandem arrays can expand rapidly within a population under strong Darwinian selection for an amplified phenotype, they can also contract quickly when selection is relaxed (Anderson & Hughes, 2009).

In the Northern hemisphere, evidence shows that Cenozoic sea-level glaciations began at least 2.5 Ma ago (Shackleton *et al.*, 1984), although new evidence (Stickley, 2009) and a recent modeling study (DeConto *et al.*, 2008) suggest that sea ice formation started well before that, ~ 20 to 45 Ma earlier. Type III AFP genes have been found in five families within the suborder Zoarcoidei (Shears *et al.*, 1993; Davies *et al.*, 1988), which suggests that the type III emerged before or during family radiation. Moreover, members of two of these families (wolffishes, OP and other eelpouts) have been shown to have genes belonging to the SP and QAE-type AFP gene subfamilies (Hew *et al.*, 1988; Scott *et al.*, 1988b; Cheng *et al.*, 2006; this study). Therefore, it is likely that the type III gene family emerged and diversified several Ma ago in the common ancestor of these taxonomic groups following at least one GDA event. As the initiation of the sea level Cenozoic glaciations is considered the trigger for independent emergence of different AFP types among unrelated fish taxa (Scott *et al.*, 1986), a similar scenario is proposed as the basis for the diversity observed among the type I AFP genes found in flatfishes of the family Pleuronectidae (Scott *et al.*, 1985, 1988a), and the AFGP genes of the notothenioids (suborder Nothothenioidei) (Cheng *et al.*, 2003; Nicodemus-Johnson *et al.*, 2011).

The last few millions years have seen a succession of glacial and interglacial episodes (Oppo *et al.*, 1995) so it is possible that AFP genes families have expanded and contracted several times since their emergence, and in a differential fashion across species. This could have led to greater gene content diversity among the multigene families of a given AFP type than that seen, for example, in other genes for which there is a constant need. This appears to be the case for the type III AFP genes (SP and QAE) in AW and SW. Indeed, RFLPs are rampant within their AFP locus and the main cause of the interspecific difference in gene dosage is the differential amplification of the tandem repeats. In contrast, there are very few RFLPs in the β-tubulin gene families from both species, and they are more similar to each other than are the AFP families. Additional examples of diversity within and among individual AFP III gene families are provided by other species. Both major plasma AFP isoforms of the AEP are of the QAE type (see AEP, Fig. 2.4) and originate from a long tandem array (Wang *et al.*, 1995b; Deng *et al.*, 2010), while the third major isoform in AEP is a fusion of two QAE isoforms (Wang *et al.*, 1995b). SP type AFP genes have not been identified in this zoarcid. This contrasts with the AFP gene organization in AW, where tandemly arrayed SP genes represent the major component of its AFP locus (Scott *et al.*, 1988b). In another zoarcid, the OP, AFP gene organization appears to be quite complex relative to that of wolffish and AEP. In this species, the ~ 150 AFP genes encode both SP and QAE types that are not tandemly arrayed, although restriction mapping of genomic clones shows that some of the genes lie within 2 kb *Hin*dIII fragments that are clustered but unevenly spaced (Hew *et al.*, 1988). The authors concluded that differences between populations, and even between individuals within the same population, could not be explained simply by the expansion

or contraction of particular repeats and suggested that multiple rounds of contraction and expansion must have occurred. In AW, evidence of an older amplification event (possibly two) can be observed within the cluster of unevenly spaced AFP genes which correspond to the minor component of the AFP gene pool that was mapped by Scott *et al.* (1988b). Indeed, some of the genes were detected within fragments flanked by similar patterns of restriction sites. In contrast, the tandem arrays of SP-type genes in AW genes would be a more recent event, as suggested by their evenly spaced distribution within the array mapped by Scott *et al.* (1988b).

According to recent phylogenetic analyses of mtDNA, AW and SW are sister species that diverged approximately ~ 1 Ma ago (McCusker & Bentzen, 2010). Their close phylogenic relationship is supported by our recovery of identical SP-type AFP gene sequences (AWG3 and SWG1; Fig. 2.4A), with others being highly similar. Interestingly, the estimated divergence time of AW and SW falls within a period when the North hemisphere glaciation cycles were at their highest intensity (Gibbard & Kolfschoten, 2004), with a new glacial event taking place at ~ 0.1 Ma intervals over the last Ma (Augustin, 2004). The ebb and flow of glaciers would have resulted in the cyclic emergence and disappearance of depth stratified environments, where ice would be more prevalent in the shallower regions. During each retreat of the glacier fronts, shallow water habitats offering low competition for resources and low predation on the young would become available for colonization by freeze resistant species such as the AW. In contrast, the ancestor to SW would have remained in deeper waters, away from the "freeze risk ecozone". Therefore it is reasonable to suggest that a major contributing factor that led to the evolution of the more freeze resistant AW and its occupation of shallower habitats

would have been the gene amplification events that generated the tandemly arrayed AFP genes found in this species today. Speciation likely occurred in parapatry, as the distribution range of these two species still overlap today (Scott & Scott, 1988).

2.5 – Conclusion

From the evidence collected within the present study, we propose that parapatric speciation – based on habitat depth and risk of freezing associated with these depths – between the AW and SW has been facilitated if not triggered by the extensive re-amplification of a portion of the AFP multigene family in the genome of the AW, which resulted in higher plasma AFP levels and lower plasma non-equlibrium FP in that species, compared with the SW. Among members of the suborder Zoarcoidae, the observation of significant gene dosage variation between and within species (Hew *et al.*, 1988; Shears *et al.*, 1993; Deng *et al.*, 2010; this study) attests to the plasticity of the type III AFP gene loci, and the rapidity of the molecular adjustments as selective pressures promoting freeze resistance played out in these populations. The observation of important gene dosage plasticity in other AFP multifamilies (Cheng *et al.*, 2003; Scott *et al.*, 1988a), and the likely universal occurrence of tandem arrays of AFP and AFGP genes within the locus (Scott *et al.*, 1988a,b; Deng *et al.*, 2010; Nicodemus-Johnson *et al.*, 2011) extends the possible role of AFP GDA in speciation of AFP-bearing fish in general, during the succession of glacials/interglacials that punctuated the climate since the onset of the Cenozoic glaciations.

– CHAPTER 3 –

" Effect of interspecific hybridization on freeze resistance capacity in wolffish and its evolutionary significance "

3.1 – Introduction

The Atlantic and spotted wolffish (*Anarhichas lupus*, AW; *A. minor*, SW; family Anarhichadidae; suborder Zoarcoidei; Nelson, 2006) are marine fishes characteristic of the demersal species assemblage of the North Atlantic continental shelves and slopes (Barsukov, 1959; Scott & Scott, 1988; Kulka & DeBlois, 1996). Predominantly benthophagic, they feed on echinoderms, mollusks, crustaceans and fish (Albikovskaya, 1983; Scott & Scott, 1988). During the past century, the populations of AW and SW declined heavily in eastern Canadian waters, possibly due to the combined repercussions of fishery activities (by-catch and habitat disruption) (O'Dea & Haedrich, 2000), and anomalous temperature variations since the 1970s (Colbourne *et al.*, 2004; Kulka *et al.*, 2004). The two species are now protected under COSEWIC's (Committee on the Status of Endangered Wildlife in Canada) Species At Risk Act (SARA), and recently became the focus of a recovery/management plan (Kulka *et al.*, 2007).

AW and SW are both found at a wide depth range. In the waters of Newfoundland and Labrador, AW can be encountered from 5 to 350 m (sometimes deeper), while SW is found mainly from 100 to 600 m (Albikovskaya, 1982; Keats *et al.*, 1985; Scott & Scott, 1988; Kulka *et al.*, 2004). AW faces the threat of freezing in the shallowest part of its depth range, which can be considered part of a "freeze risk" ecozone (see Chapter 2).

Consequently, this species produces high levels of plasma antifreeze proteins (AFPs) (Desjardins *et al.*, 2006; 2007). Contrary to the other solutes, these proteins act in a non-colligative manner, and can lower the freezing point (FP) of the fish's hyposmotic body fluids by a full degree Celcius (for a review, see Fletcher *et al.*, 2001). They do so by adsorbing to nascent ice crystals, thus halting their growth (Raymond & DeVries, 1977; Pertaya *et al.*, 2007). The fish can then contact and absorb ice without freezing to death, which is invariably the outcome in undercooled animals (Scholander *et al.*, 1957). As ice does not normally penetrate at great depths in the temperate oceans, the closely related SW is thought to be at very little risk of freezing. Consequently, this species has low levels of plasma AFPs, which do not confer freeze resistance (Desjardins *et al.*, 2006). A recent study revealed that the lower levels of circulating AFPs in SW were due to a correspondingly lower AFP gene dosage in the former species, compared with AW (see Chapter 2). More precisely, SW has roughly three times fewer AFP gene copies than in AW. A relationship between AFP gene dosage and freeze tolerance capacity has been consistently observed among AFP-producing fishes (Goddard & Fletcher, 2002; Cheng *et al.*, 2003).

AFP genes are typically organized as multigene families (Fletcher *et al.*, 2001; Cheng *et al.*, 2003). These families likely arose independently in founders of the contemporary fish clades, in response to the new threat of freezing brought forth by the sea-level Cenozoic glaciations (Scott *et al.*, 1986). The family of AFP genes found in wolffishes (which encode the so-called type III AFPs) is shared by other fish of the suborder Zoarcoidei, and varies significantly in terms of gene numbers, organization and composition among – and even within – species (Hew *et al.*, 1988; Scott *et al.*, 1988b;

Shears *et al.*, 1993; Nishimiya *et al.*, 2005, Deng *et al.*, 2010). Such variation is also observed in the type I AFP gene family of the Northern hemisphere righteye flounders (family Pleuronectidae) (Scott *et al.*, 1988a; Hayes *et al.*, 1991; Nabeta, 2009), and the antifreeze glycoprotein (AFGP) gene family of the Southern hemisphere notothenioids (suborder Notothenioidei) (Cheng *et al.*, 2003). As the strength of the selective forces promoting freeze resistance likely varied according to the numerous glacial/interglacials (~ 52) that punctuated the Quaternary (Gibbard & Cohen, 2008), the heterogeneity within these different multigene families is likely a product of alternating rounds of strong and relaxed natural selection for this trait in the different fish species (sharing a given AFP gene pool). Thus we can argue that differential amplification-deletion of AFP genes may have promoted speciation among AFP-producing fishes in response to the ever changing climate that characterizes the Quaternary Period (see Chapter 2). This may also explain why some fish found in ice-free environments (such as the SW) still produce AF(G)Ps without an apparent need for them.

Extensive amplification of pre-existing genes is often interpreted as a rapid adaptive response to environmental stressors (Hastings, 2007); in this case, the recurrent threat of freezing faced by fishes over a geologic time scale. It can also foster the emergence of new species (Ohno, 1970; Zang, 2003; Gu *et al.*, 2005). Interestingly, two thirds of the AFP gene pool in AW are contained within a large tandem array (Scott *et al.*, 1988b) that appears mainly responsible for the gene dosage difference with SW (see Chapter 2). If it is instrumental in wolffish speciation, this amplification event has to be very recent, as McCusker & Bentzen (2010) evaluated that AW and SW diverged from a

common ancestor ~ one Ma ago. This divergence time coincides with the height of the North Atlantic Pleistocene glaciations (Gibbard & Kolfschoten, 2004).

