REGULATION OF GLUCOSE UPTAKE AND TRANSPORTER EXPRESSION IN THE NORTH PACIFIC SPINY DOGFISH (*SQUALUS SUCKLEYI*)

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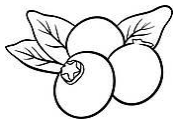
DEDICATION

This thesis is dedicated to Wyatt Stasiuk, who was taken from us far too soon.

He was the sweetest little angel and I love and miss him very much.

Rest in peace baby boy.



ABSTRACT

Elasmobranchs (sharks, skates, and rays) are a primarily carnivorous group of vertebrates that consume very few carbohydrates and have little reliance on glucose as an oxidative fuel, the one exception being the rectal gland. This has led to a dearth of information on glucose transport and metabolism in these fish, as well as the presumption of glucose intolerance. Given their location on the evolutionary tree however, understanding these aspects of their physiology could provide valuable insights into the evolution of glucose homeostasis in vertebrates. In this thesis, the presence of glucose transporters in an elasmobranch was determined and factors regulating their expression were investigated in the North Pacific spiny dogfish (*Squalus suckleyi*). In particular, the presence of a putative GLUT4 transporter, which was previously thought to have been lost in these fish, was established and its mRNA levels were shown to be upregulated by feeding (intestine, liver, and muscle), glucose injections (liver and muscle), and insulin injections (muscle). These findings, along with that of increases in muscle *glycogen synthase* mRNA levels and muscle and liver glycogen content, indicate a potentially conserved mechanism for glucose homeostasis in vertebrates, and argue against glucose intolerance in elasmobranchs. In contrast to the other tissues examined, there was a decrease in *glut4* mRNA levels within the rectal gland in response to natural feeding, a factor known to activate the gland, suggesting mRNA storage for rapid protein synthesis upon activation. A similar trend was also shown for *sglt1* in the rectal gland, and the ability of GLUT and SGLT inhibitors to prevent chloride secretion solidified the importance of glucose uptake for gland function. The exogenous factor of salinity was also investigated and high levels of *glut* mRNA were observed within the rectal glands of low salinity-acclimated fish relative to control and high salinity fish, reiterating the idea of mRNA storage when the gland is expected to be inactive. Taken together, the results of this thesis

demonstrate that glucose is an important fuel in the dogfish (and likely other elasmobranchs) and that the dogfish is fully capable of regulating its storage and circulation, contrary to prior beliefs.

RÉSUMÉ

Les élasmobranches (requins, rajidaes et raies) sont un groupe de vertébrés principalement carnivores qui consomment très peu d'hydrates de carbone et qui dépendent peu du glucose en tant que carburant oxydatif, avec l'exception de la glande rectale. Ainsi, il y a un manque d'information sur le transport et le métabolisme du glucose chez ces poissons, en plus d'une présomption qu'ils sont intolérants au glucose. Cependant, étant donné leur emplacement sur l'arbre phylogénétique, la compréhension de ces aspects de leur physiologie pourrait offrir des informations précieuses quant à l'évolution de l'homéostasie du glucose chez les vertébrés. Dans cette thèse, la présence de divers transporteurs de glucose a été déterminée chez les élasmobranches et les holocéphales. De plus, des facteurs biotiques et abiotiques qui régulent l'expression et l'activité de ces transporteurs ont été étudiés chez l'aiguillat commun du Pacifique Nord (Squalus suckleyi). En particulier, la présence d'un transporteur GLUT4 putatif, que l'on pensait auparavant avoir été perdu chez ces poissons, a été établie et il a été démontré que son niveau d'ARNm est régulé de façon positive par l'alimentation naturelle (intestin, foie et muscle), les injections de glucose (foie et muscle), et les injections d'insuline (muscle). Ces résultats, en plus de l'augmentation des niveaux d'ARNm de glycogène synthase dans le muscle, et l'augmentation du contenu de glycogène dans les muscles et le foie, indique un potentiel pour un méchanisme d'homéostasie du glucose conservé parmi les vertébrés. De plus, ces résultats plaident contre l'idée d'une supposée intolérance au glucose chez les élasmobranches. Contrairement aux autres tissus examinés, il y avait une diminution des niveaux d'ARNm pour le GLUT4 dans la glande rectale en réponse à l'alimentation naturelle, un facteur qui est connu pour son activation de la glande. Ce résultat suggère qu'il y a entreposage d'ARNm dans la glande afin de permettre à la synthèse rapide de protéines lorsqu'activée. Une tendance semblable à aussi été

démontrée pour le SGLT1 dans la glande rectale, et la capacité d'inhibiteurs de GLUT et de SGLT pour prévenir la sécrétion de chlorure a réaffirmé l'importance de l'absorption du glucose pour la fonction de la glande. La salinité en tant que facteur abiotique a également été étudiée, et de hauts niveaux d'ARNm pour le GLUT ont été observés dans les glandes rectales de poissons acclimatés à de faibles salinités, lorsque comparés à des poissons contrôles ou acclimatés à des salinités élevées. Ceci renforce l'idée qu'il y a entreposage d'ARNm lorsque la glande est supposée d'être inactive. Ensemble, les résultats de cette thèse démontrent que le glucose est un carburant important chez les aiguillats (et probablement chez les autres élasmobranches) et que ces poissons sont tout à fait capables de réguler l'entreposage et la circulation du glucose, contrairement aux croyances antérieures.

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LIST OF ABBREVIATIONS

°C	degrees Celsius
µg	microgram
µL	microlitre
µM	micromolar
µm	micrometre
µmol	micromoles
ADP	adenosine diphosphate
AIT	apical iodide transporter
ANOVA	analysis of variance
BHB	β-hydroxybutyrate
Ct	threshold cycle
cAMP	cyclic adenosine monophosphate
$CaCl_2$	calcium chloride
cDNA	complementary deoxyribonucleic acid
CFTR	cystic fibrosis transmembrane conductance regulator
CHT	choline transporter
Cl^-	chloride

DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
EF1α	elongation factor 1-alpha
g	grams
GLP	glucagon-like peptide
GLUT	glucose transporter
h	hours
HMIT	H^+-myoinositol transporter
IU	International Unit
KCl	potassium chloride
kg	kilograms
KOH	potassium hydroxide
L	litre
LDH	lactate dehydrogenase
MCT	monocarboxylate transporter
mg	milligrams

$MgSO_4$	magnesium sulphate
min	minutes
mL	millilitres
mM	millimolar
mmol	millimoles
mRNA	messenger ribonucleic acid
N	nitrogen
n	sample size
Na^+	sodium
NaCl	sodium chloride
Na_2HPO_4	disodium phosphate
$NaHCO_3$	sodium bicarbonate
Na_2SO_4	sodium sulphate
ng	nanograms
NIS	Na^+/I^- symporter
NKA	Na^+/K^+-ATPase
NKCC	$Na^+/K^+/2Cl^-$ cotransporter
nm	nanometer

nM	nanomolar
PCR	polymerase chain reaction
PE	polyethylene
qPCR	quantitative polymerase chain reaction
RM	repeated measures
RNA	ribonucleic acid
RNase	ribonuclease
RT	reverse transcription
s	seconds
SAAT	sodium-dependent amino acid transporter
SEM	standard error of the mean
SGLT	sodium-glucose cotransporter
SLC	solute carrier family
SMCT	sodium monocarboxylate cotransporter
SMIT	sodium myoinositol cotransporter
SMVT	sodium-dependent multivitamin transporter
TCA	trichloroacetic acid
TMAO	trimethylamine oxide

U	units
VIP	vasoactive intestinal peptide

CHAPTER 1

General Introduction

Elasmobranchs (sharks, skates, and rays) are some of the earliest extant vertebrates and they exhibit unique characteristics that make them an ideal model for studying physiological systems. The majority of elasmobranchs are carnivorous and thus consume very few carbohydrates. In addition, most tissues do not rely on glucose as an oxidative fuel, preferring to use ketone bodies (such as acetoacetate and β-hydroxybutyrate; BHB) even during periods where starvation is not a factor (see review by Speers-Roesch and Treberg, 2010); in mammals, the production of ketone bodies is harmful in the long-term. Nonetheless, certain species, such as the North Pacific spiny dogfish (*Squalus suckleyi*)[1], are known to feed on molluscs and crustaceans which can consist of up to 25% carbohydrates (Jones and Geen, 1977), and the rectal gland, which excretes NaCl, upon activation by a meal, has been shown to be glucose-dependent (Walsh *et al*., 2006). Fish in general have been assumed to be poor glucoregulators, leading to the belief that they are glucose-intolerant, the basis of which is the prolonged time taken to clear a glucose-load relative to mammals. However, given that the average fish metabolic rate is much lower than that of mammals, we could expect that glucose turnover rates would also be lower (see review by Moon, 2001).

Perhaps because of the above pre-conceptions, very few studies have examined glucose tolerance in elasmobranchs and only one has investigated the presence of glucose transporters (GLUTs; Balmaceda-Aguilera *et al.*, 2012) which, in other vertebrates, are responsible for the uptake of glucose into the tissues, thereby helping to regulate plasma glucose. Thus, this thesis examined the regulation of glucose uptake and transporter expression in an elasmobranch, the North Pacific spiny dogfish (*S. suckleyi*), in an attempt to further our knowledge of glucose

[1] Note that this population has also been referred to as *Squalus acanthias* in other publications but Ebert *et al.* (2010) determined that they were a separate species from the Atlantic population and thus suggested we revert to the former name of *Squalus suckleyi*.

regulation in these fish as well as the evolution of glucose homeostasis in vertebrates. Prior to stating the central hypothesis and themes of this thesis, this general introduction will develop a framework for understanding the research conducted in this thesis by reviewing key concepts such as elasmobranch metabolism, glucose homeostasis in fish, glucose transporters, and insulin.

