# FREEZE RESISTANCE IN RAINBOW SMELT (Osmerus mordax): SEASONAL PATTERNS OF GLYCEROL AND ANTIFREEZE PROTEIN ACCUMULATION AND THE in Vitro LIVER ENZYME ACTIVITY ASSOCIATED WITH GLYCEROL PRODUCTION



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# Freeze Resistance in Rainbow Smelt (*Osmerus mordax*): Seasonal Patterns of Glycerol and Antifreeze Protein Accumulation and the *in Vitro* Liver Enzyme Activity Associated with Glycerol Production.

By

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**Abstract**

Rainbow smelt (*Osmerus mordax*) inhabit inshore waters along the North American Atlantic coast. During the winter, these waters are frequently ice covered and can reach temperatures as low as –1.9°C. The freezing point of normal teleost blood is between –0.7 and –0.9°C. Therefore, to prevent freezing, smelt accumulate antifreeze proteins (AFP) and high levels of glycerol, which, unlike AFPs, lower the freezing point via colligative means. By performing a long term acclimation study, it was discovered that the upregulation of the antifreeze response (both glycerol and AFP) occurs in early fall, when water temperatures are 5-6°C. The accumulation of glycerol appears to be the main mechanism of freeze resistance in smelt as it contributes more to the lowering of the body's freezing point than the activity of the AFP (0.4 – 0.5°C versus 0.3°C for glycerol and AFP, respectively). The role of the AFP appears to be a safeguard mechanism to prevent freezing when glycerol levels are low. The significant increases in activities of the liver enzymes GPDH, AlaAT and PEPCK and their significant correlations with increasing glycerol levels, indicate that these enzymes are closely associated with, but do not regulate, the synthesis and maintenance of elevated glycerol levels in smelt for use as an antifreeze.

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## List of Abbreviations

AFGP: Antifreeze glycoprotein

AFP: Antifreeze protein

AlaAT: Alanine aminotransferase

ALD: Aldolase

AspAT: Aspartate aminotransferase

ATP: Adenosine triphosphate

$Ca^{2+}$: Calcium

CCAC: Canadian Council on Animal Care

DHAP: Dihydroxyacetone phosphate

FBP: Fructobisphosphate

G1P: Glucose-1-phosphate

G3P: Glycerol-3-phosphate

G3Pase: Glycerol-phosphatase

GPDH: Glycerol-3-phosphate dehydrogenase

G6P: Glucose-6-phospate

GAP: Glyceraldehyde phosphate

GSI: Gonadosomatic index

HK: Hexokinase

IDP: Inosine diphosphate

KCN: Potassium cyanide

LDH: Lactate dehydrogenase

$MgCl_2$: Magnesium chloride

$MnCl_2$: Manganese chloride

mRNA: Messenger ribonucleic acid

$NaHCO_3$: Sodium bicarbonate

NADH: Nicotinamide dinucleotide reduced from

NADPH: Nicotinamide dinucleotide phosphate reduced form

OAA: Oxaloacetate

PEP: Phosphoenolpyruvate

PEPCK: Phosphoenolpyruvate carboxykinase

PFK: Phosphofructokinase

PK: Pyruvate kinase

SEM: Standard error of the mean

## 1. Introduction

Ectothermic animals have the same body temperature as that of the environment and generally include all species except birds and mammals. Animals that live in the northern temperate regions of the earth are seasonally or constantly exposed to subzero temperatures. In order to survive such harsh environments, these ectothermic organisms have developed varied strategies in order to prevent death from freezing (Storey and Storey, 1988). Some rely upon behavioural means in order to avoid freezing, such as Monarch butterflies, which migrate annually to warmer climates. Others, such as frogs and turtles, undergo a period of torpor during which they remain in the ground below the frost line to prevent freezing (Storey and Storey, 1988). Animal species that do not rely on behavioural means of freeze avoidance must find alternative methods to prevent death from exposure to subzero temperatures. These species rely on several different physiological or biochemical mechanisms in order to survive the winter.

There are two main physiological/biochemical adaptations that have evolved within the animal kingdom: freeze tolerance, in which ice formation in extracellular fluids is tolerated for short periods of time; freeze resistance where ice formation within the body is lethal and animals have evolved various methods of avoiding ice formation by lowering the freezing point of body fluids (Storey and Storey, 1988). In order for an animal to tolerate freezing the formation of ice crystals must only occur extracellularly and the rate of freezing must be controlled in order to limit osmotic stress on the cells (Storey and Storey, 1988). Freeze tolerance has developed in many species of insects, both terrestrial and aquatic, and in a few species of amphibians and reptiles (Storey,

1997). Within freeze resistance strategies, animals lower their freezing points by either producing antifreeze proteins or through the accumulation of low-molecular weight carbohydrates that act as cryoprotectants (Storey and Storey, 1988).

### *1.2 Antifreeze Proteins*

The production of antifreeze proteins (AFP) has been discovered in many organisms, ranging from bacteria to plants to vertebrates (Ewart et al., 1999). However, the appearance of AFP in these organisms seems to be limited to those animals that inhabit environments in which they are periodically exposed to subzero temperatures (Ewart et al., 1999). These proteins lower the freezing point of body fluids in a non-colligative manner without causing an osmotic imbalance, and have been found to be 200-300 times more effective than molecules that lower the freezing point through colligative properties alone (Davies et al., 1988; Fletcher et al., 1998). Antifreeze proteins actively lower the freezing point of a solution by binding to the surface of ice crystals and preventing the addition of more water molecules, thereby inhibiting the growth of the crystal (Fletcher et al., 1998). Ice crystals have smooth rounded surfaces in their natural state; however, when AFPs are present, the shape of the crystal becomes faceted or long and spicular (DeVries, 1982; Fletcher et al., 1998). The depression of the freezing point of body fluids containing AFPs is proportional to the concentration of the antifreeze that is present (Fletcher et al., 1998). In teleost fishes, five different types of AFP have been discovered to date, type I-IV AFP and antifreeze glycoprotein (AFGP) (Table 1). It is hypothesized that the various types of AFP evolved recently, on an

evolutionary scale, and independently of each other as the various types are highly diverse and are narrowly distributed (Ewart et al., 1999).

**Table 1.** Summary of antifreeze protein types, teleost species in which they occur and distinguishing characteristics. (Modified from Ewart et al., 1999).

| AFP Type | Species | Characteristics |
|---|---|---|
| Type I | Flounder<br>Sculpin | Single α-helix<br>Sequence repeats |
| Type II | Sea Raven<br>Rainbow Smelt<br>Atlantic Herring | Globular<br>$Ca^{2+}$ dependent (smelt & herring) |
| Type III | Ocean Pout | Globular with one flat surface |
| Type IV | Longhorn Sculpin | Antiparallel helix bundle |
| AFGP | Atlantic Cod<br>Antarctic Nototheniids | Polymer of Ala-Ala-Thr and variants, disaccharide on each Thr. |

### *1.3 Low-Molecular Weight Cryoprotectants*

Not all animals rely upon antifreeze proteins to induce freeze resistance or freeze tolerance during exposure to subzero temperatures. Some animals accumulate unusually high levels of low-molecular weight solutes as a cryoprotectant. These solutes act to lower the freezing point of body fluids by their colligative properties, meaning that as the number of solute molecules in a liquid increases so does the melting or freezing point of that solution, in a predictable fashion unrelated to the type of molecule (Storey and

Storey, 1988). Various types of solutes are accumulated, the most common being polyhydric alcohols and sugars, such as glycerol, sorbitol and glucose (Storey and Storey, 1988; Storey, 1997). These solutes are able to diffuse through cellular membranes providing freeze protection for the whole body. As well, these naturally occurring cryoprotectants are relatively non-toxic to the cell at the high concentrations in which they occur (Storey and Storey, 1988; Storey, 1997). Glycerol is thought to be able to pass through water channels in cellular membranes, due to the orientation of the hydroxyl and hydrogen groups. This may explain why this molecule is frequently used for cell volume regulation in various organisms and possibly why it is the most commonly used cryoprotectant in insects (Storey, 1997). As well, glycerol has been found to act as a chemical chaperone in cells, aiding in the proper folding of proteins and the transport of denatured or mutant proteins across the cellular membrane (Welch and Brown, 1996; Morello et al., 2000). This action of glycerol as a chemical chaperone has been proposed as an alternative treatment to gene therapy for diseases such as cystic fibrosis and Alzheimer's, as glycerol may be able to enable the folding of the mutant proteins into the wild-type conformation, allowing them to perform their normal functions (Welch and Brown, 1996; Morello et al., 2000).

### *1.4 Freeze Tolerance in Insects*

Freeze tolerance has been found in many species of insects, especially in members of Coleoptera, Diptera, Hymenoptera and Lepidoptera, in virtually every life

stage and it enables survival in temperatures as low as -35° C. The accumulation of low molecular weight cryoprotectants is the most common method, with glycerol being the main molecule accumulated (Storey and Storey, 1988). However, there are examples of some insects employing the accumulation of AFPs in order to combat subzero temperatures (Ewart et al., 1999).