The synthesis of higher AFP levels in a wolffish subpopulation may have given rise to a new Evolutionarily Significant Unit (ESU) that could dwell in the presence of ice and re-colonize shallower inshore habitats during the retreat of glaciers. However, newly emerged ESUs or species often show incomplete reproductive isolation, and environmental disturbances (natural or anthropogenic) can increase the probabilities for these nascent species to hybridize (Hubbs, 1955; Lamont *et al.*, 2003; Mallet, 2005; Fisher *et al.*, 2006). Natural hybridization occurs more frequently among fish than in any other vertebrate group (Allendorf *et al.*, 2001). Among marine fishes, most cases of interspecific (and even intergeneric) hybridization have been reported among flatfishes, whose benthic habitats and spawning grounds often heavily overlap (Hubbs, 1955). Interestingly, despite their partially overlapping distribution range and close genetic relatedness, no hybrid of the AW and SW has been formally characterised in the wild (Templeman, 1986c; Imsland *et al.*, 2008; McCusker *et al.*, 2008). Robust pre-zygotic barriers thus seem to prevail between AW and SW, and not enough is known of wolffish biology to understand their basis. What is known, however, is that bypassing this initial barrier through artificial fertilization results in healthy (Gaudreau *et al.*, 2009) and fertile offspring (Savoie, pers. com.). Hence, the maintenance of the wolffish lineages in nature seems to be mainly achieved through pre-zygotic barriers (*e.g.* temporal, habitat, and behavioral isolation), which in turn may be preserved by the need of the AW to maintain high AFP gene copy numbers.

The present work aimed to evaluate and compare the freeze resistance capacities among F1 AW/SW hybrids and their parental species, based on AFP gene dosage and organization, AFP mRNA expression, and the actual levels of antifreeze protection afforded by the circulating plasma AFPs. The evolutionary significance of wolffish hybridization in an ever changing environment will be discussed, with consideration of anthropogenic disturbance and climate change.

3.2 – Materials and Methods

Experimental animals, conditioning and sampling

The animals used in the present study were all produced in captivity, from broodstock harvested in the Northeastern part of the St. Lawrence Gulf (Beaugé Bank and East Anticosti shelf areas, Québec, Canada). A family of purebred Atlantic wolffish (*Anarhichas lupus*; AW) and two families of reciprocal hybrids of Atlantic and spotted wolffish (*A. minor*; SW) were used. These fish, weighing an average of 47.5 ±16.6 g at the onset of the experiment, were produced and hatched during the winter of 2007 at the Centre Aquacole Marin de Grand-Rivière (CAMGR, MAPAQ, QC, Canada). The ♀*lupus* x ♂*minor* hybrids (family AH2) were derived from the same female used to produce the family of purebred AW (family A1). A different male was used to produce each of these two families, while the ♀*minor* x ♂*lupus* hybrids (family SH3) were issued from a fertilization event involving the semen of several males (Information about these families is presented in Table 3.1).

Ten fish from each of the three families were transferred to separate tanks. The animals, then of age 1^{+}, were exposed to seasonally ambient temperature and photoperiod (Fletcher, 1977) from mid-March to mid-April 2008, while fed formulated food (Gemma Skretting Canada, NB) *ad libitum*. At the end of this period, a preliminary evaluation of the plasma AFP levels was conducted for all fish. The results (see Thermal hysteresis measurements) prompted us to initiate a longer exposure trial, using new fish from the same above-mentioned families of purebred AW and reciprocal hybrids (A1: n = 10; AH2: n = 10; SH3: n = 11). The animals were exposed to seasonally ambient conditions starting on October 21st 2008. Five adult purebred SW derived from Québécois (family SQ) and Norwegian (family SN) broodstocks in 2003 were also included in the experiment, to allow for comparison (small SW contemporary to the AW and hybrid wolffish juveniles were not available).

Table 3.1. Breakdown (by family, breed and genitor) of the origin of the wolffish used in this study.

Family	Breed	Genitor provenance	Notes about genitors
A1	Atlantic wolffish (*A. lupus*; AW)	Québec (Canada)	Same ♀ as AH2
AH2	Hybrid (♀*lupus* x ♂*minor*)	Québec (Canada)	Same ♀ as A1
SH3	Hybrid (♀*minor* x ♂*lupus*)	Québec (Canada)	Several ♂ used
SQ	Spotted wolffish (*A. minor*; SW)	Québec (Canada)	---
SN	Spotted wolffish (*A. minor*; SW)	Norway	---

Blood sampling was conducted on February 20th (2009) for all fish, at a time when plasma AFP activity levels were shown to be at their peak (Desjardins *et al.*, 2006; 2007).

At that time, the purebred AW and the hybrids were of age 2^{+}, while the adult SW where of age 6^{+}. Fish were anesthetized for 10 min in a tub containing 50 mg/L $_{final}$[benzocaine]. Blood was obtained from a caudal blood vessel using 1 ml heparinized syringe, transferred to heparinized 1.5 ml microcentrifuge tubes and centrifuged to remove the blood cells (4000 g, 10 min, 4 °C). Plasma samples were stored at -70 °C until analysis. After sampling, the fish were given time to recuperate in oxygenated tubs before being replaced in their respective experimental tanks. Following the measurement of antifreeze activity (thermal hysteresis; TH) in the plasma (see Results section), the two best AFP producers in each family were selected for tissue and blood sampling, which occurred in April, when AFPs are still present in the bloodstream at high concentrations (Desjardins *et al.*, 2006; 2007). Fish were anaesthetised and blood sampled as described above. The animals were then killed by a sharp blow to the head. Tissues (liver, skin, and gill filaments) were removed from the fish as rapidly as possible, immediately frozen in liquid nitrogen, and stored at -70 °C before use in Northern blotting analyses. Extra liver pieces were flash-frozen for use in Southern blotting analyses.

The guidelines of the Canadian Council on Animal Care were followed during the experiments, and all measures were taken to keep pain and discomfort of the fish to a minimum during the blood/tissue samplings.

Thermal hysteresis measurements

Plasma antifreeze activity was measured as TH using a Clifton Nanolitre Osmometer (Clifton Technical Physics, Hartford, NY, USA) following the procedure of *Evans et al.*,

(2007). TH is defined as the difference between the melting and freezing temperatures (°C) of a test solution, and is representative of the AFP concentrations present in that solution, according to a non-linear relationship (Davies *et al.*, 1990). All TH results were expressed as mean ± SD. Statistical analyses (Generalized Linear Model and Mann-Whitney tests) were conducted on the TH data using SigmaPlot 10 (Systat Software Inc.).

At the end of the preliminary trial, the reciprocal hybrids showed similar values of plasma antifreeze activity (TH: 0.24 ± 0.04 °C for family SH3, and 0.27 ± 0.04 °C for family AH2). These values, while lower than those obtained in the AW family (0.43 ± 0.11 °C), were still superior to average winter levels previously recorded in purebred SW (0.10 °C; see Desjardins *et al.*, 2006). This motivated longer trails to be undertaken (see Resuslt section)

Northern blot analysis

A nonradioactive method was used to detect AW and hybrid wolffish AFP mRNAs from liver, skin and gill tissues on Northern blots, using a 385 bp DIG-11-dUTP-labeled QAE-type-AFP mRNA probe (Roche Diagnostics, Laval, QC, Canada). This probe hybridizes to both QAE and SP-type AFP genomic sequences (please see Chapter 2 for details related to the template and probe generation methods).

Total RNA was extracted from frozen tissues and prepared as outlined in Chapter 2. Briefly, 1 µg of total RNA aliquots were separated on a denaturing 1.2 % agarose gel containing 0.67 % formaldehyde. The RNA was then blotted onto a positively charged

nylon membrane (Roche Diagnostics) using a VacuGene XL Vacuum Blotting System® (Amersham Biosciences, Piscataway, NJ, USA), and cross-linked with UV light.

Membrane pre-hybridization, hybridization, stringency washes, blocking and detection procedures were performed as described in Chapter 2 using the kit and reagents supplied by Roche Diagnostics, according to the recommendations by the DIG Application Manual for Filter Hybridization (https://www.roche-applied-science.com/publications/print_mat/dig_application_manual.pdf). The chemiluminescent signals generated by the hybridized probe were imprinted on a Lumi-Film® Chemiluminescent Detection Film (Roche Diagnostics). Atlantic salmon (*Salmo salar*) total mRNA was used as a negative control, as this species does not have AFP genes.

Southern blot analysis

The DIG-based method was also used to detect AW and hybrid wolffish AFP genes on Southern blots. The DIG-11-dUTP-labeled AFP DNA probe was generated from the same template used to produce the mRNA probe, using the PCR DIG Probe Synthesis Kit® supplied by Roche Diagnostics and specific primers (primers 5′#2 and 3′#1; Chapter 2).

High molecular weight wolffish genomic DNA samples were obtained and prepared as described in Chapter 2. DNA aliquots (AW and the hybrids:10 µg; SW: 20 µg, for ease of visualisation) were digested with restriction endonuclease (RE) *Ase*I (New England BioLabs, Pickering, ON, Canada). The choice of this particular RE was deemed the most relevant for interspecific comparison of AFP gene organization and dosage among wolffishes based on the results obtained in our previous study (see Chapter 2). The

use of this RE allows AFP genes to be separated on relatively small DNA fragments, and precisely isolates a tandemly arrayed inverted AFP gene duplication that was amplified in AW, but not in SW (Scott *et al.*, 1988b; see Chapter 2).

Restriction digests were electrophoresed overnight on 0.8 % agarose gels (30 V; 1.5 V/cm), and blotted the following day and overnight (Southern, 1975) onto a positively charged nylon membrane (Roche Diagnostics, Laval, QC, Canada), accordingly to manufacturer's instructions. The nylon membranes were then pre-hybridized and hybridized in Easy Hyb hybridization solution at 46 °C. The hybridization step was conducted overnight in the presence of the above-mentioned DIG-11-dUTP-labeled 385 bp QAE-type III AFP probe.

Following hybridization, the membranes were subjected to washes of progressively increasing stringency, as recommended in the DIG Application Manual for Filter Hybridization. Following blocking and successive incubation with alkaline phosphatase-bearing DIG antibody and chemiluminescent CSPD alkaline phosphatase substrate, membranes were exposed to Lumi-Film® Chemiluminescent Detection Film (Roche Diagnostics) for signal recording.