1.1 Elasmobranch Metabolism

Elasmobranchs are a unique group of fish whose physiology differs from that of their teleost counterparts in a number of ways, and they are of particular interest to scientists due to their basal position in the vertebrate phylogeny. Thus, studying these animals can provide valuable insights into the evolution of various physiological systems. One characteristic of this group of fish that is of particular interest to physiologists is their osmoregulatory strategy. Rather than excreting their nitrogenous wastes as ammonia, which is the strategy employed by teleost fish, elasmobranchs convert them to urea in a manner similar to what is observed in most terrestrial vertebrates. Further, as the vast majority of elasmobranchs are marine, they retain this urea at high concentrations in their plasma (300-400 mM) which allows them to maintain a plasma osmolality slightly greater than that of the surrounding seawater which helps to reduce water loss (reviewed by Hazon *et al.*, 2003). Because urea at such high concentrations can denature proteins, elasmobranchs also retain relatively high levels of the protein-stabilising molecule trimethylamine oxide (TMAO) to counteract the negative effects of urea (Yancey and Somero, 1979).

A second aspect of their osmoregulatory system that is unique is the presence of a rectal gland, a small digitiform organ, attached to the posterior intestine, that acts to excrete excess

NaCl (Burger and Hess, 1960). Although elasmobranchs maintain high plasma osmolality, the concentration of salts is low relative to seawater and thus ion gradients exist that can cause a passive uptake of salt. Rather than actively excreting Na^+ and Cl^- back out through the gills (the mechanism employed by teleosts), they use the rectal gland to eliminate any excess salt. This also holds true for the salt load experienced following a meal. Walsh *et al*. (2006) observed increases in the activities of enzymes such as Na^+/K^+-ATPase (NKA) and lactate dehydrogenase (LDH) following a meal. Interestingly, this study also indicated that the gland is glucose-dependent, meaning that glucose is the only fuel that can sustain secretion rates on its own. An additional study by Deck *et al*. (2013) (see Appendix A) that investigated transcriptional changes in the rectal gland following a meal via suppression subtractive hybridisation showed an increase in *ldh* transcripts at 6 hours post-feeding, however *nka* transcripts decreased post-feeding and this response was suggested to be due to the storage of mRNA for rapid NKA protein synthesis upon activation, since a time lag that included both mRNA and protein synthesis might be too long relative to the rapid need for enhanced chloride secretion.

In terms of diet and fuel usage by other elasmobranch tissues, elasmobranchs are generally carnivorous, thus consuming large quantities of protein and lipids but often very few carbohydrates. Interestingly though, elasmobranchs are limited in their ability to transport fatty acids in the blood which is believed to be due to the absence of albumin, possibly a consequence of maintaining high plasma urea levels (Ballantyne, 1997), and extrahepatic tissues have a very limited capacity for the oxidation of fatty acids (see review by Speers-Roesch and Treberg, 2010). However, many elasmobranch tissues can rely on ketone bodies as fuels and as previously mentioned, some actually function better when ketone bodies are taken up over glucose. This is

likely one of the reasons for the dearth of information on glucose transport and metabolism in elasmobranchs.

1.2 Glucose Homeostasis in Fish

In fish, the ability to maintain stable plasma glucose levels varies widely between species and also between studies conducted on the same species, making it difficult to draw any general conclusions about the ability of teleosts and elasmobranchs to regulate glucose. For instance, American eels (*Anguilla rostrata*; Moon, 1983; Cornish and Moon, 1986) and adult sea bass (Zammit and Newsholme, 1979) were able to maintain stable plasma glucose levels through more than 100 days of food-deprivation, yet brown trout (*Salmo trutta*; Navarro *et al*., 1992; Navarro and Gutierrez, 1995), yellow perch (*Perca fluvescens*; Foster and Moon, 1991), catfish (*Rhamdia hilarii*; Machado *et al*., 1988), and European eels (*Anguilla anguilla*; Larsson and Lewander, 1973) were shown to experience a significant decrease in plasma glucose with fasting. Interestingly, in the European eel, plasma glucose began to increase again towards control levels as food-deprivation continued and this corresponded with a decrease in liver glycogen content (Larsson and Lewander, 1973). The American eel on the other hand maintained plasma glucose without a decrease in liver glycogen (Moon, 1983). Decreases in hepatic glycogen were observed in catfish, yellow perch, brown trout, and juvenile sea bass but in these species the mobilisation of such stores was insufficient to prevent decreases in plasma glucose throughout the fasting period (Machado *et al*., 1988; Foster and Moon, 1991; Gutierrez *et al*., 1991; Navarro *et al*., 1992). Similarly, liver and muscle glycogen content decreased significantly in the Atlantic hagfish (*Myxine glutinosa*) following one month of starvation, but decreases in plasma glucose could not be prevented (Emdin, 1982).

For elasmobranchs, there is far less information on glucose regulation but, as in teleosts, the ability to maintain stable plasma glucose levels appears to be species-specific as the Pacific spiny dogfish (*S. suckleyi*) was shown to maintain plasma glucose through 56 days of fasting (Wood *et al*., 1995) while the small-spotted catshark (*Scyliorhinus canicula*; also referred to as the lesser-spotted dogfish), experienced significant decreases in glucose levels with only short periods of food deprivation (Zammit and Newsholme, 1979; Anderson *et al*., 2002). Unfortunately, muscle and liver glycogen stores were not measured in these studies. Studies that have injected dogfish (*Squalus acanthias*; Patent, 1970) or skates (*Leucoraja erinacea*; Hartman *et al*., 1944) with glucose showed that both species were capable of clearing the glucose load, however control values were not reached until 48 hours post-injection.

Sturgeon represent a unique position on the evolutionary tree, falling in between elasmobranchs and teleosts and showing a metabolism that is intermediate intermediate these two groups of fish, but again, there are few studies on glucose regulation in these fishes. In white sturgeon (*Acipenser transmontanus*), plasma glucose decreases through 4 weeks of starvation and then is maintained at this level while liver glycogen levels continue to decrease (Hung *et al*., 1997). On the other hand, feeding this species a glucose load increases both plasma glucose and liver glycogen in a dose-dependent manner, the former of which can take between 12-24 hours to return to control values (Fynn-Aikins *et al*., 1992; Gisbert *et al*., 2003). In Adriatic sturgeon (*Acipenser naccarii*), plasma glucose and liver glycogen also decreased with fasting but only through two days, after which levels were maintained (Furne *et al*., 2012). Contrary to this, the Persian sturgeon (*Acipenser persicus*) was able to maintain plasma glucose levels through 4 weeks of fasting (Yarmohammadi *et al*., 2012). Thus, it is clear that few to no general

conclusions can be made about the ability of fish to regulate plasma glucose as there are a number of species-specific differences, possibly relating to the life-histories of each fish.

1.3 Glucose Transporters

There are two primary families of glucose transporters, the facilitated diffusion glucose transporters (GLUTs; solute carrier family SLC2A) and the sodium-glucose cotransporters (SGLTs; solute carrier family SLC5A), both of which were investigated in this thesis. The GLUT family transport glucose down its concentration gradient and consists of 14 known members (in humans) which are divided into three classes. This family is briefly reviewed here, but the reader is referred to existing reviews for more detailed descriptions (see Joost and Thorens, 2001; Scheepers *et al*., 2004; Zhao and Keating, 2007; Thorens and Mueckler, 2010; Mueckler and Thorens, 2013). Class I consists of GLUTs 1-4, and these are the "classic" glucose transporters as their primary function is to transport glucose across cell membranes (note that GLUT14 is now also considered part of Class I based largely on sequence information, but its function is yet to be determined). Class II consists of GLUTs 5, 7, 9, and 11, and these transporters, most notably GLUT5, are actually fructose-specific transporters. Class III consists of GLUTs 6, 8, 10, 12, and HMIT1 (H^+-myoinositol transporter) but little is known about the function of these transporters.

The best characterised class of GLUTs in mammals is Class I and these have also been investigated to some extent in teleost fish and birds. The following descriptions are primarily based on the mammalian literature but the tissue distributions for other vertebrate groups are shown in Table 1.1. GLUT1 (SLC2A1) is known as the erythrocyte-type transporter and the

protein is most highly expressed in the erythrocytes and brain, although both the protein and mRNA can be found in a number of tissues. This transporter plays an essential role in transporting glucose across the blood-brain barrier and has been hypothesised to increase the glucose carrying capacity of the blood by allowing it to equilibrate between the serum and erythrocyte cytoplasm (Mueckler and Thorens, 2013). GLUT2 (SLC2A2) is primarily expressed in the liver, pancreatic beta-cells, kidney, and intestine and is a low affinity transporter, meaning that the rate of transport is highly dependent on glucose concentrations. It is also bi-directional, which allows the liver and intestine to not only take up glucose, but to release it into the blood as well. In the pancreas, GLUT2 is believed to act as a glucose sensor, signaling the beta cells to release insulin at high blood concentrations. GLUT3 (SLC2A3) has a higher affinity for glucose than any of the other Class I transporters and the protein is most abundant in tissues with high glucose requirements, namely the brain and testis, although like GLUT1, the mRNA is ubiquitously expressed. GLUT4 (SLC2A4) is an insulin-responsive transporter that is primarily expressed in adipose tissue, skeletal muscle, cardiac muscle, and brain (as well as the gill in teleost fish). This transporter is typically stored within intracellular vesicles and is inserted into the plasma membrane in response to activation of the insulin receptor, which then allows increased uptake of glucose into the cells (see review by Huang and Czech, 2007). As previously mentioned, GLUTs have been characterised to some extent in teleost fish but only one study has been conducted in elasmobranchs. This study was performed by Balmaceda-Aguilera *et al.* (2012) and they were able to show that the GLUT1 protein is expressed in the glial cells of the blood brain barrier in two species of catshark (*Schroedericthys chilensis* and *Scyliorhinus canicula*). The only other mention of GLUTs in elasmobranchs came in a paper by Anderson *et*

al. (2002) who suggested that these fish may lack the insulin-responsive GLUT4 because of the delayed hypoglycaemic response observed in response to insulin injections.