Research has shown that polyol cryoprotectant synthesis occurs in the fat body of insects, using glycogen as the substrate. Triglycerides and protein stores remain constant during the period of cryoprotectant synthesis, indicating that these stores are not mobilized for antifreeze production (Storey and Storey, 1988; Joanisse and Storey, 1994a; Joanisse and Storey, 1994b). For most insects, temperature, in the form of a threshold temperature cue, is the main trigger for cryoprotectant synthesis (Storey and Storey, 1988). When using environmental cues for the upregulation of freeze tolerance it is important for animals to have an anticipatory component to their freeze tolerance because of varying weather patterns from year to year (Storey and Storey, 1988). Glycerol production in insects follows a similar pattern each year, with accumulation beginning in early autumn, production reaching a plateau over midwinter and falling off in early spring (Storey and Storey, 1988; Joanisse and Storey 1994a; Joanisse and Storey, 1994b).

As mentioned above, the majority of glycerol is produced from the breakdown of glycogen via glycolysis. Strict regulation of glycolysis must occur in order to efficiently shunt carbon intermediates into the glycerol production pathways. Studies on glycerol accumulating insects show that preparation for cold exposure entails accumulation of

large reserves of glycogen and the induction of maximal enzyme activities in the top portion of glycolysis such as glycogen phosphorylase, hexokinase (HK) and phosphofructokinase (PFK) (Figure 1) (Storey and Storey, 1988). Enzymes such as glycogen phosphorylase and PFK are cold activated at temperatures between 0 and -5°C. The upregulation of these enzymes can initiate carbon flux into glycolysis but other controls are needed to shunt the carbon intermediates out of glycolysis and into glycerol production pathways (Storey and Storey, 1988). Research on gall moth larvae (*Epiblema scudderiana*) and gall fly larvae (*Eurosta solidaginis*), both of which accumulate up to 500-600 mM glycerol as a cryoprotectant, indicate that there are two possible pathways for glycerol production, both begin at the triose phosphate branch point and requiring NADH or NADPH for reducing power (Joanisse and Storey, 1994a; Joanisse and Storey, 1994b). The first pathway involves the dephosphorylation of glyceraldeyhde phosphate (GAP) to glyceraldehyde, which is then reduced to glycerol by polyol dehydrogenase (Figure 1). The second pathway involves the reduction of dihydroxyacetone phosphate (DHAP) to glycerol-3-phosphate (G3P) via the increased activity of glycerol-3-phosphate dehydrogenase (GPDH). G3P is then dephosphorylated by glycerol-3-phosphatase (G3Pase) to produce glycerol (Figure 1) (Joanisse and Storey, 1994a; Joanisse and Storey, 1994b).

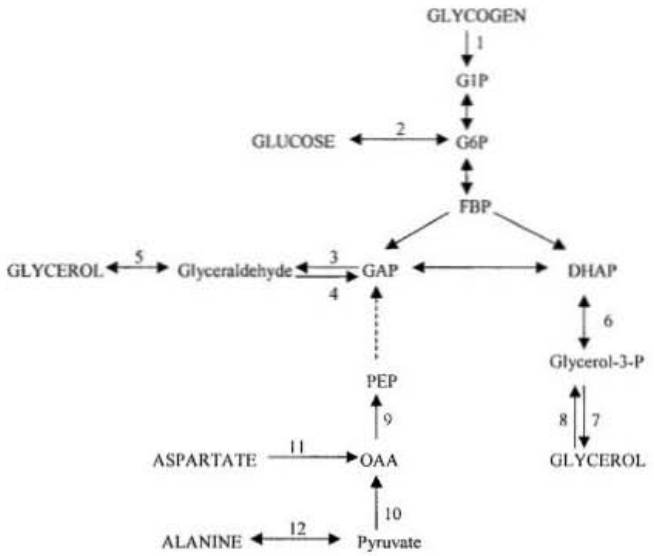



**Figure 1.** Metabolic pathway for the production of glucose and glycerol from the breakdown of glycogen and amino acids. Dotted lines indicate multiple conversion steps. Numbers refer to the following enzymes: 1) Glycogen phosphatase; 2) Glucose-6 phosphatase; 3) Glyceraldehyde-3 phosphatase; 4) Glyceraldehyde kinase; 5) Polyol dehydrogenase; 6) Glycerol-3-Phosphate dehydrogenase; 7) Glycerol-3 phosphatase; 8) Glycerol kinase; 9) Phosphoenolpyruvate carboxykinase; 10) Pyruvate carboxylase; 11) Aspartate aminotransferase; 12) Alanine aminotransferase.

### *1.5 Freeze Tolerance in Amphibians*

All freeze tolerant terrestrial vertebrates studied thus far have been found to accumulate high levels of low molecular weight cryoprotectants in order to induce freeze tolerance (Edwards et al., 2000). There are four known species of frogs in North America that have developed the ability to tolerate freezing these are: Wood frog (*Rana sylvatica*), Spring peeper (*Hyla crucifer*), Chorus frog (*Pseudacris triseriata*), and Gray treefrog (*Hyla versicolor*) (Storey and Storey, 1988; Layne, 1999; Layne and Jones, 2001). All these species spend the winter just below the leaf litter in a state of torpor and can be exposed to temperatures as low as -6°C for two to four weeks at a time (Storey and Storey, 1988; Layne, 1999). *Hyla versicolor* accumulates high levels of glycerol; whereas the other three species accumulate glucose as a cryoprotectant (Storey and Storey, 1988; Layne, 1999). The synthesis of both glucose and glycerol in frogs requires the mobilization of large reserves of glycogen in the liver. When released from the liver, the solute enters the blood stream where it is distributed to other organs throughout the body (Storey and Storey, 1988). Not all organs accumulate the same levels of cryoprotectant; studies have shown that central organs such as the liver, heart, brain and kidney accumulate the highest levels, followed by the lungs, stomach and intestine. Non-vital organs such as skeletal muscle, skin and gonad accumulate the least amount of cryoprotectant (Storey and Storey, 1988). Unlike insects, there is no anticipatory production of cryoprotectants in amphibians. The synthesis of glucose or glycerol is initiated with each period of freezing and reserves are cleared and returned to liver glycogen after each thaw (Storey and Storey, 1988). This adaptation prevents the loss of

energy that would be associated with producing continuous stores of such high levels of glycerol or glucose throughout the winter. As well, this adaptation prevents the toxic effects, similar to those of diabetes in mammals, of accumulating and maintaining high levels of glucose for extended periods of time (Storey and Storey, 1988). During freezing events the internal organs of *R. sylvatica* lose up to half of their water to the coelomic cavity, which becomes a layer of ice beneath the skin, preventing injury to tissues and organs due to ice formation (Costanzo et al., 1993).

The pathway for glucose production from glycogen in anurans is fairly simple, involving only the following enzymes: glycogen phosphorylase, phosphoglucomutase and glucose-6-phosphatase (Figure 1) (Storey and Storey, 1988). The control of glucose production in anurans lies mainly in the enhanced activity of glycogen phosphorylase and the inhibition of glycogen synthetase and phosphofructokinase (PFK), which prevent the flux of carbon intermediates into the glycolytic pathway (Storey and Storey, 1988; Edwards et al., 2000). Unlike insects, these enzymes are not cold activated, they respond only to the formation of ice within the body. It has been hypothesized that an intermediate signal (nervous or hormonal) is responsible for transferring information in order to initiate metabolic events in the liver (Storey and Storey, 1988). Discovery of the production of glycerol as an antifreeze in *H. versicolor* has only occurred within the last few years (Layne, 1999; Layne and Jones, 2001) and the metabolic processes behind the production of glycerol still remain elusive.

### *1.6 Freeze Resistance in Teleosts*

The freezing point of teleost blood ranges from –0.5 to –0.9°C (DeVries 1982). For marine teleosts that live in temperate and polar regions this poses a threat as during winter, water temperature in these oceans can drop as low as –1.9°C and these waters frequently become ice covered (Duman and DeVries, 1974) (Figure 2).

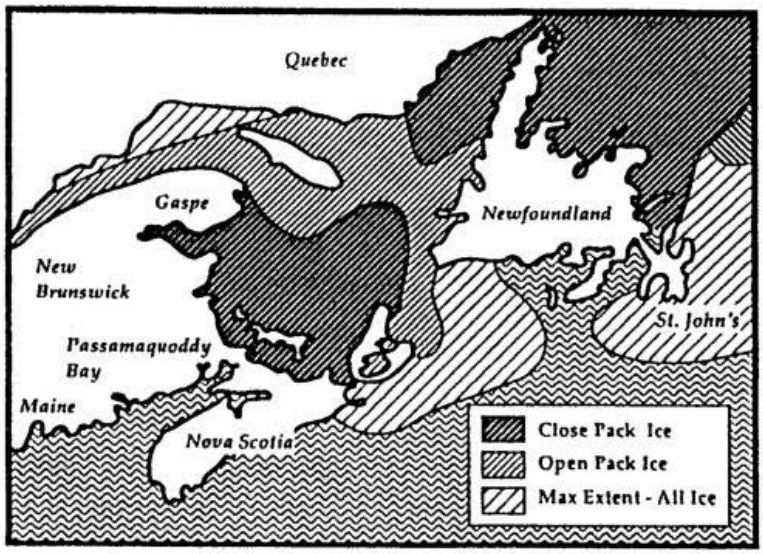



**Figure 2.** Average ice conditions in Atlantic Canada during the spring (periods of sub-zero water temperatures and ice coverage can occur at any time during the winter in this region) (Fletcher et al., 1992).

