GLYCEROL METABOLISM IN SMELT (OSMERUS MORDAX)

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GLYCEROL METABOLISM IN SMELT

(*Osmerus mordax*)

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Abstract

Rainbow smelt (*Osmerus mordax*) is a small fish that accumulates high amounts of glycerol in winter as a cryoprotectant. The principal objective of my thesis was to investigate the metabolic control of the synthesis and clearance (loss/catabolism) of glycerol in this species. As rainbow smelt lose 4-13% of their glycerol content daily, they have to vigorously synthesize this metabolite at temperatures below 0°C to reach the exceptional levels reported (> 200 mM). In this context, the two main hypotheses of my thesis were: 1) glycerol loss/catabolism might be turned-off during the accumulation period and be turned on during the decrease period; and 2) the last step of glycerol synthesis, leading from glycerol-3-phosphate (G3P) to glycerol might be direct, involving a single reaction catalyzed by glycerol 3-phosphatase (G3Pase), an enzyme not yet convincingly described in any animal species.

Activity and mRNA levels of glycerol kinase (GK), the first enzyme involved in glycerol breakdown, were measured in liver, the tissue displaying the highest activity in vertebrates. mRNA levels did not change over the cycle but GK activity did, suggesting a regulation at a post transcriptional level. Overall, GK activity was so low in liver, and in all other tissues assayed, that it likely has a minor quantitative role in glycerol clearance. Daily loss toward water was also determined over the cycle but was not lower over the accumulation period, ruling out the presence of any mechanisms to retain glycerol during this period. Taken together, these results suggest that glycerol levels are dictated primarily by the rate of glycerol synthesis.

To produce glycerol, G3P can be directly dephosphorylated by a G3Pase, as in yeast (*Saccharomyces cerevisiae*) or can enter the triacylglycerol pool and then be released as glycerol via reactions well recognized in mammals. Lipid content and activities of G3Pase and key enzymes involved in lipid metabolism were measured over the glycerol cycle in liver, the site of glycerol synthesis. Lipid content did not change over the cycle and was too low to account for a significant amount of glycerol, but activities of G3Pase and of enzymes involved in lipid degradation were up-regulated early in the cycle, at the onset of glycerol accumulation. A second set of experiments was performed using isolated smelt hepatic cells incubated in optimal conditions to produce glycerol over 72h in the presence or absence of a potent inhibitor of lipid synthesis. It was shown that neither on-board lipids nor newly synthesized lipids could be the source of the glycerol produced, pointing out for the first time a direct dephosphorylation by G3Pase as the main source of glycerol in an animal species.

The last objective of my thesis was to partially purify and characterize G3Pase from smelt liver. The enzyme is most likely a cytosolic, acidic, low molecular weight phosphatase, in contrast to other species where the enzyme was previously purified and that has an optimal activity at neutral pH.

These results are crucial in terms of understanding glycerol metabolism in rainbow smelt but also for the elucidation of pathways involved in glycerol synthesis in vertebrates as they clearly showed that glycerol is not necessarily only a by-product of lipid degradation.

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Co-authorship statement

I was responsible for intellectual design, set-up of experiments, biochemical and statistical analysis, as well as, for preparation of the manuscripts presented in this thesis. The only exception to this was the collection of data related to glycerol loss to water (Chapter 2). Connie Short was responsible for the set-up of this experiment, collection of samples and determination of glycerol concentrations. She graciously accepted to let me use her data as they completed nicely the story presented in chapter 2. I was responsible for the statistical analysis, presentation of the data and preparation of all the sections of the manuscripts related to them.

List of tables

List of figures

Abbreviations and symbols

% inhib	percentage inhibition
4-MU	4-methylumbelliferol
4-MUH	4-methylumbelliferone
a.a.	amino acids
ACS	acyl-CoA synthase
ADP	adenosine diphosphate
AFGP	antifreeze glycoprotein
AFP	antifreeze protein
Al	alcohol
AAT	alanine amino transferase
AMPL	acetone mobile polar lipids
ANOVA	analysis of variance
AspAT	aspartate amino transferase
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
CoA	coenzyme A
CPT-I	carnitine palmitoyltransferase I
DHAP	dihydroxyacetone phosphate
DMSO	dimetylsulfoxide
DNA	deoxyribonucleic cid
dNTPs	deoxyribonucleotides
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
FFA	free fatty acid
FP	freezing point
G3P	glycerol-3-phosphate
G3Pase	glycerol-3-phosphatase
G6PDH	glucose-6-phosphate dehydrogenase
GAP	glyceraldehyde-3-phosphate
GDH	glutamate dehydrogenase
GK	glycerol kinase
GPDH	glycerol-3-phosphate dehydrogenase
HC	hydrocarbons
HDL	high density lipoprotein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIS	hepato-somatic index
HOAD	hydroxyacyl CoA dehydrogenase
ILDL	intermediate density lipoprotein
KET	ketone
LDL	low density lipoprotein
MDH	mitochondrial malate dehydrogenase
ME	malic enzyme
Mw	molecular weight
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide reduced
NADPH	nicotinamide adenine dinucleotide phosphate
NMR	nuclear magnetic resonance
PCR	polymerase chain reaction
PEPCK	phosphoenolpyruvate carboxykinase
PDK	pyruvate dehydrogenase kinase
P_I	inorganic phosphate
PL	phospholipid
RLM-RACE	RNA ligase-mediated rapid amplification of cDNA ends
RNA	ribonucleic acid
RQ	relative quantity
SE	steryl ester
S.E.	standard error
ST	sterol
TG	triacylglycerol, triglyceride
TH	thermal hysteresis
TLC/FID	thin layer chromatography/ flame ionization detection
TMAO	trimethylamine oxide
v:v	volume:volume
VLDL	very low density lipoprotein
Δ	variation

TABLE OF CONTENTS

Chapter 1

Background and literature review

1.1 Adaptations to freezing temperatures in ectotherms

Ectotherms are animals in which internal temperature is dictated by their external environment. Except birds and mammals, all animal species are generally ectothermic. For species inhabiting regions continuously or seasonally exposed to subzero temperatures, the threat of internal ice formation poses a real problem, being incompatible with life processes. Species that cannot migrate to more inviting places have thus developed strategies to cope with freezing conditions. The two main strategies to do so are freeze tolerance and freeze avoidance. Both include adaptations at behavioral, physiological and biochemical levels and some of them are shared between the two strategies (Storey & Storey 1988; Storey & Storey 1996). This thesis focuses on glycerol management in a specific fish species but, in order to set the context of the study, a general overview of how ectotherms survive very low temperatures is provided.

1.1.1 Freeze tolerance

Some species can tolerate freezing of a significant amount of their water content without affecting their survival. This phenomenon has been observed in various species of the animal kingdom including insects, marine invertebrates, reptiles and amphibians. In vertebrates, the most studied species is the wood frog, *Rana sylvatica*, that can survive in a frozen state for up to four weeks at temperatures just above -6°C (Storey & Storey 1988; Storey & Storey 1992). The degree of freezing and the length of freezing events bearable depend on the species, but also on the microclimate to which organisms are exposed (Storey & Storey 1988).

Freezing is exclusively limited to extracellular water and involves mechanisms to control ice growth and also to protect and stabilize the intracellular environment from dehydration and ischemia that accompany freezing (Storey & Storey 1988). Ice formation is generally initiated at temperatures close to the freezing point of body fluids offering a slow rate of ice growth. While ice formation progresses in a regulated manner, other metabolic adjustments can be made to prevent dehydration of the intracellular environment, one of the most serious threats imposed by freezing (Storey & Storey 1996). Wood frogs, will take up to 24h to reach their maximum ice content (≈60%) at -2.5°C (Layne & Lee 1987). For many species, initiation of ice formation is done by direct contact with environmental ice crystals but some species also developed the ability to initiate it spontaneously by synthesizing specific proteins or lipoproteins that are active ice-nucleation agents (Storey & Storey 1996; Lee & Costanzo 1998). In all cases, freezing is accompanied by metabolic rate depression and many freeze tolerant species will accumulate high concentrations of low molecular weight (low Mw) cryoprotectants such as glycerol. These solutes will have a marked impact on freezing duration and on the minimal lethal temperature (Storey & Storey 1988).

1.1.2 Freeze avoidance

When organisms cannot tolerate freezing, but still have to face freezing temperatures, ice formation in body fluids must be inhibited. Adaptations supporting freeze avoidance are of three types: 1) supercooling, 2) synthesis of antifreeze proteins, and 3) accumulation of high concentrations of low Mw cryoprotectants. Organisms will generally use a

combination of those adaptations to face the freezing temperatures (Storey & Storey 1988).

1.1.2.1 Supercooling

Supercooling involves the maintenance of a fluid state of body fluids even at temperatures lower than their freezing point (FP). In species intolerant to freezing, the initiation of ice formation starts as soon as their FP is reached in the presence of ice nuclei upon which ice crystals will grow. Nuclei can result from a spontaneous aggregation of water molecules (homogeneous nucleation) or from the presence of specific ice-nucleation agents, nucleators, that attract water molecules to form nuclei (heterogeneous nucleation). Nucleators can be specific proteins, lipoproteins or microorganisms such as bacteria and have different efficiencies to initiate freezing (Lee & Costanzo 1998). Within biological systems, ice nucleation is believed to begin by heterogeneous mechanisms. In the absence of efficient nucleators, body fluids of an organism can remain unfrozen below their FP. The organism is said to be supercooled. Supercooling capacities depend on the water content, decrease as the body mass increases and can be influenced by the stage of life history (Lee & Costanzo 1998).

In fish, supercooling is only an option for species that are not in direct contact with ice crystals. This strategy is thought to be quite common for fish living permanently in deep-water such as polar cod (*Boreogadus salida*) and snailfish (*Liparis turneri*) (Davenport 2002), and was proposed for cunner (*Tautogolabrus adspersus*) from the North Atlantic Ocean that spend winter in shallow freezing water in a hypometabolic state, hidden in holes and crevices (Green 1974). Indeed, introduction of ice in their

wintering sites was shown to be lethal. Even though this species was later shown to also produce antifreeze proteins (AFPs) in winter, it may display supercooling capacities to compensate for the limited protection that AFPs offer (Hobbs et al. 2011).

1.1.2.2 Antifreeze proteins

Antifreeze proteins (AFPs), also referred to as thermal hysteresis proteins, are a group of proteins that allow organisms to resist freezing by binding to nascent ice crystals, inhibiting their growth and their destructive propagation through body fluids (DeVries 1983; Goddard & Fletcher 2002). The discovery of AFPs derived from an interest in Antarctic fish inhabiting freezing waters. How could those fish live at temperatures close to the freezing point of seawater (FP; - 1.8°C) when the FP of fish body fluids is usually not lower than -0.8°C, and supercooling is not an option, since most fish species are in constant contact with environmental ice crystals?

The first AFP was identified by DeVries and Wohlschlag (1969) from plasma of Emerald rockcod, *Trematomus bernachii*. Since then, AFPs were identified in many other fish species, and also in invertebrates, plants, fungi and bacteria (Ewart 2002). These proteins are very diverse in terms of size and structure. Just in fish, AFPs are grouped in five different categories: type I to IV AFPs (all nonglycoproteins) and antifreeze glycoproteins (AFGP) (Table 1.1).

Table 1.1. Some properties of antifreeze proteins identified in teleosts [modified from Ewart (2002) and Venketesh & Dayananda (2008)]

Protein	Mass (kDa)	Characteristics	Species
Type I	3.3-4.5	α-helical, some with repeat motif	Sculpins, Right-eye flounders
Type II	11-24	Globular, Ca^{2+} dependent (smelt and herring)	Sea raven, Smelt, Herring
Type III	6.5	Globular with one flat surface	Eel pouts
Type IV	12	Folded α-helical structure	Longhorn sculpin
AFGP	2.6-3.3	Ala-Ala-Thr polymer with dissacharide on each Thr	Cods, Antarctic Notothenioids

As with any solute, AFPs lower the FP of body fluids colligatively; however it was shown that their non-colligative mode of action, by binding to ice crystals, was much more effective (DeVries 1971; Ewart 2002). The latter mechanism results in a decrease in the FP of body fluids without any change in the melting point (DeVries 1971). The gap between FP and the melting point, called thermal hysteresis (TH), is a characteristic of AFPs and gives an indication of their "antifreeze" capacity. TH is dependent on the type and quantity of AFPs produced, and can vary within a single species depending on its geographical location and on its development stage (Goddard & Fletcher 2002). AFPs, *per se*, do not have antifreeze properties but rather control the size, shape and aggregation of ice crystals (DeVries 1983). At the molecular level, all AFPs bind to distinct and specific surfaces of ice crystals, but the general effect will remain the same: an alteration

of ice crystal morphology resulting in an inhibition of ice formation (Ewart 2002). In addition to inhib ice growth, AFPs were also shown to inhibit recrystallization, the natural gradual increase of ice crystals' size. This phenomenon was proposed to explain the presence of AFPs in some freeze tolerant species where recrystallization is more likely to cause important physical damage to tissues and cells (Storey & Storey 1988; Ewart 2002).

1.1.2.3 Low molecular weight (low Mw) cryoprotectants

Accumulation of high amounts of low Mw cryoprotectants (generally 0.2-2M) is one of the most important biochemical adaptations supporting freeze tolerance in terrestrial animals, but this is also observed in some freeze avoiding species such as supercooled insects and a few fish species (Storey & Storey 1988; Raymond 1992; Storey 1997). By increasing the osmolality of their body fluids, freeze tolerant species reduce the proportion of water that can accumulate as extracellular ice and prevent the reduction of intracellular volume below a critical minimum that would be lethal (Storey 1997). In addition, accumulation of these solutes in high concentrations lowers the FP of body fluids in a colligative manner, allowing freeze avoiding species to tolerate much lower temperatures than in normal conditions (Storey & Storey 1988).

Low Mw cryoprotectants accumulated by terrestrial animals are almost exclusively carbohydrates that can be glucose, or polyhydric alcohols such as glycerol and sorbitol, or disaccharides such as trehalose. For freeze tolerant and freeze avoiding insects, glycerol is by far the most commonly used cryoprotectant (Storey 1997). In vertebrates, freeze-tolerant frogs usually accumulate glucose, but two species of the Gray

tree frog complex, *Hyla versicolor* and *Hyla chrysoscelis*, are different in that they also accumulate glycerol as a cryoprotectant (Layne & Lee 1995; Irwin & Lee 2003). Four species of northern fish also accumulate glycerol in freezing conditions, including smelt (*Osmerus mordax* and *Hypomesus pretiosus)* and greenling (*Hexagrammos stelleri* and *Hexagrammos octogrammus)* (Raymond 1992).

Glycerol is naturally found in all animals and is very important biochemically as a precursor of glycerol-3-phosphate (G3P) that is a key metabolite in carbohydrate metabolism, providing a carbon skeleton for gluconeogenesis, but also in lipid metabolism, providing a backbone to glycerolipids (Lin, 1977). In addition to being part of central pathways, it is highly soluble and non-toxic (Storey 1997), easy to transport across cell membranes through aquaglyceroporins (Hara-Chikuma & Verkman 2006), and it acts as a chemical chaperone, favouring proper folding of proteins (Welch & Brown 1996; Gong et al. 2011). All these characteristics might explain why glycerol is such a common natural cryoprotectant.

The combination of supercooling and accumulation of low Mw cryoprotectants can result in impressive low temperature tolerance. Three species of willow gall insects from Alaska were shown to supercool to temperatures below -50°C with a parallel accumulation of up to 4.8 M of glycerol (Miller & Werner 1987).

1.1.3 Freeze avoidance in teleosts: the particular case of rainbow smelt

Synthesis of AFPs is the strategy widely used to avoid freezing by teleost fish that inhabit freezing environments (Ewart 2002). Supercooling may be an additional option for fish

that can hide in rock crevices or at depth deep enough not to be directly exposed to ice crystals (Goddard & Fletcher 2002).

Accumulation of low Mw cryoprotectants in fish was viewed with skepticism until a study by Raymond (1992) where, for the first time, unusual levels of glycerol were reported in plasma of northern fish species, including smelt and greenling (Table 1.2). Greenling did not seem to produce any kind of AFP in addition to glycerol, while all species of smelt did (Raymond 1992). To date, these species are the only ones reported that accumulate significant amounts of glycerol, smelt species being the ones showing the highest levels with concentrations as high as 412 mM measured in Arctic rainbow smelt (*O. mordax dentex*) (Table 1.2). In comparison, usual glycerol levels in mammals range between 0.04 and 0.4 mM (Lin 1977). In Arctic rainbow smelt, the major contributors to the freezing point depression were glycerol, NaCl and AFPs (0.77, 0.64 and 0.45°C, respectively) allowing fish to remain active at temperatures close to seawater FP (Raymond 1992). The higher effect of glycerol compared with AFPs on the FP was also reported by Lewis et al. (2004) in rainbow smelt (*O. mordax*). In addition to glycerol and AFPs, smelt species also accumulate lower, but significant, amounts of trimethylamine oxide (TMAO) and urea (Raymond 1994, Treberg et al. 2002a).

Table 1.2. Serum solutes and osmolalities ($mmol.kg^{-1}$) and freezing points and melting points of some northern fishes[1] (replicated from Raymond 1992)

	Alaska, -2.0°C	Japan, -1.0°C[2]			
	Osmerus mordax dentex	*Osmerus mordax dentex*	*Hypomesus pretiosus japonica*	*Hexagrammos stelleri*	*Hexagrammos Octagrammus*[3]
Na	203 ± 9 (6)	208 ± 28 (4)			
Cl	175 ± 12 (6)	178 ± 9.4 (4)			
K	2.0 ± 0.5 (6)	1.0 ± 0.7 (4)			
Ca	4.0 ± 0.7 (6)	5.0 ± 0.9 (4)			
Glucose	7.0 ± 0.5 (6)	5.3 ± 0.4 (4)			
Urea	28 ± 4.8 (6)	8.3 ± 2.3 (4)			
Amino acids	7.3 ± 0.8 (3)	18 (1)			
Glycerol	412 ± 23 (12)	205 ± 46 (8)	152 ± 24 (3)	131 ± 45 (2)	23 ± 7.4 (6)
Osmolality	1,009 ± 16 (10)	729 ± 35 (8)	664 ± 29 (10)	567 ± 65 (2)	502 ± 120 (6)
Melting pt.	- 1.86 ± 0.03 (3)	- 1.49 (1)	- 1.31 ± 0.02 (3)	- 1.02 (1)	- 0.85 ± 0.02 (4)
Freezing pt.	- 2.30 ± 0.02 (3)	- 1.95 (1)	- 1.85 ± 0.02 (2)	- 1.07 (1)	- 0.90 ± 0.01 (4)
Hysteresis	0.45 ± 0.02 (3)	0.46 (1)	0.54 ± 0.02 (2)	≤ 0.05 (1)	≤ 0.05 ± 0.01 (1)

[1]Values are expressed as averages, standard deviations, and the No. of individuals sampled. Where n = 2, the limits of range are given in place of standard deviation. Hysteresis is the difference between the freezing point and melting point and is an indication of the presence of macromolecular antifreeze.
[2]During the days prior to the sampling date, water temperatures were in the range 0 to 1°C. Thus, the fishes in Lake Saroma may not have fully acclimated to - 1°C.
[3]Tentative identification

A seasonal cycling of glycerol levels in relation to water temperature was expected based on comments from locals (Japan and Canada) who eat smelt. They mentioned their sweet taste in winter ("the sugar fish"), a characteristic of glycerol, consistent with high glycerol levels recorded in winter in comparison to fall (Raymond 1992). However, it was only in 2004, that a complete pattern of the glycerol levels was reported for rainbow smelt (*Osmerus mordax*) (Figure 1.1; Lewis et al. 2004). This pattern was determined from a small population indigenous to Newfoundland, Canada,

that migrate for reproduction in the late fall into fresh water streams near estuaries, as opposed to other populations that generally migrate and reproduce in spring between March and May (Buckley 1989). This peculiarity offered the possibility to follow glycerol levels of smelt maintained in captivity from late fall (water temperatures > 8°C) through winter (water temperatures < 0°C) and until the beginning of spring (water temperatures > 0°C). Initial plasma levels were below 0.5 mM but increased to 84 mM in December, when water temperature was close to 5°C, to finally reach a peak in February at levels higher than 200 mM, when temperature was still at -1°C. From this point, glycerol levels started to decrease to reach levels lower than 5 mM in May. Such a cycle was absent for smelt maintained at 10°C over the same period for which levels remained low, suggesting that the decrease of temperature may trigger glycerol synthesis (Figure 1.1). This latter point was confirmed thereafter by showing that glycerol production could be induced later in the season in smelt maintained at 8°C and not producing glycerol, by decreasing water temperature in a stepwise manner (Driedzic et al. 2006; Driedzic & Short 2007). AFP activity, expressed as TH, does not follow the same pattern as glycerol but rather increases more slowly from late fall and remains constant after February (Lewis et al. 2004). A change in photoperiod may trigger the glycerol decrease observed in February, possibly in association with sufficiently high levels of AFPs (Lewis et al. 2004; Driedzic & Short 2007).

Accumulation of glycerol as a low Mw cryoprotectant does not seem a natural choice for fish as, unlike many terrestrial species that are "closed" systems and remain in a "dormant" state in freezing conditions, smelt remain active and are in direct contact

with ambient water, offering an opportunity for leakage of this small and highly water soluble molecule. Indeed, Raymond (1993) showed that, even though smelt reduce glycerol loss by decreasing urine production in winter, 4 to 13% of the total content of glycerol was lost daily to water, with gills and skin being the two main sites of loss. Accumulation of such amounts of glycerol in smelt would thus rely on its active synthesis to compensate for the constant loss to the environment. Since those findings in the early 90's, interest in mechanisms involved in glycerol production during freeze avoidance increased, with a particular emphasis on rainbow smelt (*Osmerus mordax*).

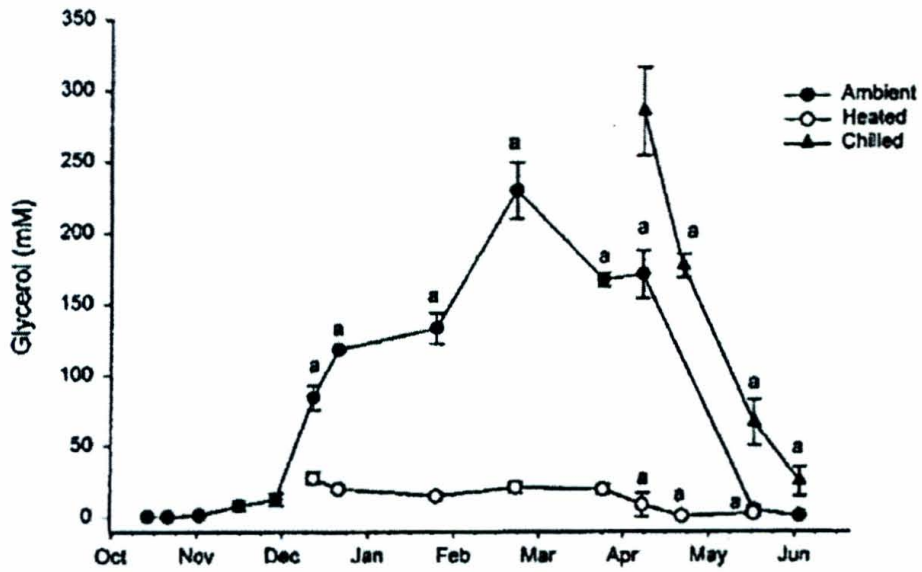

Figure 1.1. A comparison of seasonal plasma glycerol concentration in glycerol and non-glycerol producing rainbow smelt. Ambient refers to rainbow smelt maintained in seawater that followed natural temperature change. Heated refers to rainbow smelt maintained in 10°C seawater. Chilled refers to fish artificially exposed to below normal temperature. "a" indicates significance (ANOVA: $p < 0.05$) from initial sampling point within a treatment. Figure from Lewis et al. (2004).

1.2 Glycerol metabolism in rainbow smelt: focus on synthesis

1.2.1 Sources of carbons

Glycerol, also known as glycerin or 1,2,3-propanetriol, is a small 3 carbon sugar alcohol and is best known as the backbone of glycerolipids including triacylglycerol (triglycerides, TG) and most phospholipids (Figure 1.2). In mammals, glycerol is normally made available from lipolysis in adipose tissue and from hydrolysis of TG in blood lipoproteins (Lin 1977). Rainbow smelt is not a fatty fish with a total fat content ranging between 2 and 10% of the total weight depending on the tissue and on the population considered. Flesh, the main tissue of fish, does not contain more than 2.2% of fat whatever the population considered (Krzynowek & Murphy 1987). Raymond (1995) was the first to investigate the potential fuels involved in glycerol production using rainbow smelt caught in winter. Fish were maintained at -1°C and starved for 5 days and levels of glycerol, glycogen, glucose and TG were measured. Glycerol synthesis that may be maintained over this period could thus only rely on on-board fuels. Over this period, glycerol levels remained relatively high even if a slight decrease was noticed after three days of experiment. This decrease was, however, not sufficient to rule out the continuous glycerol synthesis under starvation. Plasma levels of TG were low (2.5 to 10 mg/ml) and did not decrease over the experiment but rather increased slightly by the end of it. Levels of TG in liver were relatively similar to those in plasma while they were not detected in muscle with the method used. Both levels of glucose and glycogen did, however, decrease by the end of experiment, with glycogen levels being almost depleted after three days, shortly after the stomach content was emptied (Raymond 1995). These data did not

support on-board lipids as a source of glycerol but pinpointed glucose and glycogen as two potential sources. This was confirmed in an extended experiment the following year (Raymond 1996).

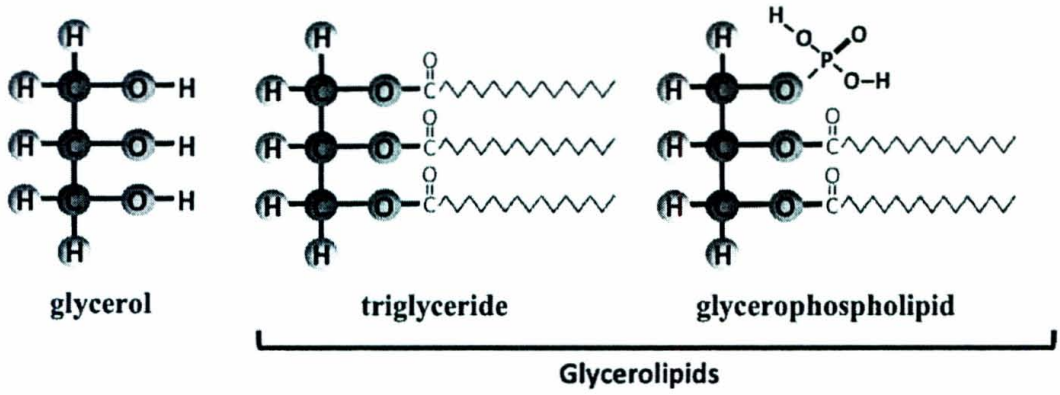

Figure 1.2. Structure of glycerol and glycerolipids highlighting their common backbone. Fatty acids are represented in light grey with their characteristic carboxyl group (C=O) and hydrocarbon chain (zigzag).

Glycogen reserves, mainly in liver, are not sufficient to provide enough glycerol in winter but are rather a short-term source when external sources of carbon are not available. Glycogen levels are not depleted in winter in normal conditions of feeding for fish tracking ambient temperatures (Treberg et al. 2002a) but are rapidly depleted in conditions where glycerol production is forced following a rapid decrease of temperature and are not sufficient to keep fish alive for extended periods of starvation (Driedzic & Short 2007). External sources of carbon through feeding are thus essential for smelt in nature.