The second family of glucose transporters investigated in this thesis is the sodium-glucose cotransporter family (SGLTs; SLC5A) that can utilize the energy stored in sodium gradients to move glucose against its gradient in secondarily active transport. Again, there are existing reviews on this group of transporters that provide more detailed descriptions (Wood and Trayhurn, 2003; Scheepers *et al*., 2004). There are 12 SGLT family members and these are divided into two subfamilies. Subfamily 1A consists of SGLTs 1,2, 4-6, SAAT1 (sodium-dependent amino acid transporter), and SMIT (sodium myoinositol cotransporter) while subfamily 1B consists of NIS (Na^+/I^- symporter), SMVT (sodium-dependent multivitamin transporter), CHT (choline transporter), AIT/SMCT1 (apical iodide transporter/sodium monocarboxylate cotransporter 1, and SMCT2 (sodium monocarboxylate cotransporter 2). For the purposes of this thesis, however, we will focus solely on SGLTs 1 and 2. SGLT1 (SLC5A1) is a high affinity transporter expressed primarily in the apical membranes of the intestine and kidney. In the intestine, powered by the Na^+ gradient created by basolateral Na^+/K^+-ATPase, it acts to take up glucose from the lumen that is then released into the blood via GLUT2. SGLT2 (SLC5A2) on the other hand is a low affinity transporter and it is ubiquitously expressed but the highest levels are found in the kidney. Here, it acts to reabsorb most of the glucose from the glomerular filtrate, the rest being taken up by SGLT1. SGLTs are not as well characterised in fish as are GLUTs but they have been identified in the kidney and intestine of teleosts (Freire *et al*., 1995; Maffia *et al*., 1996) and the kidney of elasmobranchs (Althoff *et al*., 2006; 2007).

Table 1.1 Distribution of Class I *glut* mRNA in different vertebrate classes based on previous literature.

Transporter	Common Name	Functional Information	*Elasmobranchs*	Teleosts	Birds	Mammals	References
SLC2A1	GLUT1	Erythrocyte-type	*Brain*	All Tissues Examined	All Tissues Examined	All Tissues Examined	Welch et al. (2013); Sweazea and Braun (2006); Kono et al. (2005); Balmaceda-Aguilera et al. (2012); Bell et al. (1990); Hall et al. (2014)
SLC2A2	GLUT2	Low-affinity; pancreatic glucose sensor	*?*	Kidney, Liver, Muscle, Brain, Intestine	Kidney Liver	Kidney, Liver, Intestine	Welch et al. (2013); Terova et al. (2009); Hall et al. (2006); Panserat et al. (2001); Bell et al. (1990); Kono et al. (2005); Hall et al. (2014)
SLC2A3	GLUT3	High affinity	*?*	All Tissues Examined	All Tissues Examined	All Tissues Examined	Welch et al. (2013); Bell et al. (1990); Hall et al. (2005); Zhang et al. (2003); Kono et al. (2005); Hall et al. (2014)
SLC2A4	GLUT4	Insulin-responsive	*?*	Kidney, Heart, Gill, Muscle, Intestine, Adipose Tissue	Not Present	Heart, Muscle, Brain, Kidney, Adipose Tissue	Welch et al. (2013); Bell et al. (1990); Kono et al. (2005); Capilla et al. (2004); Planas et al. (2000); Hall et al. (2014)

1.4 The Role of Insulin in Fish

In mammals, insulin is a hypoglycaemic peptide hormone that is released from the beta cells of the pancreatic islets in response to an increase in blood glucose concentrations. It acts by stimulating an increase in the concentration of GLUTs in the plasma membrane, thus enhancing glucose uptake by the tissues, and also plays other roles such as stimulating glycogenesis and inhibiting glycogenolysis. In fish, few studies have investigated the effects of insulin, however the available data suggest that it acts in a similar manner as it does in mammals (see review by Mommsen and Plisetskaya, 1991). In brown trout (*Salmo trutta*), plasma insulin levels increased within 2 hours post-feeding corresponding with a decrease in plasma glucose (Navarro *et al*., 1993). Similarly, an injected glucose-load stimulated insulin release in brown trout within one hour, leading to a subsequent decrease in plasma glucose (Blasco *et al*., 1996). On the other hand, when these fish were fasted, plasma insulin levels decreased (Navarro *et al*., 1992) and this phenomenon was also observed in juvenile sea bass (*Dicentrarchus labrax*; Gutierrez *et al*., 1991). In Persian sturgeon (*Acipenser persicus*), insulin levels trended towards a slight decrease over 4 weeks of starvation, but the decrease was not significant (Yarmohammadi *et al*., 2012). One issue with the majority of studies measuring insulin levels in fish, however, is the need to use heterologous assays where the antibodies may not bind as efficiently to the piscine insulin peptide (piscine insulins share only about 60 and 75% identity with mammalian insulin A-chains and B-chains, respectively) or could bind other hormones (eg. insulin-like growth factors), possibly skewing the results. Thus, the results of these studies may need to be interpreted cautiously, particularly in terms of the absolute values for plasma insulin concentrations.

Other studies have investigated the direct effects of insulin injections in fish. In river lamprey (*Lampetra fluviatilis*; Larsen, 1976) and Atlantic hagfish (*Myxine glutinosa*; Emdin,

1982), mammalian insulin decreased plasma glucose within 8 hours of injection and these levels remained low through 24 hours. In common carp (*Cyprinus carpio*) infused with mammalian insulin over a 90 min period, plasma glucose decreased significantly within the first hour and again these levels remained low through 24 hours at which time plasma glucose began to increase again (Van Raaij *et al*., 1995). The differences in the time courses of insulin action could be due to the location of the injection, as the lamprey and hagfish were injected intramuscularly and thus the increase in plasma insulin levels was delayed relative to the carp that were infused through the dorsal aorta. On the other hand, the differences could simply be species-specific and have more to do with the life histories of the fish rather than the nature of the injection itself, or with spurious/non-meaningful responses to mammalian insulin. In condrichthyans (elasmobranchs and holocephalans), insulin injections have also been shown to induce hypoglycaemia. Notably, in the spiny dogfish (*Squalus acanthias*), administration of either bovine or dogfish insulin resulted in immediate decreases in plasma glucose that became significant within 6 hours (Patent, 1970; deRoos and deRoos, 1979; deRoos *et al*., 1985) and this decrease was also observed in the ratfish (*Hydrolagus colliei*; Patent, 1970). Thus, at least in this species, mammalian insulin may serve as a suitable experimental substitute for homologous insulins. In *Scyliorhinus canicula*, however, plasma glucose did not decrease until 12 hours post-injection with homologous insulin (Anderson *et al*., 2002). It should be noted that the studies on *S. acanthias* used much higher doses of insulin which may account for the earlier decreases in plasma glucose relative to that observed in *S. canicula*. However, the Anderson *et al*. (2002) study did not sample between 4 and 12 hours so it is unknown whether decreases in glucose started to occur earlier on in the experiment.

Fewer studies have investigated the effects of insulin on GLUTs in fish, but those that do exist support a role similar to that observed in mammals (illustrated in Fig. 1.1). Capilla *et al*. (2004) isolated GLUT4 from salmon (*Oncorhynchus kisutch*) and showed that insulin stimulated both the insertion of the protein into the membrane of adipocytes and an increase in glucose uptake by the cells. Similarly, in brown trout (*S. trutta*), insulin increased GLUT4 protein content in red muscle (Diaz *et al*., 2007). This study also indicated that fasting (during which time insulin levels are expected to be low) caused a decrease in GLUT4 protein levels. Lastly, both *glut1* and *glut4* mRNA levels were shown to increase in response to insulin treatment in rainbow trout (*Oncorhynchus mykiss*) muscle cells (Diaz *et al*., 2009). All of these results mimic what has been observed in the mammalian literature suggesting that the function of this hormone is relatively conserved among vertebrates. However, there are no studies on the effects of insulin injection on GLUT expression in elasmobranchs.

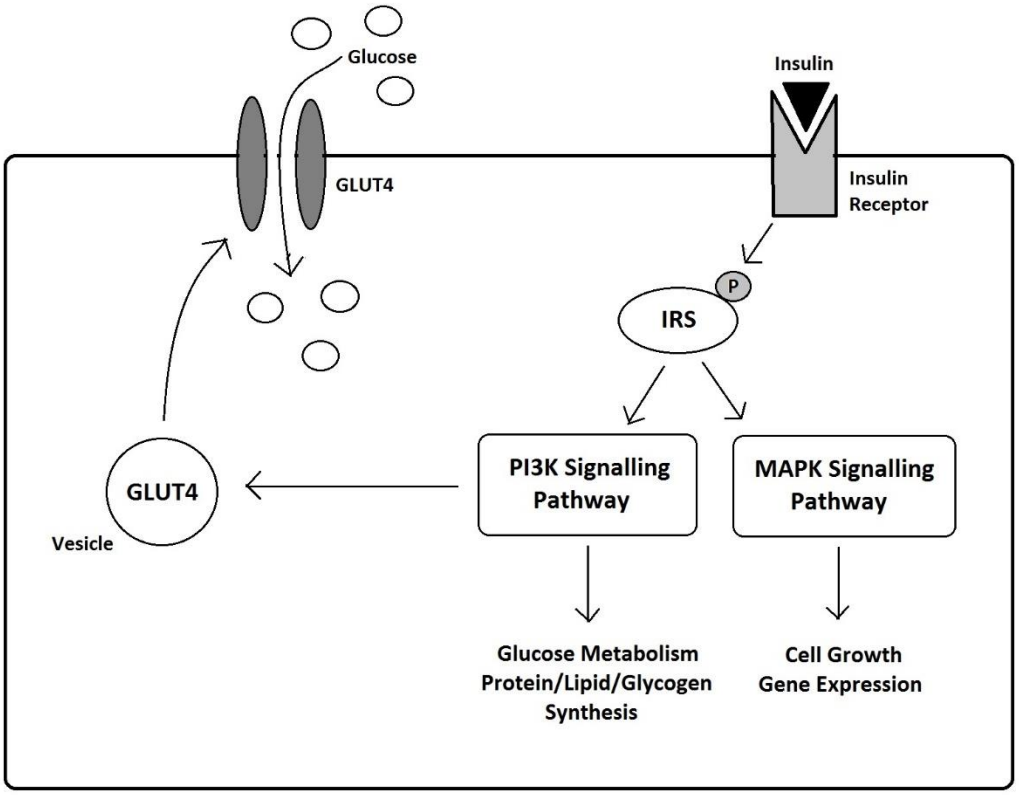

Figure 1.1 When the mammalian insulin receptor is bound by insulin, it phosphorylates insulin receptor substrates (IRS) which then activate the mitogen-activated protein kinase (MAPK) and phosphoinositide-3 kinase (PI3K) pathways leading to changes in gene expression and metabolism, as well as the translocation of glucose transporter 4 (GLUT4) to the plasma membrane.