The majority of teleosts deal with the threat of freezing by migrating further off shore to deeper, warmer waters. Fish such as cunners (*Tautogolabrus adspersus*) enter a state of torpor and seek shelter in rocks and crevices on the ocean bottom in order to avoid

contact with ice, and exist for the winter months in a supercooled state (DeVries, 1982; Scott and Scott, 1988). Fish have also developed biochemical means of avoiding freezing which allow them to remain and thrive in these icy inshore waters. The most common method is the production and accumulation of a macromolecular antifreeze. These marcromolecules were first described in the early 1970s and are now known to be polypeptides or glycopeptides, which have been named antifreeze proteins. As noted earlier, they appear in five various forms in different species of fish (Table 1) (Fletcher et al., 1998; Ewart et al., 1999). In winter flounder (*Pleuronectes americanus*) the main site of AFP synthesis is the liver, from which they are secreted into the blood stream for transport throughout the whole body to lower the freezing point of the body fluids (Fletcher et al., 1998). The expression of AFP in teleosts varies depending on the type of AFP and the environment in which the animal lives. Winter flounder and various species of sculpin enlist an anticipatory production of Type I AFP before periods of environmental freezing occurs, usually on a predetermined annual cycle. Atlantic cod (*Gadus morhua*) initiate production of AFGP in direct response to freezing events. Whereas, ocean pout (*Macrozoarces americanus*) possess high levels of AFP all year round (Davies et al., 1988). Even though AFPs are found in many teleost species, the majority of the descriptive work has been carried out on winter flounder. The high activity levels of AFP in winter flounder allow it to lower its freezing point from –0.7°C in the summer to as low as –1.5°C in the winter (Duman and DeVries, 1974). AFP seasonal production cycle appears to be endogenously controlled. Flounder from two different geographic areas (Nova Scotia and Newfoundland) maintained differences in

the timing of AFP production when held under the same environmental conditions in a laboratory setting (Fletcher, 1981). This eliminated the speculation that the production and loss of AFP were controlled solely by environmental factors such as temperature and photoperiod. However, photoperiod does appear to play an important role in the initiation of AFP production in winter. It seems that changes in photoperiod are sensed by the central nervous system. Decreasing day lengths cause the inhibition of growth hormone secretion from the pituitary, which signals the AFP genes in the liver to begin the synthesis of AFP mRNA and in turn the production of AFP is increased. In the summer the reverse happens, with increasing day lengths stimulating the production of growth hormone that inhibits the actions of AFP genes, preventing further production of the protein (Fletcher et al., 1998). In contrast, the closely related skin-type AFPs of winter flounder are not seasonally regulated (Fletcher et al., 2001).

### *1.7 Freeze Resistance in Rainbow Smelt*

Rainbow smelt (*Osmerus mordax*) is a small anadromous species of fish that is a member of the Family Osmeridae, which inhabits coastal and inshore waters in the North Atlantic, Arctic and North Pacific oceans (Scott and Scott, 1988). Smelt are usually found in large schools and are a predaceous fish whose diet is mostly carnivorous (Scott and Scott, 1988). Rainbow smelt, inhabit inshore coastal waters, usually at depths no greater than 6 m or further than 2 km from the shoreline (Buckley, 1989). During the winter, these waters can reach temperatures as low as –1.9°C and are frequently ice-

covered (Figure 2). As this species remains in these waters during the winter, it must employ a biochemical means of freeze resistance in order to prevent freezing.

Smelt produce AFP in response to these low temperatures (Duman and DeVries, 1974). Further research into smelt and their ability to resist freezing demonstrated that this AFP was a Type II AFP similar to that of the sea raven, but in smelt this protein is capable of binding $Ca^{2+}$ and requires its presence for activity (Ewart et al., 1999). However, the activity of this AFP was much lower on a concentration basis than levels of AFP in other fish such as the winter flounder and ocean pout, which have AFP activity that may depress the freezing temperature by 1.4°C during the winter months (Ewart and Fletcher, 1990).

Further research into the freeze resistance of smelt allowed the discovery that smelt accumulate unusually high levels of organic solutes, specifically glycerol, whose colligative properties allow the depression of the freezing point of the blood (Raymond, 1992). Winter caught smelt, at water temperatures of –1.5°C, had blood glycerol levels as high as 200-400 mM (normal glycerol levels in animals are usually less than 1 mM) (Raymond, 1992). Based on results from this study, the production of glycerol in smelt was thought to be initiated only upon exposure to extremely low temperatures (Raymond, 1992). The accumulation of high levels of glycerol as a method of freeze resistance is not found in any other teleost family. As mentioned previously, there are terrestrial animals that accumulate glycerol as colligative antifreeze (Storey and Storey, 1988). However, smelt are unique in that they remain active throughout the winter season, and therefore must deal with the constant loss of glycerol to their aqueous

environment, which is proposed to occur at rates of up to 10% of glycerol stores per day (Raymond, 1993). Glycerol is easily lost via the skin and gills as cellular membranes are highly permeable to this molecule (Raymond, 1992). Smelt also increase production and accumulate higher levels of other organic solutes such as urea and trimethylamine oxide (TMAO) during the winter months, which most likely aid in the antifreeze response (Raymond, 1994; Raymond, 1998; Treberg et al., 2002a).

Initial studies described glycogen as the carbon source for glycerol production, as in insects and frogs that produce glycerol as a cryoprotectant. This was based upon a strong inverse correlation between glycogen levels and plasma glycerol levels in winter-caught fish (Raymond, 1995). This was further confirmed when the $^{14}C$ label appeared in glycerol in fish that had been injected with $^{14}C$ glucose. Other supporting evidence for the use of glycogen for glycerol production is the decrease in glycogen stores in short-term starvation experiments and the increase in glycogen phosphorylase activity in liver and muscle in winter caught smelt (Raymond, 1995). However, upon closer examination, smelt in starvation experiments were maintaining high glycerol levels for up to two weeks after glycogen stores had been depleted, indicating that there must be another active glycerol synthesizing pathway present in rainbow smelt (Raymond et al., 1996). Measurements of triglyceride stores indicated that they could not be a potential source as levels remained constant and in some cases increased slightly during this period (Raymond, 1995; Raymond et al., 1996). The next logical step was to look towards proteins as the source for glycerol production via gluconeogenesis as preliminary studies showed the appearance of $^{14}C$ labeled glycerol in fish that had been

injected with labeled pyruvate. As well, there was an increase in the activity of the liver enzyme phosphoenolpyruvate carboxykinase (PEPCK), which is known to be a key regulating enzyme of gluconeogenesis in glycerol producing smelt (Figure 1) (Raymond, 1995). To strengthen this line of thinking, *in vivo* experiments were carried out using radiolabeled amino acids (alanine and glutamate) in order to confirm the use of proteins as precursors of glycerol production (Raymond and Driedzic, 1997). This study indicated that amino acids are used as precursors of glycerol synthesis via gluconeogenesis (Raymond and Driedzic, 1997). This route of glycerol synthesis is active even during periods of feeding, which correlates well with smelts' consumption of a high protein diet during the winter months, most likely to maintain amino acid stores for elevated rates of glycerol production (Raymond and Driedzic, 1997). During periods of feeding, the use of glycogen as a precursor for glycerol production is practically non-existent, indicating that smelt rely mainly upon the breakdown of amino acids for glycerol synthesis (Raymond and Driedzic, 1997).

At this time, the main goal of research on the production of glycerol as an antifreeze in rainbow smelt has taken on more of a descriptive role. Studies began to focus on the finer points of the glycerol producing pathways in hopes of pinpointing the regulating loci. Two comparative studies were successful in demonstrating that the livers of smelt are specially poised for increased glycerol production during the winter months. Driedzic et al. (1998) and Treberg et al. (2002b) accomplished this by comparing the key enzymes of gluconeogenesis and glycerol synthesis in smelt to non-glycerol producing fish caught from the same environment (tomcod and smooth

flounder) and capelin (*Mallotus villosus*), which are closely related to smelt (Driedzic et al., 1998; Treberg et al., 2002b). It was thought that capelin may also produce high levels of glycerol during the winter months as they are from the same family as smelt and also live in icy waters during the winter months, but results indicated that at temperatures of –0.7 to –1.5°C the blood glycerol level of capelin was in the normal range for teleost fish (<1mM) (Raymond and Hassel, 2000).