Genotyping

Small aliquots of genomic DNA (working concentration: 50 ng/ul) were analyzed to ascertain the hybrid or purebred status of the experimental animals. Two microsatellite markers (loci: Alu27 and Alu28) were chosen for genotyping. These markers, among others, were developed by McCusker *et al.* (2008) and have proven reliable for

identification of wolffish species and hybrids (Gaudreau *et al.*, 2009). This is possible because these microsatellites are inherited in a Mendelian way, with hybrid offspring getting one distinct allele from each parent (Gaudreau *et al.*, 2009), whilst the alleles have distinct size ranges for each species (McCusker *et al.*, 2008).

The choice of markers used in the present study (Alu27 and Alu28) was motivated by the significant interspecific size difference observed by McCusker *et al.* (2008) between the AW and SW amplicons for these loci, with SW being homozygous for both loci. We thus assumed that these two markers would be the most sensible choice for an easy and rapid diagnostic at minor costs. According to the method of McCusker *et al.* (2008), we used fluorescently-labeled primers (Bio-Rad, Mississauga, ON, Canada) in PCRs to amplify these genetic markers in all our fish, and visualized them by capillary electrophoresis in an AB 3730 48-capillary DNA Analyzer (Applied Biosystems, Burlington, ON, Canada). The results are displayed in Table 3.2. While the purebred AW (fish 1 & 2) and SW (fish 7 to 10) show values that fall in the predicted size ranges, fish 3 to 6 display a mixture of sizes belonging to both species, thereby confirming their status as hybrids.

Table 3.2. Observed amplicon sizes (bp) for the two microsatellite loci (Alu27 and Alu28) analyzed in purebred AW and SW and their reciprocal hybrids. In AW, the expected sizes were of 207-283 bp for Alu27 and of 93-253 bp for Alu28. In SW, sizes of 171 bp for Alu27 and of 123 bp for Alu28 were anticipated. The hybrids were expected to show a size combination of the above, due to the previously observed pattern of inheritance (Gaudreau *et al.*, 2009). It is noteworthy that the fluorescent label attached to each primer increases the observed size of the amplicons by 5 to 6 bp (so that Alu27 in SW has a real size of 171 bp and not 177 bp, and so on).

Fish #	**Alu27** (blue dye)		**Alu28** (green dye)		**Breed**	**Family**
1	237	237	232	240	AW	A1
2	237	281	232	240	AW	A1
3	177	281	128	224	Hybrid	AH2
4	177	281	128	224	Hybrid	AH2
5	177	237	128	236	Hybrid	SH3
6	177	237	128	220	Hybrid	SH3
7	177	177	128	128	SW	SQ
8	177	177	128	128	SW	SQ
9	177	177	128	128	SW	SN
10	177	177	128	128	SW	SN

3.3 – Results

Intermediate average plasma TH levels in wolffish hybrids are mainly due to intermediate gene dosage and expression

On average, the wolffish hybrids had plasma antifreeze activity levels (expressed here as thermal hysteresis - TH) somewhat intermediate between those of the purebred AW and SW. Following a four-month exposure to seasonally ambient temperature and photoperiod, the reciprocal juvenile hybrids (♀*lupus* x ♂*minor* – family AH2, and

♀*minor* x ♂*lupus* – family SH3) showed mean February TH values (0.42 °C) that were significantly higher ($p < 0.01$) than in the of the adult SW (0.20 °C), but significantly lower ($p < 0.01$) than in the AW (0.66 °C) (Fig. 3.1). These TH levels led to virtually identical mean non-equilibrium FPs in the hybrids (-1.05 and -1.08 °C). In comparison, mean non-equilibrium FPs in the SW and AW were -0.85 and -1.33 °C, respectively (Fig. 3.1).

The observed differences in TH averages among wolffish families were paralleled by differences in AFP gene dosage, with the hybrids showing a number of genes and a banding pattern on the *Ase*I Southern blot that appeared intermediate to those of both parental species (Fig. 3.2). A notable feature of the AFP gene profile of the hybrids is the presence of a cluster of four bands in the vicinity of 2.6 kb (see boxed bands in Fig. 3.2). That cluster, also visible in AW, corresponds to bands which contain two inverted AFP genes that belong to an extensive tandem array (Scott *et al.*, 1988b). This portion of the AFP gene pool of AW was not amplified in SW (see Chapter 2). Overall, the four clustered bands (three in AW individual 1) characteristic of AW were visibly fainter in the wolffish hybrids.

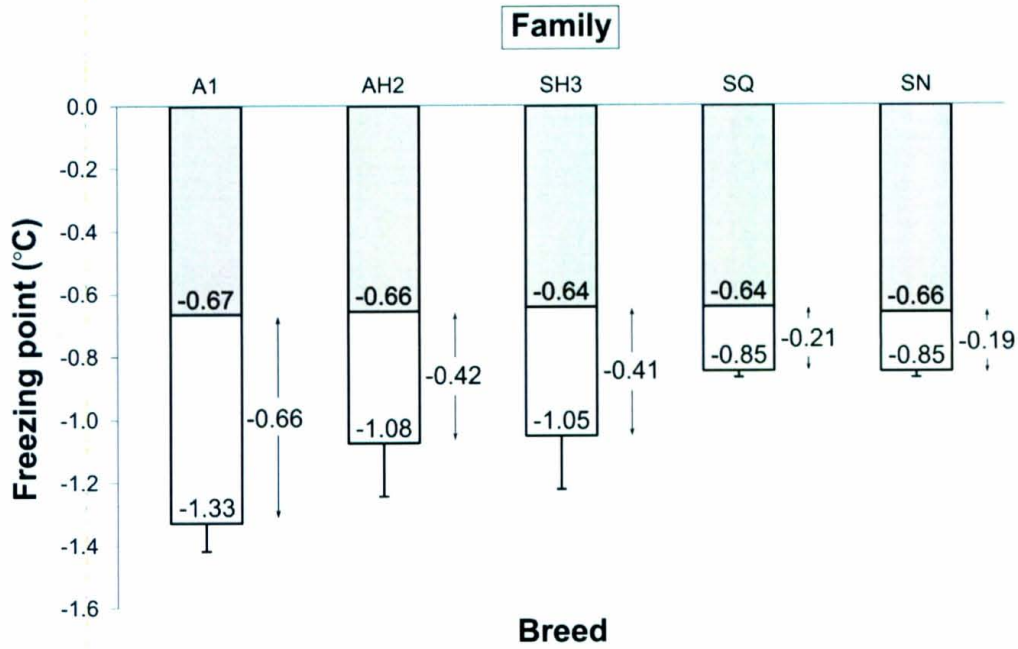

Figure 3.1. February mean plasma non-equilibrium FPs of purebred AW (family A1) and reciprocal hybrids (♀*lupus* x ♂*minor* – family AH2, and ♀*minor* x ♂*lupus* – family SH3) juveniles, without (grey bars) and with (grey + white bars) the contribution of TH (white bars). Values from purebred adult SW from Québec and Norway families QC and SN) were included for comparison.

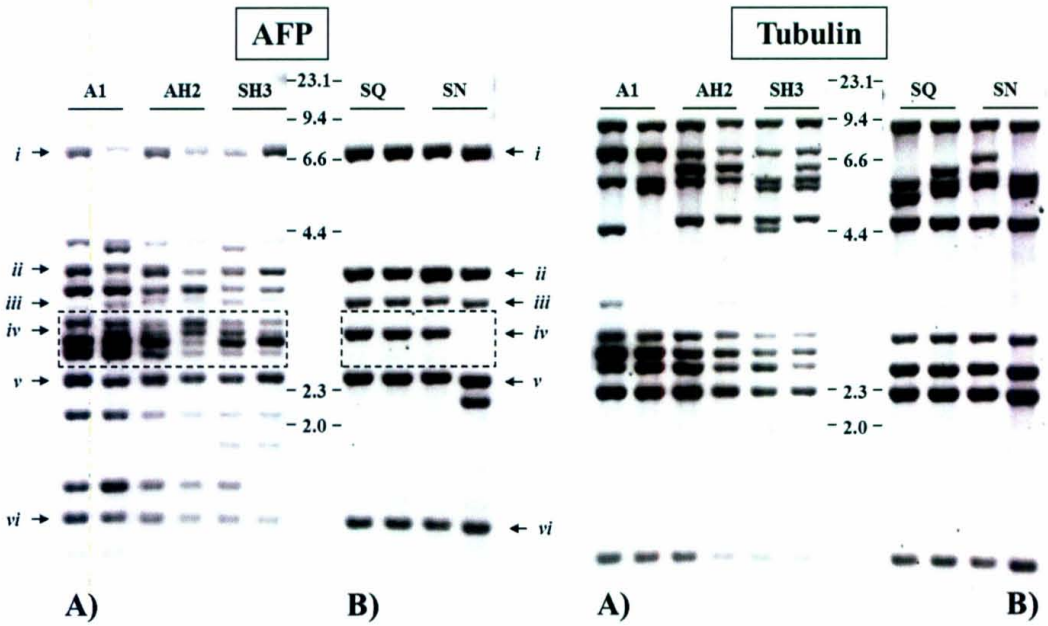

Figure 3.2. *Ase*I AFP and tubulin Southern blots for two individuals of **A)** AW (family A1), ♀*lupus* x ♂*minor* and ♀*minor* x ♂*lupus* wolffish hybrids (families AH2 and SH3), and **B)** SW (families SQ and SN). Individual fish appear in order. Bands that may represent AFP genes shared among fishes are indicated by arrows and roman numbers. The three to four AFP positive bands at ~ 2.6 kb are boxed. 10 and 20 µg of DNA was loaded per lane for AW and SW, respectively. The marker sizes are in kb. Note: the different intensity of the signals across the tubulin blot is due to the uneven spreading of one of the reactants within the hybridization bag; the loading gel is shown on the right.

The differences in AFP transcription, observed among the wolffish families (Fig. 3.3), were consistent with the gene dosage differences (Fig. 3.2), and reflect their contemporary (April) TH values (Table 3.3), as well as the average values measured in February (Fig. 3.1). The mRNA was obtained from tissues collected from the two best AFP producers (which were determined in February). Among the three tissues

investigated (liver, skin and gill filaments), transcription was found to be highest in the liver, as reported previously (see Chapter 2). This result is consistent with the liver as the main purveyor of blood-borne AFPs in most AFP-producing fishes (Fletcher *et al.*, 2001). In comparison, the peripheral tissues tested (skin and gill filaments) yielded lower amounts of transcripts (Fig 3.3), with the general trend for expression levels being to be greater in skin than gills. Here again, the levels of transcript in these epithelial tissues were the highest in AW, the lowest in SW, and intermediate in the hybrids. Since the plasma antifreeze activity initiates its decline during April (in identical experimental conditions; Desjardins *et al.*, 2006; 2007), the plasma TH values contemporary to the blot data (Table 3.3) were generally lower than in those measured in February for the selected individuals (Table 3.4).