1.5 Thesis Objectives

Because of the paucity of information on glucose homeostasis and transport in elasmobranchs, this thesis employed a combination of *in vivo* and *in vitro* experiments, along with a number of biochemical and molecular techniques, to determine some of the factors that influence glucose transport and metabolism in elasmobranchs, using the North Pacific spiny dogfish (*Squalus suckleyi*)[2] as a model. The central hypothesis tested in this thesis is that, in opposition to the prevailing view, glucose homeostasis in elasmobranchs is a dynamically regulated process in which glucose transporters are present and play a major role. Thus, specifically for the dogfish, I predict that in the face of various endogenous and exogenous challenges, they will show regulation of glucose transporter expression in many of their tissues, and that they will regulate circulating glucose levels. Furthermore, since the rectal gland is one of the few tissues in dogfish that strictly requires glucose as a metabolic fuel (for NaCl secretion), I predict that it should be especially sensitive to the inhibition of glucose transport as it cannot use other fuels, as well as potentially exhibit novel patterns in glucose transporter expression.

To this end, Chapter 2 focuses first on identifying whether glucose transporters of the GLUT family are present in elasmobranchs, and if so, to recreate a phylogenetic tree to determine their relationships to other known vertebrate GLUTs. The chapter further determines in which tissues they are expressed, and finally how the biotic factor of feeding might regulate *glut* mRNA expression. In Chapter 3, I tested the prediction that the additional biotic

[2] Note that this species is listed as 'of least concern' by the IUCN and a recent assessment by Fisheries and Oceans Canada concluded that there is not a conservation concern for the Pacific populations. Thus, the use of animals for this thesis is not expected to have a profound effect on population status.

perturbations of glucose-loading and insulin administration would lead to upregulation of *gluts* to allow for glucose homeostasis through an increase in glucose uptake and storage (as glycogen) by the tissues. The focus of Chapter 4 was on glucose uptake by the rectal gland using an *in vitro* perfusion method and it was based on the observation that the gland is in fact glucose-dependent. In this study, the gland was perfused with glucose transport inhibitors which I predicted should significantly reduce chloride secretion.

Lastly, Chapter 5 investigated the effects of the abiotic factor of changes in salinity to determine whether these could indirectly impact glucose homeostasis. This particular set of conditions was chosen since the literature is not clear on whether the rectal gland functions mostly in times of external salt loading, or salt-loading via feeding and diet (see introduction in Chapter 4). If increased rectal gland function, and hence increased glucose-utilization, occur at higher salinities, I would predict that there would be an increase in *glut* transcript levels. Through these four data chapters, this thesis provides a detailed examination of glucose transport and homeostasis in elasmobranchs under different physiological and environmental conditions, something that had yet to be studied in this evolutionarily important group of fish. Further, it provides insights into the evolution of glucoregulatory mechanisms in vertebrates.

CHAPTER 2

Phylogenetic analysis and tissue distribution of elasmobranch glucose transporters and their response to feeding

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2.1 Abstract

Elasmobranch diets consist of high quantities of protein and lipids, but very low levels of carbohydrates including glucose. Reflecting this diet, most tissues use lipids and ketone bodies as their main metabolic fuel. However, the rectal gland has been shown to be dependent on glucose as a fuel, so it was hypothesized that glucose transporters (GLUTs) would be present and upregulated in the gland during times of activation (e.g., following a meal). In this study, transcriptomes were searched to identify putative Class I GLUTs in three elasmobranchs and a holocephalan and the sequences were used to reconstruct a Bayesian phylogeny. It was determined that each of the four species possessed three of the four Class I GLUT sequences, but the identities of the isoforms present in each species differed between the elasmobranchs (GLUTs 1, 3, and 4) and the holocephalan (GLUTs 1, 2, and 3). qPCR was then used to measure mRNA levels of these *gluts* in the rectal gland, liver, intestine, and muscle of fed and starved spiny dogfish (*Squalus suckleyi*). The rectal gland data showed higher mRNA levels of *glut4* in the starved relative to the fed fish. In the muscle, both *gluts 1* and *4* were significantly elevated at 24 h post-feeding, as was the case for *glut4* in the liver. In the intestine on the other hand, *glut4* was significantly elevated by 6 h post-feeding, remaining elevated through 48 h. It is suggested that GLUT4 has taken on the role of GLUT2 in elasmobranchs as the expression patterns observed in the liver and intestine are representative of GLUT2 in other vertebrates.

2.2 Introduction

Elasmobranchs are a primarily carnivorous group of vertebrates obtaining little glucose from their diet. They also have very low hepatic glycogen stores (0.5-2% compared to 6-8% in mammals and 2-6% in teleosts), possibly due to the accumulation of lipids in the liver (deRoos and deRoos, 1979). These lipids are used for buoyancy and to produce ketone bodies, such as acetoacetate and β-hydroxybutyrate, which are the preferred fuel for many elasmobranch tissues making these species much less reliant on glucose than other vertebrates species (Ballantyne, 1997). One exception in elasmobranchs is the rectal gland, a salt secreting organ responsible for eliminating excess salts from the body. Walsh *et al*. (2006) demonstrated that among a variety of metabolic fuels tested, glucose was the only one able to sustain gland secretion on its own. These findings led to the hypothesis that glucose transporters (GLUTs) are upregulated in the gland upon activation, for instance following a meal, or exposure to a hypersaline environment. However, a thorough literature search revealed that very little is known about elasmobranch glucose transporters in general. A study by Balmaceda-Aguilera *et al*. (2012) showed that the GLUT1 protein is expressed in the brain, and there has been speculation about the presence of GLUT2 and the absence of GLUT4 in elasmobranchs (Anderson *et al*., 2002).

The transporters investigated in the current study are GLUTs 1-4, which compose Class I of the facilitated diffusion glucose transporters (solute-carrier family SLC2A) and are the best characterised GLUTs in other vertebrates (see reviews by Scheepers *et al*., 2004; Thorens and Mueckler, 2010; Wood and Trayhurn, 2003; Zhao and Keating, 2007). Briefly, GLUT1 is a ubiquitously expressed transporter that is responsible for the general uptake of glucose by tissues. In mammals, the protein is found in all tissues but is most highly expressed in the brain and in the erythrocytes, and thus it has sometimes been referred to as the erythrocyte-type

transporter. The mRNA for GLUT1 is ubiquitously expressed across tissues in all vertebrate groups examined to date. GLUT2 is a bidirectional transporter and the protein and mRNA are expressed highly in the liver, intestine, and kidney, and also in the pancreatic β-cells where it acts as a glucose sensor to signal the release of insulin. GLUT3 is a high affinity transporter whose protein is abundant in tissues with high glucose needs, namely the brain and testis, whereas the mRNA is expressed ubiquitously across tissues. GLUT4 is the insulin-responsive transporter which is stored within the cell and transported to the membrane when signalled by insulin. In mammals, the protein and mRNA are expressed highly in the heart, brain, skeletal muscle, and adipose tissue, whereas teleosts also highly express this transporter in the gills and birds are not believed to express this transporter at all, as they rely more on fructose than on glucose and thus require other transporter types, namely GLUT5.

The presence of these four transporters has been confirmed in most vertebrate groups, but has yet to be investigated in the cartilaginous fishes (elasmobranchs and holocephalans). Thus, the transcriptomes for three elasmobranchs, the little skate (*Leucoraja erinacea*), the North Pacific spiny dogfish (*Squalus suckleyi*), and the small-spotted catshark (*Scyliorhinus canicula*), and one holocephalan, the elephantfish (*Callorhinchus milii*), were searched to find potential GLUT sequences that could be used to construct the phylogeny of the transporters, as well as to assess which transporters are present in the cartilaginous fishes. Three putative GLUT sequences were found in each transcriptome, but the transporter types differed between the elasmobranchs and the holocephalan. Once identity of the sequences was confirmed, they were used to determine tissue distribution of each transporter as well as measure their mRNA levels in the rectal gland, liver, muscle, and intestine of fed and fasted spiny dogfish.

2.3 Materials and Methods

2.3.1 Animals and Feeding

Adult male North Pacific spiny dogfish (*Squalus suckleyi*) ranging in weight from 1.5-2.5 kg were obtained by angling from Barkley Sound, British Columbia (Canada) and transferred to Bamfield Marine Sciences Centre in August 2013 and June 2014. The sharks were maintained in a 155,000 L circular tank with running seawater and aeration. Following a two-week acclimation period, dogfish were fed a ration (2.5% body weight) of frozen hake every 3rd day. One group of fish was transferred to a separate tank following a meal where they were fasted for 7 days, while other groups were fasted for 7 days, fed, and then sacrificed at 6 h, 24 h, or 48 h post-feeding (N=7) by MS-222 overdose (1 g L^{-1}) and subsequent cervical dislocation. Fish were randomly allotted to each experimental group. Plasma, rectal gland, intestine, liver, and muscle samples were frozen in liquid nitrogen and then stored at -80°C for further analysis. Plasma glucose was measured using the Infinity Hexokinase Reagent according to the manufacturer's instructions (Thermo Scientific) and all standards contained 300 mM urea to account for any effects of plasma composition. All experiments were conducted in accordance with guidelines of the Canadian Council of Animal Care, and under approvals from the Animal Care Committees of the University of Ottawa and Bamfield Marine Sciences Centre.

2.3.2 Phylogenetic Analysis

Access to the skate (*Leucoraja erinacea*), elephantfish (*Callorhinchus milii*), and catshark (*Scyliorhinus canicula*) transcriptomes was gained online through SkateBase (http://skatebase.org; Wyffels *et al*., 2014; see Table 2.1 for Contig numbers), whereas the dogfish transcriptome was generously provided by Dr. Greg Goss (University of Alberta).