In comparison to livers of tomcod (*Microgadus tomcod*) and smooth flounder (*Liopsetta putmani*) the livers of smelt appear to be specially designed for the increased production of glycerol during the winter months (Driedzic et al., 1998). Enzymes of amino acid breakdown, such as alanine aminotransferase (AlaAT) and aspartate aminotransferase (AspAT) showed significantly higher activity in smelt than in the other two fish (Figure 1). As well, pyruvate kinase (PK), which is a regulatory enzyme of the lower end of the glycolysis pathway, had significantly lower activity in smelt, indicating that the liver of smelt is poised for amino acid breakdown and the shunting of carbon intermediates of glycolysis to glycerol synthesis instead of amino acid formation (Driedzic et al., 1998). When examining the enzymes of the glycerol synthesis pathways, the similarity in activity levels for GAP and polyol dehydrogenase between the three species of fish and the significantly higher activity levels of GPDH and G3Pase in smelt suggest that the production of glycerol in smelt may occur mainly through the breakdown of DHAP to G3P and not through glyceraldehyde as in insects (Figure 1) (Driedzic et al., 1998).

Results from the comparison between liver enzyme activities of smelt and the closely related capelin carried out by Treberg et al. (2002b) further emphasized that the livers of smelt are unique in their capability to synthesize high levels of glycerol. Enzymes of the upper end of the glycolysis pathway such as PFK and aldolase (ALD) were significantly higher in smelt than in capelin, indicating the smelt liver is better poised for glycerol production via the breakdown of glycogen than the liver of capelin (Treberg et al., 2002b). The activity of the key enzymes in amino acid breakdown (AlaAT and AspAT) and gluconeogenesis (PEPCK) (Figure 1) were all significantly higher in smelt than in capelin, reinforcing the fact that glycerol production occurs mainly through the breakdown of amino acids via gluconeogenesis and indicating that smelt livers have a greater gluconeogenic capacity than capelin during the winter (Treberg et al. 2002b). Finally, the extremely high levels of GPDH in smelt, which are producing up to 200 mM of glycerol at this time, as compared to the non-glycerol producing capelin, supports the results of Driedzic et al. (1998) that suggest that in smelt glycerol may be produced mainly via the breakdown of DHAP and not through the alternative pathways as in insects (Treberg et al., 2002b). This idea has also been supported by molecular work on smelt indicating that GPDH mRNA is significantly elevated in fish held at low temperatures (high glycerol levels) as opposed to fish held at warm temperatures (Ewart et al., 2001).

Recently, a study was conducted in order to examine factors of the antifreeze response in smelt over an entire winter. Fish were caught and maintained in a laboratory setting from December to May at either ambient seawater temperature or at 5°C for the

entire season (Treberg et al., 2002a). This study demonstrated that the antifreeze response was upregulated well before December (5°C) as glycerol levels had already reached levels of 80 mM and AFP activity (thermal hysteresis) was 0.2°C by the initial sampling time (Treberg et al., 2002a). Results also indicated that constant low temperatures are required for the maintenance of high levels of osmolytes and AFP as fish in the 5°C decreased their levels soon after being placed in the warmer water. However, smelt in the ambient treatment began to lose glycerol from the blood in mid-February, while water temperatures were still low, but AFP kept increasing until the end of the study. Indicating that the antifreeze response in smelt is asynchronous with respect to the timing of glycerol and AFP production (Treberg et al., 2002a). With regards to the enzyme activity levels, the results from this study agreed with previous results described by Driedzic et al. (1998). Activity of GPDH was elevated at the initial sampling date but consistently decreased for the remainder of the study, indicating that the upregulation of this key enzyme for glycerol production in smelt may occur early in the fall, before glycerol levels begin to increase in the blood and decreases to maintenance levels for the duration of the winter (Treberg et al., 2002a). There was no significant difference in the activity of any of the enzymes studied despite the level of glycerol in the blood, indicating that the decrease in glycerol levels in fish exposed to warm water is not due to decreased enzyme activity at these particular loci (Treberg et al., 2002a).

## 2. Purpose of Research

The previously mentioned long term seasonal study provided the groundwork upon which research for this M.Sc thesis was based. The main goal of the present research was to describe the full antifreeze response in rainbow smelt, from initiation of the response in early fall, to the peaks of antifreeze components and suppression of the response in the spring. To accomplish this goal, the project examined the timing and levels of glycerol and antifreeze protein in order to fully describe the pattern of freeze resistance in smelt, and to attempt to pinpoint the environmental or endogenous triggers of the antifreeze response. As well, the activities of key liver enzymes involved in the breakdown of amino acids, gluconeogenesis and glycerol production were explored in order to determine potential regulating loci of glycerol synthesis in smelt.

By achieving these goals, a unique physiological/biochemical process in teleost freeze resistance is described, which could be used as a model system to which other glycerol producing animals could be compared.

The research in this project also has application to the fields of medical science and aquaculture. Glycerol has been described as a potential chemical chaperone through which it aids in the refolding and transport of misfolded proteins across cellular membranes. These properties of glycerol could make it a potential treatment for human genetic diseases such as cystic fibrosis and Alzheimer's. In order to be successful in this form of treatment, glycerol would have to be present in molar levels. By studying how smelt are capable of increasing glycerol production 200-400 fold, it may be possible to

induce increased glycerol production in other animals, including humans, for medical purposes.

In addition to medical treatment, the induction of high levels of glycerol production in animals that cannot produce glycerol in such high amounts could be very beneficial to the aquaculture industry in northern countries such as Canada. At this time, the majority of aquaculture species that are farmed in seawater cages do not have a natural biochemical method of freeze resistance. These species, such as salmon, usually swim to deeper waters off shore to avoid subzero seawater temperatures and contact with ice. The majority of seawater cage aquaculture in Canada is restricted to sheltered bays and inlets where seawater temperature rarely drops below 0°C and ice coverage rarely occurs, in order to prevent high mortality rates of stocks due to "superchilling". If through bioengineering, it becomes possible to induce freeze resistance in these animals by inducing the production of AFP or elevated levels of glycerol as in smelt, the industry could be opened up to many other areas. As well, many of the parasites that plague cultured species are unable to survive subzero temperatures, therefore, by farming fish in cooler waters, with greater distance between farms it would become possible to culture healthier fish and spread the wealth of the industry to other areas of the country. Another suggested use of glycerol to enhance the production of cultured species is based upon glycerol's role as a stress protectant in yeast cells (Pahlman et al., 2001). It may be possible that inducing elevated levels in cultured fish could result in protection against stress and in doing so increase growth rates and disease resistance, which in turn would increase profitability in the industry.

## 3. Materials & Methods

### *3.1 Animal Collection*

Approximately 450 smelt were obtained from fresh water streams near estuaries in mid-October 2000. Water temperature was approximately 11°C at that time. Fifty smelt were caught by dip net in Gambo River and 400 smelt were caught by seine netting in Long Harbour, Placentia Bay, Newfoundland. All fish were transported in freshwater to the Ocean Sciences Centre (OSC), Memorial University of Newfoundland, where they were transferred to a 4.0 $m^3$ tank with flow through ambient seawater (11°C). Fish were fed three times a week with frozen brine shrimp for the first two weeks, after which they were switched to a diet of chopped frozen herring for the duration of the study. On November 26, 2000 (ambient temperature 6°C) 230 fish were transferred to a second 4.0 $m^3$ tank with flow through heated seawater, the water temperature in this tank was maintained at 8-11°C throughout the study. On March 14, 2001, 40 fish from the ambient tank were moved to an insulated 1.0 $m^3$ tank with flow through chilled seawater to study the effect of extreme cold temperatures on glycerol production in the smelt. These three tanks comprised the ambient, heated and chilled seawater treatments respectively and all treatments were exposed to natural photoperiod.

### *3.2 Sample Collection*

Blood was drawn from five fish from Gambo River while in the field to obtain an initial blood glycerol level reading. After transport to the OSC, five fish were randomly sampled from each treatment at approximately every 1°C drop in ambient seawater

temperature. Fish were killed with a blow to the head and blood was drawn via the caudal blood vessel with a heparinized 25-gauge needle/1 ml syringe and livers were removed, cut into sections and frozen in liquid nitrogen. The treatment of fish and sampling procedures were in accordance with CCAC guidelines. Blood samples were stored on ice for approximately 20 minutes before centrifuging for 5 minutes. Plasma was then removed, divided into two parts for glycerol and antifreeze protein analysis, and frozen in liquid nitrogen. All samples were stored at -80°C until analysis. Each fish sampled was individually numbered, and the sex, gonadosomatic index (GSI = gonad weight / somatic weight x 100), and presence or absence of food in the stomach were recorded.

Water temperature and general fish health was observed and recorded for each tank on a daily basis throughout the study. All animals exhibited signs of good health under laboratory conditions and death rates were low. The one exception was for fish held in the heated seawater treatment. These fish were of good health and had low mortality rates until February – March when they underwent early spawning due to the elevated seawater temperatures. After spawning the tank experienced high mortality rates due to the natural occurrence of high male mortality after spawning events (Buckley, 1989).

### *3.3 Plasma Glycerol Levels*

Plasma samples were analyzed for glycerol content using Sigma diagnostic kit No. 337-40A. A standard curve ranging from 5.2 μg – 0.315 μg, was run with samples,

and plasma samples were diluted in order to remain in the linear portion of the assay. Twenty μl of sample and 2 ml of triglyceride reagent was added to each tube, and each sample was run in duplicate. Tubes were incubated at room temperature for 15 minutes, after which absorbance at 540 nm was measured on a Novaspec spectrophotometer. Values were corrected with a blank. The increase in absorbance at 540 nm is directly proportional to the glycerol concentration of the sample (mM).