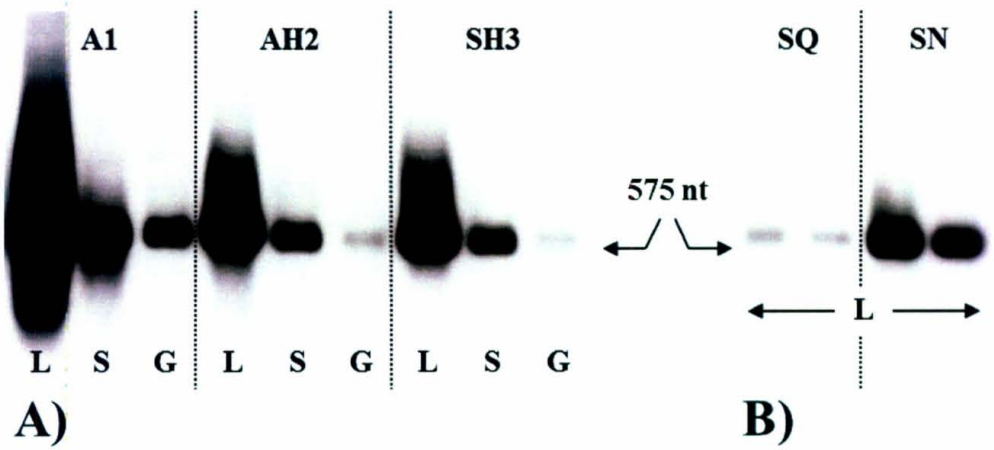

Figure 3.3. Northern blots for one individual of **A)** AW (family A1), ♀*lupus* x ♂*minor* and ♀*minor* x ♂*lupus* wolffish hybrids (families AH2 and SH3), and **B)** SW (families SQ and SN). The following tissues were assayed for the presence of AFP transcripts: liver

(L), skin (S), and gill filaments (G). This result is contemporary to the TH and FP values displayed in Table 3.3.

Table 3.3. April individual plasma TH and FP values from the two highest AFP producers from each family (AW: family A1; ♀*lupus* x ♂*minor* and ♀*minor* x ♂*lupus* hybrids: families AH2 and SH3; and SW: families SQ and SN). These values are contemporary to the Northern blot analysis results (Fig 3.3), and the individual used for these blots are indicated by an asterisk next to its TH value.

		Family				
	Fish #	**A1**	**AH2**	**SW3**	**SQ**	**SN**
TH (°C)	1	0.77*	0.42*	0.44*	0.02*	0.19*
	2	0.64	0.40	0.38	0.03*	0.17*
FP (°C)	1	-1.41	-1.07	-1.07	-0.68	-0.84
	2	-1.28	-1.08	-1.05	-0.67	-0.87

Table 3.4. Individual plasma thermal hysteresis (TH) and freezing point (FP) values (°C) for both wolffish hybrid families (AH2 and SH3), and the AW family. The two highest values per family are indicated by a (+), while the lowest is indicated by a (-). The two highest AFP producers (+) in each lane were selected for use in Northern blotting (Fig. 3.3).

Family		TH (°C)	FP (°C)
A1		0.60	-1.28
(n = 9)	-	0.48	-1.07
		0.70	-1.36
	+	0.75	-1.40
	+	0.75	-1.40
		0.57	-1.21
		0.71	-1.46
		0.65	-1.30
		0.74	-1.46
AH2		0.48	-1.13
(n = 10)		0.45	-1.10
		0.48	-1.18
	+	0.73	-1.39
	+	0.50	-1.17
		0.31	-0.95
		0.32	-0.97
		0.49	-1.15
	-	0.08	-0.74
		0.33	-0.98
SH3		0.25	-0.91
(n = 11)		0.37	-1.03
		0.37	-1.01
	+	0.80	-1.42
	+	0.60	-1.26
	-	0.20	-0.85
		0.33	-0.99
		0.47	-1.13
		0.25	-0.85
		0.44	-1.10
		0.42	-1.04

The AFP gene locus is highly polymorphic within the hybrid and AW families

The four SW individuals showed what appears to be the same amount of AFP genes (Fig. 3.2), despite the fact that these fish came from either side of the Atlantic. Moreover, a single restriction fragment length polymorphism (RFLP) was detected. In contrast, considerable levels of variation in both the banding pattern and intensity of individual bands could be observed among the blotted DNA from the reciprocal hybrid and AW families (Fig. 3.2). This was even evident between individuals from the same family. For instance, when considering the AW (family A1), three bands were missing in individual 1 compared to individual 2 (between molecular weight markers 2.3 and 4.4 kb). In the hybrids, and within that same size range, the occurrence of such gene presence-absence polymorphisms appeared to be lower, while the intensity of the individual bands was highly dissimilar. This is especially true for the hybrid family AH2, and indicates that the number of copies for all genes localized on bands localized above band "*v*" is highly variable. Interestingly, the hybrid family AH2 was generated from the same female AW that was used to produce the purebred AW family (A1), and both egg batches were fertilized using the semen of a single male (a different male genitor was used for each family, however).

These high interindividual AFP gene dosage variations observed among the AW and the hybrids may partly explain the striking variation in plasma TH measured in these fish in February (Fig. 3.1, Table 3.4). In Fig. 3.1, it is noteworthy that the SD values are higher in the reciprocal hybrids than in either purebred wolffish species. The individual TH values can be visualized in Table 3.4, which give a better measure of the intense

variation in plasma AFP levels among individuals of a given family. For instance, a difference of 0.65 °C can be seen between the highest and lowest AFP producers in the hybrid family AH2 (Table 3.4). A similar range (0.60 °C) exists between individuals of the hybrid family SH3, while the difference is smaller in the AW family (A1) with a maximum value of 0.27 °C between fish. Variations among SW individuals are even smaller.

Some AFP genes appear to be interspecifically shared

The production of wolffish hybrids offered an easy opportunity to qualitatively explore the extent to which AFP genes are shared between AW and SW. In our previous study (see Chapter 2), we isolated one sequence that was perfectly conserved between the two species (AWG3 and SWG1; GenBank Accession #s: **JQ040516**, **JQ040518**). The comparison of the wolffish DNA banding patterns on the Southern blot (Fig. 3.2) showed that a single band corresponding to the position of each of the SW bands could be seen in the hybrid DNA lanes when compared to those of the AW. In other words, the genes contributed by the SW parents to the hybrid offspring are also present in AW. Those genes, that are suspected to be interspecifically shared, are indicated by arrows and roman numbers "***i***" to "***vi***" on the Southern blot (Fig. 3.2). It is noteworthy that the band "***iv***" (also observed in SW) is located at a size that roughly corresponds to an inverted duplicate of AFP genes in AW (Scott *et al.*, 1988b; see Chapter 2). Interestingly, this band seems absent in AW individual 1. A variable number of bands for AW in that ~2.6

kb cluster was also recorded in our previous study (see Chapter 2). AW individual 1 also lacks band "***iii***", which is found in all other fishes on the blot.

The highest winter TH values of the hybrids and AW are equivalent

It is noteworthy that while the lowest AFP producers were found among the hybrids (when excluding the purebred SW families), the highest winter (February) values in those families (0.73 and 0.80 °C) ended up being similar to the highest values attained in the purebred AW family (0.75 °C). This was quite unexpected, given the apparent Mendelian inheritance of the AFP genes (Fig. 3.2). These fish, along with the highest producers from the AW and SW families, were thus selected for use in the AFP gene dosage and transcript level comparisons shown in Fig. 3.2 & 3.3. Interestingly, the hybrids showed spring (April) TH values that were more related to their respective gene dosages than were their February values. Other (unknown) factors than gene dosage may thus help explain the relatively high February TH values measured in these individuals, despite the evidently lower gene dosages when compared to the purebred AW individuals.

In all cases (and for all fish families), the TH values amounted to non-equilibrium FPs that were insufficient to grant freeze resistance in ice-laden waters. This is unusual for AW, which is normally known to express high TH under identical experimental conditions (Desjardins *et al.*, 2006; 2007, see Chapter 2). The reason for this low performance could be linked to genetics (parents with lower than average AFP gene dosage), and to the fact that these fish were produced in captivity where natural selection would not act to maintain AFP higher gene dosage. It could also be due to intra-individual

plasticity. Such variations were observed by Desjardins *et al.* (2006; 2007) where, for reasons yet unknown, some of the AW studied went from producing relatively low plasma AFP levels during one winter to expressing higher levels during the next, and inversely (see individuals 4 and 6, Fig. 3.4).

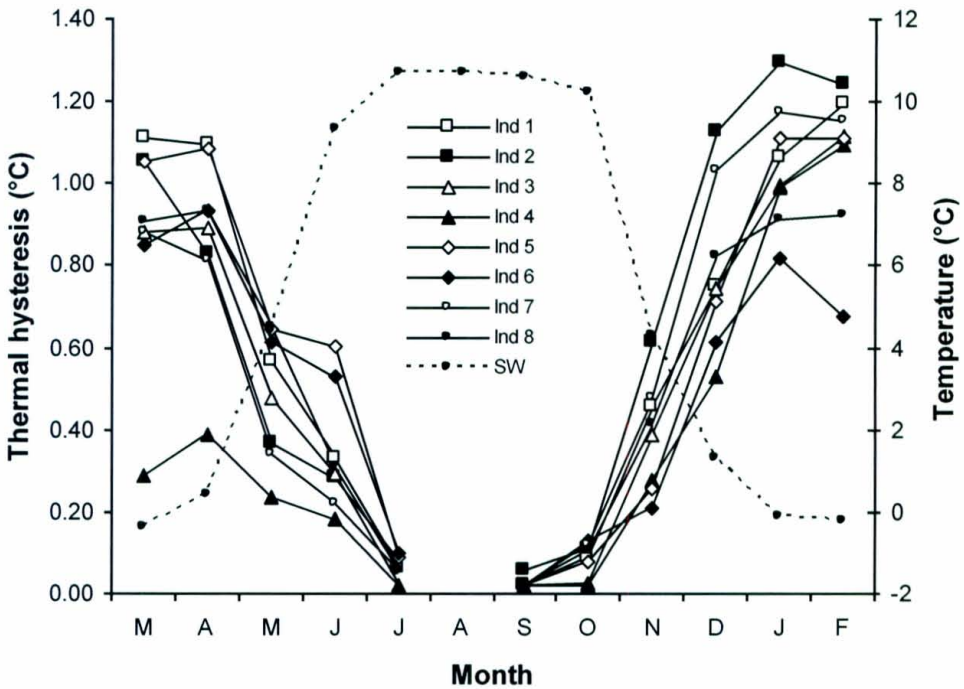

Figure 3.4. Annual plasma thermal hysteresis (TH) profiles from eight adult AW individuals studied by Desjardins *et al.* (2006). Only the average values were previously published.

3.4 – Discussion

On average, the AW/SW hybrids produced winter TH levels that were intermediate between those measured in the AW and SW. These in turn were mirrored by intermediate hepatic levels of AFP mRNAs (based on two individuals sampled in April), which were a direct consequence of an intermediate AFP gene dosage in these fish, compared with AW and SW. In marine teleosts, plasma TH levels have consistently been shown to depend on AF(G)P gene copy numbers, with lower AF(G)P gene dosages apparently failing to provide freeze resistance. Indeed, fish with lower plasma AF(G)P levels are mostly found in deeper or warmer waters, where the risk of freezing is minimal (Hew *et al.*, 1988; Scott *et al.*, 1988a; Cheng *et al.*, 2003; Nabeta, 2009; Desjardins *et al.*, 2006). With a mean non-equilibrium FP of ~ -1.1 °C (maximal individual value: -1.4 °C), the wolffish hybrids surveyed during this study would clearly be at a disadvantage within icy, shallow coastal waters (in which FP varies between -1.7 to -1.9 °C, depending on salinity).