Mammalian and teleost sequences obtained from Ensembl (http://www.ensembl.org) were used to search each transcriptome (blastn) for potential elasmobranch and holocephalan GLUT sequences. These were then aligned with known GLUT sequences from other species using MUSCLE (Edgar, 2004) and the appropriate nucleotide substitution model (GTR + G + I) identified using jModelTest (Darriba *et al*., 2012; Guindon and Gascuel, 2003). A phylogeny was then constructed using a Bayesian framework (BEAST; Drummond *et al*., 2012). The analysis was run for ten million generations with a thinning of 1000 and the first 2500 trees were discarded as burn-in.

2.3.3 Tissue Distribution

Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's instructions, treated with DNase I (Invitrogen), and used as a template for reverse transcription (RT). The RT reactions combined 2 μg RNA (quantified spectrometrically by NanoDrop) with 375 ng random hexamers (Integrated DNA Technologies), 125 ng oligo dTs (IDT), and dNTPs (Invitrogen; 0.5 mM final concentration) and the mixture was incubated at 65°C for 5 min. The reaction was then chilled on ice for 5 min and first-strand reaction buffer (Invitrogen; 1x final concentration), dithiothreitol (5 mM final concentration), RNase Out (Invitrogen; 0.5 μL), and nuclease-free water (0.5 μL) were added. The mixture was incubated at 42°C for 2 min and then combined with Superscript II reverse transcriptase (Invitrogen; 1 μL) to a total volume of 20 μL. Lastly, the reaction was incubated at 42°C for 50 min and inactivated at 72°C for 15 min.

Following RT, the cDNA was amplified by adding 1 μL of diluted template (1:5) to a 25 μL total reaction volume containing PCR reaction buffer (Denville Scientific; 1x final concentration), dNTPs (Invitrogen; 0.2 mM final concentration), 0.15 μL Choice Taq (Denville

Scientific), and gene-specific sense and antisense primers (IDT; 0.2 μM final concentrations). Primers for each gene were derived from sequences found in the dogfish transcriptome. The cycling parameters were 94°C for 30 s followed by 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30s and concluding with 15 min at 72°C. The products were then visualized on a 1.5% agarose gel.

2.3.4 qPCR

For real-time PCR, the cDNA template was prepared as described above and 1 μL of the diluted template was added to a 12.5 μL total reaction volume containing 6.25 μL Rotor-Gene SYBR Green PCR master mix (Qiagen) and 200 nM each gene-specific sense and antisense primer (Table 2.2). All primer sets were tested by first visualizing the product on a 1.5% agarose gel and having the products sequenced by Genome Quebec. This was followed by a melt curve analysis to show amplification of a single product and a calculation of the efficiency of the standard curves. We used Norma-Gene (Heckmann *et al*., 2011) to normalise the rectal gland samples as both EF1α and ubiquitin changed with our treatments in this tissue. Relative mRNA levels for intestine, muscle, and liver were normalised to *S. suckleyi* ubiquitin levels. Norma-Gene could not be used because not enough genes were run in these tissues but ubiquitin levels appeared to be constant across our treatments (ie. levels were not statistically different from one another). Fluorescence was detected using a Rotor-Gene Q Real Time PCR cycler (Qiagen) with the following cycling parameters: 95°C for 5 min followed by 40 cycles of 95°C for 5 s and 60°C for 10 s. All reactions were run in duplicate and relative mRNA levels were calculated using the delta C_t method. One-way ANOVAs and Holm-Sidak post-hoc tests were used to compare mRNA levels between treatments. When the equal variance test failed, a one-way ANOVA on Ranks with a Dunn's post-hoc test was conducted.

Table 2.1 SkateBase contig numbers for putative GLUT sequences in the Elephantfish (*Callorhinchus milii*), little skate (*Leucoraja erinacea*), and catshark (*Scyliorhinus canicula*).

	Elephantfish	**Little Skate**	**Catshark**
GLUT1	Contig33132 Contig77633	Contig13066	Contig15075
GLUT2	Contig44099 Contig3400 Contig80556	----------	----------
GLUT3	Contig17067 Contig33132	Contig90454	Contig16627
GLUT4	----------	Contig64419	Contig103256 Contig12163 Contig87962

Table 2.2 Glucose transporter (GLUT) primer sequences for the spiny dogfish (*Squalus suckleyi*).

Gene	Forward Primer	Reverse Primer
ubiquitin	GTGGTGCCAAGAAGAGGAAG	GTGACTGGCCATGAACACAC
glut1	AGTCGGATTGTTCGTCAACC	GCCGGAGTAGATGCCAATAA
glut3	GAGCCGGCATACTTGGATAG	GGACGGGATCCATAATCTCA
glut4	GGAGGAAAAGAGGAGGATGG	GGTGATGTAGACCGGCTGTT

2.4 Results

2.4.1 Phylogenetic Analysis and Tissue Distribution

Three sequences in the dogfish, little skate, catshark, and elephantfish transcriptomes were identified as possible GLUT sequences. These were used to recreate a phylogenetic tree which revealed the putative identities of each sequence (Fig. 2.1). A GLUT1-like sequence was identified in all four transcriptomes. The amino acid sequences were 77-84% identical to human GLUT1 and contained the functional motifs characteristic of Class I transporters. A GLUT3-like sequence was also found in all four transcriptomes with amino acid sequences ranging from 60-67% identical to human GLUT3. A GLUT2-like sequence was found solely in the holocephalan transcriptome and the amino acid sequence was 62% identical to human GLUT2. Lastly, a GLUT4-like sequence was found in the dogfish and skate transcriptomes which were 64-69% identical to human GLUT4. The only full sequence belonged to the little skate and it contained the PSLL and TELEY C-terminus motifs (although the latter contained one substitution) and the FQQI N-terminus motif (again, with a single substitution) characteristic of vertebrate GLUT4s. We obtained the majority of the dogfish GLUT4 sequence and this also contained the characteristic C-terminus motifs. We were able to clone *gluts 1*, *3*, and *4* in the dogfish in order to determine in which tissues the mRNA was being expressed (Fig. 2.2). *gluts 1* and *3* showed a ubiquitous expression, with relatively even amounts present in each tissue. *glut4*, however, was slightly more tissue-specific. It was expressed highly in the brain, heart, muscle, gill, and interrenal gland with lower levels present in the liver and intestine. Attempts were made to isolate a *glut2*-like gene in the dogfish despite the absence of such a sequence. Both regular and consensus primer sets were designed using the holocephalan sequence, teleost sequences, and mammalian sequences but all attempts to isolate the gene were unsuccessful.

2.4.2 Plasma and Tissue Analysis

Plasma glucose concentrations were measured and a slight decrease from 5.54 ± 0.39 SEM in the control fish to 4.98 ± 0.16 SEM at 48 h post-feeding was observed but the change was not significant (Fig. 2.3). qPCR was then used to measure the levels of *glut 1, 3* and *4* mRNA in the rectal gland, intestine, liver, and muscle of fed and fasted dogfish. In the rectal gland, *glut1* showed a trend toward a decrease in mRNA levels at 48 h post-feeding but the change was not significant (Fig. 2.4A). *glut4* on the other hand had significantly lower mRNA levels at both 24 and 48 h post-feeding (Fig. 2.4B; One-way ANOVA; $p<0.05$). In the liver and intestine, *glut1* mRNA levels were not significantly different across time points, however, *glut4* was significantly higher at 24 and 48 h post-feeding in the liver relative to the fasted fish (Fig. 2.5A; One-way ANOVA; $p<0.05$). In the intestine, there was a 5-fold increase in *glut4* mRNA levels by 6 h post-feeding and this was maintained through the 24 and 48 h time points (Fig. 2.5B; One-way ANOVA; $p<0.05$). In the muscle, there was a significant increase in both *glut1* and *glut4* mRNA by 24h post-feeding and this returned to fasted levels by 48 h (Fig. 2.6; One-way ANOVA; $p<0.05$). *glut3* did not change significantly in any of the tissues examined.

Figure 2.1 Phylogeny of the glucose transporters in vertebrates using a Bayesian framework. Numbers indicate posterior probabilities; stars show the position of the cartilaginous fish on the tree; scale bar represents the mean number of nucleotide substitutions per site.