### *3.4 Antifreeze Protein Activity*

Antifreeze protein activity analysis was based upon the methods used in Ewart et al. (2000). Plasma samples were placed in cylindrical wells on metal plate, which is placed on a cooling stage and viewed through a compound microscope. For each individual plasma sample, triplicate measurements of the freezing and melting points of a single ice crystal were obtained using a Clifton nanoliter osmometer (1000 mOsmols = -1.86°C). Blank values were obtained by measuring the freezing and melting points of deionized water, and used to correct for background error of the osmometer. Values for AFP activity are based on the thermal hysteresis of the sample (°C) and were calculated by subtracting the average melting point from the average freezing point of the sample.

### *3.5 In Vitro Liver Enzyme Activity*

Liver samples were weighed and homogenized in nine volumes of extraction buffer for three 10-second bursts with a Polytron tissue homogenizer. Extraction buffer

(pH 7.4 at 5°C) contained: 20 mM imidazole, 5 mM EGTA, 5 mM EDTA, 10 mM β-mercaptoethanol and 50 mM NaF. Maximum enzyme activities in crude homogenates were determined spectrophotometrically under optimal conditions of pH. Enzyme activities are expressed as μmoles substrate converted to product $min^{-1}$ $g^{-1}$ tissue. All assays were run on a Beckman DU640 spectrophotometer with a circulating water-jacketed cell holder maintained at 15°C. Procedures and conditions for each individual enzyme assay were taken from Driedzic et al. (1998).

Glycerol-3-Phosphate Dehydrogenase (EC 1.1.1.8): Assay medium contained 20 mM imidazole and 0.15 mM NADH at pH 7.6 at 20°C. Reaction was initiated with 2 mM of DHAP.

Phosphoenolpyruvate Carboxykinase (EC 4.1.1.32): Assay medium contained 80 mM Tris 7.4, 1 mM KCN, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 1.5 mM IDP, 1.1 mM PEP, 0.17 NADH, 19 IU $ml^{-1}$ of MDH in glycerol at pH 7.0 at 20°C. Reaction was initiated with 20 mM $NaHCO_3$.

Alanine Aminotransferase (EC 2.6.1.2): Assay medium contained 50 mM imidazole, 1 mM KCN, 200 mM alanine, 0.05 mM pyridoxal-5-phosphate and 1 IU $ml^{-1}$ of LDH at pH7.4 at 20°C. Reaction was initiated with 10 mM α-ketoglutarate.

Aspartate Aminotransferase (EC 2.6.1.1): Assay medium contained 50 mM imidazole, 1 mM KCN, 30 mM aspartate, 0.05 mM pyridoxal-5-phosphate and 7 IU $ml^{-1}$ of MDH at pH 7.4 at 20°C. Reaction was initiated with 7 mM α-ketoglutarate.

### *3.6 Data Analysis*

For each treatment, values for the five fish killed per sampling time were averaged and results are expressed as mean ± SEM (n = 5). Values of specific parameters were compared using a one-way ANOVA ($p<0.05$ significant) to obtain significant differences between treatments at a particular sampling time. Tukey's post-test was used to determine significant differences within a treatment throughout the study. Regression analysis was conducted in order to determine if there is a positive correlation between enzyme activity levels and plasma glycerol content for fish in the ambient and chilled treatments only (n = 76) in order to examine the relationship between specific enzyme activities and the production of glycerol as a response to low temperatures. Results are expressed as individual sample points ($p<0.05$ significant).

## 4. Results

### *4.1 Water Temperature*

Ambient seawater temperature varied with season, ranging from 11°C in October to −1.0°C in February. The heated seawater used in this experiment was maintained approximately between 8°C and 11°C for the entire study. The temperature of the chilled

seawater used was consistently 2°C lower than the temperature of the ambient seawater, ranging from –1.5°C to 2°C (Figure 3A).

### *4.2 Plasma Glycerol*

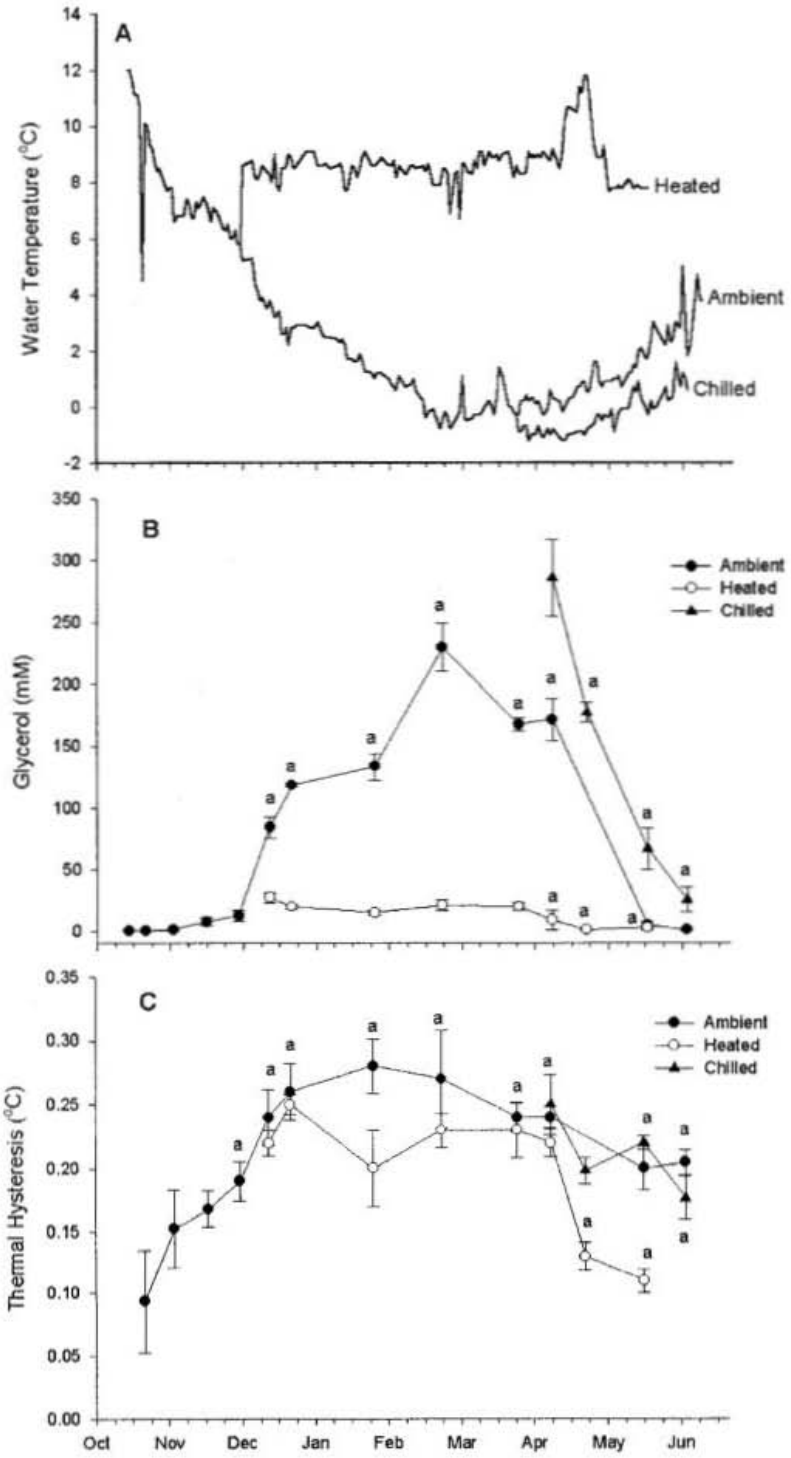
Initial glycerol levels from fish sampled in the field were 0.3 ± 0.04 mM. Fish that were maintained at ambient seawater temperature showed a significant increase to 84.4 ± 8.7 mM in December when water temperatures reached 5°C (Figure 3B). These fish reached a peak glycerol concentration of 229.9 ± 19.8 mM in February (-1°C), after which levels began to decrease. A significant decrease to 4.9 ± 2.5 mM was observed by the end of April, bringing the glycerol levels close to those obtained in early fall (Figure 3B). The glycerol levels in fish at the initial sampling date after their transfer to the heated seawater (8-10°C) was 27.2 ± 4.0 mM. Smelt maintained glycerol concentrations at this slightly elevated level until April when there was a significant decrease to 0.7 ± 0.08 mM. By exposing fish to chilled seawater temperatures as low as –1.5°C, glycerol levels were elevated to 285.8 ± 31.1 mM. After this peak in April, smelt began to steadily lose glycerol at a significant rate, regardless of the extreme cold temperatures, to a final concentration of 25.0 ± 10.4 mM by June (Figure 3B).