Interestingly, the *Ase*I-restricted DNA banding profiles of the AW/SW hybrids revealed that the SW seems to share most (if not all) of its AFP genes with AW. Indeed, a single positive band was detected on the AW and hybrid's Southern blots at the corresponding positions of each SW band. Eventually, some of these orthologs may have accumulated mutations, but the recent discovery of identical genes between the two species (see Chapter 2) suggests that the extent of any sequence divergence may be minor. This agrees with the close genetic relationship between the AW and SW, which are thought to have diverged between 1.37 and 0.83 Ma (McCusker & Bentzen, 2010). As SW has approximately one third of the AFP gene pool of the AW (see Chapter 2), the

hybrids would likely be homozygous or heterozygous for the interspecifically shared loci, but hemizygous for the two thirds of the genes that are exclusive to AW.

Of the AFP genes exclusive to AW, the majority are part of a large tandem array. This feature, partially mapped and sequenced by (Scott *et al.*, 1988b), consists of several repeated units that each contains two AFP genes in an inverted (tail-to-tail) orientation. On our Southern blots, these inverted duplicates could be isolated and visualized as individual DNA fragments, thanks to the use of the RE *Ase*I, which cuts only a few bp upstream of the initiation codons of each AFP genes (see Chapter 2). Based on the information from Scott *et al.* (1988b), these inverted duplicates are expected to be contained within three to four *Ase*I bands shown in the vicinity of 2.6 kb on the blot, with all the genes they harbour potentially sharing perfect to near-perfect identities.

Sequence homogeneity is characteristic of tandemly arrayed genes, and stems from their recent emergence and/or tendency to undergo gene conversion (Graham, 1995; Despons *et al.*, 2010). Their highly repetitive structure and evenly-spaced genic arrangement flag tandemly iterated genes as "hotspots" for meiotic homologous recombination events (Despons *et al.*, 2010). Apart from gene conversion (which does not affect the number of gene copies and promotes sequence homogenization), the main outcome of recombination between homologous chromosomes is crossing-over. Where sequence misalignment occurs, crossing-over will be "unequal", which will cause the chromosomes from both donor and receiver to be modified on the basis of gene dosage. Thus, each gamete produced by an individual fish has the potential to contain a distinct number of AFP genes. This in turn can significantly affect the number of these genes within a given wolffish population, in one direction or the other, depending on selection.

Gene tandem arrays will be maintained (and potentially expanded within a population) only if Darwinian selection intensely favors the resulting phenotype (Kondrashov & Kondrashov, 2006; Conant & Wolfe, 2008). Relaxation of these selective pressures most often results in a rapid decrease in gene dosage over generations, as a direct result of the intrinsically unstable nature of gene tandem arrays (Andersson & Hughes, 2009).

In the two AW individuals studied here, the highest variability in AFP gene dosage/organization was observed within the ~ 2.6 kb bands on our Southern blot. These bands (which contain genes that are part of the AFP gene tandem array) varied between three and four in number. Significant pattern variability was also previously observed in AW in this region of the *Ase*I blot (see Chapter 2). As the AW individuals used in the present study were produced from the semen of a single male, the absence of the second band in the ~2.6 band quartet of individual 1 indicates that a deletion happened in the gametes from both parents at that locus. Alternatively, this observation could be due to the occurrence of a mutation at the *Ase*I restriction site. However, as these sites (which are AT-rich) seem to be highly conserved among AFP III-producing fishes (Zhang *et al.*, 2009) and that the mutation would have to be present in both gametes, the first explanation appears more likely.

Other bands were observed to be missing in AW individual 1 compared to individual 2. This implies that crossing-over events are not limited to the genes of the aforementioned tandem array. Irregularly spaced gene repeats may act as recombination sites as well, in the measure where pattern(s) of repetition are still obvious, and where dispersion of the genes along a given stretch of DNA is limited. In AW, such genes belong to what Scott *et al.* (1988b) called the "minor component" of the AFP gene locus.

Their restriction map shows that these genes are flanked by sequences that retained patterns of redundancy.

In multigene families, an obvious requirement for protection against gene loss by unequal crossing-over is the dispersion of individual genes within a heterogeneous genic environment (Graham, 1995). Such may be the case for the AFP genes of SW, which may explain their apparent stable dosage and organization (see Chapter 2; this study). Interestingly, some old, well-established multigene families can resist gene dosage alterations while conserving a tandemly arrayed organization and high sequence iteration. An example is the ribosomal RNA gene family. In yeast, DNA replication control mechanisms will restore the native dosage of these genes, in the event of a disastrous loss by unequal crossing-over (reviewed in Grenetier *et al.*, 2006). Such mechanisms obviously do not act on the AW AFP locus, and its general dosage maintenance may depend entirely on natural selection in response to the environment.

In freeze-resistant fishes, variability in AFP gene copy numbers will be tolerated to the extent that the resulting organismic TH is sufficiently high to preclude death in presence of ice. An example of a fish displaying full freeze resistance, despite displaying substantial variability in its gene pool, is set by the unrelated notothenioid *Dissostichus mawsoni*, or Antarctic toothfish (Turner *et al.*, 1985; Cheng *et al.*, 2006; DeVries & Cheng, 2005). Following the sequencing of the antifreeze glycoprotein (AFGP) gene locus of an individual *D. mawsoni*, Nicodemus-Johnson *et al.* (2011) discovered that the haplotypes from this particular fish differed widely in terms of gene dosage and organization. While one haplotype contained 14 copies of the AFGP gene, the other included only eight. Each gene was positioned back-to-back with a trypsinogen

pseudogene, and found within moderately irregularly-spaced tandem repeats. Obviously, this case supports the fact that homologous unequal recombination can happen readily among linked but irregularly spaced genes, and that variation in dosage among haplotypes (and ultimately, individual fish) is not deleterious, as long as freeze resistance results.

The high TH variability measured among the reciprocal AW/SW hybrids is most likely entirely due to the intrinsic gene dosage variability characterizing the AW haplotype. The presence of a single AW haplotype in the hybrids offered a clear view of the genetic rearrangements that are rampant within the highly dynamic and polymorphic AFP locus. The dosage variability was particularly striking within the AH2 hybrids, which shared the same mother as the purebred AW, and were produced using the semen of a single male. Because of the intermediate plasma TH levels derived from their intermediate AFP gene dosage, natural AW/SW hybrids, if they did occur, could not survive in the shallower part of the freeze risk ecozone unless some of these fish inherit a haplotype with unusually high gene dosage from their AW parent. Since there is no intermediacy in freeze resistance, such hybrids could not occupy and specialize within a hybrid zone based on intermediacy for freeze resistance. Their only apparent viable fate would be to remain in the lower risk – deeper – part of the freeze risk ecozone, and/or to back-cross with one of the two founder species through introgressive hybridization. The fact that F2 hybrids were successfully produced in captivity (through artificial insemination) indicates that hybrids could potentially contribute to dosage evolution of the AFP multigene family in wolffishes, if the condition for their occurrence and survival in the wild are met. A potential evolutionary significance of the hybrids would thus be as

facilitators of AFP family gene contraction in AW in the absence of freezing risk, and/or the introduction in SW of new members of the type III AFP gene family.

The fact that no hybrids have been formally reported from the wild (Templeman, 1986c; Imsland *et al.*, 2008; McCusker *et al.*, 2008) indicates that the occurrence of hybridization between the AW and SW must be extremely rare (if it occurs at all), which suggests that well-developed pre-zygotic barriers prevail between the two species. However, these natural barriers may be weakened in the face of important environmental perturbations (Hubbs, 1955), whether they be caused by large-scale climatic changes or anthropogenic generalized environment destruction and overfishing, as both types of disturbances have the potential to influence the direction of selection for freeze resistance in marine fishes.

Since the beginning of the Quaternary Period (~ 2.6 Ma), the North Atlantic has seen 52 glacials and interglacials (Gibbard & Cohen, 2008). Within the last Ma only, one glacial event has occured every ~100 Ka (Augustin, 2004), interspersed by relatively short interglacials. It is assumed that seasonal ice formation still occurred over the oceans during these interglacials, although its intensity and range are impossible to know as yet (Lambeck *et al.*, 2002; Otto-Bliesner *et al.*, 2006). Perhaps because of the brevity of these warmer episodes (and/or other unknown factors), separate wolffish species succeeded in maintaining their integrity during their ~ one Ma of existence. However, as the inception of a next glaciation appears to be postponed to a distant future (Tzedakis *et al.*, 2012), the present interglacial is forecasted to shadow the previous ones in both length and intensity. Interestingly, warming water temperatures have been hypothesized to be the impetus for a northward or depth shift in the distribution of different northern fish species (Perry *et al.*,

2005; Dulvy *et al.*, 2008). A depth shift in wolffish distribution was also reported by Kulka *et al.* (2004) who linked it to temperature changes. The displacement of the AW to the deeper level (or out) of the freeze risk ecozone may lead to the intrinsic contraction of AFP gene dosage in AW, through the relaxation of the pressures that maintain high AFP gene dosage in this species. Moreover, if wolffish pre-zygotic barriers are climate-driven, a complete and/or longer suppression of the threat of freezing may encourage the occurrence of natural hybridization with SW (unless the hybrids are unfit for other reasons), which are sympatric to AW in deeper waters (Scott & Scott, 1988).

Another potential facilitator of interspecific hybridization and/or intrinsic reduction of the AFP gene pool in AW are anthropogenic disturbances, through habitat destruction and overfishing. The solitary, non-schooling habits of wolffishes, their slow maturation rate and their use of a K-based reproductive strategy make them particularly vulnerable (Keats *et al.*, 1985; Pavlov & Novikov, 1993; Templeman, 1986a;b; Gunnarsson *et al.*, 2006). Intensive trawling of the sea floor has been shown to profoundly alter the benthic ecosystems on which wolffish species and other apex predators depend for survival (reviewed by Thrush & Dayton, 2010). Although wolffishes are of no commercial value in Canada, they regularly occur as by-catch in diverse ground fisheries. As the coastal fish stocks suffered severe depletion due to overfishing, exploitation of the resource was extended to offshore and deeper waters. Now, in Newfoundland waters, AW is mostly caught at depths of ~ 100 m during ground fish surveys (Kulka *et al.*, 2004). Nevertheless, archeological evidence hints that predatory fishes (including AW) were more prevalent in shallow waters in the past than they are today (Steneck *et al.*, 2004). As green urchins are a major part of the diet of AW in

shallow waters (Keats *et al.*, 1986), it was suggested that the decline of AW populations is linked to the recent proliferation of sea-urchin barrens in coastal waters (Steneck *et al.*, 2004).