Heavy and radiolabeled isotopes (^{13}C, ^{14}C) were used to prove that glucose and amino acids provide carbon to generate glycerol. Injection of labeled molecules such as glucose, pyruvate or amino acids all resulted in the detection of labeled glycerol (Raymond 1995; Raymond & Driedzic 1997; Walter et al. 2006). The use of nuclear magnetic resonance (NMR) allowed the tracking of isotopes and showed that, two hours post-injection at an incubation temperatures of -0.8°C, labeled signatures were not only detected in glycerol in liver, but also remained intact with respect to carbon position in the precursor molecules. This suggested a direct metabolic pathway for glycerol synthesis (Walter et al. 2006). Liver glycogen is thus considered to be a source of glycerol during the early stages of glycerol accumulation, whereas dietary glucose and amino acids are essential to maintain rates of glycerol synthesis (Clow et al. 2008). The importance of amino acids is consistent with the protein-rich stomach content reported by Raymond for a smelt population collected in winter (1995).

1.2.2 Metabolic pathways involved and initiation of glycerol production

Carbohydrates (glucose/glycogen) result in glycerol synthesis through an abbreviated glycolysis while amino acids do so through glyceroneogenesis. The key role of glyceroneogenesis in glycerol synthesis is supported at the protein and transcript levels. At the protein level, a positive correlation was observed between activities of amino acid transaminases, alanine amino transferase (AAT) and aspartate amino transferase (AspAT), and glycerol levels, as well as, between activity of phosphoenolpyruvate carboxykinase (PEPCK) and glycerol levels (Lewis et al. 2004). At the transcript level,

amounts associated with mobilization of amino acids (PEPCK, MDH, AAT, GDH) were all transiently higher in isolated hepatic cells producing glycerol than in cells not producing any (Hall et al. 2011). Increases in transcript levels encoding for PDK, an inhibitor of pyruvate dehydrogenase, in both isolated hepatic cells model (*in vitro*) and liver of smelt (*in vivo*) exposed to a controlled decrease in temperature, that produce glycerol, were also reported. This suggested a channeling of pyruvate and oxaloacetate derived from amino acids (a.a.) into glycerol rather than to oxidation via the citric acid cycle (Hall et al. 2011; Hall et al. 2012).

Whether carbon units originate from carbohydrates or amino acids, in both cases the conversion to glycerol requires a transition through the three-carbon intermediates glyceraldehyde-3-phosphate (GAP) or dihydroxyacetone phosphate (DHAP). From there, two main pathways can lead to glycerol (Figure 1.3). The first pathway (GAP pathway) involves glyceraldehyde-3-phosphatase and polyol dehydrogenase. The second one (DHAP pathway) involves glycerol-3-phosphate dehydrogenase (GPDH) and the conversion of the resulting G3P to glycerol by a direct dephosphorylation through glycerol-3-phosphatase (G3Pase) or possibly by a transition through the glycerolipids pool.

In freeze-avoiding gall moth (*Eplima scudderiana)* larvae, which accumulate up to 2M glycerol in winter, both pathways have been shown to be activated with key enzymes activities starting to increase in the fall (Joannisse & Storey 1994). In smelt, however, the DHAP pathway is most likely the one used. Activities of enzymes involved in both pathways were measured in liver of rainbow smelt accumulating glycerol, and in other

species inhabiting the same environment but not producing glycerol (tomcod, *Microgadus tomcod*, and smooth flounder, *Liopsetta putmani*). Activities of enzymes involved in glycerol production through DHAP were higher in smelt than in species not producing glycerol while enzymes involved in glycerol production through GAP were not. Furthermore enzymes activities of the GAP pathway were lower in smelt than those through DHAP (Driedzic et al. 1998). The same pattern was observed in the liver of rainbow smelt in comparison to capelin (*Mallotus villosus*), a very closely related species that does not accumulate glycerol in winter (Raymond & Hassel 2000; Treberg et al. 2002b).

Hepatic GPDH proved to be a key enzyme in glycerol synthesis with very high activities measured in smelt in comparison to other species not accumulating glycerol (Driedzic et al. 1998; Treberg et al. 2002b). Activity in liver was shown to increase sharply in December, at the onset of glycerol synthesis, and to remain high over the rest of the season (Lewis et al. 2004). GPDH mRNA levels also presented a sharp increase in liver in December but levels did not remain high after this point returning back to their initial levels in February (Liebscher et al. 2006; Robinson et al. 2011).

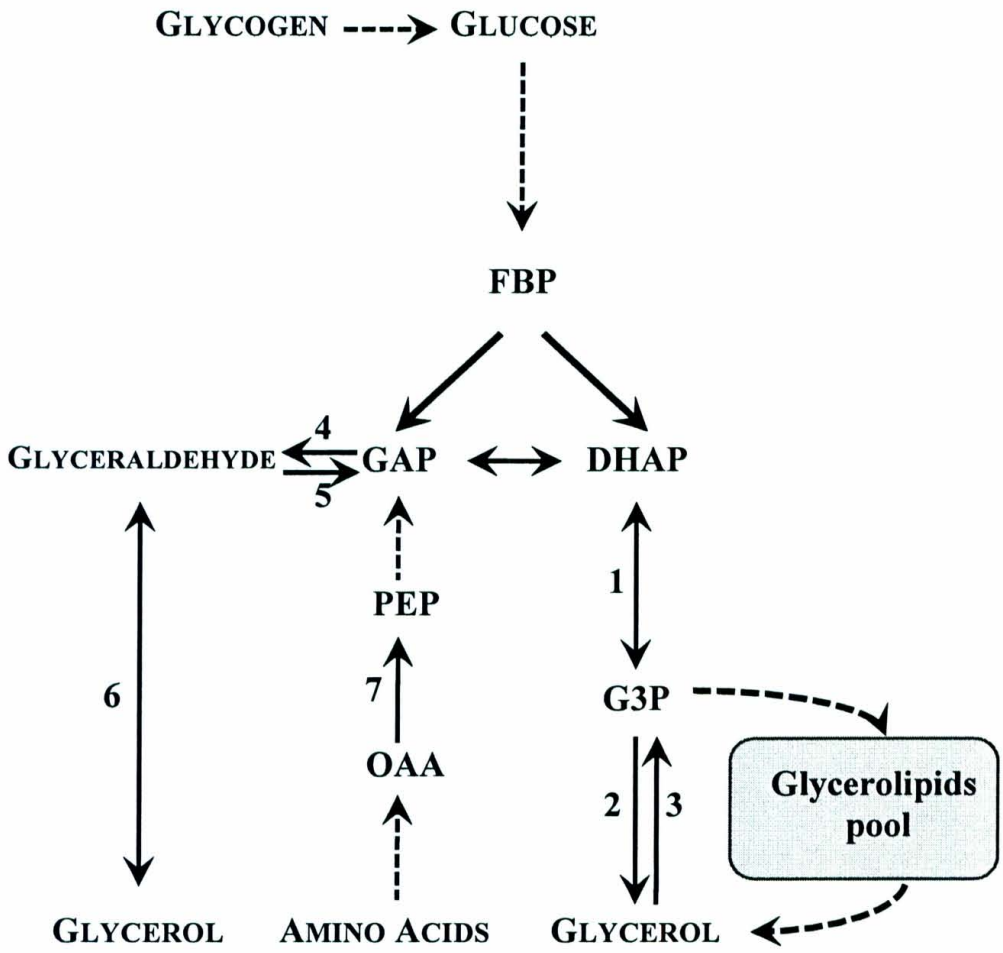

Figure 1.3: Potential metabolic pathways involved in glycerol synthesis from carbohydrates and amino acids. Numbers correspond to the following enzymes: 1, glycerol-3-phosphate dehydrogenase; 2, glycerol-3-phosphatase; 3, glycerol kinase; 4, glyceraldehyde-3-phosphatase; 5, glyceraldehyde kinase; 6, polyol dehydrogenase; 7, phosphoenolpyruvate carboxykinase. Metabolites are: FBP, fructose-1,6-bisphosphate; GAP, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone-phosphate; OAA, oxaloacetic acid; PEP, phosphoenol pyruvate and G3P, glycerol-3-phosphate. Dashed lines indicate multiple enzyme-catalyzed steps

1.2.3 Liver as the major site of glycerol synthesis

Liver was rapidly found to be the major site of glycerol production, primarily because of its major role in metabolism, being central in the synthesis and breakdown of small and complex molecules such as TG, glycogen, a.a. and proteins. All studies investigating activities and/or mRNA levels of enzymes involved in glycerol synthesis in liver support this contention (reviewed by Driedzic & Ewart 2004 for review). As well, evidence from liver slices (Driedzic et al. 1998) and isolated hepatic cells (Clow et al. 2008), that are able to produce significant amounts of glycerol when incubated at cold temperatures, support this central contention. However none of the previous work excluded other tissues as potential sites of glycerol synthesis. A study conducted by Robinson et al. (2011) recently investigated the potential role of other tissues by measuring GPDH activity, one of the key enzyme involved in glycerol synthesis, in white muscle, heart, brain and kidney of rainbow smelt producing and not producing glycerol (maintained at ambient and constant warm temperatures, respectively). GPDH activity was higher in both liver and white muscle of smelt producing glycerol while it was the same between groups in all other tissues. In the cold acclimated group, activity was however much higher in liver (> 175 $\mu mol.min^{-1}.g_{tissue}^{-1}$) than in all other tissues investigated (12, 2.97, 0.94 and 7.09 $\mu mol.min^{-1}.g_{tissue}^{-1}$ for white muscle, heart, brain and kidney, respectively). Although some synthesis still may occur in other tissues, it should be limited by the lower activities of GPDH, clearly pointing to liver as the major site of glycerol production.

1.3 Glycerol metabolism in smelt: the missing pieces

1.3.1 Regulation of mechanisms involved in glycerol breakdown/clearance

For glycerol to accumulate in winter, inputs must exceed outputs. In rainbow smelt, outputs include glycerol catabolism through metabolic pathways and direct loss to the surrounding environment. The daily rate of loss of glycerol to water was first estimated to range between 4 and 13% of the total amount (Raymond 1994). This estimation was based on an experiment conducted at one sampling time, likely close to the peak of glycerol judging by the high levels of glycerol recorded. This raises the question as to whether the rate of glycerol loss could be controlled during the seasonal cycle. This possibility is suggested by findings in other systems. For instance, yeast can regulate glycerol efflux when important intracellular levels are needed to face osmotic stress (Luyten et al. 1995). In mammals, glycerol movement across cell membranes occurs via aquaglyceroporins (AQP3, 7 and 9) (Hara-Chikuma & Verkman 2006), the expression of which can be regulated, as shown in fasting rat hepatocytes (Cabrey et al. 2003). AQP9 is also present in smelt hepatic cells and its mRNA levels were shown to increase in isolated hepatic cells incubated at cold temperature shortly after their transfer to -0.4°C (Hall et al. 2011). All this suggests that some molecular mechanisms may be in place in key sites of smelt such as skin and gills to limit glycerol loss during the critical accumulation period of glycerol, but this remains to be validated.

The importance of metabolic pathways involved in glycerol catabolism has not received as much attention as those involved in its synthesis. Down-regulation of glycerol catabolism would be expected over the accumulation period when retention may be

already challenged by direct loss to water. Up-regulation may however happen over the decrease period of glycerol levels to participate in glycerol clearance. This contention is supported by a recent study by Robinson et al. (2011). These authors measured the activity of the mitochondrial form of GPDH (mGPDH) over the glycerol cycle in liver of smelt maintained at ambient and warm temperature (producing and not-producing glycerol, respectively). mGPDH catalyses the irreversible dehydrogenation of G3P to DHAP (as opposed to cGPDH involved in reversible reactions), and thus can only relate to glycerol breakdown (Figure 1.3). Activity of mGPDH remained constant over the accumulation period of glycerol but increased significantly during the decrease period with the maximum activity measured in May when glycerol levels were back to normal. This pattern was not observed in fish not producing glycerol. These results support a channeling of glycerol toward DHAP by the end of the glycerol cycle and potentially toward glycogen, as previously proposed by Driedzic & Short (2007). The first step of glycerol breakdown, involving the phosphorylation of glycerol toward G3P by glycerol kinase (GK) might be the rate-limiting step in the channeling of glycerol to glycogen. This possibility has never been investigated in smelt and is the subject of part of this thesis.

1.3.2 G3P to glycerol: relative implication of glycerolipids and G3Pase

An important question remains concerning the very last step of glycerol synthesis from G3P. The most efficient pathway would be a direct dephosphorylation of G3P by a phosphatase, G3Pase. To date, such an enzyme has only been unequivocally identified

and sequenced in a limited number of species that accumulate glycerol in response to different environmental stressors. These species include bacteria, *Bacillus licheniformis*, fungi, *Saccharomyces cerevisiae* and *Candida albicans* and a plant, *Arabidopsis thaliana* (Table 1.3). As previously mentioned, G3Pase was also proposed to participate in glycerol synthesis in gall moth larvae as G3Pase-like activity was successfully measured. However activity measured was constant and at its lowest levels in fall and winter, when glycerol was building up, while it increased significantly in March when glycerol levels were already reduced by more than four (Joannisse & Storey 1994). G3Pase-like activity was also measured in smelt liver and was higher than in liver of other species inhabiting the same environment but not producing glycerol (Driedzic et al. 1998). Furthermore, mass action ratios of G3Pase expressed as a ratio between its products (glycerol and P_I) *vs* its substrate (G3P) increased while glycerol was accumulating in smelt following a controlled decrease of temperature (Driedzic et al. 2006). All these results support the existence of a G3Pase in smelt, as would be expected in a species that needs to actively produce glycerol to accumulate high amounts while losing some constantly toward its environment. Hall et al. (2011) detected the up-regulation of mRNA levels of a smelt gene encoding for an *uncharacterized* phosphatase, that may be G3Pase, following incubation of isolated hepatic cells at cold temperature, producing glycerol. But expression of different genes involved in lipid metabolism was also up-regulated suggesting that these may be alternative/additional routes to glycerol synthesis.

Table 1.3. Evidence supporting the direct dephosphorylation of G3P to glycerol by a phosphatase (G3Pase) in organisms that accumulate glycerol in response to different stressors.

Domain/ Kingdom	Species	Stressor	Evidences	Reference
procaryota/ Bacteria	*Bacillus licheniformis*	Anaerobic	Activity/Identification	Skraly & Cameron 1998
Eucaryota/ Fungi	*Candida albicans*	Osmotic	Activity/Identification	Fan et al. 2005
	Saccharomyces cerevisiae	Osmotic	Activity/Identification	Norbeck et al. 1996
Eucaryota/ Viridiplantae	*Dunalellia tertiolecta*	Osmotic	Activity	Belmans & Laere 1988
	Dunalellia salina	Osmotic	Activity	Sussman & Avron 1981
Eucaryota/ Plantae	*Arabidopsis thaliana*	Osmotic	Activity/Identification	Caparrós-Martín et al. 2007
Eucaryota/ Animalia	*Eplima scudderiana*	Temperature	Activity	Joanisse & Storey 1994
	Osmerus mordax	Temperature	Activity	Driedzic et al. 1998

In theory, G3P synthesized could enter the glycerolipid pool and their subsequent degradation would result in the release of glycerol (Figure 1.3). On the basis of the available evidence this pathway cannot be ruled out even though it may not be as efficient in terms of energetics as the one involving G3Pase, as different enzymes are needed just for the synthesis of glycerolipids. Furthermore, the degradation of glycerolipid releases not only glycerol but also free fatty acids (FFA), which cannot accumulate in the cells. In mammals, only a very small fraction of the FFA released following lipolysis under starvation is actually oxidized to fuel the organism with energy. Most FFA are actually recycled back to TG, G3P newly synthesized through glyceroneogenesis being used as the new backbone (Reshef et al. 2003; Hanson et al.

2006). Such a pathway may be an alternative to G3Pase in smelt. It would not require a high demand of new FFA if there was recycling, and would not require an enzyme (G3Pase) that has yet to be convincingly described in any animal species.

1.4 Objectives of research

The primary objectives of my research were; 1) to determine the respective roles of glycerol loss and of GK in glycerol levels over the glycerol cycle; and 2) to determine the respective contributions of G3Pase and glycerolipids in glycerol synthesis. Having shown that G3Pase was indeed critical, further work went on to partially characterize this enzyme.

Rainbow smelt of a particular population of Newfoundland can be kept and monitored in captivity from late fall to spring. These smelt offer opportunities to study glycerol metabolism over a complete cycle of glycerol levels, including the accumulation and decrease periods. Furthermore, individuals maintained in warmer conditions (> 8°C) will not produce glycerol, and can serve as a control group. Isolated hepatic cells and their capacities to produce glycerol in cold conditions are also well suited for this research as the extracellular environment can be precisely controlled.

This work is significant since it could give new insights into the mechanisms involved in freeze avoidance in a very unique group of fish. Further, the overall work done on smelt could greatly contribute to a better understanding of glycerol metabolism in higher vertebrates where glycerol is largely considered as a by-product of lipid degradation. Finally, confirmation that G3Pase may have a major role in glycerol

production and eventually its identification are relevant not only in freeze-avoiding species that accumulate unusual amounts of glycerol but also in mammals. The direct dephosphorylation of G3P to glycerol has been proposed as an alternative pathway in heart and brain of oxygen-limited rats, although neither the protein nor the transcript have been indentified yet in any vertebrate (de Groot et al. 1994; Nguyen et al. 2007). Identification of such an enzyme in vertebrates would open new avenues of research in medical sciences concerning the roles of glycerol and whether or not glycerol levels always reflect the rate of breakdown of glycerolipids.

Chapter 2

Glycerol loss to water exceeds glycerol catabolism via glycerol kinase in freeze resistant rainbow smelt (*Osmerus mordax*)

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Glycerol loss to water exceeds glycerol catabolism via glycerol kinase in freeze-resistant rainbow smelt (Osmerus mordax)
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2.1 Abstract

Rainbow smelt accumulate high amounts of glycerol in winter. In smelt, there is a predictable profile of plasma glycerol levels that starts to increase in November (< 5 mM), peaks in mid-February (> 200 mM) and thereafter decreases to reach the initial levels in the beginning of May. The aim of this study was to investigate the respective role of the two main mechanisms that might be involved in glycerol clearance from mid-February: 1) breakdown of glycerol to glycerol-3-phosphate through the action of GK and 2) direct loss to the environment. Over the entire glycerol cycle, loss to water represents a daily loss of about 10% of the total glycerol content of fish. GK activities were very low in all tissues investigated and likely have a minor quantitative role in the glycerol cycle. These results suggest that glycerol levels are dictated by the rate of glycerol synthesis (accelerated and deactivated during the accumulation and decrease stages, respectively). Although not important in glycerol clearance, GK in liver might have an important metabolic function for other purposes such as gluconeogenesis, as evidenced by the significant increase of activity at the end of the cycle.

2.2 Introduction

Rainbow smelt (*Osmerus mordax*) is an unusual fish species with regards to the strategies it uses in winter to remain fully active at temperatures lower than the usual freezing point (FP) of teleost blood. As with many other species inhabiting the same environment, rainbow smelt produce antifreeze proteins (Ewart & Fletcher 1990) but also accumulate high amounts of glycerol (Raymond 1992; Treberg et al. 2002; Lewis et al. 2004). Accumulation of glycerol, in addition to other solutes like urea and TMAO, may participate in the decrease of the FP by colligative mechanisms (Raymond 1992) but it may also serve as a chemical chaperone to promote or preserve protein folding (Gong et al. 2011) or, as it is the case in some insects, contribute to promote supercooling (Karow 1991).

It was first reported that plasma glycerol levels of rainbow smelt caught in late fall were very low compared to the levels measured in winter when water temperature was colder (Raymond 1992; Raymond 1994). Tracking of glycerol levels from an indigenous Canadian population during the cold period, from December (5°C, decreasing water temperature) to the beginning of April (1°C, increasing water temperature), showed that glycerol levels peaked between late January and late February and decreased from there even if water temperature remained low (Treberg et al. 2002a). These data were confirmed by Lewis and collaborators who reported a complete cycle of glycerol levels (Lewis et al. 2004). This cycle started in late fall with an increase of glycerol levels in parallel to the decrease in water temperature. Levels increased until late February when

they peaked at concentrations higher than 200 mM and then decreased until the beginning of May, when values were back to levels measured in November (lower than 5 mM).

A drop of temperature was determined as the trigger of glycerol accumulation, while a change in the photoperiod is more likely the trigger for the decrease in plasma glycerol reported from late January/late February (Driedzic & Short 2007; Clow et al. 2008). Liver is the major site of production for the entire animal (reviewed in Driedzic & Ewart 2004) with glucose, glycogen and amino acids serving as fuels (Raymond 1995; Raymond & Driedzic 1997, Walter et al. 2006) and the cytosolic form of GPDH (cGPDH) functioning as a key enzyme in the control of glycerol synthesis (Driedzic et al. 2006; Lewis et al. 2007) (Fig. 1.3). Furthermore, glycerol accumulation requires a very active synthesis, as it is constantly challenged by direct losses to the surrounding water (Raymond 1996). Physiological mechanisms involved in the glycerol decrease phase are not as well documented. Robinson et al. (2011) assessed the role of the mitochondrial form of GPDH (mGPDH) that catalyses an irreversible dehydrogenation of G3P to DHAP (as opposed to cGPDH involved in reversible reactions), and thus is only related to glycerol breakdown (Figure 1.3) (Klingenberg 1970; Lin 1977). Their results clearly showed an increase of the mGPDH activity in April and May with average activities higher during the decrease period of glycerol, suggesting a channeling of glycerol toward the gluconeogenic pathway as plasma glycerol returned to low levels.

The first enzyme directly involved in glycerol breakdown is glycerol kinase (GK). This enzyme phosphorylates glycerol to G3P that can then enter glycerolipid synthesis (*e.g.* TG and PL) or be converted back to DHAP by cGPDH or mGPDH and eventually

flux back to glucose (Figure 1.3). In mammals, normal plasma glycerol levels range between 0.04 and 0.4 mM (Lin 1977) with higher levels reported in cases subject to GK deficiency (Sjarif et al. 2004). Glycerol levels generally reflect the state of fat mobilization. It is released following lipid degradation and is phosphorylated back to G3P by GK mainly in the liver, which has the highest levels of GK (Lin 1977). In mammals, GK activity can be regulated dependent on the physiological status of the animal. Insulin injections resulted in a decrease of glycerol levels in plasma *in vivo* in rabbits (Hagen 1963), while it was shown to induce GK activity in liver tissue and adipose cells of rats (Koschinsky et al. 1971). High fat diets also resulted in an increase of GK activity in adipocytes of obese Zucker rats, maybe as a consequence of the well-known increase of glycerol levels in plasma following this kind of diet (Stern et al. 1983). These indirect links between glycerol and GK levels were observed in species where glycerol is a product of lipid degradation. They show that GK activity can be regulated depending on the physiological needs of the animal considered. Smelt survival in freezing waters relies on the accumulation of glycerol to levels more than five hundred times what is considered normal in mammals. It would thus seem probable that GK activity, in this particular species, would be regulated. Activity might thus be down regulated during the accumulation period while it might be up regulated during the decrease period of glycerol. To assess this contention, GK activity and transcript expression were measured in liver over the glycerol cycle from late fall to early spring. These data were later complemented with measures of seasonal GK activity in brain, gill, heart and white muscle.

A balance between glycerol inputs and outputs sets glycerol levels. For glycerol levels to decrease, outputs must exceed inputs. In addition to biochemical pathways involving GK, direct loss in water might be an important part in the glycerol decrease phase. Raymond (1993) estimated these daily losses between 4 and 13% of the total content of glycerol. Those estimates were obtained from three different smelt populations at one point of the cycle that was likely close to the peak of glycerol concentrations as levels reported for all of them where higher than 200 mM. Glycerol movement across cell membranes in mammals occurs via aquaglyceroporins (AQP3, 7 and 9; Hara-Chikuma & Verkman 2006). The transcript expression of AQP9 was up regulated in hepatocytes of fasting rats, most likely to allow for a more efficient transport of the glycerol into liver for gluconeogenesis following release from lipid degradation in adipocytes (Cabrey et al. 2003). This suggests that there might exist some mechanisms that could allow rainbow smelt to regulate the rate of glycerol loss in the environment. Daily loss of glycerol might thus be lower during the accumulation period while it might be higher during the decrease period of glycerol levels. This chapter tested this hypothesis by estimating glycerol loss to the environment at different points during the glycerol cycle. Moreover, estimates of glycerol loss could be compared to glycerol salvaging via GK. Thus, the two principal mechanisms involved in glycerol output were investigated to give new insights into factors regulating glycerol levels in smelt.

2.3 Materials and Methods

2.3.1 Animal collection and sampling

Rainbow smelt (*Osmerus mordax*) were collected by seine netting from Mount Arlington Heights, Placentia Bay, Newfoundland, in October 2007 and 2008, transported to the Ocean Sciences Centre, Memorial University of Newfoundland (St. John's, Canada), and transferred to 3000 litre tanks with flow-through seawater. In 2007, all fish were first held in one tank tracking ambient temperature (9.3°C the day of transfer) and thereafter one-half of those fish were transferred to a second tank maintained at 8-10°C immediately after the first sampling in November. In 2008, fish were immediately transferred to two 3000 litre tanks. For both years and for the rest of the experiment one group was maintained at warm temperature fluctuating around 9°C and 11°C for the 2007-2008 and 2008-2009 seasons, respectively, (warm smelt) and one group tracked ambient temperature (ambient smelt). Fish were kept on a natural photoperiod with fluorescent lights set by an outdoor photocell and fed a diet of chopped herring twice a week to satiation.

2.3.2 Experimental protocols

2.3.2.1 GK activity and transcript expression

From November 2007 to May 2008, blood and liver were sampled monthly from six fish per tank (except for March and April where samples were taken bi-weekly). In November 2007, only ambient smelt were sampled, as the second tank was yet to be set up. In May

2008, only ambient smelt were sampled as warm smelt started to die from the end of April. Occurrence of high mortalities in March-April is a recurrent phenomenon for smelt maintained at warm temperature and is thought to be the result of early spawning, males being predominant and one-time spawners in this population. Blood was used to measure glycerol levels in plasma, and liver was used to clone glycerol kinase transcript (GK mRNA) and to measure seasonal transcript expression and GK activity.

From November 2008 to May 2009, blood, liver, white muscle, brain, gill and heart were sampled monthly from six fish per tank. Only ambient smelt were sampled in April and May. Blood was used to measure seasonal glycerol levels in plasma and white muscle, brain, gill and heart were used to measure seasonal activity of GK as a complement to the previous year. A few samples of liver (n= 3-5) were also run for GK activity in November, and from February to May in both groups to confirm the pattern observed the season before.

In all cases, fish were killed with a sharp blow to the head and tissues immediately harvested. Samples were frozen in liquid nitrogen and stored at -80°C until analysis. Blood was extracted via caudal puncture using heparinized syringes and centrifuged at 10,000 × g for 10 min at 4°C. Plasma was then separated from the blood cell pellet and stored at -80°C until analysis.

2.3.2.2 Seasonal rate of glycerol loss in water

The rate of glycerol release in water was estimated at different points of the glycerol cycle during the 2007-2008 and 2008-2009 seasons. Five points between February 2008

and April 2008 (Feb 4th, Feb 20th, Feb 27th, March 25th and April 9th) and six points between December 2008 and February 2009 (Dec 11th, Dec 23rd, Jan 27th, Feb 10th, Feb 12th, Feb 24th) were selected. The first group captured the glycerol decline period, whereas the second group tracked the glycerol increase period. As such, two different populations of fish were used in these studies.