### *4.3 Antifreeze Protein Activity*

Initial thermal hysteresis values obtained in October were 0.09 ± 0.03°C. A significant increase, in the ambient temperature group, to 0.18 ± 0.01°C was observed in

November when water temperatures were approximately 6°C. A peak value of 0.28 ± 0.02°C was reached in January (1°C). Antifreeze protein activity remained at this level for the duration of the study for fish in the ambient and chilled treatments. There was no significant difference in antifreeze protein activity between the three treatments until April when fish in the heated treatment experienced a significant decrease in thermal hysteresis to 0.10 ± 0.01°C (Figure 3C).

**Figure 3.** Temperature profile (A) for smelt held in a long-term acclimation study from October 2000 to June 2001 and seasonal pattern of plasma glycerol levels (mM) (B), antifreeze protein activity (thermal hysteresis °C) (C) (● ambient, o heated, ▲ chilled). Symbols represent mean ± SEM (n=5), "a" indicates significance (ANOVA: $p<0.05$) from initial sampling point within a treatment.


A
Water Temperature (°C)
Heated
Ambient
Chilled
B
Glycerol (mM)
Ambient
Heated
Chilled
C
Thermal Hysteresis (°C)
Ambient
Heated
Chilled
Oct
Nov
Dec
Jan
Feb
Mar
Apr
May
Jun

### *4.4 Glycerol 3-Phosphate Dehydrogenase*

Initial activity of liver GPDH in smelt sampled in October was 76. 2 ± 11.7 μmol $g^{-1}$ $min^{-1}$. In smelt from the ambient treatment, activity significantly increased in December to 172.7 ± 36.9 μmol $g^{-1}$ $min^{-1}$, after which it decreased to the initial range of activity. Another significant increase was observed in February when activity returned to 173.6 ± 17.8 μmol $g^{-1}$ $min^{-1}$, after which activity once again decreased to initial levels. Activity for GPDH in fish sampled from the chilled seawater was not significantly different from activity levels in fish in ambient seawater. However, activity levels from smelt sampled from the heated seawater treatment gradually increased from initial values (104.4 ± 11.3 μmol $g^{-1}$ $min^{-1}$) to a peak in February, when activity levels reached 277.6 ± 28.0 μmol $g^{-1}$ $min^{-1}$. Enzyme activity remained at this elevated level until April, when it began to decrease, approaching initial levels for activity for smelt in the heated treatment (Figure 4A).

### *4.5 Phosphoenolpyruvate Carboxykinase*

In October, activity levels for PEPCK in smelt were 2.9 ± 0.3 μmol $g^{-1}$ $min^{-1}$. During the study there were no significant differences found in activity levels in smelt from the ambient, heated or chilled seawater treatments. There was a gradual increase in activity throughout the study, which reached a significant increase from initial levels in April when fish from the ambient and heated treatment had activity levels of 5.8 ± 0.3 and 4.7 ± 0.4 μmol $g^{-1}$ $min^{-1}$ respectively (Figure 4B).

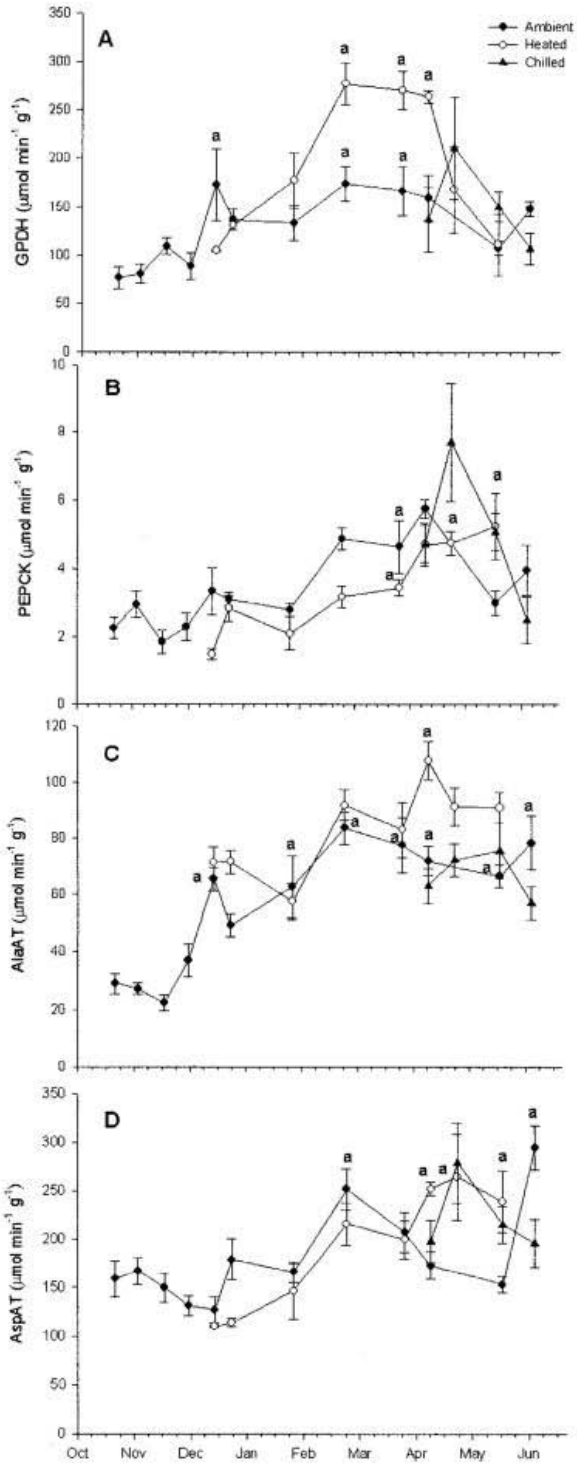
### *4.6 Alanine Aminotransferase*

Activity levels for AlaAT in early fall were recorded at 28.9 ± 3.5 μmol $g^{-1}$ $min^{-1}$. There was no significant difference observed in enzyme activity in smelt sampled from the ambient, chilled or heated seawater treatments. Activity levels gradually increased throughout the study, with significant increases in the fish from the ambient seawater in December, when levels reached 65.5 ± 4.1 μmol $g^{-1}$ $min^{-1}$. Another significant increase in enzyme activity was observed in February for fish in the ambient treatment when levels reached 83.8 ± 5.7 μmol $g^{-1}$ $min^{-1}$, AlaAT activity was maintained at this elevated level for the duration of the study (Figure 4C).

### *4.7 Aspartate Aminotransferase*

Initial enzyme activity levels for smelt in October were recorded at 158.8 ± 18.8 μmol $g^{-1}$ $min^{-1}$. Enzyme activity remained at this level for fish in both the heated and ambient seawater treatments until February when a significant increase in activity (251.9 ± 21.5 μmol $g^{-1}$ $min^{-1}$) was observed in fish from the ambient seawater, which peaked again in late May to 294.4 ± 25.5 μmol $g^{-1}$ $min^{-1}$. Fish exposed to heated seawater throughout the study, showed an increase in enzyme activity from initial values in April, when levels increased to and maintained at 264.6 ± 44.4 μmol $g^{-1}$ $min^{-1}$ for the duration of the study (Figure 4D).

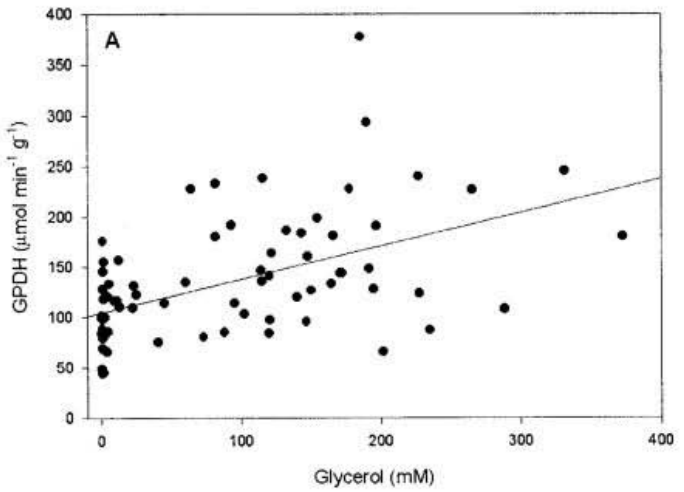
**Figure 4**. Seasonal pattern of in vitro enzyme activity (μmol $g^{-1}$ $min^{-1}$) of A) GPDH, B) PEPCK, C) AlaAT and D) AspAT, in the livers of rainbow smelt held in a long term acclimation study in ambient (●), heated (o) and chilled (▲) seawater from October 2000 to June 2001. Values represented as mean ± SEM (n=5), "a" indicates significance (ANOVA: $p<0.05$) from initial sampling point within a treatment ($p<0.05$).