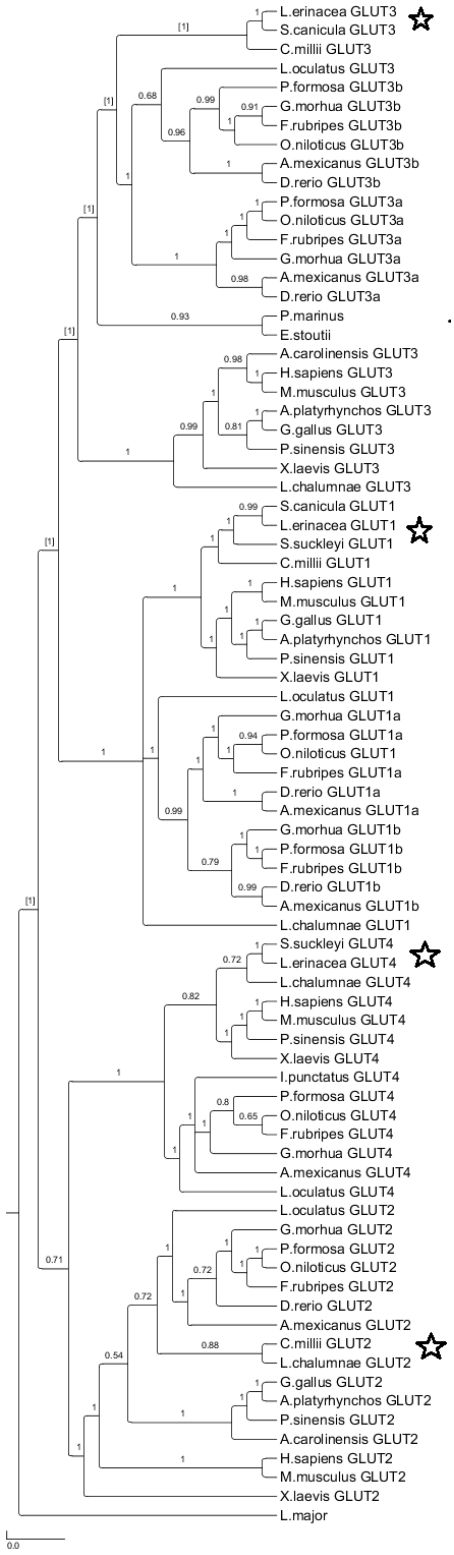
L.erinacea GLUT3
S.canicula GLUT3
C.millii GLUT3
L.oculatus GLUT3
P.formosa GLUT3b
G.morhua GLUT3b
F.rubripes GLUT3b
O.niloticus GLUT3b
A.mexicanus GLUT3b
D.rerio GLUT3b
P.formosa GLUT3a
O.niloticus GLUT3a
F.rubripes GLUT3a
G.morhua GLUT3a
A.mexicanus GLUT3a
D.rerio GLUT3a
P.marinus
E.stoutii
A.carolinensis GLUT3
H.sapiens GLUT3
M.musculus GLUT3
A.platyrhynchos GLUT3
G.gallus GLUT3
P.sinensis GLUT3
X.laevis GLUT3
L.chalumnae GLUT3
S.canicula GLUT1
L.erinacea GLUT1
S.suckleyi GLUT1
C.millii GLUT1
H.sapiens GLUT1
M.musculus GLUT1
G.gallus GLUT1
A.platyrhynchos GLUT1
P.sinensis GLUT1
X.laevis GLUT1
L.oculatus GLUT1
G.morhua GLUT1a
P.formosa GLUT1a
O.niloticus GLUT1
F.rubripes GLUT1a
D.rerio GLUT1a
A.mexicanus GLUT1a
G.morhua GLUT1b
P.formosa GLUT1b
F.rubripes GLUT1b
D.rerio GLUT1b
A.mexicanus GLUT1b
L.chalumnae GLUT1
S.suckleyi GLUT4
L.erinacea GLUT4
L.chalumnae GLUT4
H.sapiens GLUT4
M.musculus GLUT4
P.sinensis GLUT4
X.laevis GLUT4
I.punctatus GLUT4
P.formosa GLUT4
O.niloticus GLUT4
F.rubripes GLUT4
G.morhua GLUT4
A.mexicanus GLUT4
L.oculatus GLUT4
L.oculatus GLUT2
G.morhua GLUT2
P.formosa GLUT2
O.niloticus GLUT2
F.rubripes GLUT2
D.rerio GLUT2
A.mexicanus GLUT2
C.millii GLUT2
L.chalumnae GLUT2
G.gallus GLUT2
A.platyrhynchos GLUT2
P.sinensis GLUT2
A.carolinensis GLUT2
H.sapiens GLUT2
M.musculus GLUT2
X.laevis GLUT2
L.major
0.0

Figure 2.1

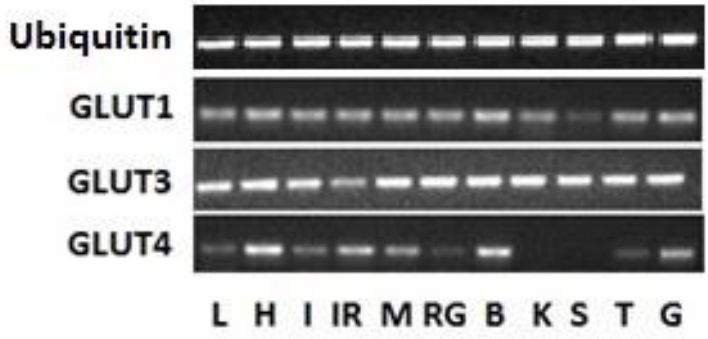

Figure 2.2 Representative tissue distribution of ubiquitin and glucose transporter (*glut*) mRNA in *S. suckleyi*. L- liver, H- heart, I- intestine, IR- interrenal, M- muscle, RG- rectal gland, B- brain, K- kidney, S- spleen, T- testis, G- gill.

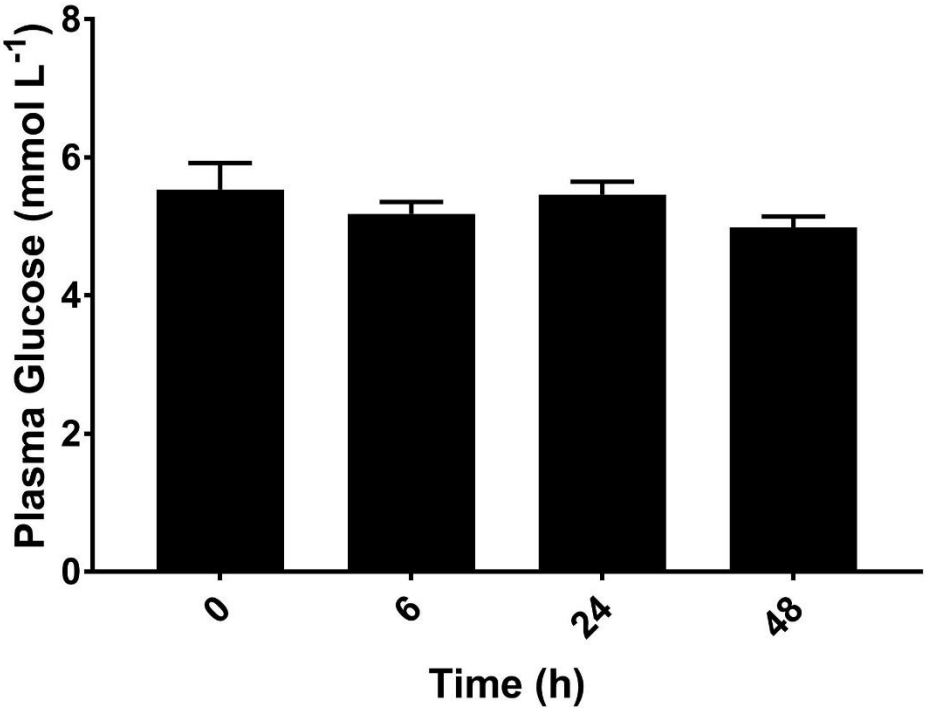

Figure 2.3 Plasma glucose concentrations (mmol L^{-1}) following a meal in *S. suckleyi*. Time 0 fish were starved for 7 days. Data are means + SEM (n=7).

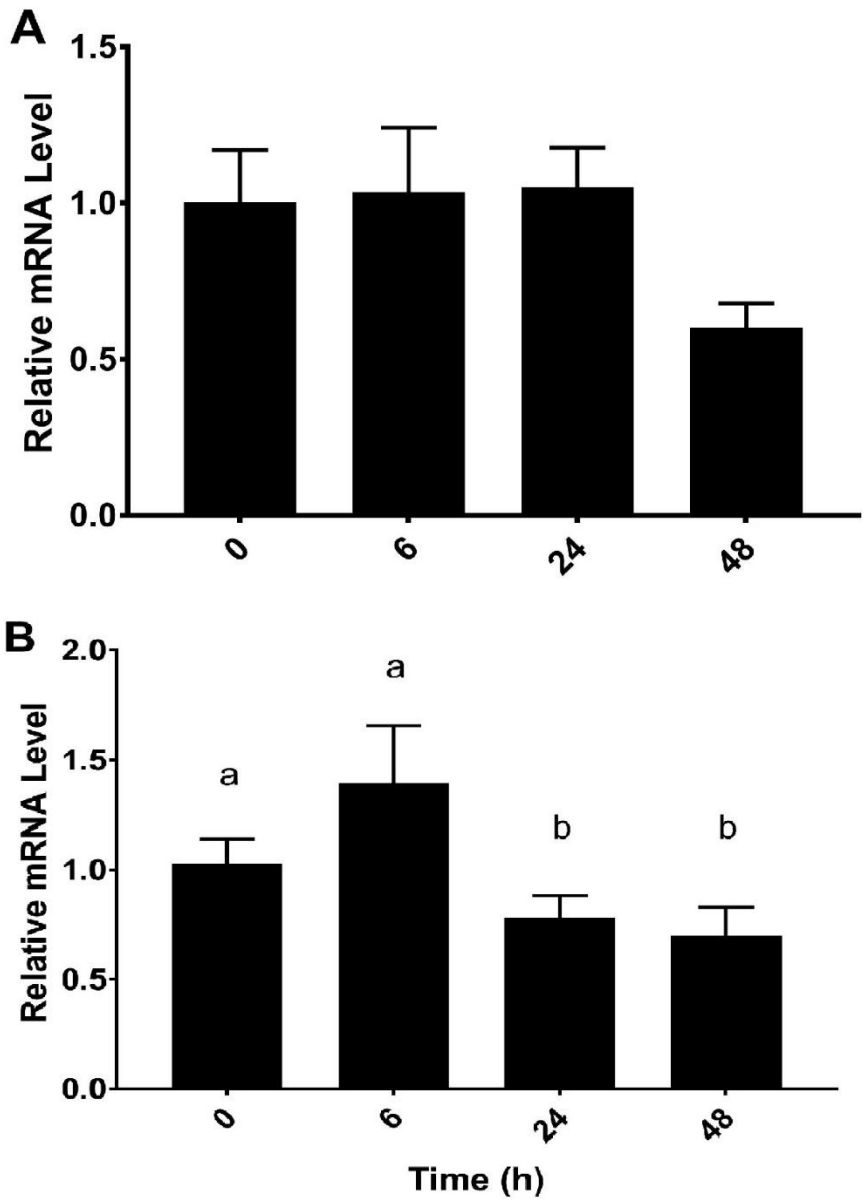

Figure 2.4 *glut1* (A) and *glut4* (B) mRNA levels in the rectal gland of *S. suckleyi* at various times post-feeding. Time 0 fish were starved for 7 days. Data are means + SEM. Different letters indicate significant differences between time points (One-way ANOVA; n=7; $p<0.05$).