For all experiments, four plastic containers (21 cm (L) x 16.5 cm (W) x 13 cm (H)) were placed on a platform in a one cubic meter tank with flow-through seawater that tracked ambient temperature. This maintained the containers at the same temperature as the holding tank. Each container had its own air and water lines. On the day of the experiment, two ambient smelt were placed in each container, the water line was removed and a lid with holes to allow air circulation and water sampling was secured. Water level was set to fill the container to 6.5 cm in height. One ml of water was sampled from each container at this time and after 1, 3, 6, 12 and 24h. Water samples were frozen and kept at -80°C for further glycerol analysis. After 24h, plasma was sampled from each fish and processed as previously described and total volume of water was determined. For each container, the rate of accumulation was expressed in μmol per day and in μmol per gram fish per day ($\mu mol.g_{fish}^{-1}.day^{-1}$). For the latter, the rate of glycerol accumulation in $\mu mol.day^{-1}$ was divided by the combined mass of the two fish present in the container. The daily percentage of glycerol loss (% $loss.day^{-1}$) was calculated based on the premise that the plasma glycerol level is similar to the glycerol content in all tissues in the fish (Driedzic & Short 2007). The following formula was applied:

% loss.day^{-1} = total amount of glycerol accumulated in the container (μmol) × 100 / [[plasma glycerol $_{fish1}$ (μmol.ml^{-1}) × mass$_{fish1}$ (g)] + [plasma glycerol $_{fish2}$ (μmol.ml^{-1}) × mass$_{fish2}$ (g)]].

Length (cm), mass (g) and sex were recorded for all fish in all experiments. Animal protocols were approved by the Institutional Animal Care Committee, Memorial University of Newfoundland, St. John's, Canada.

2.3.3 Seasonal transcript expression and activity of GK

2.3.3.1 Preparation of RNA from liver

Total RNA was extracted from liver using Trizol Reagent (Invitrogen, Burlington, ON, Canada). Extracted RNA was quantified by UV absorption at 260 nm and its quality (integrity and purity) verified prior to cDNA synthesis. Integrity and purity were verified by agarose gel electrophoresis and by calculating the OD_{260}/OD_{280} ratio, respectively. Ten μg of RNA was treated with DNAse using the TURBO DNA-*free* kit (Ambion, Austin, TX) and treated RNA was quantified a second time prior to cDNA synthesis. One μg of DNase-treated RNA was reverse transcribed using random hexamers and M-MLV reverse transcriptase (Invitrogen, Burlington, ON, Canada). cDNA so generated was used for cloning and real-time quantitative PCR (qPCR). DNase treatment and reverse transcription were performed following manufacturers' instructions.

2.3.3.2 GK and 18S cloning

A partial sequence of smelt 18S rRNA was obtained using PCR from freshly synthesized cDNA while the complete sequence of the GK transcript was obtained following a combination of PCR and RLM-RACE. Touchdown PCR was used to obtain the 18S rRNA amplicon and the central fragment of GK used for RLM-RACE. For GK, a second PCR was necessary to obtain enough product for subsequent steps. This second PCR was the same as the first except for the use of nested primers and 5 µl of the first reaction as a template. All PCRs were performed in a total volume of 50 µl containing 1X dyNAzyme buffer, 0.2 µM of each forward and reverse primer, 0.2 mM of dNTPs mix and 2 U of dyNAzyme II DNA polymerase. Twenty ng of cDNA were used as template for the 18S rRNA and the first GK reaction while 5 µl of the first reaction were used as template in the second GK reaction. Conditions of the PCR were as follow: 94°C for 2 min of initial denaturation, 30 cycles of 94°C for 30 sec, 60°C↓0.5°C per cycle for 30 sec and 72°C for 90 sec, 10 cycles of 94°C for 30 sec, 45°C for 30 sec and 72°C for 90 sec, and a final extension of 10 min at 72°C. All primer sequences used to obtain the 18S rRNA and GK sequences are listed in Table 2.1. 18S rRNA primers were designed from *Salmo salar* 18S rRNA sequence (Genbank accession number: AJ427629) while GK primers were designed from *Danio rerio* (Genbank accession number: NP_001108056.1) and a conserved region of other vertebrates' consensus sequences.

Amplicons of 1298 bp and 826 bp were amplified for 18S rRNA and GK, respectively. Those products were electrophoresed on a 1.5% agarose gel, excised and

purified using a QIAquick gel extraction kit (QIAGEN Inc., Missisauga, ON, Canada). They were then cloned into pGEM-T Easy vectors (Promega, Madison, WI, USA) following the manufacturer's instructions and triplicate clones were sequenced on both strands at the Genomics and Proteomics Facility, Core Research Equipment and Instrument Training (CREAIT) Network, Memorial University, St. John's, NL, Canada.

The 5'- and 3'-ends of the GK transcript were cloned using the GeneRacer kit for RLM-RACE (Invitrogen, Burlington, ON, Canada) following manufacturer's instructions. For both ends, two consecutive reactions were necessary to obtain enough product (the second reaction used nested primers and 5 µl of the previous reaction as template). Primers used are listed in Table 2.1. For each reaction, one primer was furnished with the kit; the second was designed from the central fragment of GK previously sequenced. Resulting products were then processed in the same way as the central GK product previously sequenced (gel extracted, cloned and sequenced).

Table 2.1. Sequences of primers used for glycerol kinase (GK) and 18S rRNA cloning (PCR and RLM-PCR) and qPCR.

Transcript	Direction*_Sequence (5'-3')	Application	Position from 5'-end
18S rRNA	F_AAACGGCTACCACATCCAAG	PCR1	
18S rRNA	R_CCGAGGACCTCACTAAACCA	PCR1	
18S rRNA	F_GAAAGCGAAAGCATTTGCCA	QPCR	
18S rRNA	R_GGCATCGTTTATGGTCGGAAC	QPCR	
GK	F_AAGGATGGGTRGARSARG	PCR1 (central)	
GK	R_GTYTGGAARCAVACAGCYT	PCR1 (central)	
GK	F_AAGGATGGGTGGAGGAAGATC	PCR2 (central)	265
GK	R_TGCAATGGCAACTGAACCCTC	PCR2 (central)	1074
GK	F_CTCGCTCTCTGGTATTCCAATCTC	RLM-RACE1 (3'end)	857
GK	F_CACGTATGGGACTGGCTGTTTTCT	RLM-RACE2 (3'end)	950
GK	R_GCGTCCGCAAATCTAGCCACACA	RLM-RACE1 (5'end)	452
GK	R_CTCTCTGGTTAGTCACTCCGATTG	RLM-RACE2 (5'end)	376
GK	F_TGACCACCGTGGCTTACAAAC	QPCR	1021
GK	R_CAGAAGCAGCCAACTTCTCCA	QPCR	1153

* F= Forward; R= Reverse

2.3.3.3 Sequence analysis

Sequences were analyzed using Vector NTI Advance 10 (Invitrogen, Burlington, ON, Canada). Percentages of identity with other available sequences were obtained at the nucleotide and at the amino acid levels using the Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990). A multiple alignment of 21 GK protein sequences (16 vertebrates and 5 invertebrates) was performed using MUSCLE version 3.8.31 (Edgar 2004) and used to construct a phylogenetic tree. The tree was constructed using MEGA version 4.0.2 (Tamura et al. 2007) following the Neighbor-Joining method (Saitou & Nei

1987) with Poisson correction. Bootstrap analysis was performed with 1000 replicates.

2.3.3.4 Quantitative real-time PCR (qPCR)

GK expression was quantified as levels of GK mRNA. Total RNA was extracted and treated as previously described and the cDNA synthesized was used for quantification by qPCR with normalization to 18S rRNA using SYBR Green I dye chemistry and the 7300 Real Time PCR system (Applied Biosystems, Foster City, CA). Primers used for 18S rRNA and GK are listed in Table 2.1 and were designed from the sequences previously cloned. For both transcripts, the amplification, efficiency and specificity of the reactions were confirmed before running the samples. Amplification efficiencies (%) of the reactions were estimated using a five point cDNA serial dilution and were 97.9% (r^2, 0.997) and 99.9% (r^2, 0.999) for 18S rRNA and GK, respectively. Specificity of each reaction was verified by running a melt curve analysis, and by running the reaction products on a 2% agarose gel. Both methods showed a single specific product of the expected size for each gene. Samples were then run in duplicate in separate reactions. qPCR reactions were performed in a total volume of 25 µl containing 1X power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), forward and reverse primers (50 nm and 250 nm for GK and 18S rRNA, respectively), and 10 ng of cDNA. The qPCR program consisted of 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min. The threshold cycle (C_T) was determined using the 7300 PCR Detection System SDS software version 1.2.3 from Applied Biosystems (Foster City, CA) and the relative quantity of GK mRNA was determined following the Pfaffl method (Pfaffl 2001). This mathematical model takes

into account the respective efficiencies of the gene of interest and the normalizer gene considered. The individual with the lowest normalized expression level was set as the calibrator sample (assigned RQ value = 1). Data are presented as mean relative quantity, RQ (± standard error) relative to the calibrator.

2.3.3.5 GK activity

GK catalyses the following reaction: Glycerol + ATP → Glycerol-3-Phosphate + ADP

Commonly used assays to measure GK activity based on NAD/NADH coupled reactions were found unsuitable for smelt liver due to high background activities that could not be dampened. The assay used was based on a colourimetric procedure that results in the production of quinoneimine dye that absorbs light at 540 nm (McGowan et al. 1983). Briefly, tissues were thawed on ice and homogenized using a polytron in 4 volumes of ice-cold Triethanolamine buffer 0.1 M at pH 7.4. Assays were run in duplicate in a tris-HCl buffer (50 mM, pH 7.5) containing ATP (1 mM), $MgCl_2$ (5 mM), 4-amino-antipyrine (1 mM), N-ethyl-N-(3-sulfopropyl)-m-anisidine (2 mM), glycerol phosphate oxidase (5 $U.ml^{-1}$) and peroxidase (2 $U.ml^{-1}$). The concentration of the homogenate was chosen to provide a linear response over 15 min. Reactions were run at 30°C and GK activity was monitored, after a 5 min incubation period, for 15 min at 540 nm on a Beckman DU640 spectrophotometer to detect the liberated dye (Extinction coefficient = 14.1 $mM^{-1}.cm^{-1}$). The assay temperature was selected to allow for the detection of GK activity in a reasonable time and is thought to be acceptable as we were interested in relative rather than absolute activities. GK activities are expressed as Unit per g protein ($U.g^{-1}$), one Unit being the formation of 1 μmol of dye per minute at 30°C. Samples for

which any activity was detected were attributed the minimal activity detectable with this method (Δ absorbance of 0.00012 per min).

2.3.4 Glycerol and protein assays

Glycerol concentrations were determined using the free glycerol determination kit from Sigma (Oakville, ON, Canada) following manufacturer instructions. Protein concentrations in tissues were measured from homogenates used for GK assays with the Bio-Rad Protein Assay (Bio-Rad, Mississauga, ON, Canada).

2.3.5. Statistical analysis

For all specific parameters followed, values are presented as means (± S.E.) and significant differences between treatments at a particular sampling point were assessed using *t*-tests. One-way ANOVAs with Tukey's post-hoc test were used to determine differences between sampling points within a treatment.

2.4 Results

2.4.1 Water temperature and glycerol level profiles

Profiles of water temperature are presented in Figure 2.1A for both the 2007-2008 and 2008-2009 periods. Temperature of ambient water was higher between November and mid-January in 2008-2009 than in 2007-2008. For both periods, temperature decreased until March to reach minima at -0.4°C and 0°C for 2007-2008 and 2008-2009, respectively. Temperature increased from there to reach 1.1°C and 2.1°C at the end of the

experiment in the beginning of May 2008 and 2009, respectively. For technical reasons temperature of the warm tank was higher in 2008-2009 with values fluctuating around 11°C in contrast to 9°C in 2007-2008.

In both cases, glycerol levels accumulated in plasma of ambient rainbow smelt to levels higher than 200 mM in mid-February while they remained below 10 mM in warm smelt (Figure 2.1B). Plasma glycerol profiles in fish held in ambient water were very similar in both experiments with the highest levels recorded in the middle of February (216.6 ± 15.4 and 221.4 ± 23.4 mM in 2008 and 2009, respectively). The only significant differences between studies were observed in January and April when levels were significantly lower in the 2008-2009 study (*t*-test, $p < 0.05$). For rainbow smelt held at warm temperature, glycerol levels were higher in 2008-2009 with average values reaching levels greater than 7 mM in January and February while they never exceeded 4 mM in 2007-2008 (ANOVA, $p < 0.05$) (Figure 2.1B).

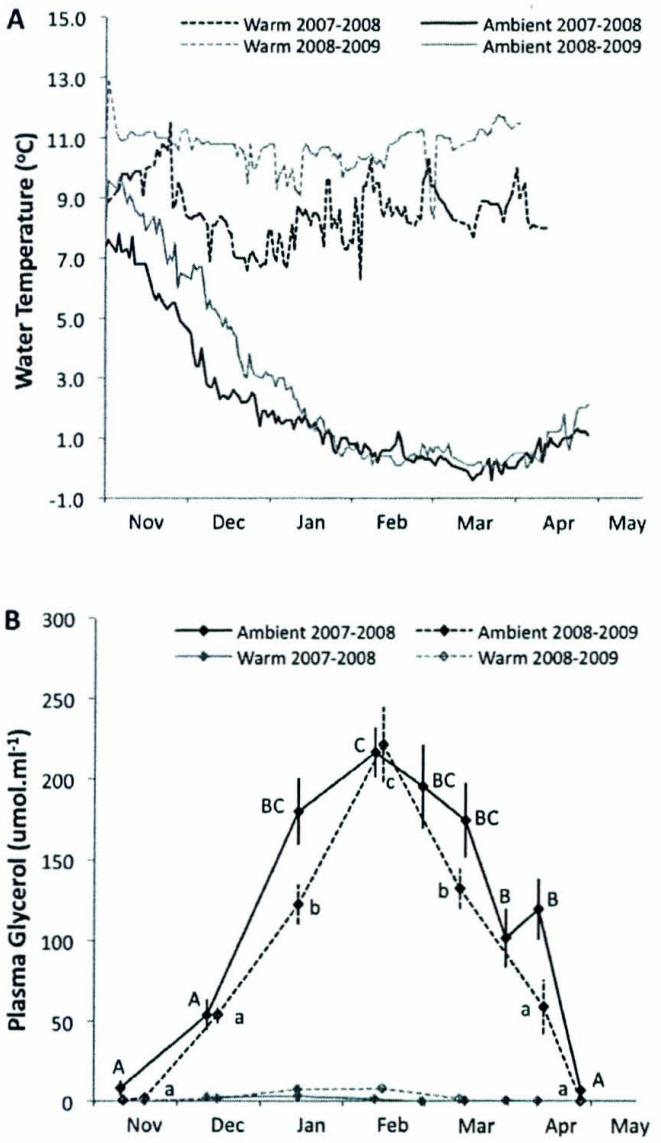

Figure 2.1. Seasonal profiles of water temperature (A) and plasma glycerol levels (B) for smelt held under controlled (warm > 8°C) and ambient (ambient) temperatures for 2007-2008 and 2008-2009 experiments. Glycerol values are presented as mean ± S.E. (n=6). Different upper and lower case letters represent glycerol values significantly different within 2007-2008 and 2008-2009, respectively, for smelt tracking ambient temperature.

2.4.2 Seasonal activity of glycerol kinase (GK)

The complete profile of GK activity in the liver is presented for 2007-2008 in Figure 2.2A. In warm smelt, GK activity did not change over the whole period with an average activity of 79.4 ± 9.5 $mU.g_{protein}^{-1}$. In ambient smelt, activity remained constant until April when it started to increase significantly to reach a maximum in May at levels almost 25 times higher than those recorded in November (18.4 ± 2.1 and 452.9 ± 74.7 $mU.g_{protein}^{-1}$ for November and May, respectively). When comparison was possible, GK activity of ambient smelt was not different from activity of warm smelt except at the beginning of April where activity was higher in ambient smelt. The increase of GK activity detected from the beginning of April corresponded to the period where water temperature started to increase again (Figure 2.2A). Average activity during the accumulation period of glycerol (from November to February) was 43.0 ± 6.4 $mU.g_{protein}^{-1}$ while it was 197.7 ± 29.3 $mU.g_{protein}^{-1}$ during the decrease period (from February to May).

A few liver samples were processed for the 2008-2009 experiment for GK activity to confirm the pattern observed during the decrease period of the previous 2007-2008 experiment. Values were in the same range as the ones reported in 2007-2008 and again a clear significant increase was observed from April with the maximum GK activity measured in May (380.8 ± 46.8 $mU.g_{protein}^{-1}$). GK activities measured in November (43.8 ± 13.6 $mU.g_{protein}^{-1}$), February (142.2 ± 46.4 $mU.g_{protein}^{-1}$), March (108.6 ± 62.0 $mU.g_{protein}^{-1}$) and April (204.4 ± 30.2 $mU.g_{protein}^{-1}$) were not different from one another; activities measured in May 2009 were significantly different from November, February

and March but were not different from April, resulting in a close pattern to the one reported in Figure 2.2A for 2007-2008. When considering each month separately, GK activities were not different between the two studies. As in 2007-2008, GK activity of warm smelt was not different from activity of ambient smelt for the months considered (November (71.6 ± 36.6 $mU.g_{protein}^{-1}$), February (40.3 ± 17.5 $mU.g_{protein}^{-1}$) and March (52.6 ± 37.3 $mU.g_{protein}^{-1}$)) and did not change with respect to time.

Four other tissues were investigated in 2008-2009. GK activity was not detected in gill homogenates, and brain homogenates yielded very high non-specific background so that it was not possible to get an accurate quantitation of enzyme activity. GK activity was, however, easily detectable in heart and white muscle homogenates (Figure 2.3). In heart of ambient smelt producing glycerol, activity did not change over the cycle with an average activity of 89.6 ± 5.6 $mU.g_{protein}^{-1}$ (Figure 2.3A). In warm fish, GK activity was higher in March than in all other months. At this point, activity was also higher in the warm than in the ambient group. GK activity in white muscle did not change over time in either warm or ambient fish but was higher overall in warm fish with average activities of 144.6 ± 10.5 and 182.6 ± 19.3 $mU.g_{protein}^{-1}$ for ambient and warm smelt, respectively (Figure 2.3B).

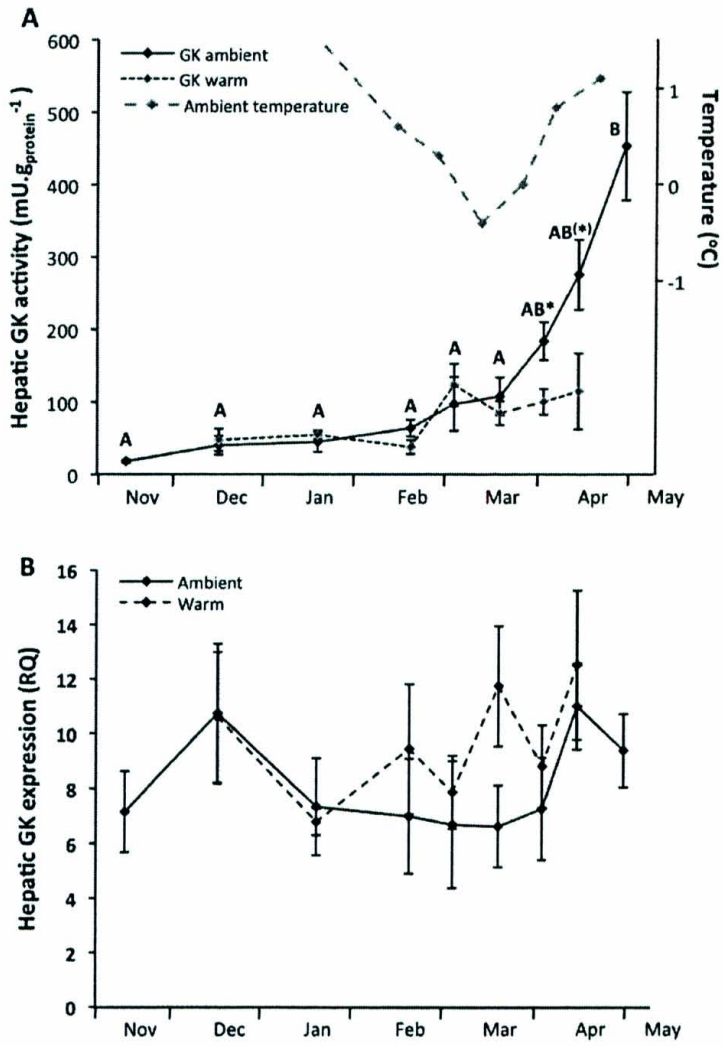

Figure 2.2. Seasonal activity (A) and transcript levels (B) of glycerol kinase (GK) in smelt liver (2007-2008 experiment). Panel A also includes a partial temperature profile. GK activity is presented as mean ± S.E. (n=6). Those levels are presented as mean relative quantity (RQ) ± S.E. for GK normalized to 18S rRNA and calibrated to the individual with the lowest normalized expression (n=6). Different letters indicate GK activities significantly different within ambient smelt. * and (*) represent values that are significantly different ($p < 0.05$) and close to significantly different ($p < 0.06$), respectively, between warm and ambient smelt for the sampling date considered.

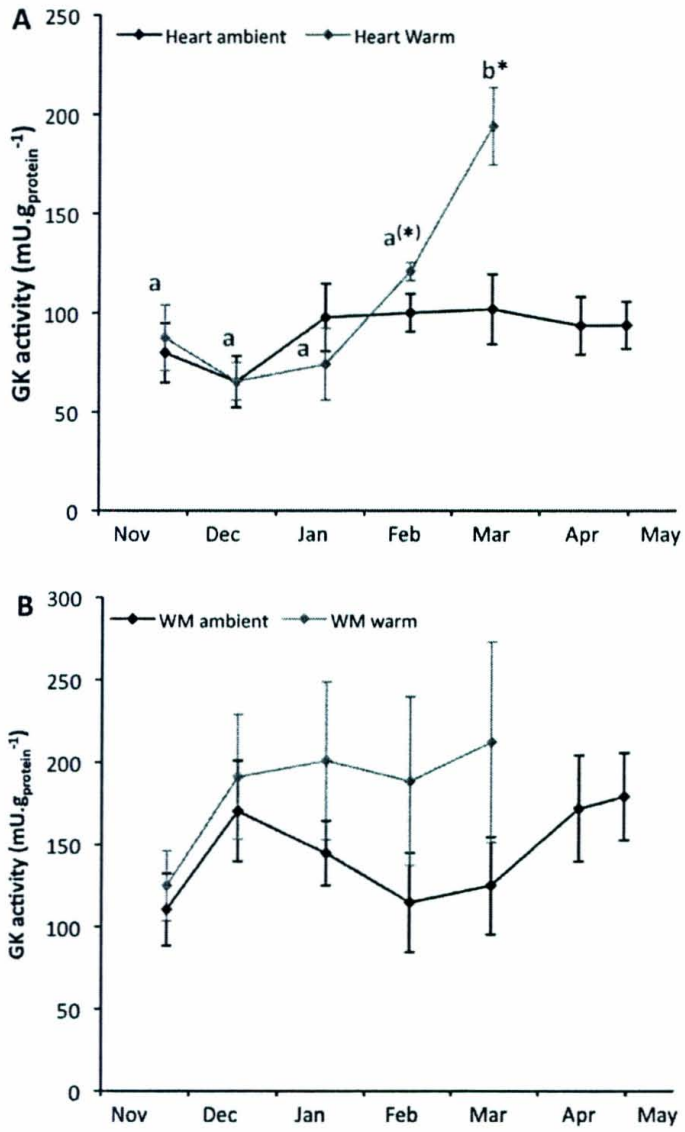

Figure 2.3. Seasonal activity of glycerol kinase (GK) in smelt heart and white muscle (2008-2009 experiment). Enzyme activity is presented as mean ± S.E. (n=6). Different letters indicate values that are significantly different ($p < 0.05$) for the group considered. * and (*) represent values that are significantly different ($p < 0.05$) and close to different ($p < 0.06$), respectively between warm and ambient smelt for the sampling date considered.

2.4.3 GK transcript expression in liver

2.4.3.1 18S and GK cloning

A partial 18S rRNA sequence of 1298 bp was cloned (Genbank accession number: HM856872). This sequence is very well conserved among vertebrates with sequence identities of 99.8% and 95% compared to *Plecoglossus altivelis* and *Homo sapiens*, respectively.

By combining RT-PCR and RLM-RACE PCR, a full-length cDNA was cloned for glycerol kinase (Genbank accession number: HM856871). It is a 1869 bp cDNA that contains a 101 bp 5'-UTR, a 1599 bp open reading frame (ORF) and a 169 bp 3'-UTR. At the nucleotide level, this sequence had the highest identity to GK of *Salmo salar* (84.2%), followed by *Danio rerio* (79.3%) and *Xenopus tropicalis* (73.2%). The 1599 bp ORF encodes for a 533 amino acids (a.a.) protein that clusters with other GK fish sequences within the vertebrate groups (Figure 2.4). At the a.a. level, smelt GK had the highest identity to *Salmo salar*, *Danio rerio* and *Xenopus laevis* (87%, 85% and 78% respectively). The 533 a.a. protein features the two expected conserved domains for glycerol kinase; FGGY-N and FGGY-C superfamily domains. These domains adopt a ribonuclease H-like fold and are structurally related to the N- and C-terminal domains of GK respectively (data not shown).

2.4.3.2 Seasonal expression of GK transcript in liver

18S expression was stable over the season and was thus determined as a suitable housekeeping gene for this experiment. Expression of the GK transcript did not change

over the tested time period for either ambient or warm smelt and was not different between both groups (Figure 2.2B). Levels of GK transcripts are very low as shown by the need of two PCR in a row to be able to amplify sufficient products (for size > 400 bp), and the high Ct values measured during the qPCR experiment.