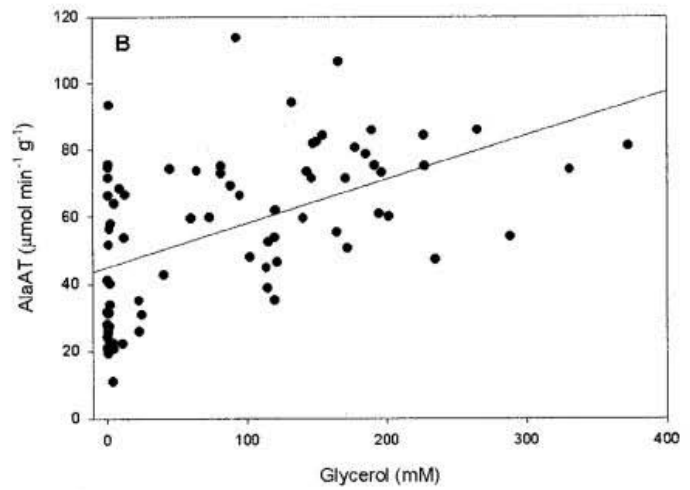

A
B
C
D
Ambient
Heated
Chilled
GPDH (μmol min-1 g-1)
PEPCK (μmol min-1 g-1)
AlaAT (μmol min-1 g-1)
AspAT (μmol min-1 g-1)
Oct
Nov
Dec
Jan
Feb
Mar
Apr
May
Jun

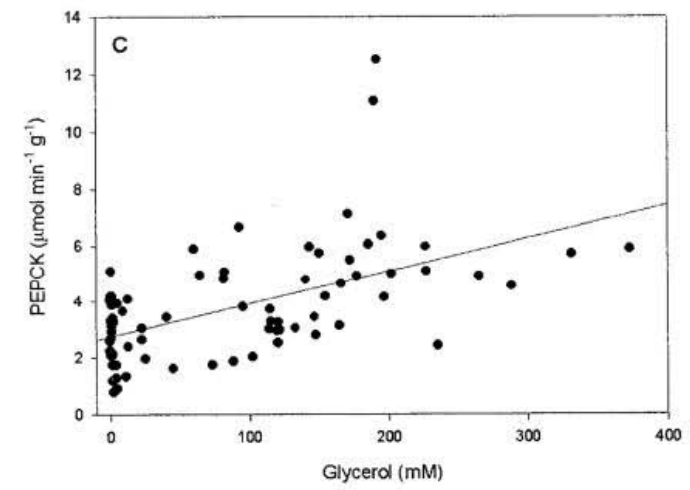
4.8 Regression Analysis

The purpose of performing regression analysis was to determine if there was a significant relationship between various enzyme activities and the increased production of glycerol in response to low temperatures. In order to achieve this, only fish held in the ambient and chilled treatments, were used for the regression analysis. Fish from the heated treatment were not analysed as glycerol levels were low and there may have been additional demands on the glycerol production pathway that were not associated with temperature, such as the production of triglycerides during the period when fish, especially the females, were becoming gravid. A relationship to glycerol production in the liver becomes apparent when enzyme activities for fish in the ambient and chilled water treatments are plotted against plasma glycerol levels for the same fish. A significant positive correlation was found between enzyme activity and plasma glycerol content for GPDH, PEPCK, and AlaAT (p values of $2.77 \times 10^{-6}$, $3.97 \times 10^{-7}$ and $1.12 \times 10^{-6}$ respectively (Figure 5).

**Figure 5**. Regression analysis comparing plasma glycerol level (mM) and in vitro liver enzyme activity for A) GPDH, B) AlaAT and C) PEPCK ($\mu$mol $g^{-1}$ $min^{-1}$) in fish held in ambient and chilled treatments. Symbols represent individual fish (n=76); ($r^2$ = 0.26, 0.28, 0.30; $p < 0.001$ in all cases).


A
GPDH (µmol min-1 g-1)
Glycerol (mM)
0
50
100
150
200
250
300
350
400


B
AlaAT (µmol min-1 g-1)
Glycerol (mM)
0
20
40
60
80
100
120


C
PEPCK (µmol min-1 g-1)
Glycerol (mM)
0
2
4
6
8
10
12
14

## 5. Discussion

As previous studies have demonstrated, rainbow smelt employ both the accumulation of low molecular weight cryoprotectants and antifreeze proteins as a means of preventing freezing during the winter months (Ewart and Fletcher, 1990; Raymond, 1992; Treberg et al., 2002a). However, the specific contributions and interactions of these two biochemical means of freeze resistance to the antifreeze response in rainbow smelt throughout the whole season and the specific metabolic factors that regulate the increased production of glycerol still remained elusive. The present study was the first to depict the antifreeze response over a full season, from early fall to early summer. Therefore, new information on the seasonal pattern of the individual contributions of glycerol and AFP to the antifreeze response is now available. As well, further insight has been gained in the area of the metabolic processes and potential regulating loci responsible for the increased production of glycerol throughout the winter months.

### *5.1 Plasma Glycerol & Antifreeze Protein Activity*

Results from this study confirm the ideas proposed Treberg et al. (2002a), which suggested the antifreeze response in smelt is upregulated well before there is any threat of freezing. Both AFP activity and plasma glycerol levels underwent a significant increase in early December when water temperatures were 5-6°C. Glycerol levels increased to 85 mM and the increase in AFP activity resulted in a 0.2°C decrease in the freezing point of the blood. These values are in the same range obtained in the study by Treberg et al. (2002a). The results from both these studies contradict the original suggestions that the

production of glycerol in smelt occurs only after exposure to subzero temperatures (Raymond, 1992). The accumulation of glycerol appears to be the main mechanism of freeze resistance in smelt as during mid-winter it contributes a greater degree of the lowering of the body's freezing point than the activity of the AFP (0.4 – 0.5°C versus 0.3°C for glycerol and AFP respectively). However, the earlier upregulation of AFP activity in the fall and the maintenance of activity in the spring of the year, well after glycerol levels had returned to normal values suggests that the production of AFP may serve as a safeguard against unexpected freezing events that may occur when glycerol levels are low.

The seasonal pattern of glycerol accumulation in the blood of smelt appears to be quite similar to that of insects. Production begins in early fall, reaching a peak mid-winter after which levels begin to decrease, with complete loss occurring in the spring (Storey and Storey, 1988; Joanisse and Storey, 1994a; Joanisse and Storey, 1994b). This information places the one species of glycerol-producing anuran, *Hyla versicolor*, in a category of its own, as it does not experience an anticipatory increase in glycerol levels. Glycerol synthesis in *H. versicolor* is stimulated by the formation of ice within the body (Layne, 1999; Layne and Jones, 2001). From the data obtained in this study, it is not possible to determine the triggers of the seasonal production and loss of glycerol production in smelt. The production of glycerol in the fall could be triggered by exposure to a temperature of 5-6°C, a photoperiod cue or a combination of both environmental factors. The loss of glycerol from the blood in the spring does not appear to be regulated by temperature as there was a dramatic decrease in blood glycerol levels in all three

treatments at the same point of time, regardless of the water temperature. This could indicate that the cessation of glycerol production is triggered by a photoperiod cue, or controlled by a genetically pre-determined endogenous cycle, similar to the control of AFP production in winter flounder (Fletcher et al., 1998). As in other studies, these results indicate that the maintenance of high levels of glycerol requires exposure to low temperatures. However, even though fish exposed to abnormally high temperatures for the winter season (8-10°C) did show a significantly lower level of glycerol in their blood (10 –20 mM), the levels remained slightly higher than those found in smelt during the summer (<1 mM) which fall within the normal range for glycerol in teleost fish. This could be due to an endogenous cycle being switched on in early fall that mobilizes the breakdown of carbon sources in the body into glycerol producing pathways, with the amount that is retained in the blood being dependent upon temperature. The pattern of glycerol production, accumulation and loss in rainbow smelt appears to follow the same annual cycle, as the timing of the cycle in this study follows a similar pattern as what was described in a previous seasonal study by Treberg et al. (2002a).

The seasonal pattern of AFP in smelt follows the same general pattern as that of glycerol accumulation, however, the increase in the fall of the year is more gradual than that of glycerol and synthesis is upregulated a couple of weeks earlier. The main difference between the two components of the freeze response is the decrease in temperature throughout the season is correlated with the increase in glycerol levels, but not with AFP activity. Fish in the heated treatment had the same level of activity as those in the ambient and chilled treatments. This can be explained by the fact that production

of AFP in other teleost fish, such as winter flounder, has been found to be controlled by a pre-determined genetically controlled cycle. Winter flounder from two distinct populations, which were held under the same environmental conditions in the laboratory maintained their own endogenous AFP production cycles, demonstrating that the cycle is not controlled solely by environmental factors (Fletcher et al., 1998). Further research on winter flounder has shown that the production of AFP is regulated by the central nervous system, which responds to changes in photoperiod. Decreasing day lengths cause the inhibition of growth hormone secretion from the pituitary, which signals the AFP genes in the liver to begin the synthesis of AFP mRNA and in turn the production of AFP is increased. In the summer the reverse happens, with increasing day lengths stimulating the production of growth hormone that inhibits the actions of AFP genes, preventing further production of the protein (Fletcher et al., 1998). Results from the present study do suggest that the cessation of AFP production in smelt may be influenced by temperature after a certain point. AFP activity dropped off in the fish held in heated seawater in April, but activity levels were maintained in fish in the ambient and chilled treatments, where water temperatures were still below 2°C.