3.5 – Conclusion

The observation that SW shares most, if not all, of its genes with the AW strengthens the hypothesis that speciation in wolffishes was based on freeze resistance and prompted by the extensive amplification in an ancestor to AW of an inverted duplicate of AFP genes. Following the evaluation of the plasma AFP levels displayed by the artificially produced AW/SW hybrids studied here, we conclude that such intermediate antifreeze activity could not grant these fish a performance advantage within the shallowest part of the freeze risk ecozone. However, long-term relaxation of Darwinian selection for freeze resistance may contribute to the erosion of the natural reproductive barriers between AW and SW and/or allow the intrinsic compression of the highly dynamic AFP gene pool in the AW (and perhaps the integration of new AFP genes in the genome of the SW). Thus, the maintenance of the anthropogenic and/or natural forces that appear to be directing the displacement of the AW to deeper and/or non-freezing waters (where the probabilities of encounting ice are lowered, and those of encountering the sympatric SW are increased) could potentially favour AFP gene dosage contraction in wolffishes.

– CHAPTER 4 –

" Temporal and spatial expression of type III AFP genes in wolffish species "

4.1 – Introduction

Since the freezing point (FP) of seawater (~ -1.7 to -1.9 °C) can be a full degree lower than in their body fluids, marine teleosts of temperate and polar regions are at risk of freezing if they encounter ice while in an undercooled state. As freezing invariably results in death, some fish avoid this threat by living in deep waters, where their biological fluids will remain in a metastable state (Scholander *et al.*, 1957). However, as temperate and polar shallow coastal waters are resource-rich ecosystems, fish that can safely exploit the freeze risk ecozone are clearly at an advantage.

The attractiveness of these environments is reflected in the convergent evolution of freeze resistance in many distinct fish clades, through the emergence and evolution of distinct families of antifreeze proteins (AFPs) and glycoproteins (AFGPs). Five structure-based families of these proteins have been characterised so far (AFGPs and type I, II and III AFPs), all of which are encoded within distinct multigene families (Fletcher *et al.*, 2001). These proteins grant freeze resistance by lowering, non-colligatively the FP of a fish down to that of the surrounding seawater. Despite their distinct evolutionary origins, these proteins all seem to inhibit ice growth by the same general mechanism: by adsorbing to the surface of nascent ice crystals, effectively preventing the binding of more water molecules to the ice (Raymond & DeVries, 1977).

Unlike the coastal waters of Antarctica, where sea ice is perennial, freezing conditions occur on a seasonal basis in the Arctic and temperate coastal regions. Consequently, fish inhabiting these waters will show seasonal variations of their AF(G)P blood levels, with higher levels of AF(G)Ps present during winter, and moderate to none during summer (Fletcher, 1977, 1981; Fletcher *et al.*, 1982; Fletcher *et al.*, 1985; Enedsolven *et al.*, 2003; Lewis *et al.*, 2004; Desjardins *et al.*, 2006). For instance, the shortening of day length (decreasing of photoperiod) is the impetus for the synthesis of type I AFPs in the winter flounder (*Pseudopleuronectes americanus*; family Pleuronectidae). In contrast, the Atlantic cod (*Gadus morhua*) needs exposure to cold temperatures in order to exhibit measurable levels of circulating AFGPs, regardless of the photoperiod (Fletcher *et al.*, 1987). In fish of the suborder Zoarcoidei, such as the eelpouts (family Zoarcidae) and the wolffishes (family Anarhichadidae), photoperiod may be the zeitgeber of the antifreeze response (Fletcher *et al.*, 1985; Desjardins *et al.*, 2006), although experimental evidence is wanting. Interestingly, these fish seem unable to halt the synthesis of their type III AFPs, and low to appreciable levels can be measured in their plasma during summer and when water temperature is kept warm year-round in the laboratory (Fletcher *et al.*, 1985; Enedsolven *et al.*, 2003; Desjardins *et al.*, 2006).

Evidence suggests that AF(G)Ps evolved by gene sub- or neofunctionalisation following the duplication of coding sequences whose products exhibited no to very weak antifreeze activity (Chen *et al.*, 1997b; Ewart *et al.*, 1998; Cheng & Chen, 1999; Baardsness & Davies, 2001; Deng *et al.*, 2010). Consequently, it is likely that extensive gene duplication/amplification (GDA) of these nascent sequences (and concomitant

higher expression) compensated for the initial sub-optimal performance of the ancestral AF(G)Ps. Apart from providing a shortcut to the achievement of freeze resistance in cold water marine teleosts, another outcome of GDA was the multiplication of templates for adaptive evolution. Consequently, the AF(G)Ps found in fishes today are fully functional and often highly diverse in sequence within a given multigene family (Hew *et al.*, 1984, 1988; Nishimiya *et al.*, 2005; Scott *et al.*, 1985, 1988a; Cheng *et al.*, 2003; Nicodemus-Johnson *et al.*, 2011). In fishes of the suborder Zoarcoidei, the type III AFP genes and their products have been classified within two subfamilies: the QAE- and the SP-type genes. These appellations originate from the propensity of the proteins to either bind QAE (anion-exchange) or SP (cation-exchange) Sephadex resins during ion-exchange chromatography (Hew *et al.*, 1984). Members (genes and proteins) of both subfamilies have been reported in fishes of the families Zoarcidae (Hew *et al.*, 1984, 1988; Wang *et al.*, 1995a,b; Nishimiya *et al.*, 2005) and Anarhichadidae (Cheng *et al.*, 2006; see Chapter 2).

The Atlantic wolffish (*Anarchichas lupus*; AW) and the spotted wolffish (*A. minor*; SW), are bottom-dwelling Anarhichadids that inhabit the Northwest Atlantic waters. The AW can be found in shallow waters and produces high levels of type III AFPs in its plasma, while the deeper-dwelling SW produce trace amounts (Desjardins *et al.*, 2006, see Chapter 2). In general, the plasma AFPs in AW and SW will rise to higher concentrations during winter and fall to low or trace levels during summer (Enedsolven *et al.*, 2003; Desjardins *et al.*, 2006). It is not known how these plasma AFP levels relate to the amount of AFP transcripts produced by these fish on a seasonal basis, nor if the two

families of isoforms are differentially expressed. The only species studied in that respect is the winter flounder, a fish that produces type I AFPs. Two of the three subfamilies of its AFP genes were found to be differently regulated (Gong *et al.*, 1996; Mia *et al.*, 1998a,b), and the levels of plasma AFPs were tightly related to the amount of translatable mRNA in the liver (Pickett *et al.*, 1983). The present study thus has two goals: 1) to evaluate the general levels of type III AFP gene transcripts in winter and summer in different tissues from both AW and SW, and 2) to investigate the spatial and temporal expression of the members of QAE- and SP-type gene subfamilies in wolffishes.

4.2 – Material & Methods

Animals, experimental conditions and tissue sampling

The Atlantic wolffish (AW) and spotted wolffish (SW) used in the present study were derived from the same stock used in Chapter 2. The fish of the two species were maintained in separate tanks (2 m x 2 m x 0.3 m) at seasonally ambient temperature and photoperiods (Fletcher, 1977) in the Ocean Science Centre facilities (Memorial University of Newfoundland), and fed formulated food (Ewos, Marine) *ad libitum*.

Blood and tissue sampling took place in early February 2007, during a time when plasma AFP activity levels are at their peak (Desjardins *et al.*, 2006; 2007). A second sampling took place in July, when the AFP blood levels were at their lowest. Two fish of each species were used. Prior to dissection, the fish were immersed in a smaller tank for lethal anesthesia (benzocaine $_{final}$[50 mg/L]). After 10 min, blood was sampled from a

caudal blood vessel and treated as described by Desjardins *et al.* (2006) in order to retrieve the plasma. The anaesthetized fish were then given a sharp blow on the head, to ensure that they were dead. Various tissues (liver, skin, gill filaments, stomach, intestine, heart, white muscle, kidney, spleen, and brain) were sampled and flash-frozen to prepare for total RNA extractions. All samples (including the plasma samples) were stored at -70 °C before use.

The guidelines of the Canadian Council on Animal Care were followed during transport and care of the fish, and all measures were taken to keep pain and discomfort in our experimental animals to a minimum during sampling.

Thermal hysteresis measurements

Plasma antifreeze activity was measured as thermal hysteresis (TH) using a Clifton Nanolitre Osmometer (Clifton Technical Physics, Hartford, NY, USA) following the procedure of Evans *et al.* (2007). TH is defined as the difference between the melting and freezing temperatures (°C) of a test solution, and is representative of the AFP concentrations present in that solution, according to a non-linear relationship (Davies *et al.*, 1990).

Northern blot analysis

A nonradioactive method was used to detect AW and SW AFP transcripts on Northern blots, using a 385 bp DIG-11-dUTP-labeled QAE-type-AFP mRNA probe (Roche

Diagnostics, Laval, QC, Canada). This probe hybridizes to both QAE and SP-type AFP mRNA sequences (please see (see Chapter 2) for details relative to the template and probe generation methods).

Total RNA was extracted from frozen tissues and prepared as previously described (see Chapter 2). Briefly, 1 ug of total RNA aliquots were separated on a denaturing 1.2 % agarose gel containing 0.67 % formaldehyde. The RNA was then blotted onto a positively charged nylon membrane (Roche Diagnostics) using a VacuGene XL Vacuum Blotting System® (Amersham Biosciences, Piscataway, NJ, USA), and cross-linked with UV light. Analyses were first run on the summer samples. Only the mRNA from the tissues that showed significant levels of expression in these summer blots was used to generate the blots for the winter to summer comparison of AFP gene transcript levels.

Membrane pre-hybridization, hybridization, stringency washes, blocking and detection procedures were performed as outlined in Chapter 2, using the kit and reagents supplied by Roche Diagnostics. The chemiluminescent signals generated by the hybridized probe were captured on a Lumi-Film® Chemiluminescent Detection Film (Roche Diagnostics).

RT-PCR conditions

First-strand cDNA was generated from the various mRNA samples through reverse transcription, using SuperScript II™ RT and an oligo$(dT)_{20}$ primer (Invitrogen Canada

Inc.) according to the manufacturer's protocol. Atlantic salmon (*Salmo salar*) total mRNA was used to generate a negative control (this species does not have AFP genes). One tenth of each RT reaction was then included in PCRs using the primer combinations indicated below (see primer sequences and references in Table 4.1).