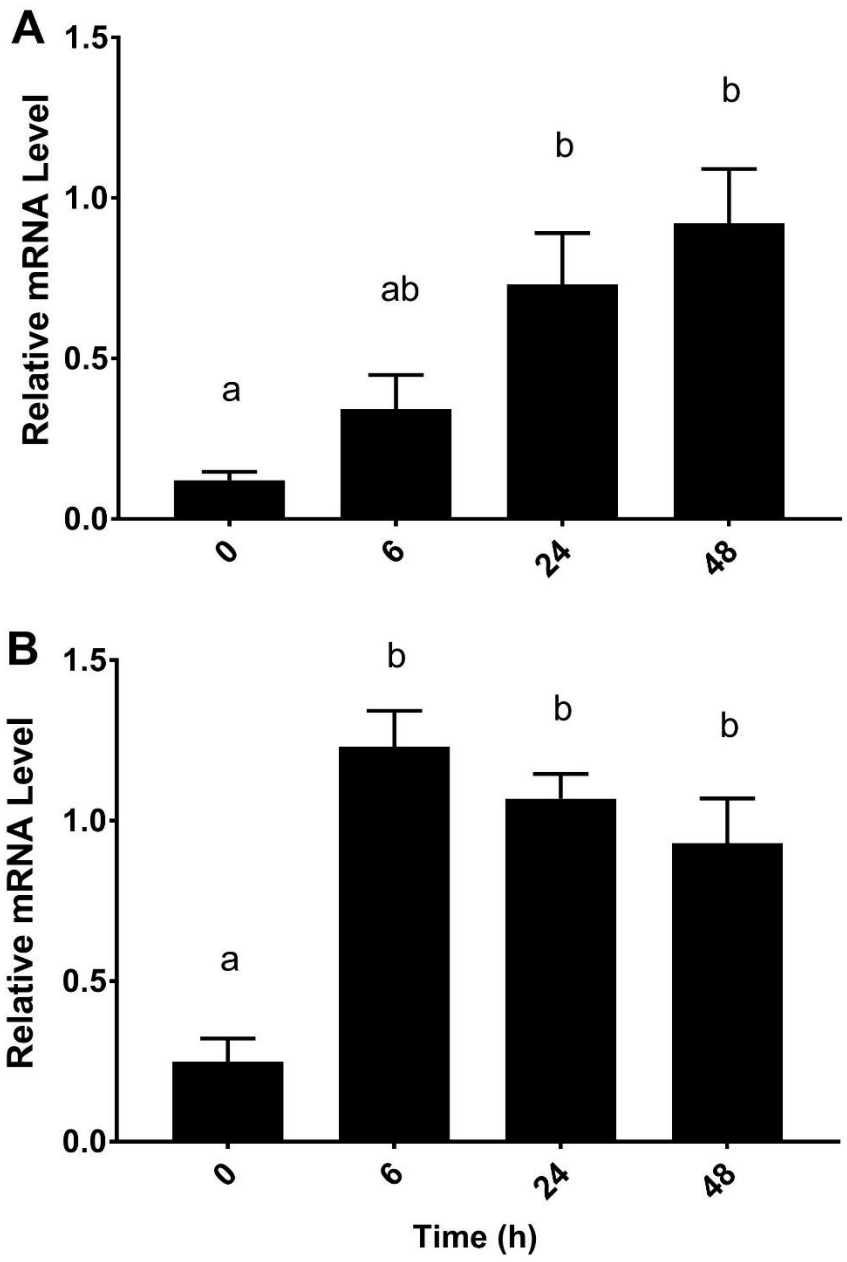

Figure 2.5 *glut4* mRNA levels in the liver (A) and intestine (B) of *S. suckleyi* at various times post-feeding. Time 0 fish were fasted for 7 days. Data are means + SEM. Different letters indicate significant differences between time points (One-way ANOVA; n=7; $p<0.01$).

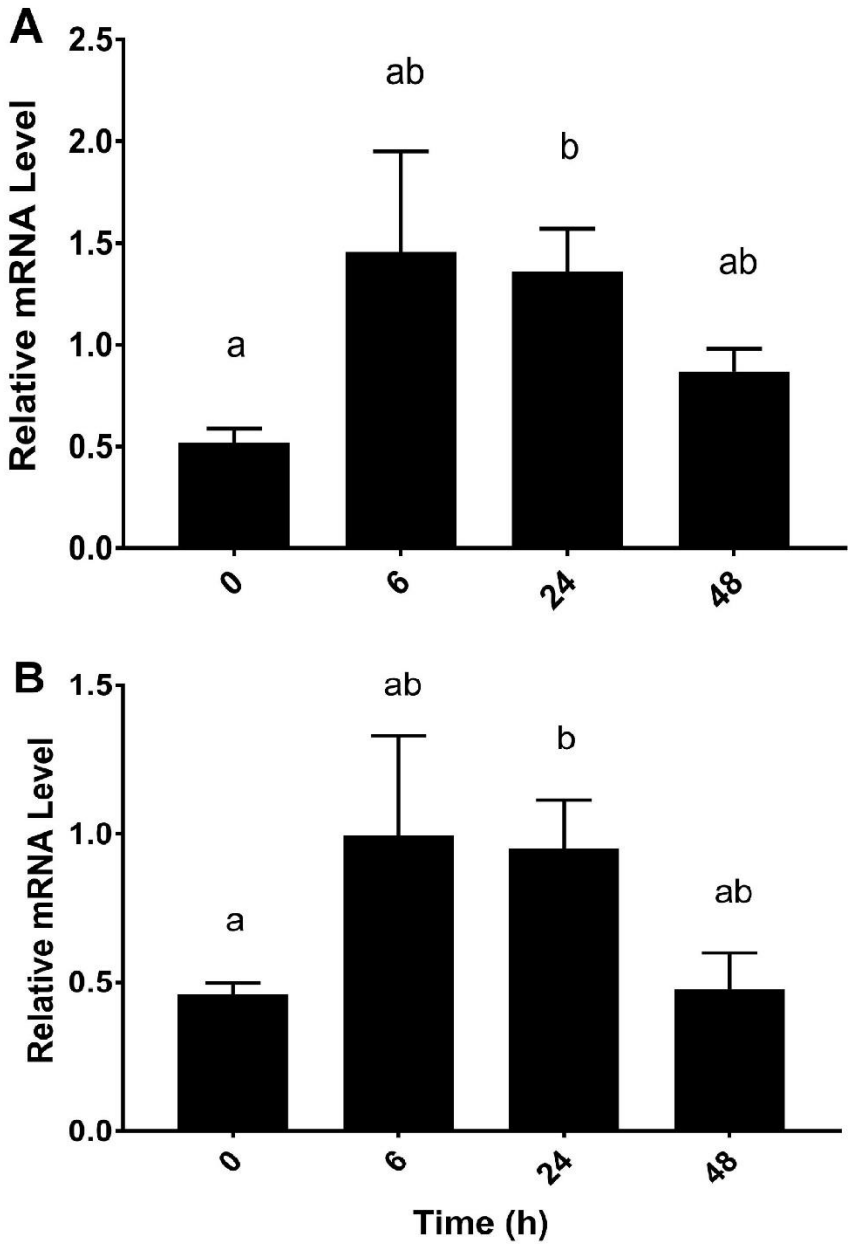

Figure 2.6 *glut1* (A) and *glut4* (B) mRNA levels in the white skeletal muscle of *S. suckleyi* at various times post-feeding. Time 0 fish were starved for 7 days. Data are means + SEM. Different letters indicate significant differences between time points (One-way ANOVA; n=7; $p<0.05$).

2.5 Discussion

This study sought to identify Class I GLUTs from selected elasmobranch and holocephalan species as they have not been thoroughly investigated in cartilaginous fish, but are likely important transporters, particularly in the rectal gland which requires glucose as a metabolic fuel. Three potential GLUT sequences were present in the transcriptomes of both the elasmobranchs and holocephalans but the identities of the transporters differed. Both groups possess GLUT 1- and 3-like sequences, but we were unable to retrieve a GLUT2 sequence for the elasmobranchs and a GLUT4 sequence for the holocephalan, and attempts to isolate these transporters were unsuccessful. This does not provide definitive evidence against the presence of GLUT2 in the elasmobranchs and GLUT4 in the holocephalans as the transporter may have diverged to the point that it is unrecognizable or could have become a pseudogene. However, it may speak to differing selection pressures in the two taxa and reflect differences in the energy metabolism of these fish. For instance, since glucose is of limited importance in these species they could have either lost or developed a new function for one of the transporters. Given that GLUTs 2 and 4 appear to be duplications of one another (as suggested by the phylogeny), it is not implausible that GLUT2 in the holocephalans and GLUT4 in the elasmobranchs took on both roles while the "lost" transporter was converted into a protein that would be of more use to them. It is interesting to note that GLUT4 appears to also have been lost in birds. This loss is believed to be the cause of their apparent insulin resistance and the higher plasma levels of glucose relative to other vertebrates (reviewed by Braun and Sweazea, 2008). It would be interesting to determine whether this is also the case in holocephalans.

The transporter phylogeny presents GLUTs 1 and 3 branching off into one clade while GLUTs 2 and 4 branch off into a separate clade, supporting the 2R genome duplication

hypothesis (Sidow, 1996). However, only one GLUT sequence is known for the cyclostomes. We believe it is likely that they possess a GLUT4-like transporter as both the hagfish and lamprey are responsive to insulin (Emdin, 1982; Larsen, 1976), but the loss or functional divergence of a second transporter is also possible. In addition, most teleosts only possess one each of GLUTs 2 and 4, whereas there are both 'a' and 'b' forms for GLUTs 1 and 3, suggesting that duplicates of these genes may have been lost due to a lack of necessity. Interestingly though, two GLUT4 isoforms have been reported in the muscle of Atlantic salmon (*Salmo salar*; Menoyo *et al*., 2006) which may reflect the tetraploid nature of the salmonids. GLUTs 2 and 4 are very tissue-specific and have definitive roles, whereas GLUTs 1 and 3 are ubiquitously expressed and play a more general role in glucose uptake. Thus, the maintenance of two forms of GLUTs 1 and 3 may allow for greater versatility across tissues and under varying conditions. For instance, studies have shown changes in the expression of these two transporters in response to hypoxia and/or changes in salinity in various tissues (Balmaceda-Aguilera *et al*., 2012; Hall *et al*., 2005; Zhang *et al*., 2003). Unfortunately, all three studies only measured one of the GLUT 1 or 3 transcripts so we cannot determine whether the two forms have varying roles or even be sure that the same transcript was picked up in each tissue. This would be an interesting avenue for future studies.