### *5.2 In Vitro Liver Enzyme Activity*

The liver is a key organ of carbohydrate metabolism and one of the main gluconeogenic organs in teleost fish (Suarez and Mommsen, 1987; Moon and Foster, 1995). Factors such as exercise, stress and environmental parameters such as photoperiod, season, and temperature can result in changes in the rates of carbohydrate

metabolism, specifically the levels of liver enzyme activity (Suarez and Mommsen, 1987; Moon and Foster, 1995). Results from this study support these claims as the liver enzymes responsible for amino acid breakdown, gluconeogenesis and glycerol production are significantly higher during the winter months, when smelt are producing elevated levels of glycerol. Previous research in this area has demonstrated that the livers of smelt are specifically designed to produce increased levels of glycerol during the winter and the main substrate for glycerol production in smelt are amino acids, which are shunted into the glycerol production pathway via gluconeogenesis (Raymond and Driedzic, 1997; Driedzic et al., 1998; Treberg et al., 2002b).

Enzyme activities obtained in this study are generally comparable to previous studies that employed similar analysis methods and laboratory setup. In the present study activities for AlaAT are slightly higher than those obtained in Driedzic et al. (1998) and activities for AspAT are approximately 3 fold higher than those obtained in Treberg et al. (2002a).

The initial peaks in the enzyme activities of GPDH and AlaAT correspond with the initial upregulation of glycerol that occurs in the fall of the year, at approximately 5-6°C. This suggests that these enzymes may play an initial regulatory role in the rapid synthesis of glycerol in the fall of the year. These results confirm the suggestion of Treberg et al. (2002a), that the regulatory events of glycerol production most likely occur in the early fall, well before the fish are exposed to subzero temperatures (Treberg et al., 2002a). After this initial peak, GPDH levels decrease but are maintained at a slightly elevated level for the duration of the study, whereas AlaAT continues to increase

throughout the study. As well, PEPCK undergoes a gradual increase in enzyme activity from winter to early summer. These elevated activities found in GPDH, AlaAT and PEPCK, as well as the highly significant correlations between these enzyme activities and glycerol levels in the smelt held at low temperatures suggest that they are associated with increased glycerol production during the winter months. It is most likely the activity of these enzymes that is responsible for the maintenance of elevated levels throughout the winter season. In studies on the gall fly (*Eurosta solidainis*) and the gall moth larvae (*Epiblema scudderiana*), which produce glycerol as a method of freeze tolerance, a similar seasonal increase in GPDH levels (3 fold) occurs between early fall and mid-winter (Joanisse and Storey, 1994a; Joanisse and Storey, 1994b). PEPCK has been designated as one of the key regulatory enzymes that allow the flow of carbon intermediates into the gluconeogenic pathway (Suarez and Mommsen, 1987). Therefore, it is not surprising that this enzyme is closely associated with the increased gluconeogenesis that occurs in smelt in order to maintain elevated glycerol levels. In addition, rainbow trout that are fed high protein diets have elevated blood amino acid levels and increased rates of gluconeogenesis from alanine (Suarez and Mommsen, 1987). Smelt are known to consume a highly carnivorous diet in the wild, and fish used in this study were fed a high protein diet. If the biochemical processes of gluconeogenesis in smelt resemble those of rainbow trout, then there should be increased breakdown of alanine to supply the need for increased substrate for glycerol production. This assumption is supported by the elevated activity levels of AlaAT in smelt from this study, which are approximately 5 fold higher than activity levels of AlaAT in smelt from

previous studies (Driedzic et al., 1998). As well, the seasonal increase in activity of AlaAT corresponds with the seasonal increase in plasma glycerol levels indicating that this enzyme is closely linked with the upregulation of glycerol production during the winter months.

Despite the general trend of a seasonal increase among the enzymes studied, such as the results obtained in Treberg et al. (2002a), there was no significant difference in enzyme activity between fish that were producing glycerol (ambient and chilled treatments) and those that were not (heated treatment) for the enzymes studied, except for GPDH, in which the levels in the heated fish underwent a significant increase between February and April. This indicates that the decrease in glycerol levels that occurred in smelt moved to the heated seawater treatment is not as a result of the down regulation of enzyme activity at these specific loci. The decrease in plasma glycerol levels may be due to increased excretion rates via skin, gills or the kidneys to the surrounding environment or through the catabolism of glycerol within the body. There was a steady increase in GPDH activity levels in smelt from the heated treatment, which reached peak levels that were 2-3 fold higher than levels in glycerol producing fish, during the times of peak glycerol production. This suggests that the decrease in glycerol levels is not occurring through the dumping of unnecessary glycerol, but through the breakdown of the molecule within the body as GPDH in the liver is capable of functioning in both the synthesis and catabolism of glycerol. If this is the situation, the breakdown of glycerol in smelt occurs via a different pathway than is proposed for the gall fly, as GPDH activities remain constant and glycerol kinase activity is virtually undetectable in the spring of the year.

Catabolism is hypothesized to occur via the glyceraldehyde-3-phosphate pathway (Figure 1) (Joanisse and Storey, 1994a; Joanisse and Storey, 1994b). However, to determine this, enzyme activity levels for enzymes responsible for the catabolism of glycerol need to be measured in smelt, as well as excretion rates from smelt held in similar conditions. Elevated activity of GPDH when glycerol levels were low may suggest this enzyme is functioning for other purposes besides glycerol production for use as a colligative antifreeze. Increased GPDH could be serving to produce elevated levels of glycerol for triacylglycerol (lipid) synthesis, or increased activity of the α-glycerophosphate shuttle, which is used to transfer reducing equivalents into the mitochondria for ATP production (Joanisse and Storey, 1994a).

Another possible scenario to explain the elevated levels of GPDH in fish from the heated seawater treatment is the use of GPDH in the breakdown of glycerol for its use as a substrate for gluconeogenesis. Smelt in the heated treatment underwent spawning earlier than smelt exposed to the natural water temperatures, most likely due to the elevated water temperatures to which they were exposed. This spawning event occurred from late February to March, which coincides with the period of time when the extremely high levels of GPDH activity are found in these fish. Smelt cease to feed several weeks before they undergo spawning, and in order to survive they must begin to mobilize energy stores, such as lipids, to supply the increased energy demands that accompany a spawning event. The majority of lipids in animals are stored in the form of triacylglycerol, which when broken down produce fatty acids and glycerol. Smelt are unable to convert fatty acids into carbohydrates, due to the fact that acetyl CoA cannot be

converted into a gluconeogenic precursor in animals. Therefore, to use lipids as a potential energy source in periods of starvation, smelt must rely upon the phosphorylation of glycerol, which then enters the gluconeogenic pathway to produce carbohydrates for energy usage (Matthews and Van Holde, 1990). It could be the increased catabolism of glycerol via G3P to supply energy stores during spawning that caused the unexpected elevation in GPDH activity in smelt from the heated treatment in the spring of the year. Further research into this area is needed before definite reasons can be found for this event.

## 6. Summary

This study was the first to depict the seasonal cycle of plasma antifreeze levels in rainbow smelt over a full season, from early fall through to early summer. A full picture of the timing of the upregulation and cessation of the response has been described, separating and defining the individual roles of glycerol and AFP. The accumulation of high levels of glycerol appears to be the main method of freeze resistance, with the production of AFP acting as a safeguard against freezing when glycerol levels are low. However, further work in this area needs to be completed before the exact triggers and controls of the antifreeze response can be pinpointed in rainbow smelt.

With respect to *in vitro* liver enzyme activity, this study was successful in obtaining enzyme activity profiles for key enzymes in the amino acid breakdown, gluconeogenic and glycerol production pathways in smelt over a full season. These results indicate that GPDH and AlaAT may play key roles in the initial up-regulation and

rapid synthesis of glycerol that occurs in early fall. As well, the maintenance of elevated levels of these two enzymes as well as PEPCK, a key regulatory enzyme of gluconeogenesis, are closely associated with the increased seasonal production of glycerol in rainbow smelt. As well, the highly significant correlations between activity levels of GPDH, AlaAT and PEPCK and glycerol levels in fish from the ambient and chilled treatments provides strong evidence that there is metabolic upregulation at these specific loci in fish producing glycerol for use as an antifreeze. However, these results do not indicate proof of rate of control for glycerol production in smelt and further research is required in order to pinpoint the exact regulatory loci for glycerol production in smelt. Once the regulatory loci have been described, researchers can then use the production of elevated levels of glycerol in rainbow smelt as a model system, upon which the induction of increased glycerol production in other organisms can be based. Obtaining this knowledge will open doors in the fields of medical research where using high levels of glycerol and its mechanisms as a molecular chaperone may enable it to be used as a alternative method of treatment for human genetic disorders such as cystic fibrosis, Alzheimer's or cancer. As well, advances could be made in the aquaculture industry, by inducing increased glycerol production in non-freeze resistant cultured species. This would allow the expansion of the industry to areas that experience seasonally low temperatures and ice coverage, which presently are unable to culture fish in open seawater cages due to the threat of losing stock to superchill or death from freezing.

## 7. References

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