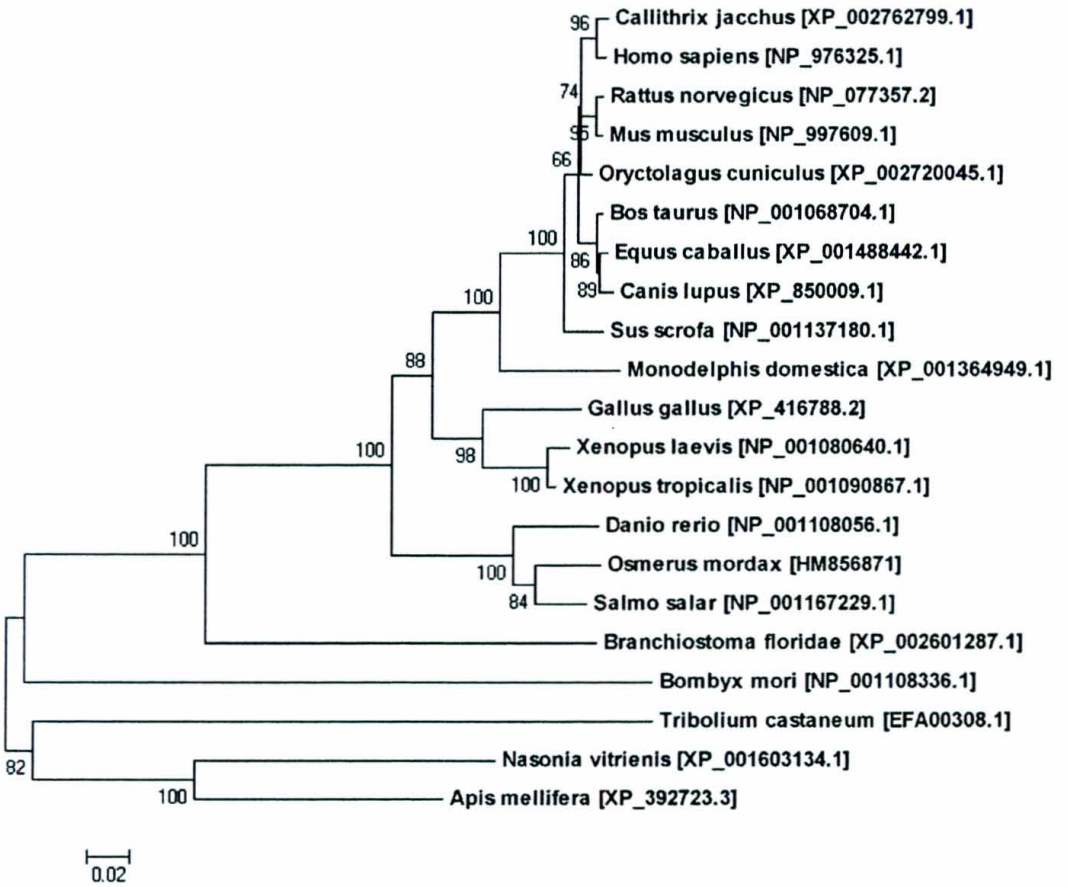

Figure 2.4. Neighbour-joining tree (Saitou and Nei 1987; constructed in MEGA) showing relationship between smelt glycerol kinase (GK) protein sequence and other GK sequences available in GenBank (accession numbers are indicated in brackets). Numbers represent bootstrap values. The scale bar represents the number of substitutions per amino acid site.

2.4.4 Glycerol loss in water

Plasma glycerol levels confirm that the periods considered are related to a decrease period from February 2008 to April 2008 and to an increase period from December 2008 to February 2009 (Figure 2.5A). Water temperature ranged between 1.2°C and 0.3°C during the decrease period and between 6.5°C and 0.3°C during the increase period (Figure 2.5A). The highest temperature was recorded at the beginning of the increase period and slowly decreased from there to reach 0.3°C at the end of February. Glycerol accumulated in the water in a linear fashion over the 24 h incubation period (data not shown). Over both periods considered, glycerol loss was positively linearly correlated to the glycerol concentration in plasma (Figure 2.5B). The relationship between glycerol loss and plasma glycerol was not significantly different between the increase or decrease in glycerol time periods even though the two groups of fish differed in mass with the February 2008 to April 2008 group being 75.2 ± 5.3 g and the December 2008 to February 2009 group being 49.8 ± 1.3 g. As the two rates were similar the data sets have been combined to yield the following relationship:

$$Glycerol\ loss\ (\mu mol.g_{fish}^{-1}.day^{-1}) = 0.047\ x\ plasma\ glycerol\ (\mu mol.ml^{-1}) + 1.290$$

When expressed as a daily percentage of glycerol loss (%), most values were below 10%. The one exception was at the beginning of the increase period where the % was the most variable. In early December, the average % loss was higher, close to 15% (Figure 2.5C).

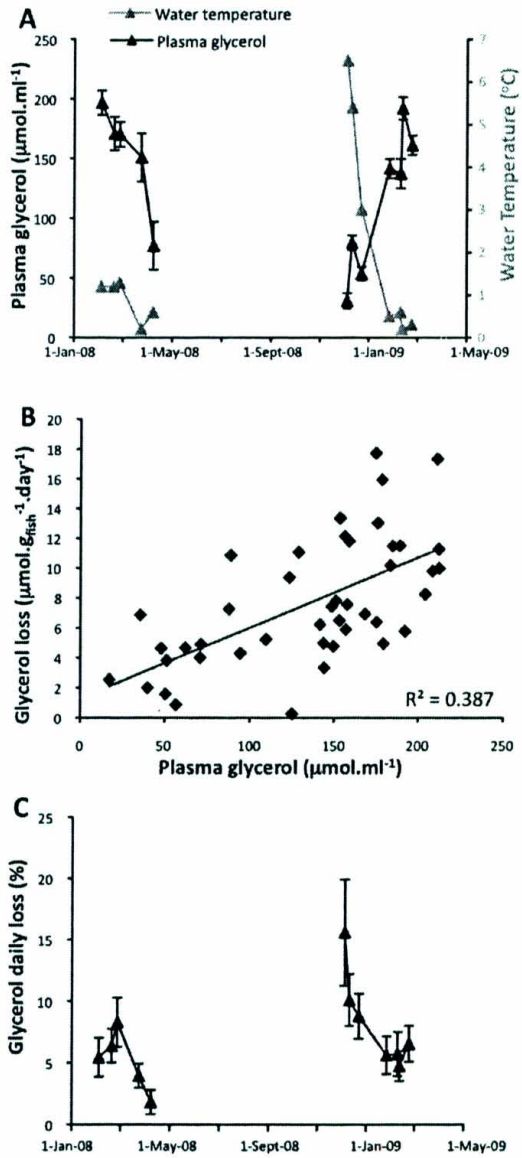

Figure 2.5. (A) Rate of glycerol loss from smelt (mean ± S.E.) and associated water temperature at different points of the glycerol cycle (n=4 trials per point), (B) Rate of glycerol loss as a function of glycerol concentration in smelt plasma for the increase and decrease periods of the cycles, and (C) Daily percentage of glycerol loss at different points of the glycerol cycle (n=4 trials per point).

2.5 Discussion

The first notable result of this study is the similarity between glycerol cycles measured in 2007-2008 and 2008-2009 with the same peak detected in mid-February at levels higher than 200 mM. The cycles differ slightly with January and April concentrations higher in 2008 than in 2009. Those differences, however, did not have any effect on the overall rate of glycerol accumulation and decrease between both seasons. These patterns are also very similar to the one reported by Lewis and collaborators (Lewis et al. 2004), all of them closely fitting one on each other. The predictable decrease in glycerol levels observed in February supports the hypothesis that the decrease of glycerol levels from mid-February is not triggered by temperature but by some other factor such as photoperiod (Driedzic & Short 2007).

Glycerol loss to water was determined during both the glycerol accumulation and glycerol decrease periods. Fish in experimental chambers did not show any signs of agitation and the rate of glycerol accumulation was linear to 24 h. As such, I considered the measurements of rate of glycerol loss to the environment to be a normal physiological process and not one induced by experimental stress. Dates chosen for those experiments covered an important part of both periods with plasma glycerol values ranging from 17 to 211 mM and from 212 to 50 mM for the accumulation and decrease periods, respectively. A positive linear correlation between plasma glycerol levels and the rate of glycerol loss is observed for both periods with no significant difference between both increase and decrease periods. As such, no obvious mechanism seems to be in place to retain glycerol during the accumulation period.

Glycerol loss to the environment appears to be by diffusion since it is dictated by the concentration difference between plasma and water levels. Raymond (1993) previously reported that the major sites of glycerol loss are gill and skin with only small losses in urine. Although the data obtained here are consistent with a passive diffusion we cannot rule out the possibility of a more complex scenario perhaps involving facilitated diffusion via aquaglyceroporins or even different processes in gill, skin, and kidney. For instance, glycerol movement into heart appears to be by simple diffusion alone; however, in red blood cells there is both a simple and a facilitated component to glycerol uptake (Clow and Driedzic, 2012).

According to the formula deduced from the linear relationship between glycerol loss ($\mu mol.g_{fish}^{-1}.day^{-1}$) and the concentration of glycerol in plasma (mM), a fish with a plasma concentration of 100 mM (or 100 $\mu mol.ml^{-1}$) would lose around 6 μmoles of glycerol per g fish per day. Thus, a 60 g fish would lose 360 μmol per day. Given that glycerol levels in all tissues are similar to plasma, a 60 g fish would contain about 6000 μmol of glycerol. The rate of glycerol loss to water would be sufficient to clear the animal of glycerol in 16.6 days (6000/360) assuming glycerol synthesis is highly reduced. It is recognized that this is an over-simplification of the rate of glycerol loss since as plasma glycerol levels decrease so will the rate of loss. Regardless, as discussed below, on the basis of this first approximation analysis, there appears to be no need to involve GK activity to facilitate glycerol removal. This position is further developed below.

When expressed as a proportion of total glycerol in fish, my results fit nicely with the ones reported by Raymond who estimated daily loss between 3 and 13% (Raymond

1993). Mostly all proportions estimated in this study were lower than 10%, the only exceptions being at the beginning of the glycerol cycle where very high daily losses were recorded with values that exceeded 15%. These high values at the beginning of the increase period might be due to the higher water temperatures. This kind of relation between temperature and glycerol loss was previously reported for algae accumulating high amounts of glycerol when subject to osmotic stresses. The proportion of glycerol loss increased drastically with an increase in temperature (Wegmann et al. 1980). Higher glycerol loss at higher temperatures might be the result of a higher blood flow through gills generally associated with higher temperature and/or to a different membrane structure at higher temperature making small molecule exchanges easier.

The *in vitro* activity of GK appears to be a good proxy for *in vivo* rates of glycerol metabolism. This viewpoint is based on the following findings. Activity of GK was measured in rat liver during optimization of the assay and was slightly higher than 1 $U.g_{liver}^{-1}$ at 37°C (data not shown). This activity is lower but comparable to the total capacity of glycerol clearance in rat liver set between 2 and 4 $U.g_{liver\text{-}1}$ at 37°C (Lin 1977). Furthermore, at 30°C, GK activity of the same rat was 0.66 $U.g_{liver}^{-1}$ and was more than 40 times higher than GK activity measured at the same time in liver of two smelts (data not shown). As such, GK activity in liver of smelt is unexpectedly low in comparison to animals that rarely experienced glycerol plasma concentrations higher than 0.4 mM (Lin 1977).

The quantitative contribution of GK to the decrease in glycerol levels at the end of the glycerol cycle is minimal. For instance GK activity in April is about 300 $mU.g_{protein}^{-1}$

when plasma glycerol levels are around 100 μmol.ml^{-1}. This activity over-estimates glycerol metabolism *in vivo* as GK measurements were done at 30°C. Assuming a Q_{10} of 2, the *in vivo* activity would be 37.5 mU.$g_{protein}^{-1}$ at 0°C. For a typical 60 g fish with an average liver weight of 1 g and an average protein content per g of liver of 0.12 g, the maximal rate of glycerol metabolism in liver would be 6.48 μmol glycerol per day (0.0375 x 1 x 0.12 x 60 x 24). This is in contrast to glycerol loss to the water estimated to be 360 μmol per day for a 60 g fish.

Regardless of a minimal role in overall glycerol clearance, the seasonal profile of hepatic GK activity reveals changes that suggest an important metabolic function for this enzyme. GK activity remained constant and at its minimum while glycerol accumulates from November to mid-February with an average activity of 43 mU.$g_{protein}^{-1}$, while the average activity was 198 mU.$g_{protein}^{-1}$ over the decrease period. Activity levels reached a maximum in excess of 400 mU.$g_{protein}^{-1}$ at the final sampling point. The seasonal profile of GK activity supports the theory that, from February, some glycerol is directed toward the synthesis of glucose as suggested by the increase of glycogen in this tissue at the end of the season (Driedzic & Short 2007) and by the parallel increase of mGPDH activity that catalyses the formation of DHAP from G3P (Robinson et al. 2011). GK activity started to rapidly increase at the same time, as there was a small increase in water temperature above minimal winter temperatures, suggesting that this enzyme is under very fine temperature control.

GK transcript expression in liver did not show any change over the season and was not different between ambient and warm smelt. Only one sequence that encodes for a

functional GK was cloned from smelt liver but the possibility of different GK paralogues present in liver cannot be excluded. Primers designed for qPCR might anneal to other paralogues having the same conserved region and would result in a dilution of the results, and thus a failure to detect any change in a specific transcript. Alternatively, regulation of GK activity might happen after mRNA synthesis. A change in protein content can occur at a post-transcriptional level without any change in the mRNA levels following different mechanisms that regulate mRNA activity (Wickens et al. 2002). A change in GK activity can also occur with no change in the protein content at a post-translational level. Regulation of enzyme activity through phosphorylation/dephosphorylation is one of the most common post-translational modifications that can lead to protein activation or deactivation, similar to an on/off switch (MacDonald 2004). This might regulate GK activity in smelt liver as the hypothetical protein reported in this paper features potential phosphorylation sites (data not shown). Whatever the mechanism in which protein activity is regulated, GK expression was very low consistent with the low activity of enzyme.

As glycerol accumulates to the same levels in all tissues (Driedzic & Short 2007), GK activity was measured elsewhere to assess any significant role of extra-hepatic function of this enzyme during the glycerol cycle. GK activity was measured in heart, brain and gills, as they are important tissues for basic function, and in white muscle (WM), as it is a substantial portion of the body (estimated to be 40%). No activity was detected in gills and the technique used did not allow for accurate measurements in the brain. The latter result is not unexpected, as low activities were reported in brain of

mammals (Lin 1977); however, some GK activity was expected in gills, as this tissue is a major site of glycerol loss in smelt (Raymond 1993). GK activities measured in white muscle and heart of smelt are not unexpected as comparable activities were previously reported in other fish species (Newsholme & Taylor 1969). However, activities similar to the ones measured in liver are quite surprising. In mammals, liver is responsible for about three-fourths of the total body capacity of glycerol clearance (Lin 1977). The similarity of GK activity amongst the different tissues in smelt might reflect a difference between mammals and fish with respect to the function of glycerol. The lack of change in GK activity in both white muscle and heart over the glycerol cycle support a minor role of this enzyme in the control of glycerol levels, and it highlights the important metabolic function of GK in liver where activity is up-regulated at the end of the glycerol cycle.

2.5.1 Perspectives and significance

I have investigated the respective roles of direct loss of glycerol to water and GK in the glycerol cycle repeatedly observed in winter in smelt. The loss of glycerol to water is consistent with a passive diffusion process with no mechanisms to retain glycerol during the accumulation stage. This study does not rule out the possibility of more complex processes being involved at the level of gill, skin, and/or kidney, as there may be tissue differences in the mechanisms of glycerol movement across cell membranes.

Based on GK activities, no metabolic mechanism is in place to accelerate the decrease of glycerol from mid-February. Glycerol levels seem to be dictated primarily by the rate of glycerol synthesis that must be accelerated during the accumulation period and

deactivated during the decrease period. The direct dephosphorylation of G3P to glycerol has been proposed to be the pathway used in smelt to directly produce glycerol (Driedzic et al 1998; Driedzic et al. 2006), as it is the case in some algae and yeasts producing high amounts of glycerol in response to environmental stresses (Sussman & Avron 1981; Norbeck et al. 1996). Activity of such an enzyme has been suggested in oxygen-limited rat heart and brain, although neither the protein nor the transcript have been identified in a vertebrate (De Groot et al. 1994; Nguyen et al. 2007). Isolation and characterization of such an enzyme would be of interest, as it would open a new avenue of research concerning the role of glycerol in vertebrates.

Although having a minor role in glycerol clearance, GK in liver appears to have an important metabolic function as evidenced by the increase in activity late in the cycle in this tissue only. An elevated GK activity at the end of the cycle might be related to the restoration of glycogen stores in liver suggested previously (Driedzic & Short 2007). Regulation of GK activity may be controlled by small increases in water temperature above the winter minima. How this is achieved is yet to be resolved.

Chapter 3

Respective roles of lipids and of glycerol-3-phosphatase in the accumulation of high levels of glycerol in rainbow smelt (*Osmerus mordax*)

3.1 Abstract

Smelt is a small fish indigenous to Atlantic Canada that accumulates up to 200 mM glycerol in winter as a cryoprotectant. Glycerol is synthesized in liver from glucose, glycogen and amino acids that all lead to a transition through glycerol-3-phosphate (G3P). Determination of the pathway involved in glycerol synthesis from G3P was at the centre of this work. Glycerol could be produced from G3P following a direct dephosphorylation by a phosphatase (G3Pase) or by cycling through the glycerolipid pool followed by lipolysis. Levels of on-board glycerolipids and activity of G3Pase and of different enzymes involved in lipid metabolism were measured over the glycerol cycle. Levels of glycerolipids did not change over the cycle and were too low to significantly participate in glycerol production but activities of enzymes involved in both potential pathways were up-regulated at the onset of glycerol accumulation. As such, this experiment did not delineate either pathway as the main source of glycerol. A second experiment was thus conducted with isolated hepatic cells incubated at cold temperature for 72 h. These cells released glycerol linearly and showed: 1) that on-board glycerolipids were not sufficient to produce the glycerol released even though phospholipids could account for up to 16% of it, 2) that *de novo* synthesis of glycerolipids could not be the source of glycerol as glycerol was produced at the same rate following inhibition of this pathway and 3) that G3Pase activity measured was sufficient to allow the synthesis of glycerol at the rate observed. These results are the first to clearly point out G3Pase as the principal source of glycerol in an animal species.

3.2 Introduction

Numerous terrestrial animal species accumulate unusually high amounts of glycerol in winter as a cryoprotectant. This is the case of freeze tolerant species such as willow gall insects (Miller & Werner 1987) or some species of the Gray tree frog complex (Layne & Lee 1995; Irwin & Lee 2003), and of freeze avoiding species such as gall moth larvae (Joannisse & Storey 1994). Glycerol also accumulates in rainbow smelt (*Osmerus mordax*) (Raymond et al. 1992), a teleost fish. The synthesis of antifreeze proteins is the common strategy against freezing in fish, whereas accumulation of glycerol is not anticipated based on the chemical properties of glycerol. This small sugar alcohol ($C_3H_8O_3$) is highly soluble in water and lipid and easy to transport across cells membranes through aquaglyceroporins (Hara-Chikuma & Verkman 2006), thus making direct loss to the environment most likely to occur in species that are in constant contact with the surrounding water. Four fish species, however, accumulate high amounts of glycerol during winter at subzero temperatures including two species of smelt (*Osmerus mordax* and *Hypomesus pretiosus*) and two species of greenling (*Hexagrammos stelleri* and *Hexagrammos octogrammus)* (Raymond 1992). In rainbow smelt, glycerol is the most important component participating in the depression of the freezing point (FP) of body fluids, before NaCl and antifreeze proteins (Raymond 1992; Lewis et al. 2004).

In a small population of rainbow smelt indigenous to Newfoundland, glycerol levels follow a predictable cycle that starts with an accumulation period from late fall (< 5 mM) to the middle of February (> 200 mM), in parallel with the decrease of water temperature. Thereafter there is a period of glycerol decrease that is most likely induced

by a change in the photoperiod and extends until the beginning of May (< 5 mM) (Lewis et al. 2004; Ditlecadet et al. 2011).

Recent work has been done to determine how direct loss of glycerol to water and its catabolism through glycerol kinase (GK) could affect glycerol levels over the cycle (Ditlecadet et al. 2011). Concordant with Raymond's data (1993), results showed that about 10% of the total glycerol content of fish was lost daily in surrounding water but also that no mechanisms were in place to retain glycerol during the critical accumulation period. GK is unlikely to significantly affect glycerol levels due to its very low activities even though activities increased by the end of the cycle, supporting the channelling of some glycerol to glycogen as proposed previously (Driedzic & Short 2007; Robinson et al. 2011). Taken together, these results show a requirement for glycerol to be actively synthesized during the accumulation period to compensate for the continuous losses to the environment, and also that the glycerol level is primarily dictated by its rate of synthesis. This situation makes smelt a relevant model to study metabolic pathways involved in glycerol synthesis in vertebrates.

Glycerol is well known as a by-product of lipolysis, being the backbone of triglycerides (TG) and of most phospholipids (PL) (Lin 1977). Lipid reserves, such as TG, would be the most direct source of glycerol, but Raymond (1995) showed that TG levels did not decrease, over 5 days of starvation, in liver or plasma of smelt caught in winter and producing glycerol. Total fat content of smelt was reported to range between 2 and 10% of the total weight depending on the tissue and on the species considered. Muscle, the main tissue of fish, did not contain more than 2.2% of fat whatever the

population of smelt considered (Krzynowek & Murphy 1987), and TG were not even detected in this tissue by Raymond (1993). These data do not allow ruling out a role of TG in glycerol synthesis, especially at the onset of glycerol accumulation in late fall, but they are a good indication that on-board TG reserves are unlikely to be sufficient to produce the important levels needed in winter.

Carbon sources necessary for the synthesis of glycerol were later shown to be glucose, glycogen and amino acids (Raymond 1996; Raymond & Driedzic 1997; Treberg et al. 2002a; Walter et al. 2006), with dietary glucose and amino acids being essential to maintain rates of glycerol synthesis (Clow et al. 2008). In all cases, synthesis is localized in liver and involves a transition through the C_3 intermediate DHAP. From there, DHAP is converted to G3P by GPDH that proved to be a key enzyme in controlling glycerol synthesis (Lewis et al. 2004; Liebscher et al. 2006). The remaining question concerns the last step of the pathway leading to the formation of glycerol from G3P and is at the centre of this work.

Two pathways can result in the synthesis of glycerol from G3P: one that involves a direct dephosphorylation by a phosphatase (G3Pase) and one that involves the transition of G3P through the glycerolipid pool (TG + PL) prior to the release of glycerol following their degradation (Figure 1.3). In yeast (*Saccharomyces cerevisiae*), that produces glycerol to avoid dehydration in response to osmotic stress, two isoforms of G3Pase were identified, as supported by the isolation and sequencing of two phosphatases specific to G3P (Norbeck et al. 1996). Mutants for both genes encoding for those isoforms lacked G3Pase activity, and produced only small amounts of glycerol in comparison to wild

types, confirming the essential role of this pathway in glycerol synthesis in this species (Påhlman et al. 2001). This pathway was thus naturally proposed as the source of glycerol in species (including smelt) that accumulate unusual amounts of glycerol in response to various stressors, even though G3Pase has yet to be identified in any animal species.

In smelt, the existence of G3Pase is supported by the report of its activity at levels higher than in any other sympatric species not producing glycerol (Driedzic et al. 1998) as well as by mass action ratio data across G3Pase (i.e. [glycerol][P_I] \ [G3P]) that increased in association with an increase in glycerol production (Driedzic et al. 2006). *In vitro*, phosphatases can dephosphorylate different substrates. As such, the report of G3Pase-like activity is not sufficient to validate a pathway that is still hypothetical in animals. Even though the second pathway, involving glycerolipids, would be more demanding energetically, as it requires not only different enzymes but also FFA and co-factors such as or NADPH, it would be premature to rule it out since all of the enzymes necessary for the pathway should be in place. Furthermore, in mammals, only a very small fraction of the FFA released following lipolysis under starvation is actually oxidized to fuel the organism with energy. Most of the released FFA is recycled back to TG, with G3P synthesized through glyceroneogenesis as new backbone (Reshef et al. 2003; Hanson et al. 2006). This recycling is also costly energetically, but could take place in smelt liver to produce glycerol constantly, as glyceroneogenesis is activated in winter (Lewis et al. 2004). Hall et al. (2011) detected the up-regulation of mRNA levels of different genes involved in lipid metabolism in isolated hepatic cells incubated at cold

temperature, producing glycerol, but the expression of an *uncharacterized* phosphatase, which may encode for G3Pase, was regulated in the same way.

In order to provide a better understanding of the respective roles of glycerolipids and G3Pase in the synthesis of glycerol, I utilized two approaches. The first one investigated the profile of G3Pase activity and of different components of lipid metabolism in smelt liver over the complete glycerol cycle. This approach offered a representation of how levels of potentially key players are modulated in winter in parallel to changes in glycerol levels. The second approach took advantage of the capacity of isolated smelt hepatic cells to produce glycerol when incubated at low temperature. This allowed me to compare more accurately the relation between the rate of glycerol synthesis and its two potential sources in a closed system, without any confounding effects of other sources that may dilute the information in a whole organism. Findings highlight the seasonal regulation of both G3Pase activity and lipid metabolism in liver of smelt but also the impossibility for glycerolipids to be at the origin of all the glycerol produced, pointing out for the first time the direct dephosphorylation of G3P by G3Pase as the most likely pathway to synthesize glycerol in an animal species.

3.3 Materials and Methods

3.3.1 Animals

Rainbow smelt (*Osmerus mordax*), hereafter referred as smelt, were collected by seine netting from Mount Arlington Heights, Placentia Bay, Newfoundland, in October 2008, transported to the Ocean Sciences Centre, Memorial University of Newfoundland (St.

John's, Canada), and transferred to two 3000 litres tanks with flow-through seawater. One group was maintained at warm temperature fluctuating around 11°C, hereafter referred to as warm smelt. A second group tracked ambient temperature, hereafter referred to as ambient smelt. Fish maintained at ambient temperature that reaches 0°C accumulate glycerol, whereas fish maintained at warm temperature do not (Lewis et al. 2004). Fish were kept on a natural photoperiod using fluorescent lights set by an outdoor photocell and fed a diet of chopped herring twice a week to satiation.

3.3.2 Hepatocyte preparation

Hepatocytes were isolated from smelt maintained at warm temperature as these cells produce more glycerol when incubated at low temperature than cells isolated from smelt acclimated to cold winter temperatures (Clow et al. 2008). Animals were chosen randomly with no consideration of the sex and killed by a sharp blow to the head. Liver was immediately exposed and hepatocyte suspensions were prepared by perfusing the liver with collagenase as described by Mommsen et al. (1994) and adapted for smelt by Clow et al. (2008).

3.3.2.1 Liver washing

The hepatic vein of the exposed liver was cannulated (retro-cannulation) and the venous input was nicked for further drainage. Blood was washed out using a perfusion medium (PM) adjusted at pH 7.6 that contained (in mM) 176 NaCl, 5.4 KCl, 0.81 $MgSO_4$, 0.44 KH_2PO_4, 0.35 Na_2HPO_4, 5 $NaHCO_3$, 10 HEPES and 1 EGTA.

3.3.2.2 Hepatocyte isolation

Liver was perfused with a collagenase solution (PM without EGTA + 0.3 $mg.ml^{-1}$ collagenase) until the tissue was soft enough to be gently teased apart (25-30 min). Until this point, each step was performed at room temperature. Cell suspensions were then kept on ice when possible.

Cells were filtered twice (250 and 50 μm), washed using a BSA solution (PM with 2% BSA and 1.5 mM $CaCl_2$) and counted using a Neubauer hemocytometer. Viability was assessed by Tryptan blue exclusion and any preparations with viability lower than 90% were rejected from further experiments. Concentration of the cell suspensions was adjusted to 40×10^6 $cells.ml^{-1}$ with the BSA solution. One hundred and fifty μl of each suspension (25-40 mg of cells) were aliquoted into separate 20 ml glass scintillation vials containing 2 ml of incubation medium (BSA solution + 5 mM glucose) and incubated in the conditions described below for up to 72 h. Viabilities at any sampling points were usually above 90% and any viability below this was rejected.