The forward primers 5′ ALL and 5′ ALL-2 sit on the start of exon 1 and ~ 100 upstream of the start codon within the 5′ UTR, respectively. The specific reverse primers 3′ SP and 3′ QAE were designed to anneal in a region of divergence between the SP- and QAE-type AFPs (Fig. 4.1), and are expected to allow for the detection of all isoforms within each AFP gene subfamily. Comparisons of nucleotide sequences from wolffish (family Anarhichadidae) and ocean pout (family Zoarcidae) found only one polymorphic site within each of these SP- and QAE-specific regions. The subfamily-specific primers were optimized using both pure SP (AWri; GenBank accession number: M22125) and QAE (AWG1; GenBank accession number: JQ040521) clones as template. Highly stringent PCR conditions were used, in order to ensure specific amplification. A third primer, 3′ QAE-X, was used to detect a specific QAE-type sequence, AWE1, which was previously isolated and cloned from AW liver cDNA (see Chapter 2). Among the published sequences available on the databases, this wolffish QAE-type AFP sequence is most closely related to a genomic sequence from ocean pout (OPb). However, the end of its second exon (which corresponds to the mature sequence of the protein) is distinct from those of other known type III AFP sequences. This was where the specific 3′ QAE-X primer was designed (including part of the 3′ UTR), from a quasi-identical AW genomic sequence, AWG1 (see Chapter 2). Finally, the primer pair 5′ ACT/3′ ACT was used to amplify β-actin in salmon cDNA.

All RT-PCR reactions were performed using an Eppendorf Mastercycler® (Eppendorf Canada, Mississauga, ON, Canada), and were conducted in the presence of Taq DNA polymerase (Invitrogen Canada Inc., Burlington, ON, Canada). The conditions were as follow:

1) Primer set 5′ ALL/3′ SP: The PCR was initiated with a "hot start" to minimize the formation of primer-dimers. The initial denaturation step was carried out at 95 °C for 2 min, and a "touch-down" procedure was used for increased specificity, where the temperature of the first cycles decreased from 74 to 66 °C (in 2 °C decrements). Thirty more cycles were performed at that latter annealing temperature. For all cycles, the annealing step lasted 30 sec, and the conditions for denaturation and elongation were 95 °C (30 s) and 72 °C (60 s), respectively, ending with a final 72 °C elongation step of 10 min. The expected amplicon size was ~ 200 bp.

2) Primer set 5′ ALL/3′ QAE: The conditions were identical to those above, with the exception of the starting annealing temperatures, which decreased from 74 to 60 °C. The expected amplicon size was ~ 200 bp.

3) Primer set 5′ ALL-2/3′ QAE-X: the initial denaturation step was carried out at 95 °C for 2 min, followed by 40 cycles of denaturation, annealing and elongation that lasted 95 °C (30 s), 60 °C (30 s), and 72 °C (60 s) each. The PCR ended with a final 72 °C elongation step of 10 min. The expected amplicon size was ~ 435 bp.

4) Primer set 5′ ACT/3′ ACT: The conditions were identical to those above with the exception of the annealing temperature, which was 65 °C. The expected amplicon size was ~ 675 bp.

The resulting RT-PCR reactions were run on a 1.5% agarose gel in the presence of ethidium bromide, and the resolved bands were visualised with UV light.

Table 4.1. PCR primers used to amplify AFP nucleotide sequences from AW and SW cDNA, and their annealing position on reference sequences (see also Fig. 4.1 for the location of the annealing sites for all primers). Primers used to amplify β-actin sequences from salmon cDNA are also shown.

Primer name	Sequence (5′– 3′)	Ref. seq. Accession nos.	Position
5′ ALL	tctcagccacagccatgaagtc	**J03924**	256-277
3′ SP	gatttgggacatctctgcgaac	**J03924**	630-620
3′ QAE	taatcggggaatgtcctcggc	**J03923**	741-721
5′ ALL-2	gttaagtcctcccacatactg	**J03924**	131-151
3′ QAE-X	gatggtgaaaacgtatgcgtag	**JQ040521**	586-565
5′ ACT	cgccgcactggttgttgaca	**AF012125**	15-34
3′ ACT	agcaggagatgggcaccgc	**AF012125**	689-671

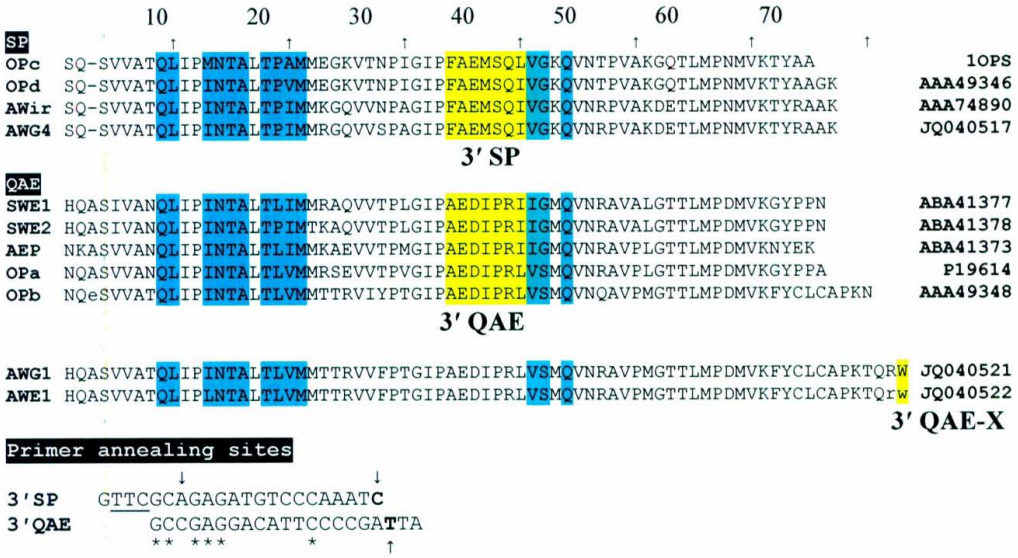

Figure 4.1. Alignments of SP- and QAE-type mature amino acid sequences from different species of the suborder Zoarcoidei (SW: spotted wolffish; AEP: Antarctic eelpout; OP: ocean pout; AW: Atlantic wolffish). The residues corresponding to the primer annealing sites are highlighted in yellow, and those involved in ice-binding are highlighted in cyan. Lower case letters in the amino acid sequences represent an integrated primer sequence (*i.e.* "rw" in AWE1) or an inferred amino acid (*i.e.*, "e" in OPb).The aligned nucleotide sequences corresponding to the annealing sites of the specific SP and QAE primers (3′ SP and 3′ QAE, respectively) are also shown, with arrows indicating the known polymorphic sites. The asterisks indicate conserved nucleotides between the two sequences, and the underlined triplet, the reading frame.

4.3 – Results

Plasma TH levels were low during summer, and relatively consistent between the two individuals of both wolffish species. The mean TH value was higher in AW (0.28 °C) than in SW (0.18 °C), and these values were proportional to the contemporary levels of AFP mRNAs detected within the liver RNA population of these fish (Fig. 4.2). As liver is

the main contributor of circulting AFP in non-notothenioid fishes (Fletcher *et al.*, 2001; Cheng *et al.*, 2006), the translation of these transcripts is likely directly responsible for the higher TH measured in the plasma of AW compared to SW.

When compared to winter (February) data collected on fish from the same lot (see Chapter 2) (Fig. 4.3), the summer TH values were lower in both species, showing a predictable decline. Interestingly however, the corresponding summer levels of AFP mRNA were comparable to those measured in winter in some tissues, namely the liver (Fig. 4.3A,B, top). Another tissue that retained significant summer expression of the AFP genes in the two wolffish species is the stomach (Fig. 4.3A,B, top), although the levels of transcript appeared significantly lower. In the other tissues, a general summer decrease (and sometimes disappearance) of the amounts of AFP gene transcripts was also visible. Overall, the most notable feature in AW and SW is the continuous expression of high (AW) to moderate (SW) levels of AFP mRNAs in the liver and stomach.

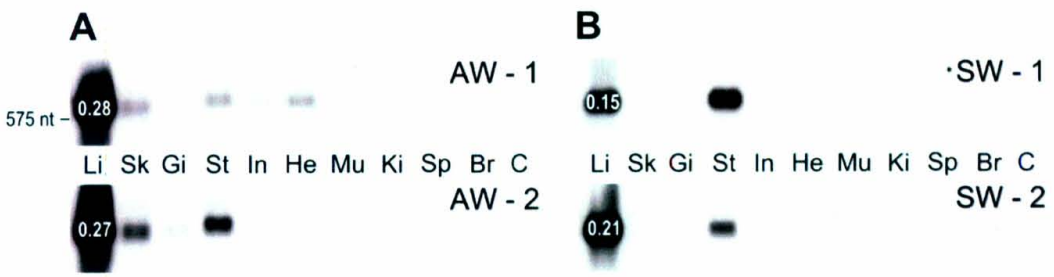

Figure 4.2. Northern blots on total RNA extracted from **A)** two AW and **B)** two SW individuals during summer. The contemporary plasma TH values (°C) are superimposed on the liver hybridisation signals. The tissues sampled were liver (Li), skin (Sk), gill filaments (Gi), stomach (St), intestine (In), heart (He), white muscle (Mu), kidney (Ki), spleen (Sp), and brain (Br). Atlantic salmon liver RNA was used as a negative control.

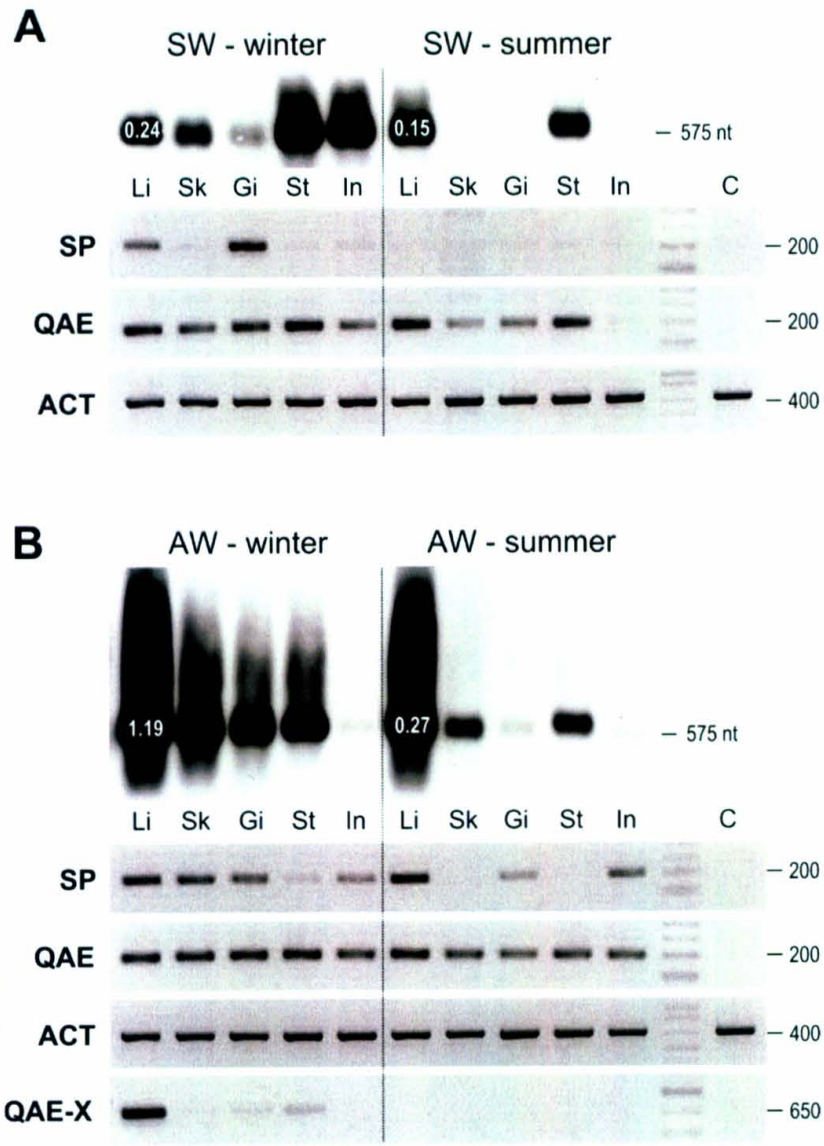

Figure 4.3. Comparison of winter and summer total AFP mRNA levels (top) and RT-PCR signals (bottom) for SP, QAE, and AWG1-like (QAE-X) sequences in A) AW and B) SW. Individuals 2 (AW) and 1 (SW) from the blots on Fig. 4.2 were used, respectively. The winter individuals correspond to individuals 1 (AW) and 2 (SW) used in Chapter 2. Only the tissues that showed significant levels of activity were used in these analyses, namely liver (Li), skin (Sk), gill filaments (Gi), stomach (St), and intestine (In). Atlantic salmon total liver RNA was used as a negative control in the RT-PCRs. Actin (ACT) was used as a control for the presence of mRNA in each reaction.