3.3.3 Experimental set-up

3.3.3.1 G3Pase activity and lipid metabolism over the glycerol cycle

Blood and liver of six ambient and six warm individuals were sampled monthly from November 2008 to May 2009 with the exception that in April and May 2009 only ambient smelt were sampled as warm smelt started to die from the end of March. Occurrence of high mortalities in March-April is a recurrent phenomenon for smelt

maintained at warm temperature and is thought to be the result of early spawning, males being predominant and one-time spawners in this population. For each individual, one piece of liver was immediately frozen in liquid nitrogen and kept at -80°C until enzyme activities were measured. A second fraction was sampled from 5 individuals per group and was placed in a lipid free glass tube with 2 ml of chloroform under nitrogen to avoid lipid oxidation. Teflon sealed tubes were held at -80°C until total lipid extraction and analysis. Blood samples were collected with syringes that were rinsed with 2% EDTA and centrifuged at 10,000 × g for 10 min at 4°C. Plasma collected was processed as liver samples for glycerol levels and lipid extraction.

G3Pase activity was measured in all livers sampled. Lipid metabolism was investigated through the measurement of activities of key enzymes in liver and through the quantification of nine lipid classes in both liver and plasma. Activity of lipase, hydroxyacyl CoA dehydrogenase (HOAD), carnitine palmitoyltransferase I (CPT-I), malic enzyme (ME) and glucose 6-phosphate dehydrogenase (G6PDH) were measured in all livers sampled. Lipase, HOAD, and CPT-I are all directly involved in lipid degradation. ME and G6PDH are both indirectly involved in lipid synthesis as major sources of NADPH necessary during fatty acid synthesis. The lipid classes quantitated were: steryl ester (SE), hydrocarbon (HC), ketone (KET), triacylglycerol (TG), free fatty acid (FFA), sterol (ST), alcohol (Al), acetone mobile polar lipid (AMPL) and phospholipid (PL).

3.3.3.2 Respective roles of G3Pase and glycerolipids in glycerol synthesis in isolated hepatocytes

The first experiment showed that activities of G3Pase and a number of enzymes of lipid metabolism were regulated in ambient smelt producing glycerol. As such, the metabolic route to glycerol required further clarity. Isolated cell models involve a lower level of complexity than a whole organism and were thus utilized to approach this question.

Hepatocytes isolated from warm smelt produce glycerol linearly for up to 72 h when placed at 0.4°C in the presence of glucose (Clow et al. 2008). Based on this, I investigated the effect of the inhibition of lipid synthesis on glycerol production over this period. Inhibition was induced using triacsin C, a fungal metabolite and potent inhibitor of long-chain fatty acyl-CoA synthases (ACS) (Tomoda et al. 1987).

An initial experiment was done to evaluate the effect of triacsin C on the activity of palmitoyl-CoA synthase (pACS) in smelt hepatocytes. Hepatocytes of warm fish (n=3) were incubated at 0.4°C in the incubation medium described above with 10 μM triacsin C in DMSO (0.1% v/v) (T+) or with DMSO only (T-) for 0, 6, 12, and 72 h. At each time point, an aliquot of the hepatocyte suspension was transferred to a centrifuge tube and centrifuged at 1,000 x g for 10 min at 4°C. The supernatant was discarded and the cell pellet kept at -80°C until pACS activity was measured. The percentage of inhibition (% inhib.) was calculated for each time point and for each suspension considered using the following formula:

$$\% \text{ inhib.} = 100 \times [(pACS_{T-} - pACS_{T+}) / pACS_{T-}]$$

After demonstrating that triacsin C significantly decreased pACS activity in smelt hepatocytes, an experiment was designed to estimate its effect on glycerol synthesis. The effect of triacsin C was determined on six fish with duplicate assays. Cell suspensions were prepared from individual animals, aliquoted into 16 different vials, and incubated at 0.4°C in either incubation medium with 10 μM triacsin C (8 T+) or in control medium (8 T-). Four vials per treatment were immediately sampled and considered as pre-incubation samples, while the remaining vials were sampled after 72h incubation (T_{72}). For each sampling, the contents of two vials were transferred to Eppendorf tubes and centrifuged at 1,000 × g for 10 min at 4°C. Supernatants were transferred to new tubes and both fractions were kept frozen at -80°C until glycerol and G3Pase analyses. The content of the two other vials was centrifuged in lipid free glass tubes at 1,000 × g for 10 min at 4°C and the supernatant was transferred to new lipid free glass tubes. Chloroform was added to both fractions and all tubes were placed under nitrogen and sealed with Teflon before being placed at -80°C until further lipid analysis.

3.3.4 Analysis

3.3.4.1 Enzyme assays

Liver samples or cell pellets were thawed on ice and homogenized using a polytron in 4 volumes of ice-cold triethanolamine buffer 0.1M at pH 7.4. All assays were run in duplicate at 15°C with the exception of lipase that was run at 25°C. In all cases, concentration of the homogenate was chosen to provide a linear response over the period specified below. Except for the cell experiments where enzyme activities were expressed

per g cells, all activities were assessed per g liver and thereafter normalized to g fish taking into consideration the liver mass for each individual animal. This method of analysis was selected in order to gain insight into the capacity of the whole liver to produce glycerol for the whole animal and to take into consideration any change of relative liver mass over the glycerol cycle. The relative mass of liver, represented by the hepatosomatic index (HSI (%); [mass of liver / mass] of fish × 100) was significantly higher in ambient smelt in December than for any month over the glycerol cycle except January (1.59 ± 0.18, 2.60 ± 0.15, 2.19 ± 0.17, 1.73 ± 0.21, 1.45 ± 0.21, 1.64 ± 0.13 and 1.79 ± 0.10 % for November, December, January, February, March, April and May, respectively). In warm smelt, HSI was only significantly lower in March than in December, January or February with average values of 2.01 ± 0.25, 2.67 ± 0.24, 2.83 ± 0.17, 2.45 ± 0.15 and 1.58 ± 0.21 % for November, December, January, February and March, respectively. HSI was higher in warm smelt than in ambient in January and February (*t*-test; $p < 0.05$).

Lipase activity was measured using 4-methylumbelliferol (4-MUH) as substrate by a method modified by Lemieux et al. (2003) from Roberts (1985). Briefly, a liposomal dispersion was prepared by dissolving 2 mM 4-MUH and 2 mM soybean lecithin in chloroform methanol (2:1). This mix was evaporated under a nitrogen stream and dissolved back in 150 mM NaCl by sonication. The reaction assay consisted of 3 ml of 1mM Tris-HCl (pH 7.5), 20 µl of liposomal dispersion and 20 µl of homogenate diluted to give a linear response for up to 10 min. The change in fluorescence was recorded at 25°C every 20 sec for 10 min using excitation/emission of 365/450 nm. A standard curve

of 4-methylumbelliferone (4-MU) was prepared and lipase activity assessed from it as mmol 4-MU produced per min per g liver and thereafter converted to $mmol_{4\text{-}MU}.min^{-1}.g_{fish}^{-1}$.

HOAD assay was run in a triethanolamine buffer (100 mM triethanolamine; pH 7.0) containing 5 mM EDTA, 1 mM KCN, 0.115 mM NADH and 0.05 mM acetoacetyl CoA. Disappearance of NADH was monitored at 340 nm for 5 min and HOAD activity assessed as Units per g fish ($U.g_{fish}^{-1}$), one Unit corresponding to the disappearance of one µmol of NADH per min at 15°C (NADH extinction coefficient at 340 nm = 6.22 $mM.cm^{-1}$).

CPT-I assay was run in a tris-HCl buffer (75 mM; pH 8.0) containing 1.5 mM EDTA, 0.25 mM DTNB, 0.035 mM palmitoyl CoA and 2 mM L-carnitine (omitted for controls). Formation of a yellow DTNB-CoA complex was monitored at 412 nm for 5 min and CPT-I activity assessed as Unit per g fish ($U.g_{fish}^{-1}$), one Unit corresponding to the appearance of one µmol of DTNB-CoA complex per min at 15°C (DTNB extinction coefficient at 412 nm = 13.6 $mM.cm^{-1}$).

ME assay was run in a triethanolamine buffer (65 mM; pH 7.4) containing 5 mM $MnCl_2$, 3.5 mM L-malic acid and 0.35 mM NADP. Appearance of NADPH was monitored at 340 nm for 5 min and ME activity assessed as Unit per g fish ($U.g_{fish}^{-1}$), one Unit corresponding to the appearance of one µmol of NADPH per min at 15°C (NADPH extinction coefficient at 340 nm = 6.22 $mM.cm^{-1}$).

G6PDH assay was run in a tris-HCl buffer (55mM, pH 8.0) containing 3.3 mM $MgCl_2$, 6 mM NADP and 100 mM glucose-6-phosphate. Appearance of NADPH was

monitored at 340 nm for 5 min and G6PDH activity assessed as Unit per g fish ($U.g_{fish}^{-1}$), one Unit corresponding to the appearance of one µmol of NADPH per min at 15°C (NADPH extinction coefficient at 340 nm = 6.22 $mM.cm^{-1}$).

G3Pase assay was run in an acetate buffer (100 mM; pH 5.5) containing 10 mM G3P. Released inorganic phosphate (P_I) was determined at three time points over 30 min with a colourimetric assay based on Rockstein and Herron (1951). Briefly, 40 µl of the reaction mix was added to 80 µl of 3.3% ammonium molybdate in 5 N sulphuric acid: distilled water (2:1; v:v) to stop the reaction. Colour was initiated by adding 40 µl of 260 mM $FeSO_4$ and absorbance measured after 5 min on a plate reader at 620 nm. A standard curve of P_I was run from different concentrations of Na_2HPO_4 and G3Pase activity assessed from it as Units per g fish ($U.g_{fish}^{-1}$) or as Units per g cells ($U.g_{cells}^{-1}$), one Unit corresponding to the appearance of one µmol of P_I per min at 15°C.

pACS assay was run in a Tris-HCl buffer (100 mM; pH 8.0) containing 5 mM DTT, 150 mM KCl, 15 mM $MgCl_2$, 10 mM ATP, 1 mM coenzyme A and 0.25 mM palmitic acid. After 5 and 20 min incubation, 100 µl of the reaction mix was stopped by adding 360 µl acetonitrile and 40 µl of 1M phosphoric acid. The mixture was filtered using a syringe filter (50µm) and 100 µl of the filtrate was used to determine the amount of palmitoyl-CoA formed using HPLC according to Tomoda et al. (1987). Measurements were made on a C18 WATERS column using WATERS HPLC system. The conditions of the HPLC were as follows: 10 min linear gradient from 40-60% acetonitrile in 25 mM KH_2PO_4 followed by 10 min isocratic run with 60% acetonitrile in 25 mM KH_2PO_4; flow rate of 1 $ml.min^{-1}$; detection UV at 254 nm. The retention time of palmitoyl-CoA was 15

min. A standard curve of palmitoyl-CoA was run and pACS activity assessed from it as Units per g cells ($U.g_{cells}^{-1}$), one Unit corresponding to the appearance of one µmol palmitoyl-CoA per min at 15°C.

3.3.4.2 Lipid analysis.

Lipids were extracted using chloroform/methanol according to Parrish (1999). This method was used principally to measure TG levels. This avoided using the usual assay that involves a measure of glycerol, as the high levels of glycerol accumulated in winter may affect the accuracy of the results. Other major classes of lipids were determined at the same time, offering valuable information concerning PL, the second source of glycerolipids. Lipid classes were determined using thin-layer-chromatography with flame ionization detection (TLC/FID) using a MARK V Iatroscan (Iatron Laboratories, Tokyo, Japan) as described by Parrish (1987). Extracts were spotted on silica-gel-coated rods and a three-step development system was used to differentiate lipid classes. The first separation consisted of a 20 min development in hexane:diethyl ether:formic acid (99:1:0.05) and allowed separation of SE, HC and KET. The second separation consisted of a 40 min development in hexane:diethyl ether:formic acid (80:20:1) and allowed separation of TG, FFA, ST and Al. The last development consisted of 2 x 15 min in 100% acetone followed by 2 x 10 min in chloroform:methanol:water (5:4:1) and allowed separation of AMPL and PL. After each development system, the rods were scanned and the chromatograms were analyzed using PEAKSIMPLE 2.83 software system (Shell USA, Fredericksburg, Virginia). Each lipid class was quantified using the following lipid standards as reference: nanodecane (HC), cholesteryl stearate (SE), 3-hexdecanone

(KET), tripalmitin (TG), palmitic acid (FFA), cetyl alcohol (Al), cholesterol (ST), monopalmitoyl (AMPL) and phosphatidylcholine dipalmitoyl (PL). For each class, levels measured in liver were determined per g tissue and thereafter normalized to g fish taking into consideration the liver mass for each individual animal ($\mu g.g_{fish}^{-1}$). Levels measured in plasma were expressed as mg per ml ($mg.ml_{plasma}^{-1}$).

3.3.4.3 Glycerol and protein measurements

Glycerol concentrations were determined by using the free glycerol determination kit from Sigma (Oakville, ON, Canada) following the manufacturer's instructions. In experiment 1, glycerol concentrations measured in plasma were expressed as $\mu mol.ml^{-1}$. In experiment 2, glycerol content measured in the medium and pellet were summed and expressed per g cell ($\mu mol.g_{cell}^{-1}$).

3.3.4.4 Statistical analysis

For all specific parameters followed over the glycerol cycle, values are presented as means ± S.E., and significant differences between treatments at a particular sampling point were assessed using Student's *t*-tests. One-way ANOVAs with Tukey's post hoc test were used to determine differences between sampling points within a treatment. Data were log-transformed when homoscedasticity was not met. Data obtained from isolated cell experiments are presented as mean ± S.E. Significant differences in pACS activity over time were assessed for each treatment using repeated measures one-way ANOVAs. *t*-tests with repeated measures were used to assess difference in activity between

treatments for a particular sampling point. Significant differences between Pre-Incubation, T- and T+ were assessed using repeated measures one-way ANOVAs.

3.4 Results

3.4.1 G3Pase and lipid metabolism over the glycerol cycle

3.4.1.1 Water temperature and glycerol levels

The profile of water temperature is presented in Figure 3.1A. In the ambient tank, temperature started to decrease in November to reach a minimum at 0°C by mid-March and increased from there to reach 2.1°C at the end of the experiment at the beginning of May 2009. Water temperature in the warm tank fluctuated around 11°C during the entire period.

Plasma glycerol levels increased from 0.71 ± 0.47 to 221.4 ± 23.4 mM between November 2008 and February 2009 in fish held at ambient temperature. After this point, glycerol levels decreased until the end of the experiment to reach 0.05 ± 0.02 mM at the beginning of May (ANOVA; $p < 0.05$) (Figure 3.1B). Glycerol levels in plasma of warm smelt were higher in January than in November (ANOVA; $p < 0.05$) but remained below 8 mM over the entire time period with average values of 3.9 ± 0.10 mM (Figure 3.1B).

3.4.1.2 Enzyme activities in liver

G3Pase activities are presented in Figure 3.2A. In liver of warm smelt, activity did not change over the whole period with an average activity of 21.7 ± 2.2 $mU.g_{fish}^{-1}$. In ambient smelt, activity was higher in December and January than in November with respective

activities of 31.0 ± 4.3, 32.5 ± 1.8 and 11.4 ± 1.3 $mU.g_{fish}^{-1}$. Activities measured after January were intermediate to activities measured in November and December/January (ANOVA; $p < 0.05$) (Figure 3.2A). Activities were not different between warm and ambient smelt for any sampling points.

Lipase activity increased in ambient smelt from November (52.3 ± 6.3 $mmol_{4-MU}.min^{-1}.g_{fish}^{-1}$) to reach maximum level in January (97.9 ± 11.2 $mmol_{4-MU}.min^{-1}.g_{fish}^{-1}$). Levels remained constant after this point until the end of the experiment with an average activity of 73.0 ± 5.8 $mmol_{4-MU}.min^{-1}.g_{fish}^{-1}$ (ANOVA; $p < 0.05$) (Figure 3.2B). In warm smelt, lipase activity was higher in December and January than in March with average values of 99.9 ± 22.0; 90.9 ± 5.0 and 42.3 ± 7.5 $mmol_{4-MU}.min^{-1}.g_{fish}^{-1}$, respectively (ANOVA; $p < 0.05$) (Figure 3.2B). Activities were not different between warm and ambient smelt for any sampling points.

For both groups, HOAD and CPT-I activities were linearly correlated (linear regression $p < 0.05$; data not shown). No changes of activities were detected for either HOAD or CPT-I in warm smelt (Figure 3.2C and 3.2D). In ambient smelt, activity increased early in the cycle, reaching its highest point in December with average activities of 115.8 ± 13.5 $mU.g_{fish}^{-1}$ and 12.7 ± 2.2 $mU.g_{fish}^{-1}$ for HOAD and CPT-I, respectively. After this point, activity decreased to reach levels not different from levels measured in November (ANOVA, $p < 0.05$) (Figure 3.2C and 3.2D). For both enzymes, no difference was noted between warm and ambient smelt for any sampling point considered, but a strong tendency was detected in December for CPT-I, with average activity higher in ambient than in warm smelt (*t*-test; $p = 0.066$).

ME activity was higher in February than in November in ambient smelt with average activities of 49.8 ± 4.3 $mU.g_{fish}^{-1}$and 31.9 ± 4.2 $mU.g_{fish}^{-1}$, respectively (Figure 3.2E). Activities measured in warm smelt were more variable than in ambient smelt and no significant differences were detected over the period considered (a tendency was noted; ANOVA; $p = 0.068$). ME activities were higher overall in warm smelt with significantly higher activities measured in December, January and February (Figure 3.2E).

G6PDH activity did not change over the glycerol cycle in ambient smelt (Figure 3.2F). In warm smelt, activity was higher in January than in November, December or March (ANOVA; $p < 0.05$). Activities measured in warm smelt were overall higher than activities measured in ambient smelt and were significantly higher in January (*t*test ; $p < 0.05$).

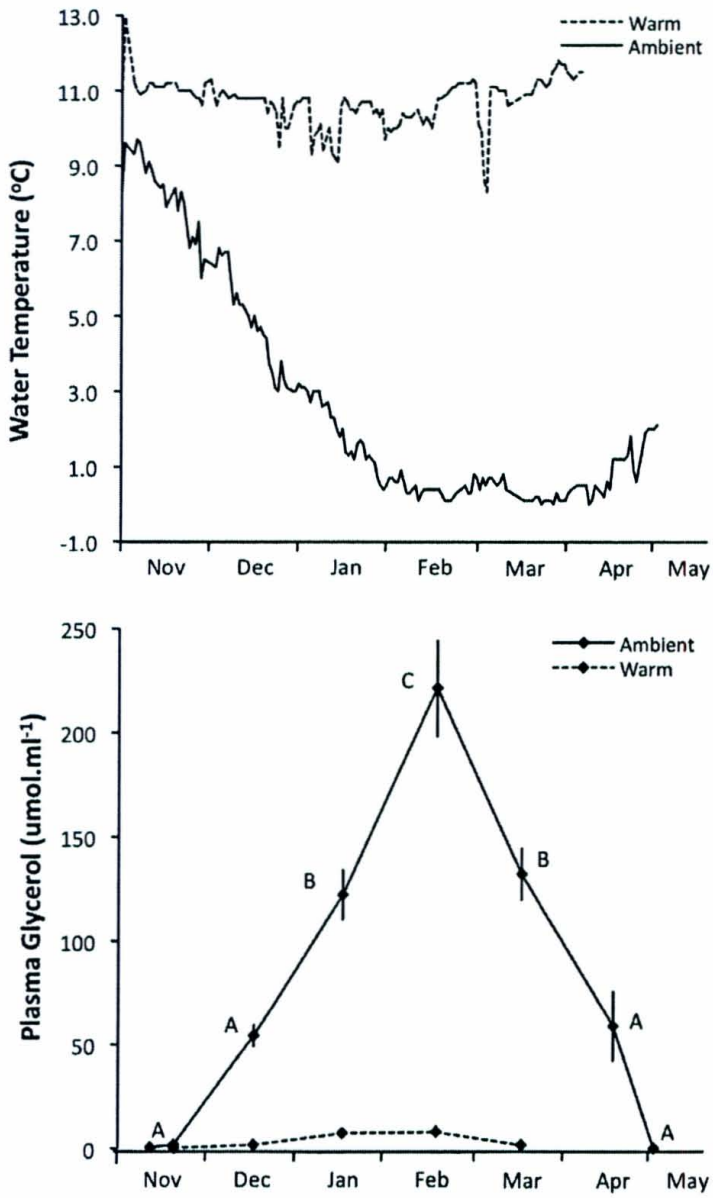

Figure 3.1. Seasonal profiles of water temperature (A) and plasma glycerol levels (B) for smelt held under controlled (warm, > 8°C) and ambient (ambient) temperatures. Glycerol values are presented as mean ± S.E. (n=6). Upper and lower case letters represent glycerol values significantly different within ambient and warm smelt, respectively.

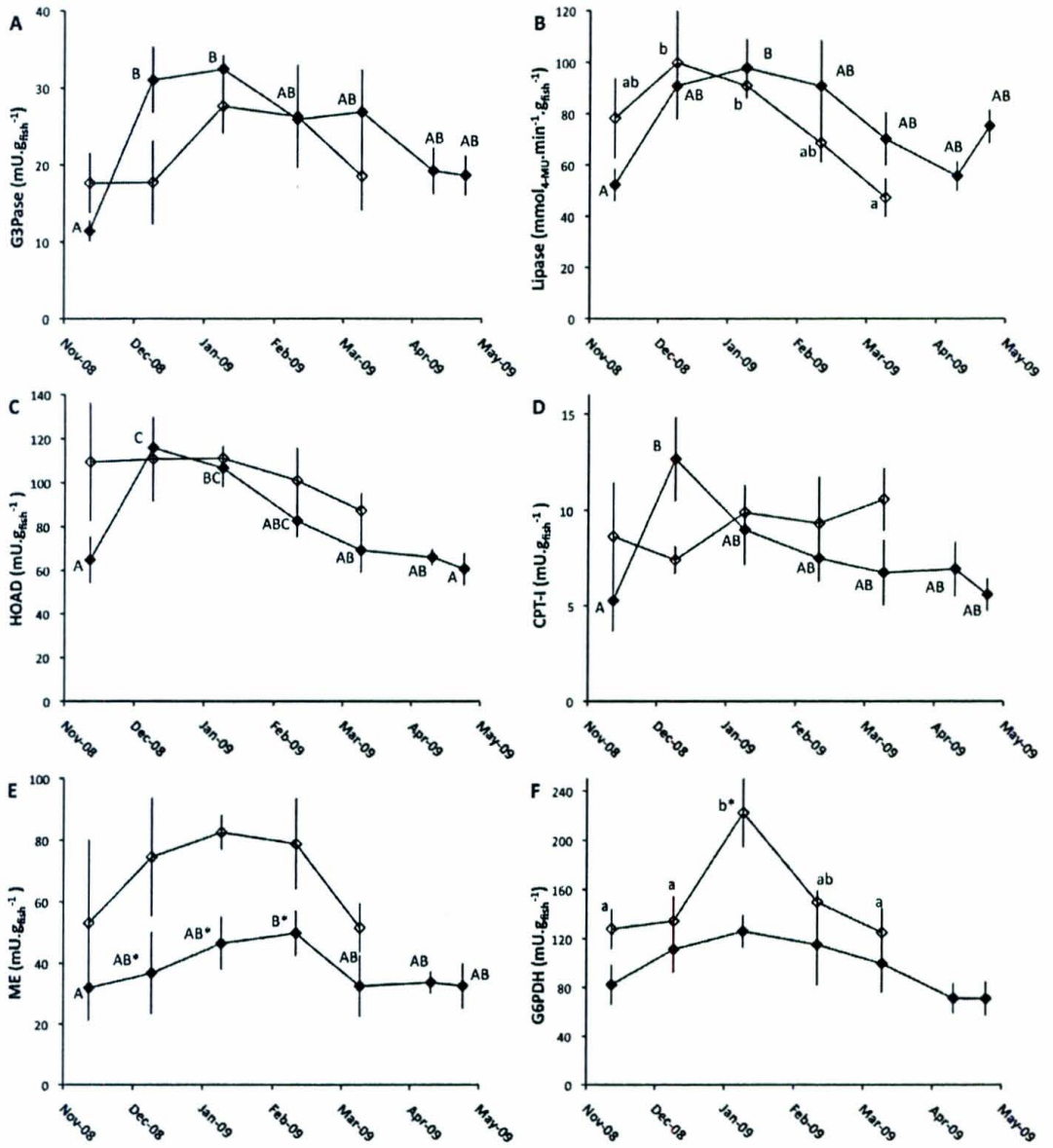

Figure 3.2. Seasonal activities of G3Pase (A), lipase (B), HOAD (C), CPT-I (D), ME (E), G6PDH (F) in liver of smelt maintained at ambient (filled) and warm (open) temperatures. Activities are presented as mean ± S.E. (n=5-6). Different letters indicate activities significantly different within ambient (upper case) and warm (lower case) smelt, respectively. * represents values that are significantly different ($p < 0.05$) between warm and ambient smelt for the sampling date considered.

3.4.1.3 Lipid content in liver

Due to technical issues, a number of samples were lost in the process of lipid extraction reducing the number of samples to 2-3 in some groups. The low number of samples tested decreased the power of statistical analysis and as such, may not be sufficient to detect subtle differences between groups. These data however give a good idea of the lipid levels present in liver and offer valuable information.

TG, ST, AMPL and PL were detected in all liver samples. Levels of none of these lipid classes significantly changed over the glycerol cycle in warm or ambient smelt. Average levels were thus pooled within a group and are presented in Table 3.1. PL were the most abundant followed by TG, ST and AMPL. No difference was detected between warm and ambient smelt for any sampling points except for ST where levels were significantly higher in warm than in ambient smelt (73.5 ± 8.4 and 54.2 ± 6.7 $\mu g.g_{fish}^{-1}$, respectively).

3.4.1.4 Lipid content in plasma

TG, ST, AMPL and PL were detected in all plasma samples. An additional group of lipids was detected in substantial amounts, eluting between SE and KET standards but closer to KET. TLC/FID was shown to separate successfully different groups of KET (Parrish et al. 1998). Furthermore, KET was also reported in cod plasma (Alkanani et al. 2005). Therefore, the additional group detected was tentatively identified as KET. Average levels measured over the glycerol cycle are presented for each group in Table 3.1. In warm smelt no differences were detected in levels of PL, ST and KET over the

experiment with averages values of 16.4 ± 1.5, 2.25 ± 0.18 and 1.99 ± 0.11 $mg.ml_{plasma}^{-1}$. Levels of TG and AMPL did however change with significantly lower levels of TG in November than in January (2.60 ± 0.34 and 6.61 ± 1.54 $mg.ml_{plasma}^{-1}$, respectively) and significantly lower levels of AMPL in November than in February or March (0.88 ± 0.06 and 1.24 ± 0.13 and 1.55 ± 0.29 $mg.ml_{plasma}^{-1}$, respectively (ANOVA; $p < 0.05$). In ambient smelt, no changes were detected in levels of TG and AMPL between November and May with average values of 5.30 ± 0.38 and 1.24 ± 0.07 $mg.ml_{plasma}^{-1}$, respectively. Levels of PL, ST and KET did however change over the glycerol cycle. PL and KET levels were higher in December than in May (25.56 ± 0.86 vs. 15.08 ± 1.09 $mg.ml_{plasma}^{-1}$ for PL and 3.36 ± 0.21 vs. 2.05 ± 0.09 $mg.ml_{plasma}^{-1}$ for KET) while ST levels where higher in December than in February or May (2.92 ± 0.26, 1.92 ± 0.08 and 1.70 ± 0.07 $mg.ml_{plasma}^{-1}$, respectively) (ANOVAs; $p < 0.05$).

Table 3.1. Average levels (± S.E.) of five classes of lipids measured in liver and plasma of ambient and warm smelt from November 2008 to May 2009 and November 2008 to March 2009, respectively. Data collected monthly were pooled within a group (n = 28 and n = 22 for ambient and warm smelt, respectively). * indicates that significant differences were detected over the period considered. n.d. = not detected. Glycerol equivalent concentrations of TG and PL were estimated in liver based on a molecular weight of 800 and 750 $g.mol^{-1}$, respectively.

	Ambient smelt		**Warm smelt**	
	Liver ($\mu g.g_{fish}^{-1}$)	*Plasma* ($mg.ml_{plasma}^{-1}$)	*Liver* ($\mu g.g_{fish}^{-1}$)	*Plasma* ($mg.ml_{plasma}^{-1}$)
AMPL	40.2 ± 5.8	1.24 ± 0.07	65.6 ± 8.3	1.17± 0.08*
KET	n.d.	2.05 ± 0.13*	n.d.	1.99 ± 0.11
ST	54.2 ± 6.7	2.32 ± 0.16*	73.5 ± 8.4	2.25 ± 0.18
TG	156.9 ± 37.5	5.30 ± 0.38	188.8 ± 30.7	4.47 ± 0.59*
equivalent glycerol	*0.20 $\mu mol.g_{fish}^{-1}$*		*0.24 $\mu mol.g_{fish}^{-1}$*	
PL	532.0 ± 75.0	18.8 ± 1.0*	586.7± 72.8	16.4 ± 1.5
equivalent glycerol	*0.71 $\mu mol.g_{fish}^{-1}$*		*0.78 $\mu mol.g_{fish}^{-1}$*	

3.4.2 Respective roles of G3Pase and glycerolipids in glycerol synthesis in isolated hepatocytes

3.4.2.1 Effect of triacsin C on pACS activity

Triacsin C significantly inhibited pACS activity in isolated hepatocytes incubated at cold temperature. Over 72 h, pACS activity increased almost two times in absence of triacsin C while it decreased by 3.5 times in its presence, being already significantly reduced after

6 h incubation (Figure 3.3). pACS activity was inhibited by 52.8± 16.9, 61.5 ± 13.6 and 85.00 ± 1.36 % after 6, 12 and 72h of incubation, respectively.

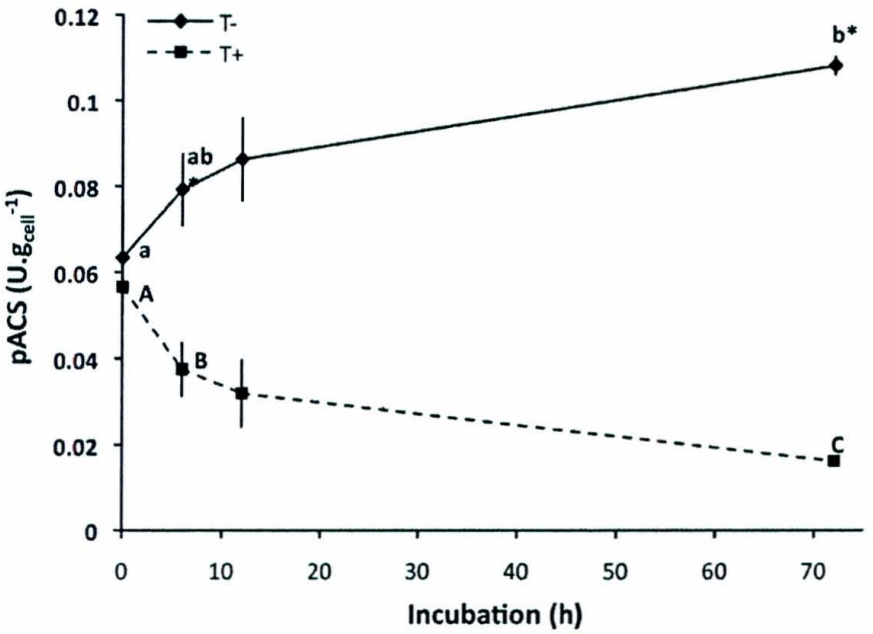

Figure 3.3. Palmitoyl acyl-CoA synthase activity (pACS) measured in isolated hepatocytes from smelt incubated at 0.4°C in the presence (T+) or absence (T-) of 10 μM triacsin C. Data are presented as mean ± S.E. (n=3; except for time 12h; n=2). Different letters indicate pACS activities significantly different within T+ (upper case) and T- (lower case), respectively (time 12h was not included in statistical analysis as equal numbers were necessary for ANOVA with repeated measures). * represent values that are significantly different ($p < 0.05$) between T+ and T- for the sampling point considered (time 12h was not included in statistical analysis as equal numbers were necessary for *t*-test with repeated measures).

3.4.2.2 Effect of triacsin C on glycerol production, G3Pase activity and lipids content

Glycerol accumulated significantly in all preparations incubated for 72h at 0.4°C, with no difference between treatments (Table 3.2). On average, 67.7 ± 17.0 and 78.7 ± 20.6 $\mu mol.g_{cell}^{-1}$ of glycerol were produced for T- and T+, respectively. The rates of glycerol production were linearly correlated between treatments within a given hepatic cell preparation but varied greatly between preparations with values ranging between 0.20 and 2.19 $\mu mol.h^{-1}.g_{cell}^{-1}$ (Figure 3.4).

TG, PL, ST and low amounts of AMPL were detected in all cell pellets. AMPL levels were too low to be detected accurately in most of the preparations and ST content did not change over the experiment in any of the preparations with average values of 0.92 ± 0.02, 0.84 ± 0.07 and 0.81 ± 0.04 $mg.g_{cells}^{-1}$ for Pre-Incubation, T- and T+, respectively. After 72h, TG levels were not different between both treatments. Initial TG levels were, however, significantly higher than after 72 h incubation in presence of triacsin C (Table 3.2). After 72h incubation, PL levels in the cells decreased significantly and in the same manner in both treatments (Table 3.2). Low amounts of PL were detected in the supernatant in all groups with a significant increase detected after 72h incubation in both T- and T+ (0.62 ± 0.09, 1.86 ± 0.12 and 2.11 ± 0.19 $mg.g_{cells}^{-1}$, for Pre-Incubation, T- and T+, respectively). These levels represent less than 20% of the decrease of PL measured in cell pellets. Very low amounts of FFA were also detected in the supernatant after 72h while they were not detectable in the supernatant in the Pre-Incubation group. FFA levels measured were significantly higher in the presence of triacsin C than in the control group

with average levels of 1.65 ± 0.25 and 1.12 ± 0.19 $mg.g_{cells}^{-1}$, respectively. G3Pase activity remained the same for all groups (Table 3.2).

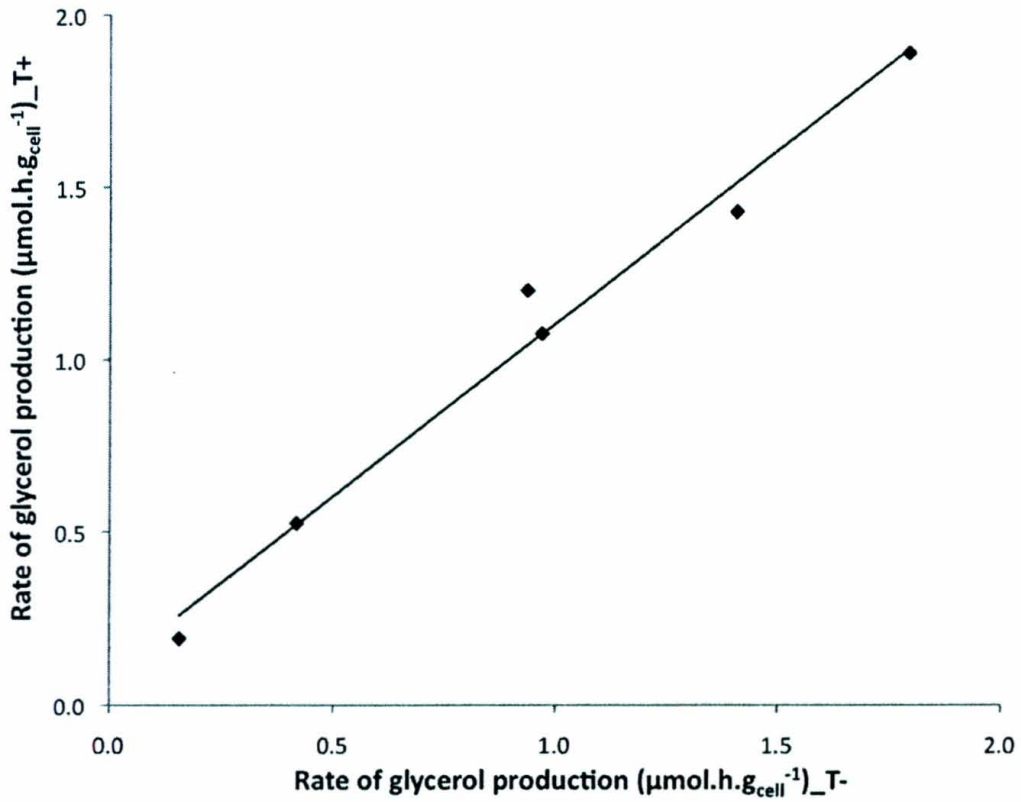

Figure 3.4. Linear correlation between rates of glycerol production by hepatic cells isolated from smelt and incubated at 0.4°C with (T+) or without (T-) 10 µM triacsin C. Each dot represents data obtained from a different hepatic cell preparation (n=6 females).

Table 3.2 Initial (Pre-Incubation) and final (T_{72}) glycerol concentrations, glycerol-3-phosphatase activities (G3Pase), triacylglycerol (TG) and phospholipid (PL) contents measured in preparations of hepatic cells isolated from smelt and incubated at 0.4°C in presence (T+) or absence (T-) of 10 µM triacsin C. Data are presented as mean ± S.E. (n=6). Different letters indicate significantly different values. Glycerol equivalent concentrations of TG and PL were estimated based on a molecular weight of 800 and 750 $g.mol^{-1}$, respectively.

	Pre-Incubation	**T_{72}/T-**	**T_{72}/T+**
Glycerol ($\mu mol.g_{cells}^{-1}$)	10.06 ± 0.37^{a}	77.8 ± 17.1^{b}	88.8 ± 20.7^{b}
G3Pase ($U.g_{cells}^{-1}$)	1.18 ± 0.26	1.00 ± 0.14	1.21 ± 0.19
	Glycerolipids		
TG ($mg.g_{cells}^{-1}$)	1.38 ± 0.19^{a}	1.31 ± 0.26^{ab}	1.03 ± 0.19^{b}
equivalent glycerol ($\mu mol.g_{cells}^{-1}$)	*1.72*	*1.64*	*1.29*
PL ($mg.g_{cells}^{-1}$)	20.9 ± 1.4^{a}	12.7 ± 2.3^{b}	11.5 ± 1.8^{b}
equivalent glycerol ($\mu mol.g_{cells}^{-1}$)	*27.9*	*16.9*	*15.1*

3.5 Discussion

3.5.1 G3Pase and lipid metabolism over the glycerol cycle

The glycerol cycle reported in this study for the season 2008-2009 was very similar to the cycles reported in 2000-2001 (Lewis et al. 2004) and 2007-2008 (Ditlecadet et al. 2011). Maximal glycerol levels reported here were 221.4 ± 23.4 mM in mid-February compared to approximately 215 and 250 mM at the same period for 2007-2008 and 2000-2001, respectively.

G3Pase activity increased early in the cycle, with the highest levels recorded in December and January at the onset of the increase of glycerol levels. Levels started to decrease after these points to reach the initial values by the end of the cycle. G3Pase activity did not change in fish maintained at warm temperature and was not significantly different from activities measured in cold fish. The increase of G3Pase activity observed in December and January may be a response to the decrease in temperature, enzyme activities being negatively affected by decreasing temperatures. Increasing the amounts of enzymes may be a way to compensate for the decrease of activities induced by the decreasing water temperature, suggesting this enzyme is important enough to be up-regulated at the onset of the glycerol cycle in smelt. This pattern of G3Pase activity is in agreement with that reported by Lewis et al. (2004) for GPDH, supporting a channelling of DHAP toward glycerol production through a direct dephosphorylation of G3P (Figure 1.3).

Such an increase of G3Pase early in the glycerol cycle was not observed in the gall-moth larvae, *Epiblema scudderania*, for which G3Pase was also suggested as one of the pathways used to produce glycerol from G3P (Joannisse & Storey 1994). A second pathway, not involving G3P but glyceraldehyde, is however also involved in glycerol synthesis in this insect, which is not the case in smelt (Figure 1.3) (Driedzic et al. 1998).

Glycerol concentrations were shown to be the same for all tissues for the whole smelt (Raymond et al. 1992, Driedzic & Short, 2006). Over the accumulation period, from November to February, the rate of glycerol accumulation can thus be estimated to be close to 1.2 nmoles.min^{-1}.g_{fish}^{-1} (215 000 nmol.g_{fish}^{-1} / (120 days x 24h x 60min). The

actual rate of synthesis should thus be higher than 1.2 nmoles.min^{-1}.g_{fish}^{-1} considering the daily 10% loss to the water (Chapter 3). In November, G3Pase activity was 10 mU.g_{fish}^{-1}, the lowest activity measured over the accumulation period. This activity would allow the production of 10 nmoles.min^{-1}.g_{fish}^{-1} glycerol in optimal conditions at 15°C. Over this period, average water temperature was about 4.5 °C. Assuming a Q_{10} of 2, the actual average activity of G3Pase would be close to 5 mU.g_{fish}^{-1}. As such, this activity may be sufficient to produce glycerol at the rate observed over the accumulation period without any need of up-regulation, but it may be limiting considering it is very close to the rate of synthesis needed. The sharp increase of activity observed at the onset of glycerol accumulation in December and January could be an actual requirement to support glycerol synthesis over this critical period.

Enzymes involved in lipid metabolism presented activities generally as high or higher in fish maintained at warm temperature, and not producing glycerol, than in fish tracking ambient temperature. Lipid metabolism may be in demand in both groups in relation to different metabolic needs in response to the temperatures at which they are exposed. This point is discussed later.

Similar to what was seen for G3Pase, activities of enzymes involved in lipid degradation (Lipase, HOAD and CPT-I) increased at the onset of glycerol accumulation. The assay used to measure lipase activity was directed to triglyceride lipases (EC 3.1.1.3) and, as such, should reflect the potential of smelt liver to lyse TG even though its actual contribution cannot be estimated due to the synthetic nature of the substrate used (Roberts 1985). CPT-I is involved in the entry of acylated FFA into the mitochondria

while HOAD is directly involved in their oxidation in the mitochondria matrix. Both CPT-I and HOAD are key to the oxidation of FFA supporting the high correlation observed between their activities. Up-regulation of these three enzymes early in the cycle supports the importance of lipids when water temperature decreases. This increase may be related to a switch in liver to a fatty acid based aerobic metabolism, redirecting glucose to glycerol production. This would also indirectly participate in glycerol production with each TG broken-down releasing one molecule of glycerol in addition to three FFA that may be oxidized to fuel the organism with ATP. Such an increase in enzyme activity was not detected in warm smelt for which both CPT-I and HOAD levels were high over the entire experiment.

ME activity was up-regulated in ambient smelt, with increasing activity between November and February, while G6PDH activity was up-regulated in warm smelt, with an important peak of activity in January. These enzymes are indirectly involved in lipid synthesis, fuelling the cells with the NADPH necessary to donate H^+ for FFA synthesis. Activities of both ME and G6PDH were, however, significantly lower in ambient than in warm smelt over the whole season, suggesting a higher potential for lipid synthesis in warm than in cold smelt. Lewis et al. (2004) suggested lipid synthesis may be activated in smelt maintained at warm temperature. This contention was based on the higher activities of GPDH measured in warm smelt between February and April, GPDH being involved in the formation of G3P that could be used for TG synthesis. Warm smelt are generally more advanced in sexual maturation than smelt maintained at ambient temperature. Lipid

synthesis might thus be activated in warm smelt in association with gonad production and would be supported by overall high activities of GPDH, ME and G6PDH measured.

Levels of all of the lipid classes detected remained unchanged in smelt liver over the course of the experiment and were very similar between warm and cold smelt. TG levels were very low overall, and variable depending on the fish. As in previous studies, fish were sampled without any consideration of the sex or age, as these factors do not seem to affect the pattern of glycerol levels in winter. However, they might play an important role in the variation in lipid levels. The important information gained from these data is the low levels measured. Levels of TG measured in liver over the glycerol cycle would not produce more than 0.19 μmol of glycerol per gram of fish if they were all broken down. This is less than 0.1% of the 215 μmol glycerol accumulated in the whole fish in February, not even taking into consideration the continuous loss of glycerol to water. As such, TG reserves *per se* cannot be the source of glycerol, confirming a report by Raymond (1993). TG do not seem to have a significant role at the onset of glycerol synthesis, as in the case of glycogen (Treberg et al. 2002a; Driedzic & Short 2006). This does not rule out the possibility that an input of dietary lipid could support or significantly participate in glycerol production.

In plasma, TG, ST, AMPL, PL and a tentatively identified KET lipid group were detected, and the levels of these changed over the season in both cold and warm fish, but in a different manner. In cold fish, only levels of ST, PL and KET changed with higher levels measured in December in comparison to the end of the season. In warm fish, only levels of TG and AMPL changed with higher levels measured in January and February,

respectively, in comparison to November. In plasma, lipids are transported associated with specialized proteins in the form of lipoproteins that can be of five groups depending on their composition and size: chylomicrons, VLDL, IDL, LDL and HDL. The similarity in the response noted for the classes detected in both groups may reflect a change in the lipoprotein composition. At present, a clear link cannot be made between these plasma lipids and glycerol production in ambient smelt.

Taken together, these results suggest that G3Pase activity is important for glycerol synthesis, and that TG reserves do not have an important role at the onset of glycerol accumulation in December. The unchanged activities of enzymes involved in lipid synthesis (ME and G6PDH) do not support an active synthesis of FFA for the formation of new glycerolipids following an active lipolysis. However, the data do not rule out any potential recycling of released FFA into new glycerolipids, a pathway that may provide glycerol coupled to G3Pase. The second experiment using isolated smelt hepatic cells as a model was conducted in order to answer this question.

3.5.2 Glycerol synthesis in isolated hepatic cells

Hepatic cells isolated from smelt maintained at warm temperature (not producing glycerol) and incubated at cold temperature (0.4°C) produce glycerol in a linear manner for up to 72 h (Clow et al. 2008). The abrupt drop of temperature faced by these cells *"in vitro"* may constitute an additional challenge in comparison to *"in vivo"* studies when water temperature decreases gradually over months. Even though the timing of the reorganization of some metabolic pathways may be altered, glycerol synthesis is rapidly

triggered by the low temperature, offering an opportunity to investigate the potential pathways by which G3P is used to produce glycerol. In this experiment, isolated cells only received glucose as external fuel, as glycerol production was shown to be at its maximal rate in this condition (Clow et al. 2008). In such conditions, the potential sources of glycerol over a short-term period may be: 1) the direct dephosphorylation of G3P by G3Pase, 2) the lipolysis of on-board glycerolipids, and 3) a continuous cycle involving *de novo* synthesis of glycerolipids from newly synthesized G3P and recycled FFA, followed by lipolysis.

3.5.2.1 Effect of triacsin C on lipid synthesis

Triacsins are fungal metabolites produced by *Streptomyces sp.* and are potent inhibitors of long-chain fatty acyl-CoAs synthases (ACS; EC 6.2.1.3), triacsin C being the most active compound reported (Tomoda et al. 1987). ACS catalyse the synthesis of acyl-CoA from FFA and CoA prior to its channelling toward lipid synthesis or oxidation. In rat, five forms were reported and the fate of a particular acyl-CoA is thought to depend on which of the ACS catalyzes its synthesis. Triacsins are believed to be strong inhibitors of ACS involved in lipid synthesis and more precisely in *de novo* synthesis of glycerolipids, *i.e.* synthesis of glycerol lipids from new G3P (Coleman et al. 2003). Inhibition of ACS by triacsin C was shown to strongly affect *de novo* synthesis of glycerolipids in different models of isolated cells (Raji cells, Tomoda et al. 1991; human fibroblasts, Igal et al. 1996 and 1997; rat hepatic cells, Muoio et al. 2000). The preliminary experiment confirmed that triacsin C (10 μM; 0.4°C) enters smelt hepatic cells and strongly affects ACS activity, as was reported in all previous animal cell models assayed under similar

conditions. ACS activities measured in the control cells were in the same range of activities as the ones reported for other models (Tomoda et al. 1997; Igal et al. 1997). In smelt, triacsin C progressively enters hepatic cells, inhibiting ACS activity by 52% after only 6 h incubation to reach a maximal inhibition of 85% at the end of the experiment. The maximal inhibition reported here was higher than that reported by Tomoda et al. (1991) in Raji cells and by Muoio et al. (2000) in rat hepatic cells at 10 μM triacsin C (maximal inhibition being measured when ACS extract is directly exposed to triacsin C). Igal et al. (1997) reported 68% inhibition when human fibroblasts were exposed to 5 μM triacsin C after 6 h. The higher inhibition reported at an equivalent incubation period may be due to a faster entry of triacsin C at 37°C than at the low temperature used in this experiment. In all experiments that allowed for a direct comparison between the effect of triacsin on ACS activity and on glycerolipid synthesis, inhibition was always higher on the rate of glycerolipid synthesis than on ACS activity. In human fibroblasts, where ACS activity is inhibited by 68% after 6 h incubation of the cells with 5 μM triacsin C, incorporation of glycerol into TG and PL was inhibited in parallel by 95 and 70%, respectively (Igal et al. 1997). In rat hepatic cells, incorporation of glycerol into TG and PL was inhibited by about 65 and 30% respectively after only 1 h incubation in the presence of 10 μM inhibitor while the maximal inhibition of ACS measured was not higher than 55% (Muoio et al. 2000). In Raji cells, IC_{50} of triacsin C was reached at 6.3, 1.3 and 0.3 μM for ACS activity, cell proliferation and total lipid synthesis, respectively. This suggests that low amounts of triacsin C more strongly affects glycerolipid synthesis than ACS activity measured *in vitro* (Tomoda et al. 1991). All these data, that support a

strong inhibition of ACS by triacsin C, support an even stronger inhibition of *de novo* synthesis of glycerolipids. Even though the inhibition of ACS in smelt hepatic cells was not 85% over the 72 h period incubation, *de novo* synthesis of glycerol lipids was most likely even more strongly impaired by triacsin C in smelt hepatocytes under the conditions of this study.

3.5.2.2 Effect of glycerolipids synthesis inhibition on glycerol production

Triacsin C did not affect glycerol production, ruling out *de novo* synthesis of glycerolipids as a possible source of glycerol in isolated hepatic cells. Glycerol levels produced after 72 h in the control conditions were very similar to the levels reported by Hall et al. (2011) who conducted their experiments at the same period of the glycerol cycle under the same conditions (77.8 ± 17.1 *vs* 69.1 ± 10.3 $\mu mol.g_{cells}^{-1}$, respectively). The large standard errors (S.E.) reported in both groups of cells are due to the variability of glycerol production depending on the cell population (*i.e.* biological variability) and not to discrepancies between vials (*i.e.* technical variability) from the same population, as suggested by the almost perfect correlation observed between rates of glycerol production in T+ and T- for each population of cell used. This variability between cell populations is also suggested by the large S.E. reported by Clow et al. (2008). Even though the rate of production is variable depending on the cell population, glycerol levels measured after the incubation period were all significantly higher in all preparations and as such, constitute proper models to investigate the respective roles of glycerolipids and G3Pase in glycerol production. *De novo* synthesis and subsequent degradation of glycerolipids

being ruled out, the two remaining sources of glycerol in smelt hepatic cells might be on-board glycerolipid and/or G3P via G3Pase.

3.5.2.3 On-board glycerolipids in isolated hepatic cells

TG levels were overall very low with concentrations lower than 1.5 $mg.g_{cells}^{-1}$. Average TG level did not change significantly in the control group while it decreased by 25% when cells were exposed to triacsin C. Activation of lipolysis by triacsin C has never been reported. Assuming TG degradation may have occurred in both groups of cells, TG broken-down may have been replaced by *de novo* synthesis in the control group while the inhibition of ACS would block this replacement in cells exposed to triacsin C, supporting the decrease of TG in this group. Without consideration of the decrease of TG levels in the group exposed to triacsin C following 72 h of incubation, initial levels of TG reported would be sufficient to provide only 2.5% of the glycerol produced. This rough estimation is based on an average molecular weight of 800 $g.mol^{-1}$ of TG with one µmol of TG needed per µmol of glycerol produced ([1.38 $mg_{TG}.g_{cells}^{-1}$ ÷ 0.8 $mg.\mu mol^{-1}$] x 100 ÷ 70 µmol glycerol produced). As such, on-board TG cannot be the source of glycerol. TG may be seen as the only reasonable candidate to fuel the cells with glycerol, as they constitute the lipid reserves of cells. PL, mostly phosphoglycerides, are not considered as a lipid reserve as their main function is to form cellular membranes, however, the decrease of PL levels observed in both groups of cells (T- and T+) was sufficiently large that they must be considered as a potential source of glycerol in this model. PL levels decreased by 40-45% over the incubation period with initial levels comparable to levels observed in whole livers. Applying the same approach used to estimate the amount of

glycerol that could be generated by TG, the amount of PL used could generate about 15 μmol glycerol or about 17% of the total glycerol released, assuming all PL were glycerophospholipids ([9 $mg_{PL}.g_{cells}^{-1}$ ÷ 0.75 $mg.\mu mol^{-1}$] x 100 ÷ 70 μmol glycerol produced). Such a decrease in the amount of PL would most likely not occur *in vivo* and as such, should not be a source of glycerol of equivalent importance at the whole animal level. Such a drastic decrease may be a response to the shock the cells received following the rapid transfer from acclimation temperature of the fish used (> 8°C) to the low temperature at which cells were incubated (0.4°C). Low amounts of PL and FFA were released in the supernatant following both treatments; with higher levels of FFA released when cells were exposed to triacsin C. PL and FFA levels measured in supernatant are not sufficient to make up for the large decrease measured in pellets (about 35% of it) suggesting most FFA released by lipolysis were oxidized by the cell. This would be consistent with the cells switching to a lipid-based metabolism to spare glycogen/glucose for glycerol production.

3.5.2.4 G3Pase activity in isolated hepatic cells

G3Pase activity remained constant over the experiment in both groups of cells and was not affected by triacsin C. Activity of this enzyme did not seem to be regulated over the 72 h incubation period. Hall et al. (2011) detected the up-regulation of transcript level of an *uncharacterized* phosphatase in isolated smelt hepatic cells producing glycerol in comparison to cells not producing any. They hypothesized it may encode for G3Pase. mRNA levels encoding for this protein were higher in cells incubated at 0.4°C (cold cells producing glycerol) than in cells incubated at 8°C (not-producing glycerol) after all

incubation periods assayed (4.7 fold at 24 h, 8.3 fold at 48 h and 20 fold at 72 h) and were significantly higher after 24 h (2.4 fold) than before incubation, or after 48 and 72 h in cold cells. Assuming this particular up-regulated mRNA encodes G3Pase, an increase at the protein level may happen earlier during the incubation period in parallel to the peak of mRNA detected after 24h and go back to initial values after 72 h, and therefore could have been missed in my experiment. Alternatively an increase in G3Pase protein may take more than 72 h to occur in smelt hepatic cells despite transient increases in mRNA. Further work would be needed to test Hall and colleagues' hypothesis. G3Pase activity measured would, however, be sufficient to produce glycerol at the rate observed. Indeed, 1 $\mu mol.min^{-1}.g_{cells}^{-1}$ glycerol could be produced at 15°C. Assuming a Q_{10} of 2, actual G3Pase activity would be closer to 0.35 $\mu mol.min^{-1}.g_{cells}^{-1}$ at 0°C, still more than sufficient to produce the average 0.02 $\mu mol.min^{-1}.g_{cells}^{-1}$ glycerol measured, without assuming any glycerol is the result of PL degradation.