In order to investigate if the transcription of the AFP genes part of the two main subfamilies of type III AFP sequences were differently regulated and expressed, we performed RT-PCR analyses using primers specific to the published sequences of the SP- and QAE-type AFP genes (see M & M section for details). We found that the members of the two known wolffish type III AFP gene subfamilies did indeed exhibit differences in their temporal and spatial patterns of expression (Fig. 4.3A,B, bottom). Overall, both species seemed to express QAE-type AFP transcripts in virtually all tissues. These genes were also expressed year-round, although not always in significant amounts (here, the reader is invited to compare the RT-PCR signals to those visible on the corresponding Northern blots). More precisely, in the tissues where no or faint expression is evident (Fig. 4.3A,B, top), it is unlikely that the transcript levels are of physiological significance for organismal freeze resistance. As RT-PCRs will amplify traces of transcript (Fig. 4.3A,B, bottom), they can only show the presence/absence of transcript, irrespective of the actual amounts.

When compared to the pattern observed on the QAE RT-PCR gels, the RT-PCR signals for SP-type sequences showed marked seasonal changes. While the banding pattern for the SP-type amplicons was sightly different from winter to summer in AW (Fig. 4.3B, bottom), bands were only visible during winter in SW (Fig. 4.3A, bottom). For the latter observation, and because the amount of transcript varies little seasonally, we deduced that most of the expressed AFP genes in SW (Fig. 4.3A, top) must belong to the QAE-type gene subfamily (Fig. 4.3A, bottom). It also appeared that the presence of AFP gene transcripts in the stomach was mostly due to the transcription of QAE genes in both

species, as was evident by the absence of a corresponding visible SP-type signal in the RT-PCRs. Finally, the SP genes did not contribute large amounts of transcript in the liver of SW, as the summer and winter AFP mRNA levels were comparable, without a corresponding SP-type liver signal present in the summer RT-PCR. In AW, however, the liver expresses genes belonging to both AFP subfamilies all year.

Interestingly, an RT-PCR assay using a 3′ primer specific to a discrete group of genes part of the QAE-type gene subfamily showed an expression pattern distinct from that generated from the complete QAE-type gene pool in AW (no assay was conducted in SW). Expression of these genes occurred exclusively in winter, and in only three tissues: the liver, skin and gill filaments (Fig. 4.3B, bottom, QAE-X gel). A gene belonging to that group (AWG1), and a nearly identical cDNA sequence (AWE1), were previously characterised from AW (see Chapter 2).

4.4 – Discussion

Total AFP transcript levels are not heavily influenced by season

The data gathered within the present study demonstrates that wolffish continue to synthesise AFPs in the summer, even though the proteins are not required (Desjardins *et al.*, 2006). The presence of AFPs in the plasma during the summer was directly related to the persistence of high levels of AFP mRNAs in the liver of both species, which is the main contributor of circulating AFPs in fish (Fletcher *et al.*, 2001). This result implied that transcription of these genes (as a whole) is maintained at long day lengths and warm

temperatures. Indeed, although mRNA seasonal profiles from other zoarcoids were not available for comparison, summer plasma TH values (ranging from a low < 0.1 to an impressive 1.35 °C) were reported among members of three other zoarcoid families (Zoarcidae, Pholidae, and Stichaeidae; Fletcher *et al.*, 1985; Enevoldsen *et al.*, 2003). We thus hypothesise that similar maintenance of high levels of AFP transcripts would be observed in other type III AFP-producing fishes (which all belong to the suborder Zoarcoidei). The high aestival hepatic transcriptional activity of the AFP genes in wolffishes contrasted with observations in the winter flounder, for which the levels of liver AFP transcripts were shown to be greatly reduced during summer (Pickett *et al.*, 1983).

In a previous paper surveying the annual profiles of plasma AFP levels in AW and SW, we hypothesised that photoperiod was the zeitgeber for the increase of plasma AFP concentrations in these species. Indeed, AW and SW kept at temperatures above 10 °C but exposed to ambient photoperiod still showed a significant (albeit slight) plasma TH increase during winter (Desjardins *et al.*, 2006). These results also indicate that low temperatures were instrumental to the establishment of the full winter antifreeze response in these fish, when comparisons were made with fish exposed to both ambient photoperiod and temperature. Experimental evidence from the winter flounder points to the importance of cold temperatures for the retention of the liver AF(G)P mRNAs and their products in the bloodstream of the fish, as warm water temperatures likely increase the turnover and excretion rates of the proteins (Fletcher *et al.*, 1981; Dunker *et al.*, 1995). During the course of our present experiment, the temperature to which the wolffish

were exposed varied between 6.7 and 11.9 °C and 0.1 and 1.8°C, before the summer and winter samplings, respectively.

At the time, the molecular basis for the reduction in the blood summer TH levels was unknown. Interestingly, and in contrast with previous data on winter flounder, the present observation of high (apparently unaltered) AFP mRNA levels in the liver of both wolffish species during the summer suggests that photoperiod and temperature do not have a significant impact on the levels of total hepatic mRNA transcript in these species. Therefore, the summer decrease of circulating AFPs might be affected at a different level by these environmental factors, possibly at the post-transcriptional level (Keene, 2007). The simplest explanation, though, resides in the balance between plasma AFP synthesis, degradation and clearance. This, however, still does not explain the occurrence of the slight winter plasma TH increase in AW and SW kept at warm temperatures year-round (Desjardins *et al.*, 2006), if they did express similarly stable liver AFP mRNA levels within that latter experimental setting.

The expression of SP- and QAE-type transcripts show spatial and temporal variations in wolffish species

The use of specific primers for SP- and QAE-type sequences in RT-PCRs provided the first insight into the existence of differential transcriptional control of the type III AFP genes in wolffish. While the QAE-type genes showed ubiquitous temporal and spatial expression in both species (*i.e.*, bands were observed at the anticipated amplicon size in both winter and summer samples, and for virtually all tissues), the SP-

type genes showed a more patchy expression pattern. These genes are apparently not transcribed during summer in SW. Nevertheless, the total amount of AFP transcripts in the liver were comparable to winter levels in that species, thereby suggesting that the plasma AFP levels in SW are mainly contributed by the hepatic expression of QAE-type genes.

The genome of a single fish contains numerous AF(G)P genes, and the evolution of these multigene families likely has a complex history (Hew *et al.*, 1988; see Chapter 2). In a previous study we observed that AW possesses ~ three times more AFP genes than in SW (see Chapter 2). We hypothesised that this difference in gene dosage was mostly due to the presence in AW of a large tandem array of SP-type genes (Scott *et al.*, 1988b), which seems exclusive to that species (see Chapers 2 & 3). In the present study, and contrary to SW, we found that the SP genes are expressed year-round in the liver of AW. We thus postulate that the highest levels of hepatic expression of the AFP genes in AW are mostly due to the year-round transcription of the SP genes part of the tandem array reported by Scott *et al.* (1988b).

Interestingly, the differential regulation of the AFP genes in wolffish and other zoarcoids promises to be even more complex, as an underlying level of expression control was observed within the QAE-type genes of AW. Indeed, the expression of a specific subgroup of QAE-type genes (what we will call here AWG1-like genes; see Chapter 2), showed an RT-PCR banding pattern (QAE-X) distinct from that of the QAE-type genes as a whole. In AW, these genes were expressed only in the liver, skin and gill filaments during winter (no assays were done in SW). This result shows that type III AFP genes

may also be differently regulated within each subfamily, which suggests a complex evolutionary history for the type III AFP gene multifamily.

The involvement of an intrinsic seasonal modulator of plasma TH in type III AFP-producing fishes may be another implication for this last result, although more experimental evidence is needed. A recent study by Takamichi *et al.* (2009) demonstrated a cooperative action between the QAE- and SP-type AFPs purified from a distant relative of the wolffishes, the zoarcid notchfin eelpout (*Zoarces elongatus*). These researchers showed that the presence of the QAE-type isoform (as little as 1% in solution) was essential for the expression of TH by the SP-type isoforms. This difference in specific TH is likely linked to differences in the respective binding sites of these proteins (Garham *et al.*, 2010). Because the SP-type isoforms may contribute a greater proportion of the blood-borne AFPs in AW (contrary to SW), the presence of higher levels of circulating QAE-type AFPs in the plasma during winter may confer higher activity to the ever present and over-expressed SP-type isoforms in the fish's plasma. They may also partly explain the higher plasma TH levels measured in winter in AW kept above 10°C (Desjardins *et al.*, 2006).

Due to the specific diversity and proportion of SP- and QAE-type sequences observable within individual type III AFP gene pools reported in zoarcoids so far (Hew *et al.*, 1984, 1988; Scott *et al.*, 1988; Nishimiya *et al.*, 2005; Cheng *et al.*, 2006), the regulation of AFP gene expression may have evolved as a tailored solution for each species of the suborder. So far, only the open reading frames of a small number of type III

AFP sequences are available for analyses. To understand how the transcription of these genes is regulated would warrant an in-depth study of their respective promoter regions.

4.5 – Conclusion

We found that seasonal variation in total levels of type III AFP mRNA was low in both wolffish species, especially in the liver, which export the AFPs to the blood stream. However, our RT-PCR analyses revealed that some level of spatial and temporal differentiation in the regulation of QAE- and SP-type AFP genes occurs, which warrants further investigation. Although the RT-PCR gels only showed the presence/absence of a particular group of transcripts, and thus must be interpreted with caution, these preliminary results nonetheless provide an insight into the complexity of the expression of SP- and QAE type genes within type III AFP-producing fishes. The use of Q-PCR would allow for a more quantitative analysis (once suitable house-keeping genes are identified and tested in wolffish).

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