3.5.3 Conclusions and perspectives

This study is the first to clearly point out that the metabolic pathway involving G3Pase is the main source of glycerol in smelt during the winter season. Adjustments of lipid metabolism occur in winter as suggested by regulation of different enzymes, but is more likely associated with adaptations to cold in general than to the specific production of glycerol. It is likely that the liver is switching to a fatty acid based aerobic metabolism and redirecting glucose to glycerol production. On-board glycerolipids cannot be a source for all the glycerol produced, although PL may contribute to it significantly in isolated

cells. The next step would be to purify and identify G3Pase in smelt as it was previously done for yeasts (Norbeck et al. 1996). This would open new areas of research concerning the role of glycerol in higher vertebrates where the existence of the G3Pase pathway was also proposed to explain glycerol release in rat brain and heart following oxygen limitation (De Groot et al. 1994; Nguyen et al. 2007).

Chapter 4

Partial purification and characterization of glycerol-3-phosphatase from rainbow smelt (*Osmerus mordax*) liver

4.1 Abstract

High amounts of glycerol accumulated as a cryoprotectant in winter by rainbow smelt (*Osmerus mordax*) were previously shown to be synthesized through a direct dephosphorylation of glycerol-3-phosphate by a phosphatase, G3Pase. Such a protein is well characterized in a few species including yeasts, bacteria and plants, but was never identified in any animal species. This work presents the first attempt to purify G3Pase from smelt liver, the main site of glycerol synthesis for the whole animal. A partial purification was performed, and some characteristics of the protein determined including optimal pH, K_M and cation requirements. Smelt G3Pase is most likely a cytosolic, low molecular weight acidic phosphatase, showing an optimal activity at pH 5.5, and a molecular weight ranging between 25 and 60 kDa, with a requirement for Mg^{2+}. This range of size is similar to that reported for species from which G3Pase was previously identified but the optimal pH is very different, activity being maximal at pH 5.5 while it is at neutral pH for all other G3Pase identified to date.

4.2 Introduction

Rainbow smelt (*Osmerus mordax*) accumulate high amounts of glycerol in winter as a cryoprotectant, reaching concentrations greater than 200 mM (Raymond et al. 1992, Lewis et al. 2004). Glycerol is a highly soluble 3 carbon small polyhydric alcohol that can be easily transported into cells through aquaglyceroporins (Storey 1997; Hara-Chikuma & Verkman 2006). As smelt do not have any mechanisms in place to retain glycerol during the critical accumulation period, about 10% of the total glycerol content of the fish is continuously lost daily across gills and skin towards surrounding water. It forces smelt to vigorously synthesize glycerol to compensate for the losses and to allow glycerol accumulation (Raymond et al. 1994; Ditlecadet et al. 2011).

In smelt, glycerol is synthesized in the liver using glucose, glycogen and amino acids as sources of carbon (Raymond 1996; Raymond & Driedzic 1997; Treberg et al. 2002b; Walter et al. 2006). In all cases, a transition through the 3-carbon intermediate dihydroxyacetone phosphate (DHAP) is required before its conversion to glycerol-3-phosphate (G3P) by the cytosolic form of glycerol-3-phosphate dehydrogenase (cGPDH), a key enzyme in glycerol synthesis (Lewis et al. 2004; Liebscher et al. 2006). From G3P, two routes could result in glycerol production. G3P can enter the glycerolipid pool that includes triacylglycerols (TG) and most phospholipids (PL) that are all built on the scaffold of a glycerol molecule. Glycerol would then be released following catalysis of glycerolipids by lipases. The second route involves a single enzyme, glycerol-3-phosphatase (G3Pase) that directly dephosphorylates G3P to glycerol.

To date, G3Pase has been unequivocally identified in only a few species including fungi, *Saccharomyces cerevisiae* (Norbeck et al. 1996) and *Candida albicans* (Fan et al. 2005), bacteria, *Bacillus licheniformis* (Skraly & Cameron 1998), and plant, *Arabidopsis thaliana* (Caparrós-Martín et al. 2007). All these species produce glycerol in response to abiotic stressors that can be a change in osmotic pressure [(*C. albicans* and *S. cerevisiae*), anaerobia (*B. licheniformis*)] or related to dehydratation (*A. thaliana*). In *S. cerevisiae*, two isoforms were identified and mutants for both genes lacked G3Pase activity and produced only small amounts of glycerol in comparison to wild types, supporting the essential role of this pathway in glycerol synthesis in this species (Påhlman et al. 2001). All G3Pase proteins identified are low-molecular weight phosphatases (25-30 KDa) and show an optimal activity at neutral pH (6.5-7.0). When substrate preference was assessed, G3P was shown to be the preferred substrate (Norbeck et al. 1996 for *S. cerevisiae*; Caparrós-Martín et al. 2007 for *A. thaliana*).

In animals, the direct dephosphorylation of G3P, as a source of glycerol, was naturally proposed in species that accumulate high amounts of glycerol in response to cold, such as the gall moth (*Epiblema scudderiana*) larvae (Joanisse & Storey 1994) and rainbow smelt (Driedzic et al. 1998). G3Pase was also suggested as an alternative pathway to lipolysis in rat heart (De Groot et al. 1994) and brain (Nguyen et al. 2007) in response to ischemic events leading to glycerol release. G3Pase-like activity was measured in all the animal species referenced above; however, the possibility that this reflects non-specific phosphatase activity cannot be ruled out. In smelt liver (Raymond 1995; Raymond & Driedzic 1997; Walter et al. 2006) and rat brain (Nguyen et al. 2007),

glucose was shown to be a source of glycerol, supporting a transition through G3P before glycerol release and not simply the production of glycerol from existing glycerolipid pools. In addition, the short time frame available (5 min) in the rat brain experiment suggested G3P produced from glucose was unlikely to enter the glycerolipid pool prior to the release of glycerol (Nguyen et al. 2007). These results were however criticized by Clausen et al. (2011) as the relative contribution of glycerophospholipids and glucose was never investigated in ischemic rat brain. As such, the presence of a direct dephosphorylation of G3P by a specific G3Pase is still under debate, and has never been clearly demonstrated in any animal species.

Recent work with isolated smelt hepatic cells went a step further, demonstrating for the first time that the direct dephosphorylation of G3P by a phosphatase was the most likely pathway in producing high amounts of glycerol in response to cold in this species (Chapter 3). Results obtained showed that: 1) on-board glycerolipids were not sufficient to be at the origin of the glycerol released over 72 h incubation at cold temperature (even though PL might account for up to 17% of glycerol production); 2) *de novo* synthesis of glycerolipids from G3P could not be involved, as inhibition of this pathway did not lead to a reduction of the glycerol produced; and 3) G3Pase-like activity measured *in vitro* would be sufficient to produce glycerol at the rate observed.

Purification, identification and characterization of the enzyme at the origin of G3P dephosphorylation in vertebrates is thus crucial for a better comprehension of the biochemical mechanisms involved in glycerol synthesis in this group. Clear identification of a specific G3Pase would open a new area of research in higher

vertebrates, in which glycerol accumulation may be much more than a simple indicator of PL degradation following a major insult such as ischemia. The present chapter presents the first efforts to purify and characterize G3Pase from smelt liver.

Following sub-cellular localization of G3Pase in smelt hepatocytes, an attempt to purify G3Pase from the active fraction was performed following the methods used to isolate G3Pase from yeast by Norbeck et al. (1996). After several unsuccessful attempts to isolate smelt G3Pase following these methods, partial purification of G3Pase from yeast was done following the same conditions to assess any technical issues. After ruling out technical problems, efforts were made to find out the optimal conditions necessary to retrieve G3Pase activity following chromatography. As such, the methods used to partially purify G3Pase from smelt hepatocytes were modified from Norbeck et al. (1996). Modifications included elution buffer composition as well as the assay conditions.

4.3 Materials and methods

4.3.1 Animals and sampling

Rainbow smelt (*Osmerus mordax*) were fished under ice in Horwood Pond (water temperature 4.5°C), Newfoundland, Canada in March 2011, transported to the Ocean Sciences Centre, Memorial University of Newfoundland (St. John's, Canada) and transferred to seawater tanks maintained close to 8°C. Fish were kept on a natural photoperiod using fluorescent lights set by an outdoor photocell and fed a diet of chopped herring twice a week to satiation.

4.3.2 Localization of G3Pase in liver cells

Except where mentioned, chemicals were supplied by Sigma-Aldrich (St. Louis, MO).

4.3.2.1 Differential centrifugations

Eleven fish were sacrificed by a sharp blow to the head and 5-6 livers were pooled and ground with a glass pestle in four volumes of ice-cold media containing 500 mM sucrose, 30 mM Hepes, 5 mM EDTA, and 5 mM EGTA at pH 7.4 according to Ballantyne et al. (1994). Homogenates (n = 2) were passed through cheesecloth and centrifuged at 800 × g for 10 min at 4°C to remove any cellular debris. Two hundred µl of the clear homogenate were immediately frozen and the rest centrifuged at 9,000 × g for 10 min at 4°C to pellet mitochondria. The resulting pellet (M) was kept on ice and the supernatant was centrifuged at 20,000 × g for 30 min at 4°C to pellet lysosomes and peroxisomes. The resulting pellet (L/P) was kept on ice and the supernatant ultracentrifuged at 100,000 × g for 60 min at 4°C to pellet microsomes (ER). The final supernatant was considered as the cytosolic fraction (Cyt). All pellets were resuspended in media containing 300 mM KCl, 30 mM Hepes, 10 mM K_2HPO_4, 2 mM EDTA, 1 mM $MgCl_2$, 2 mM $CaCl_2$ at pH 7.4 and the volume of each fraction was determined. All fractions were kept at -80°C until further analysis. Centrifugations were all done at 4°C and fractions were kept on ice whenever possible.

4.3.2.2 Enzymatic markers and G3Pase activity

Marker enzymes were used to verify the proper separation of the cell content: citrate synthase (CS) for mitochondria, catalase for peroxisomes, glucose-6-phosphatase

(G6Pase) for microsomes and lactate dehydrogenase (LDH) for the cytosolic fraction. Activity of these enzymes and of G3Pase was measured in the starting homogenate, representative of initial activities, and in all fractions subsequently collected (M, L/P, ER and Cyt). All assays were run in duplicate at 15°C. The concentration of the homogenate was chosen to provide a linear response over 5 min, except for G3Pase that was measured over 30 min.

Assay conditions were as follows: CS was run in Tris-HCl (100 mM, pH 8.0) containing dithiobis-2-nitrobenzoic acid (DTNB; 0.1 mM), acetyl CoA (0.1 mM) and oxaloacetate (0.15 mM). Formation of a yellow DTNB-CoA complex was monitored at 412 nm for 5 min and CS activity expressed as total Units per fraction (U) taking into account the respective volumes measured for each fraction. One Unit corresponds to the appearance of 1 μmol of DTNB-CoA complex per min at 15°C (DTNB extinction coefficient at 412 nm = 13.6 $mM.cm^{-1}$).

Catalase was run in potassium phosphate buffer (100 mM, pH 7.0) containing 0.04 % (w:v) H_2O_2. Disappearance of H_2O_2 was followed at 240 nm for 5 min, and catalase activity expressed as total Units per fraction (U), one Unit corresponding to the disappearance of 1 μmol of H_2O_2 per min at 15°C (H_2O_2 extinction coefficient at 240 nm = 43.6 $mM.cm^{-1}$).

LDH activity was run in potassium phosphate buffer (100 mM, pH 7.0) containing reduced nicotinamide adenine dinucleotide (NADH, 0.16 mM) and pyruvate (0.4 mM). Disappearance of NADH was monitored at 340 nm for 5 min and LDH activity expressed as total Units per fraction (U), one Unit corresponding to the

disappearance of 1 μmol of NADH per min at 15°C (NADH extinction coefficient at 340 nm = 6.22 $mM.cm^{-1}$).

Phosphatase assays, G3Pase and G6Pase, were run in an acetate buffer (100 mM; pH 5.5) and Tris-HCl buffer (20 mM; pH 7.3), respectively, containing 10 mM G3P or G6P. Released inorganic phosphate (P_I) was determined at three time points over 30 min with a colourimetric assay based on Rockstein and Herron (1951). Briefly, 40 μl of the reaction mixture was added to 80 μl of 3.3% ammonium molybdate in 5 N sulphuric acid: distilled water (2:1; v:v) to stop the reaction. Colour was initiated by adding 40 μl of 260 mM $FeSO_4$ and absorbance measured after 5 min on a plate reader at 620 nm. A standard curve of P_I was run from different concentrations of Na_2HPO_4 and phosphatases activities expressed as total Units per fraction (U), one Unit corresponding to the appearance of 1 μmol of P_I per min at 15°C.

For each fraction, activity of a particular enzyme was presented as a % from initial activities measured in the starting homogenate using the following formula:

% Activity = [(initial activity - activity measured in the fraction of interest) / initial activity] × 100.

4.3.3 Partial purification of G3Pase from yeast, *Saccharomyces cerevisiae*

After several unsuccessful attempts to isolate smelt G3Pase following the method used by Norbeck et al. (1996), partial purification of G3Pase from yeast was done following the exact same conditions to assess any technical issues. Only two of the three chromatographies used to completely purify the protein were assayed. As such, only a

partial purification was performed. The only variant from the initial protocol was the strain of yeast used. I used a haploid strain (W303-1A, genotype MATa ade2-1 ura3-1 his3-11 trp1-1 leu2-3 leu2-112 can1-100; Petrezselyovaa et al. 2010). This strain, commercially available from American Type Culture Commercial (ATCC; Manassas, VA, US), was also used in a previous study presenting kinetic properties of the glycerol synthesis pathway, including G3Pase, in this species (Cronwright et al. 2002).

4.3.3.1 Yeast cultures and yeast extracts preparation

Cultures were grown at 30°C in a medium containing 1% yeast extract, 2% bacto peptone and 2% glucose. Growth was monitored by measuring optical density at 610 nm (A_{610}) and cells were harvested at A_{610} = 1-1.5. Suspensions were centrifuged at 10,000 × g for 10 min at 4°C and cell pellet washed twice in ice-cold TRED buffer (10 mM triethanolamine, 1 mM EDTA, 1 mM dithiotreitol (DTT), pH 7.5). Pellets obtained were flash frozen in liquid nitrogen and kept at -80°C until further analysis.

Yeast extracts were prepared following resuspension of pellet in ice-cold TRED buffer containing 0.5 µl of a protease inhibitor mix (70 $mg.ml^{-1}$ phenylmethylsulfonyl fluoride, 1.4 $mg.ml^{-1}$ leupeptin, and 1 $mg.ml^{-1}$ pepstatin) per ml prior to chromatography. Suspensions were sonicated 6 times for 15 s with 20 s interval of cooling on ice and were finally centrifuged for at 10,000 × g 10 min at 4°C to remove cell debris. Supernatant was used directly for size-exclusion chromatography.

4.3.3.2 Size-exclusion chromatography

Three ml of yeast extract (0.32 mg protein.ml^{-1}) were loaded onto a 120 ml Superdex 200 column (GE Healthcare, Québec, Qc, Canada) equilibrated with TRED buffer. The column was eluted with the same buffer at a flow rate of 1 ml.min^{-1}, and 1 ml fractions were collected after 40 min of elution. Elution of proteins was monitored by continuous absorbance at 230 and 280 nm, and G3Pase activity was localized over the protein spectrum following a modification of the G3Pase assay used for smelt G3Pase. For yeast, the assay was conducted at pH 7.0 in the presence of $MgCl_2$, as suggested by Norbeck and colleagues (1996) (Tris-HCl buffer 20 mM, 5 mM $MgCl_2$; pH 7.0). Released P_I over time was measured as previously described. The most active fractions were pooled (7-10 ml) and subjected to strong anion-exchange chromatography.

4.3.3.3 Anion-Exchange Chromatography

Pooled active fractions were loaded onto a 2 ml UNOsphere Q column (Bio-Rad laboratories Inc., CA, US) equilibrated with TRD buffer (10 mM triethanolamine, 1 mM DTT, pH 7.5). The column was washed with 45 ml of TRD buffer and eluted with a 30 ml linear gradient of 0-0.15 M NaCl in the same buffer followed by 5 ml buffer containing 1 M NaCl. 0.6 ml fractions were immediately collected after loading the sample and G3Pase activity was measured. All steps of the purification were carried out in a cold room (6-8°C) and, whenever possible, samples were kept on ice.

4.3.4 Partial purification of G3Pase from smelt liver

After G3Pase was localized in the cytosolic fraction, 4-5 ml cytosolic fractions were prepared as previously described and held at -80°C until further analysis. Work done using yeast pointed out an inhibitory effect of DTT on smelt G3Pase activity, as well as, a requirement for Mg^{2+} to optimize further detection of G3Pase activity. Therefore DTT was omitted from the buffer and assay buffer was supplemented with 5 mM $MgCl_2$.

4.3.4.1 Size exclusion chromatography

Cytosolic fractions were thawed on ice and 0.5 µl of protease inhibitor mix (70 $mg.ml^{-1}$ phenylmethylsulfonyl fluoride, 1.4 $mg.ml^{-1}$ leupeptin, and 1 $mg.ml^{-1}$ pepstatin) were added per ml prior to chromatography. Three to four ml of sample (8-15 mg $protein.ml^{-1}$) were loaded onto a 120 ml Superdex 200 column equilibrated with TRE buffer pH 7.5 (triethanolamine 10 mM, EDTA 1 mM). The column was eluted with the same buffer at a flow rate of 1 $ml.min^{-1}$, and 1 ml fractions were collected after 40 min of elution. Elution of proteins was monitored by continuous absorbance at 230 and 280 nm, and G3Pase activity was measured in each fraction collected. The most active fractions were pooled (7-10 ml) and used for anion-exchange chromatography.

4.3.4.2 Anion-exchange chromatography

Two different approaches were utilized, a membrane-based procedure using a step NaCl gradient and, as for yeast, a resin-based procedure using a linear NaCl gradient. In both cases, strong anion-exchangers were used.

The step NaCl gradient chromatography was performed using Pierce strong anion-exchange spin-columns (Pierce Biotechnology, Rockford, IL, US) according to the manufacturers' instructions. These columns use the membrane-adsorber technology as a chromatographic matrix to fractionate proteins based on their charge differences. This technology offers shorter diffusion times than resin-based chromatography and as such, constitutes an interesting alternative to conduct pilot studies. Briefly, 4.8 ml of the pooled active fraction (3 mg protein) obtained from the previous chromatography were loaded onto the membrane and unbound proteins eluted by centrifugation at 1,000 × g for 1 min at 4°C. Bound proteins are eluted depending on their charge adding increasing concentration of NaCl (0.015 M, 0.03 to 0.15 M with 0.01 M increment, and 1 M) followed by centrifugation at 1,000 × g for 1 min at 4°C. G3Pase activity was then measured in the collected fractions.

As G3Pase activity was detected successfully following the step NaCl gradient chromatography, a resin-based chromatography was used with a linear NaCl gradient that allowed the loading of the whole pooled fraction obtained after size-exclusion chromatography. The pool of active fractions (12 ml) was concentrated to 3 ml using an Amicon Ultra-15 filtration unit (Millipore, MA, Canada) according to the manufacturers' instructions. The concentrate was loaded onto a 2 ml UNOsphere Q column (Bio-Rad laboratories Inc., CA, US) equilibrated with TR buffer (Triethanolamine 10 mM, pH 7.5). The column was washed with 45 ml of TR buffer and eluted with a 30 ml linear gradient of 0-0.15 M NaCl in the same buffer followed by 5 ml buffer containing 1 M NaCl. One

ml fractions were immediately collected after loading of the sample and G3Pase activity measured as previously described.

4.3.4.3 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Ten µl of starting material and of active fractions collected from smelt samples following size-exclusion and anion-exchange chromatographies were subjected to SDS-PAGE on 4-20% polyacrylamide gels for 45 min at 200 V. Proteins, separated based on their molecular weight, were visualized following Coomassie blue staining. Protein size was determined by comparison to Precision Plus Protein™ marker (10-250 KDa, (Bio-Rad laboratories Inc., CA, US).

4.3.5 Optimal pH, cation requirements and Km of smelt liver G3Pase

Some G3Pase characteristics were determined from the desalted cytosolic fraction of pooled smelt liver (n=4-5). Desalting was done using Econo-Pac® 10 DG columns (Bio-Rad laboratories Inc., CA, US) equilibrated with TRE buffer, according to the manufacturer's instructions.

G3Pase activity was measured in different buffers covering a pH range from 3.5 to 8.5. Sodium acetate buffer 100 mM and imidazole buffer 20 mM were used to cover pH 3.5-5.5 and 6.5-8.5, respectively. In all cases, buffer was supplemented with 5 mM $MgCl_2$ and G3Pase activity determined as previously described.

Activity of G3Pase was then assayed at its optimal pH with different concentrations of $MgCl_2$, $MnCl_2$, $ZnCl_2$ and $CaCl_2$ (0, 0.5, 1, 2.5, 5, and 10 mM in assay buffer).

K_M of G3Pase was then determined under the optimal conditions (pH and cation requirements) by measuring its activity using G3P concentration ranging from 0.5 to 10 mM.

4.4 Results

4.4.1 Localization of G3Pase in liver cells

Total recovery of each enzyme marker used to verify the proper separation of the cell content was close to 100% (Table 4.1). CS was detected in all fractions but was predominant in the mitochondrial fraction. Catalase and LDH activities were detected in all fractions with the exception of the endoplasmic reticulum/microsomal fraction. Catalase was found mostly in the mitochondrial and lysosomal/peroxisomal fractions while LDH was mostly found in the cytosol. G6Pase activity was detected in all fractions except the cytosol, with most activity detected in the lysosomal/peroxisomal fraction. G3Pase activity followed LDH distribution, with the amounts of activities detected within a fraction being highly correlated between the two enzymes (linear regression; $r^2 = 0.98$, $p < 0.05$).

Table 4.1. Percentage activity recovered for citrate synthase (CS), catalase, glucose-6-phosphatase (G6Pase), lactate dehydrogenase (LDH) and glycerol-3-phosphatase (G3Pase) in four subcellular fractions obtained following differential centrifugations of smelt liver homogenates (n=2). M, mitochondria; L/P, liposome/peroxisome; ER, endoplasmic reticulum and; Cyt, cytosol. Data are presented as % activity relative to activity in initial homogenate ± S.E. (n.d.: not detectable).

Marker / *Fraction*	**CS**	**Catalase**	**G6Pase**	**LDH**	**G3Pase**
M	90.4 ± 12.8	42.7 ± 1.0	30.5 ± 6.6	13.1 ± 1.5	13.5 ± 6.0
L/P	15.5 ± 4.3	31.9 ± 7.2	49.2 ± 2.9	30.6 ± 8.1	26.7 ± 4.4
ER	2.3 ± 0.13	n.d.	9.8 ± 2.5	n.d.	n.d.
Cyt	22.0 ± 3.0	15.0 ± 1.1	n.d.	53.0 ± 4.6	57.9 ± 0.8
Total	130.2 ± 5.6	89.6 ± 7.1	89.6 ± 11.3	96.7 ± 11.3	98.1± 11.3

4.4.2 Partial purification of yeast G3Pase

Yeast G3Pase activity was successfully detected after both chromatographies following the original protocol from Norbeck et al. (1996). The peak of activity was narrowed after the second chromatography (Figure 4.1). This eliminates the possibility of any technical issues to explain the poor recovery of G3Pase from smelt hepatocytes when treated in the same conditions.

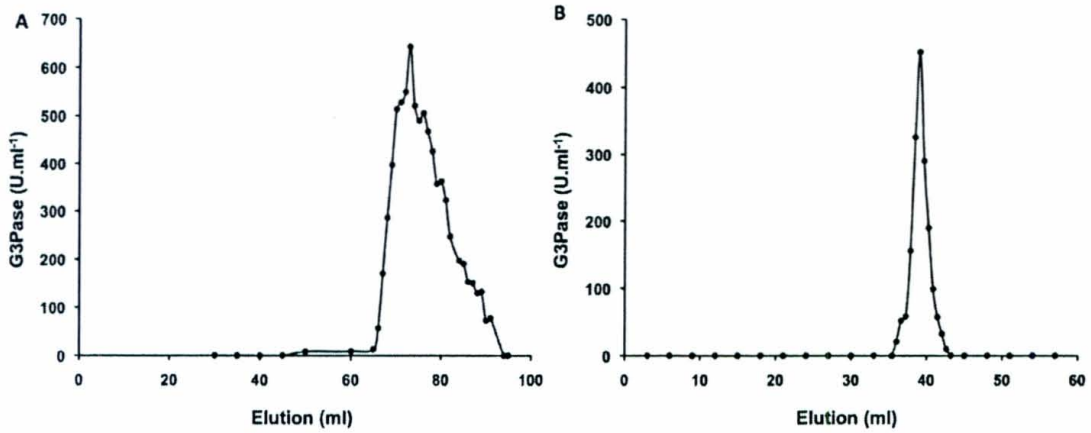

Figure 4.1. G3Pase activity profiles obtained following size exclusion (A) and anion-exchange chromatography (B) of yeast extract, *Saccharomyces cerevisiae* (Strain W303-1A)

4.4.3 Partial purification of smelt liver G3Pase

Following some modifications from the method used by Norbeck et al. (1996), smelt G3Pase activity was easily detectable after size-exclusion chromatography (Figure 4.2). SDS-PAGE revealed that a major part of both the smallest (< 25 kDa) and largest proteins (> 100 kDa) were excluded (Lane B and C; figure 4.3). This procedure resulted in purification of smelt liver G3Pase by a factor close to 3 from the starting cytosolic material (Table 4.2).

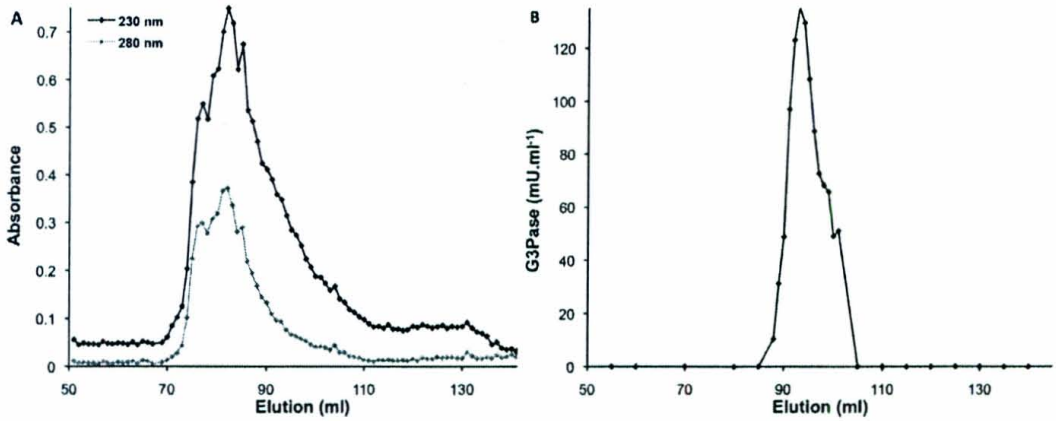

Figure 4.2. Typical protein spectrum at 230 and 280 nm (A) and corresponding G3Pase activity profile (B) obtained following size exclusion chromatography (Superdex 200) of smelt liver cytosolic fraction.

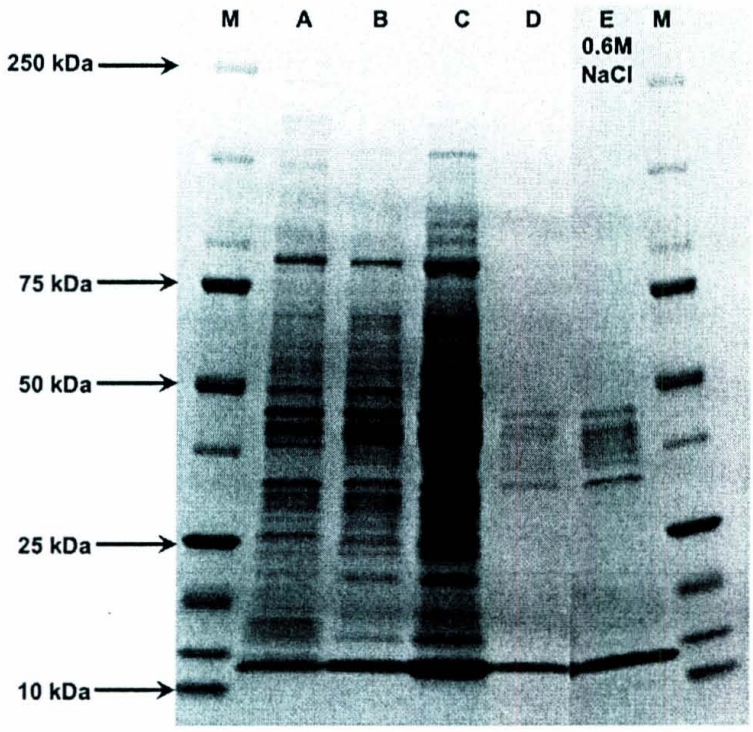

Figure 4.3. SDS-PAGE of different levels of G3Pase purification from smelt liver obtained successively after differential centrifugation (A), size-exclusion (B and C) and strong anion-exchange chromatographies following linear gradient of NaCl (D) or step gradient of NaCl (E; 0.6 M NaCl). C represents the same fraction as in B but after protein concentration. M: molecular marker.

Table 4.2. Purification of G3Pase from smelt liver cytosolic fraction following two successive chromatographies: size-exclusion chromatography (Superdex 200) and strong anion-exchange chromatography using step NaCl gradient.

	Cytosolic fraction	Superdex 200	Anion-Exchange
Total activity mU	2296	983	181
Total protein mg	50	6.7	1.4
Specific Activity $mU/mg_{protein}$	46.9	146.7	129.3
Purification Fold	1	3.1	2.7
Yield %	100	43	8

Pooled fractions from size-exclusion chromatography were further subjected to anion-exchange chromatography by step NaCl gradient. The highest peak of G3Pase activity was detected at 0.06 M with elution occurring from 0.06 to 0.11 M NaCl (Figure 4.4). Following this separation, G3Pase activity was lost over 3 hours. After this second chromatography, the calculated factor of purification from the starting material was not higher than after the size-exclusion chromatography and the yield of activity was only of 8% (Table 4.2). Even though the purification did not seem improved, SDS-PAGE of the fractions in which G3Pase activity was detected showed a focusing of the protein content, with a size range reduced to 25-60 kDa (Lane E; Figure 4.3). As such, the low yield of activity is more likely due to the rapid loss of activity in suboptimal conditions.

When anion-exchange chromatography was used with a linear gradient of 0-0.15 NaCl, G3Pase activity recovered was even lower than after a step gradient and was also

rapidly lost. SDS-PAGE, ran with the only fraction where G3Pase activity was detected, resulted in the same pattern of proteins as when a step gradient was used and confirmed the size range of G3Pase in smelt liver (Lane D; Figure 4.3).

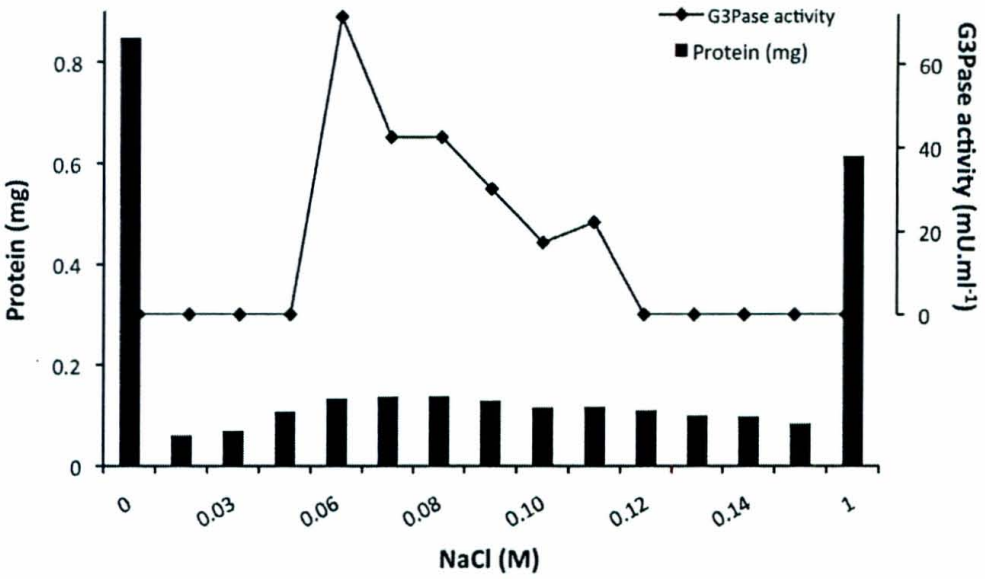

Figure 4.4. Smelt liver G3Pase partial purification following strong anion-exchange chromatography using step NaCl gradient. Columns and solid line represent protein elution and corresponding G3Pase activity measured, respectively.

4.4.4 Optimal pH, cations requirements and Km

The highest G3Pase activity was detected at pH 5.5 (Figure 4.5A). Activity was inhibited by Ca^{2+} but was enhanced by both Mg^{2+} and Mn^{2+} (Figure 4.5B). Mg^{2+} induce a 5-6 fold increase for concentrations higher than 1 mM while Mn^{2+} induce a 2-2.5 fold increase for concentrations higher than 0.5 mM. Activity was increased by 6 fold in the presence of

0.5 mM Zn^{2+} but decreased from this point with increasing concentrations of Zn^{2+} (Figure 4.5B).

G3Pase showed a hyperbolically shaped saturation curve with respect to the concentration of glycerol-3-phosphate. By fitting the data directly to a hyperbola an apparent K_M of 2.61 ± 0.65 mM was found for G3P (Figure 4.5C).

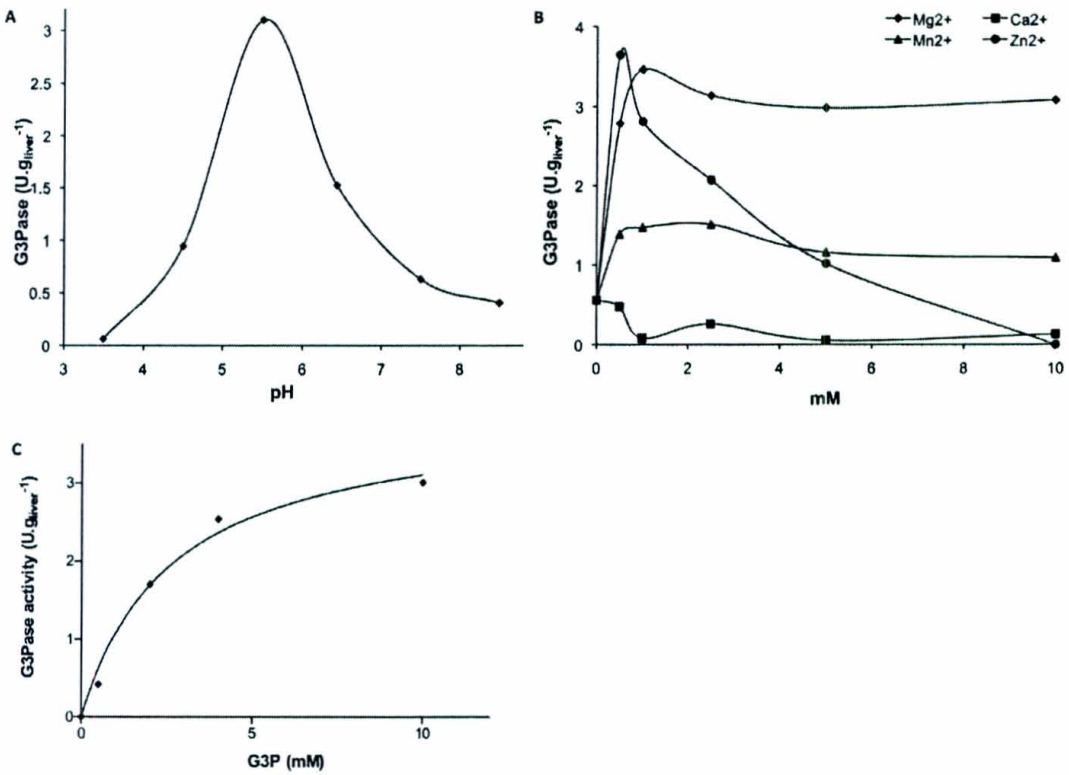

Figure 4.5 Optimal pH (A), cation requirements (B) and activity as a function of G3P concentration (C) of smelt liver G3Pase. Measurements were done in the cytosolic fraction obtained following differential centrifugations of pooled livers (5-6).

4.5 Discussion

The differential centrifugation procedure used did not result in a perfect separation of smelt liver organelles. A substantial part of the liposomal fraction was recovered in the mitochondrial pellet following centrifugation at 8,000 × g, suggesting this starting centrifugation should have been refined. Most mitochondria (up to 90%) were, however, recovered in the expected pellet, confirming that the method developed by Ballantyne (1994) is appropriate for mitochondria enrichment, its initial purpose. G6Pase activity did not fractionate as expected with most of the activity recovered after "low" centrifugations while this enzyme was chosen as a marker of the endoplasmic microsomal fraction, a fraction expected to be recovered after ultracentrifugation. G6Pase has a critical role in glucose homeostasis and is intimately linked to the endoplasmic reticulum membrane (Van Schaftingen & Guerin 2002). During homogenization, endoplasmic reticulum is broken down to smaller vesicles, microsomes, which can be separated from the cytosol following ultracentrifugation. Lewis & Tata (1983) pointed out that significant recovery of G6Pase activity (up to 75%) in the microsomal fraction required repeated homogenization. Following only one-step homogenization, much of the endoplasmic reticulum was not properly disrupted and was actually "entangled to mitochondria". As such, results I obtained for G6Pase may be due more to a rapid sedimentation of improperly disrupted endoplasmic reticulum than to an improper choice of marker for the microsomal fraction. The point of this experiment was not to purify each fraction but rather to determine the distribution of G3Pase in relation to the respective distribution of each fraction. The high correlation observed between LDH and G3Pase distribution

supports the contention that, in smelt liver, G3Pase is located in the cytosolic fraction and is not associated with any organelles larger than 0.1 μm including mitochondria, lysosomes, peroxisomes and microsomes. Activity measured in ultracentrifuged extract from *B. licheniformis* also pointed out the cytosolic fraction as the source of G3Pase (Skraly & Cameron 1998) but not activity measured in different fractions of rat heart where the lysosomal fraction was determined as the most likely source of G3Pase (de Groot et al. 1994). This latter point will be further discussed later in relation to the pH preference of G3Pase.

G3Pase was only partially purified following size-exclusion and ion-exchange chromatographies. Efforts made however allowed the removal of a substantial fraction of total proteins and reduced the range of size of the active enzyme to between 25 and 60 kDa. This range fits with the size of G3Pase purified from other species: 30 kDa in *S. cerevisiae* (Norbeck et al. 1996), 25 kDa in *B. licheniformis* (Skraly & Cameron 1995) and, 28 kDa in *C. albicans* (Fan et al. 2005). Activity was rapidly lost after ion-exchange chromatography in the conditions used, but further separation would be required for complete purification.

The choice of the procedure was based on the work done with yeast, *S. cerevisiae*, for which G3Pase was successfully purified and characterized (Norbeck et al. 1996). Yeast was cultured and G3Pase was successfully partially purified following the first two steps of the method published that included size-exclusion and anion-exchange chromatography with NaCl gradient. The third chromatography, anion-exchange chromatography with pH gradient was not performed in the present study, as the goal of

the procedure was to assess if the lack of stability of smelt G3Pase following ion-exchange chromatography in Norbeck et al. conditions was due to a difference in the protein between the two species or to technical issues. Total number of smelt G3Pase units loaded on the size-exclusion column was very similar to the one used by Norbeck and colleagues (2296 *vs* 2973 U for smelt and yeast, respectively) and the yields obtained after the size exclusion chromatography were the same. In addition, the G3Pase suspension obtained following size-exclusion chromatography proved to be very stable over time when kept on ice. A second chromatography separation may thus allow for a better purification with a sufficient yield to eventually purify G3Pase from smelt liver. The only limiting factor would be to find conditions optimal for the preservation of functional activity for this particular enzyme that seems to have different properties than the one previously purified from yeast or bacteria. Several modifications were indeed needed prior to a successful recovery of G3Pase following the first chromatography. DTT, a reducing agent added in elution buffers for the purification from yeast, was shown to inhibit G3Pase activity from smelt liver and was excluded from smelt elution buffers (data not shown). Strong-anion chromatography was not suitable to release smelt G3Pase while yeast enzyme was successfully separated following the equivalent procedure. Smelt G3Pase may be more negatively charged than yeast G3Pase, resulting in a stronger retention of the protein on the strong anion-exchange resin and in a need of higher salt concentrations to release the enzyme that may be unsuitable for the protein functionality. Finally, activity of smelt G3Pase is optimal at acidic pH while G3Pase activity of bacteria, fungi or algae is optimal at neutral pH. More work will be needed to

design conditions and the best sequence of chromatography methods for a complete separation of smelt liver G3Pase. A large variety of chromatographies could be tested including weak anion-exchange, strong cation-exchange chromatographies and affinity chromatography.

Following the progressive determination of different characteristics of smelt G3Pase, and the difficulty encountered in purifying it, general characteristics were determined from ultracentrifuged liver homogenates. The most obvious difference in comparison to species in which G3Pase was purified is certainly its optimal pH. Optimal pH of this enzyme is acidic with the highest activity measured at pH 5.5 while it was neutral in all other species where it was assayed (6.5-7.0) with the exception of rat heart where the optimal pH was also acidic at pH 5.0 (de Groot et al. 1994). In rat heart, G3Pase activity was determined in the same fractions as used in the current study for smelt liver following differential centrifugations but pellets obtained after each centrifugation were washed prior to any measurements. G3Pase activity measured in all fractions would thus be associated with the fraction purified only and not to any cytosol remaining after supernatant collection. G3Pase was measured in most fractions investigated but the lysosomal fraction was considered the main source, as suggested by higher relative specific activity in this fraction. The authors further supported this contention by the preference of the enzyme for low pH, activity being two fold higher at pH 5.0 than at pH 7.2 when measured in crude heart homogenate. However when they measured activity of G3Pase in all fractions collected at pH 5.0 and 7.2, activity measured in the lysosomal fraction did not change as a function of pH while activity

increased by 3 fold in the cytosolic fraction in the acidic conditions. These results are actually very interesting suggesting that the acidic phosphatase hydrolyzing G3P in rat heart crude homogenates may actually be located in the cytosol rather than in the lysosomal fraction, as it is the case in smelt. Acidic phosphatases are ubiquitous in nature. In the liver of vertebrates, two classes were reported based on their molecular weight (M_W): high M_W (> 100 000 KDa) and low M_W (20 000 – 35 000 KDa) forms. High Mw forms seem to be non-specific and associated with the lysosomal fraction while low Mw forms display specific activities and are found in the cytosolic fraction (Khan et al. 1996). Presence of acidic phosphatases in the cytosolic fraction is thus not an unexpected nor a new discovery even though their exact role does not seem to be fully understood.

Acid phosphatases which are active in the presence of metal ions such as Zn^{2+}, Mg^{2+} and Mn^{2+} have been reported in several mammalian and non-mammalian tissues but little is known about those enzymes due to a paucity of examples available in the literature (reviewed in Saeed 2009). Zn^{2+} is generally reported as an activator of some specific phosphatases that are regrouped as Zn^{2+}- dependant acid phosphatases. This ion was shown to increase smelt G3Pase activity at low concentration but to inhibit it at concentrations higher than 1 mM. Mg^{2+} is most likely a requirement for proper function of smelt G3Pase as activity is strongly increased in its presence. It was suggested that a G3P-Mg^{2+} complex might actually be the real substrate of G3Pase in algae, *Dunaliella salina* (Sussman & Avron 1981). In this particular species however, activity was undetected in the absence of this cation. Effect of Mn^{2+} and Ca^{2+} were also tested on

algae G3Pase and resulted in the same effect observed with smelt G3Pase, *i.e.* in activation and inhibition, respectively.

4.5.1 Conclusions and perspectives

Results collected indicate that G3Pase from smelt liver is different from G3Pase purified from bacteria or fungi but may be very similar to the one detected in rat, another vertebrate species, and may be part of a group of cytosolic, low Mw acidic phosphatases. Further work is needed to purify and identify this enzyme and validate these contentions. Characterization of this protein in smelt following protein sequencing may allow identification of similar proteins in higher vertebrates and would give more support to the hypothesis that a direct dephosphorylation of G3P may be the source of glycerol in response to ischemic events in rat tissues as proposed by de Groot et al. (1995) and Nguyen et al. (2007) but not supported by Clausen et al. (2011).

Chapter 5

Summary and perspectives

5.1 Summary

This thesis investigated glycerol metabolism in rainbow smelt (*Osmerus mordax*) by addressing two primary questions: 1) how glycerol outputs, including direct loss to the environment and biochemical catabolism, affect glycerol levels and 2) what are the respective roles of G3Pase and glycerolipids in glycerol synthesis?

5.1.1 Glycerol outputs do not drive glycerol levels

This thesis presents the first effort to determine how glycerol catabolism may affect glycerol levels in smelt by investigation of GK activity. Liver was the only tissue where activity presented a pattern of regulation coherent with the pattern of glycerol, *i.e.* lowest levels measured over the accumulation period and increasing levels measured over the decrease period (Figure 2.1). However, even at their highest, GK activities were very low and unlikely to have significant biological role in regards to glycerol levels (Chapter 2). Surprisingly, the rate of loss did not change over the glycerol cycle, discarding the presence of any mechanisms to retain glycerol (Chapter 2). Overall, these results show that glycerol levels are not dictated by regulation of glycerol catabolism through biochemical means or by regulation of the losses toward the environment but rather by regulation of the inputs, *i.e.* by the rate of glycerol synthesis. Even though the predictable decrease of glycerol levels observed mid-February remains unexplained, these findings nicely support the next hypothesis that "glycerol synthesis may occur through a rapid and direct dephosphorylation of G3P by a phosphatase, G3Pase".

5.1.2 Glycerol is mainly produced by a direct dephosphorylation of G3P

The hypothesis of a direct dephosphorylation of G3P by a phosphatase (G3Pase) to produce glycerol in response to cold was not novel to this thesis as it was previously proposed for the larval stage of *Epiblema scudderiana* (Joannisse & Storey 1994) and also for smelt as early as the late 90's (Driedzic et al. 1998). Except for these reports of G3Pase-like activity, no further work was done to validate the role of the protein. Glycerol being well accepted as a by-product of glycerolipid degradation in vertebrates, and G3Pase being not identified in any animal species, a deeper investigation of the respective roles of these two potential routes was necessary and at the centre of this thesis (Chapter 3). Determination of activities of G3Pase and of different key enzymes involved in lipid metabolism over the glycerol cycle did not clearly point out one as the most likely main source. G3Pase was up-regulated at the onset of the glycerol cycle, as was lipase and enzymes involved in lipid degradation. A second experiment, involving isolated smelt hepatocytes producing glycerol at cold temperature, offered a better model to investigate both routes without the confounding effects that may dilute information at the whole animal level. This experiment clearly showed that neither on-board nor newly synthesized glycerolipid could be the source of the glycerol released over the incubation period, even though phospholipids may account for 17% of the glycerol released in this particular model. All together, these results support an up-regulation of lipid metabolism in winter in smelt liver, as proposed by Hall et al. (2011) who detected up-regulation of several genes involved in lipid metabolism in smelt producing glycerol in comparison to smelt maintained at warm temperatures, that do not accumulate glycerol. This up-

regulation of lipid metabolism may, however, be related to an adaptation to cold temperature in general rather than to glycerol production. As such, these experiments support the importance of a direct dephosphorylation of G3P in glycerol synthesis with an up-regulation at the critical onset period of glycerol accumulation.

5.1.3 G3Pase from smelt liver is a cytosolic acidic low M_W phosphatase

Having shown that glycerol was mainly produced through a direct dephosphorylation of G3P, I directed my work towards purification of G3Pase. The method used to isolate G3Pase from yeast rapidly showed its limitation in regards to smelt protein, both proteins being most likely very different. A complete purification was not achieved but allowed to determination of some important characteristics of smelt G3Pase: 1) it is an acidic phosphatase, 2) it is located in the cytosolic fraction, 3) it is a low M_W protein and 4) Mg^{2+} is required for optimal activity. All these characteristics are not shared with previously identified G3Pases, all of them having a neutral optimal pH. Low M_W and Mg^{2+} requirements were, however, determined for all of them when assayed. More work remains to be done to identify smelt G3Pase. The determination of its sequence will allow an appropriate comparison of protein between species and would help to resolve if they originated from a common ancestral or from completely independent genes.

5.2 Significance of these results and future directions

5.2.1 Significance in regards to cold adaptation

Smelt and the few other fish that accumulate important amounts of glycerol as cryoprotectant are very unique species. As opposed to any other terrestrial species that are closed systems and can synthesize a stock of glycerol once, fish are in intimate contact with their environment and are subject to constant loss of glycerol in water, forcing them to actively synthesize it to allow accumulation. Work done in Chapter 2 supports glycerol levels being primarily dictated by the rate of synthesis, glycerol loss to the environment being proportional to plasma glycerol levels over the cycle and glycerol catabolism being too low to have any significant role. Why, when water temperatures are still at their lowest, do smelt stop synthesizing glycerol? The significant increase of GK and mGPDH activities (Chapter 2 and Robinson et al. 2011, respectively) suggests a redirection of some glycerol toward synthesis of DHAP that could be used for glucose or glycogen synthesis. This hypothesis is further supported by the increase in glycogen levels reported by Driedzic & Short (2007) by the end of the glycerol cycle. The reason of this possible redirection of G3P toward glycogen rather than glycerol synthesis at this moment of the cycle remains obscure. Glycerol might be not needed any more to confer an appropriate protection against freezing temperatures, as AFP levels synthesized by smelt continue to accumulate even after mid-February (Lewis et al. 2004). This hypothesis may be tested using greenling as models as this species also produce glycerol as a cryoprotectant in winter but does not synthesize any kind of AFP in parallel

(Raymond 1992). However, determination of an equivalent pattern of glycerol levels would first need to be validated for an appropriate comparison with smelt.

Identification of G3Pase from smelt liver is now crucial to gain further understanding of mechanisms regulating the rate of glycerol synthesis in smelt over the glycerol cycle. In addition, validation of a direct dephosphorylation of G3P as the main source of glycerol must be done in other animal species accumulating glycerol in response to freezing temperatures, including insects and frog species. This would give more credibility to the hypothesis that this pathway is conserved in animal species in response to cold.

5.2.2 Significance in regards to glycerol metabolism in vertebrates

Results collected in this thesis are not only relevant to cold adapted species but are actually very interesting for higher vertebrates, where the accepted pathway for glycerol synthesis is via lipolysis. Glycerol as has been widely accepted as a by-product of glycerolipids. In intact heart, glycerol levels are often used as indicator of lypolysis. However, in ischemic rat heart, when pH tends to become acidic, it was shown that a direct dephosphorylation of G3P may have a significant role in the resulting release of glycerol (de Groot et al. 1997). This pathway was also proposed as a source of glycerol in ischemic rat brain in which glycerol was shown to be derived from glucose and cycling of G3P through the lipid pool was not supported (Nguyen et al. 2007). Validation of this pathway in animals would give a new dimension to glycerol metabolism; glycerol being not just a by-product of lipolysis any longer but being also a purposely-synthesized

product in different stressful situations. When glycerol is synthesized purposely, a new question would be then raised: "what is the exact role of glycerol in these conditions?"

In 2001, Brisson and colleagues published a paper entitled "Glycerol: a neglected variable in metabolic processes?" These authors provided a summary list of the different potential roles of glycerol reported in various species including roles in energy metabolism and biomass synthesis, cryoprotection, osmoregulation, athletic performances, regulation of glycemia, health and disease, therapeutic and diagnostic applications. The very interesting point of this paper was the effort made to highlight the most likely underestimated importance of glycerol but this paper was somewhat confusing, mixing roles glycerol could have *in vivo* and *in vitro.* The chaperone property was, however, recurrent to support some of its roles, in particular *in vitro*. Chaperone properties of glycerol are well known, this molecule being one of the most common chemical chaperones. Chaperone properties of glycerol were determined in various diseases models including Alzheimer's disease, cancer and cystic fibrosis (reviewed in Papp and Csermely 2006). In smelt, protein 3-dimentioned structures are enhanced in the presence of glycerol (Gong et al. 2011). Acute exposure to glycerol was also shown to enhance resistance to heat and oxidative stress not just from chaperone activity but also by a beneficial induction of the proteasome, involved in protein degradation, and of endogenous molecular chaperone mortalin/mtHSP70 (Deocaris et al. 2008). Such properties could actually explain why glycerol would be synthesized purposely in stressful situations when protein integrity is threatened.

5.2.3 Future directions

Complete purification of G3Pase would be a tremendous step toward the determination of a novel pathway for glycerol synthesis in animals. Cytosolic low Mw acid phosphatases are proposed to be very specific in terms of substrate. Purification of G3Pase from smelt, that is most likely part of this group of proteins, would allow further support for this contention, by testing for substrate preferences. Access to a purified protein also offers great opportunities to investigate mechanisms involved in the regulation of this particular enzyme at the protein and at the gene level. At the protein level, mass spectrophotometry of the purified enzyme could be used to identify phosphorylation sites and other post-translational modifications that may affect activity. Microsequencing can be conducted and amino acid sequence obtained could be used to retrieve the corresponding nucleotide sequence to assess G3Pase mRNA levels. Thereafter, transcriptional regulation of the gene could be analyzed. BLAST could be used at the protein and gene levels to determine closely related molecules in other species, and more particularly in higher vertebrates that may have a phosphatase with similar characteristics. This work could be easily extended to some other species producing glycerol in response to stress, in an obvious manner (*e.g.* one species of freeze tolerant tree frog or the freeze tolerant gall-moth larvae) or in a subtler manner (*e.g.* rat brain and heart in response to ischemic events). Localization of the regulatory loci could be used to induce glycerol production in other organisms and may be valuable in areas as various as medical research or aquaculture for which its chaperone and cryoprotective properties may be relevant.

Chapter 6

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