In addition to reconstructing the phylogeny, the mRNA levels of *gluts 1*, *3*, and *4* in various dogfish tissues were examined and some interesting patterns emerged. The *glut 1* and *3* mRNA was present in all tissues examined which is consistent with what has been observed in a number of other vertebrates. On the other hand, *glut4* was more tissue-specific. The highest mRNA levels were found in the heart, brain, skeletal muscle, and gill, which again is similar to the expression pattern seen in other vertebrates. Interestingly though, *glut4* mRNA was also

expressed in the liver and intestine, tissues that express *glut2* in most other vertebrates. This observation reinforces the idea that GLUT2 may have been lost in elasmobranchs due to a lack of necessity (limited glucose intake) and its role taken over by GLUT4. Further investigation on the characteristics of this transporter in the different tissues is warranted. One interesting observation was the lack of *glut4* mRNA in the elasmobranch kidney as both GLUT2 and GLUT4 are expressed in the kidney in mammals and teleosts. This lack of expression may reflect the limited importance of the elasmobranch kidney in salt secretion due to the presence of the rectal gland.

The final aspect of this study was the measurement of *glut* mRNA levels in specific dogfish tissues in response to feeding and fasting. The four tissues investigated were the rectal gland, liver, muscle, and intestine. In the rectal gland, the mRNA levels for both *gluts 1* and *4* decreased post-feeding, although only the results for *glut4* were significant. Prior to degradation, mRNAs are deadenylated (removal of the poly-A tail) and decapped (removal of the 5' cap). However, previous studies have described the translational repression and storage of mRNAs that are yet to be decapped and this can occur during times of stress (reviewed by Coller and Parker, 2004). Previous transcriptome work in the rectal gland by Deck *et al*. (2013) (see Appendix A) illustrated that mRNA was being stored in fasted glands for a number of genes, most notably Na^+/K^+-ATPase, which we know has lower protein abundance and enzyme activities in starved glands (Dowd *et al*., 2008; Walsh *et al*., 2006). Thus it is possible that during fasting, the rectal gland is storing the mRNA of genes that are essential for immediate activation of the gland upon feeding. Protein levels of GLUTs were unable to be measured in the dogfish as the mammalian antibodies available failed to bind, but since we know that the gland is glucose-

dependent, it would not be surprising that it would store mRNA for the GLUTs so as to allow for rapid protein synthesis upon activation.

In the intestine, there was a significant increase in *glut4* mRNA at 6 h post-feeding and expression remained elevated through the 48 h. The intestine is one of the first tissues to be exposed to the nutrients from the incoming meal, so it is appropriate that it should rapidly upregulate essential transporters. It would be interesting to determine exactly what causes this upregulation as the meal has very little glucose. Also, a significant increase in *glut4* mRNA in the liver was observed, only this increase was slightly delayed relative to the intestine, not being elevated until 24 h post-feeding. However, the liver would not be exposed to the incoming nutrients as early as the intestine, and given the slow digestion rate of most elasmobranchs, the upregulation at 24 h appears to be an appropriately timed response and this could explain the slight decrease in plasma glucose that was observed. What is interesting about the expression patterns observed in the intestine and liver is that this is what would be expected of *glut2* in other vertebrates, possibly providing more evidence for the loss of that transporter in these fish. Lastly, significant increases in both *gluts 1* and *4* in the muscle were observed at 24 h post-feeding and this returned to fasting levels by 48 h. In brown trout (*Salmo trutta*) muscle, fasting has been shown to decrease *glut4* levels whereas insulin increases *glut4* levels (Diaz *et al*., 2007). Furthermore, insulin has been shown to regulate the expression of both *gluts 1* and *4* in the muscle of rainbow trout (Diaz *et al*., 2009). Amino acids are insulinotropic substances in most vertebrates and are believed to be more insulinotropic than glucose in elasmobranchs due to their carnivorous nature. Thus, it is likely that these increases are due to the actions of insulin, but future studies investigating the effects of insulin infusion on GLUT levels in elasmobranch muscle are warranted.

This is only the second report of GLUTs in elasmobranchs (the first being the study by Balmaceda-Aguilera *et al*., 2012 that established the presence of GLUT1 in the shark brain) and the first study to identify and show tissue distribution for multiple GLUTs. It was also determined that elasmobranchs possess a GLUT4-like transporter, contrary to prior beliefs put forth by Anderson *et al*. (2002). This work and the basal position of the elasmobranchs on the vertebrate tree has allowed the provision of valuable insights into the evolution of these transporters, although much work remains to be done. Future studies should aim to characterise the function of each transporter and determine what conditions modulate their expression in the different tissues.

2.6 Acknowledgements

Many thanks to the research coordinator at Bamfield Marine Sciences Centre, Dr. Eric Clelland, and to Rachel Munger for assistance with sampling. Also, thanks to Dr. Greg Goss from the University of Alberta for providing the dogfish transcriptome. This work was funded by an NSERC Discovery Grant to PJW who is also supported by the Canada Research Chair Program.

CHAPTER 3

Effects of glucose and insulin administration on glucose transporter expression in the North Pacific spiny dogfish (*Squalus suckleyi*)

Chapter 3 was performed in collaboration with Dr. Gary Anderson and has been submitted to General and Comparative Endocrinology.

3.1 Abstract

Elasmobranchs (sharks, skates, and rays) in general are a carnivorous group of fish, consuming few carbohydrates. Further, they tend to exhibit delayed responses to glucose and insulin relative to mammals, leading to the presumption of glucose-intolerance. In this study, plasma glucose, muscle and liver glycogen, and glucose transporter (GLUT) mRNA expression were measured in response to glucose and insulin injections in order to investigate the glucoregulatory capabilities of the spiny dogfish (*Squalus suckleyi*). The glucose-load aimed to double plasma concentrations, and within 6 hours half of this load had been cleared, with control levels being restored by 24 hours post-injection. It was determined that plasma clearance was due to increased uptake by the tissues as muscle and liver glycogen contents increased significantly and were correlated with the upregulation of *glut* mRNA levels. Following an insulin injection, plasma glucose steadily decreased through 18 hours before beginning to climb back towards control levels. The decrease was relatively minimal, however, and no increases in tissue glycogen content were observed. *glut4* mRNA levels did significantly increase in the muscle in response to insulin, but no changes occurred in the liver. The responses observed mimic what occurs in mammals and teleosts, thus suggesting a conserved mechanism for glucose homeostasis in vertebrates.

3.2 Introduction

The majority of elasmobranchs (sharks, skates, and rays) are carnivorous and thus consume very few carbohydrates which likely has contributed to the dearth of information on glucose transport and metabolism in this group of vertebrates. In addition, most tissues do not need to rely on glucose as an oxidative fuel, preferring to use ketone bodies even during periods where starvation is not a factor (reviewed by Speers-Roesch and Treberg, 2010), whereas in mammals the production of ketone bodies is harmful in the long-term. In particular, the brain, which in most other vertebrates is a glucose-dependent organ, was shown to take up the ketone body β-hydroxybutyrate from the blood but not glucose (deRoos, 1994). The heart has also been shown to have a very high capacity for the oxidation of ketone bodies and thus uses them as aerobic fuels (Watson and Dickson, 2001). This has led to the idea that elasmobranchs are glucose intolerant. However, certain species, such as the North Pacific spiny dogfish (*Squalus suckleyi*) feed on molluscs and crustaceans which can consist of up to 25% carbohydrates (Jones and Geen, 1977). Further, the rectal gland, which is activated by a meal, has been shown to be glucose-dependent (Walsh *et al*., 2006) and as such it is important for us to understand how glucose is transported and metabolised in these fish. Furthermore, elasmobranchs appeared early on in the vertebrate lineage and thus could provide valuable insight into the evolution of glucose homeostasis.

In a previous study, glucose transporters (GLUTs) were identified in three elasmobranch species and increases in mRNA levels were observed in response to feeding in the muscle, liver, and intestine of the spiny dogfish (*S. suckleyi*; Chapter 2; Deck *et al*., 2016). However, the meal consumed was composed of fish and thus contained very few carbohydrates. A study by Patent (1970) determined that dogfish (*Squalus acanthias*) take up to 48 h to clear a glucose load that

has been injected intra-arterially, however no parameters other than plasma glucose were measured. The prolonged time needed to restore plasma glucose levels was also demonstrated in the skate (*Leucoraja erinacea*) but again, no other parameters were measured (Hartman *et al*., 1944). In mammals, an increase in plasma glucose is accompanied by the release of insulin which stimulates the transport of previously synthesised GLUT 1 and 4 proteins from intracellular storage vesicles to the plasma membrane of myocytes and adipocytes to enhance glucose uptake (Calderhead *et al*., 1990; Huang and Czech, 2007). Furthermore, insulin causes an increase in both GLUT1 (Walker *et al*., 1989) and GLUT4 (Bourey *et al*., 1990) protein and mRNA levels in skeletal muscle cells. In rainbow trout muscle cells, insulin induced a significant increase in both *glut1* and *4* mRNA levels (Diaz *et al*., 2009) and GLUT4 protein levels (Diaz *et al*., 2007), similar to the effects observed in mammals. In mammalian adipocytes, GLUT4 protein and mRNA have been shown to increase in response to insulin in some studies with no changes in GLUT1 (see review by Pessin and Bell, 1992) but in other studies, GLUT4 protein and mRNA decreased while GLUT1 protein and mRNA increased in response to insulin (Flores-Riveros *et al*., 1993; Sargeant and Paquet, 1993). These discrepancies may be due to the conditions under which the adipocytes were allowed to differentiate or the length of the insulin treatment period. Elasmobranchs lack true adipose tissue, however they do maintain large hepatic lipid stores and in a prior study we determined the presence of both *glut 1* and *4* mRNA in the dogfish liver (Chapter 2; Deck *et al*., 2016). In regards to insulin, three studies in elasmobranchs have shown a decrease in plasma glucose in response to bolus insulin injections (Patent, 1970; deRoos and deRoos, 1979; Anderson *et al*., 2002). The response to insulin, however, was delayed relative to that observed in mammals which led the authors of the latter study to speculate that elasmobranchs lack the insulin-responsive GLUT4 although this does not

appear to be the case as this transporter was isolated in a prior study (Chapter 2; Deck *et al*., 2016).

As the earlier study investigated *glut* mRNA levels in response to a protein load and the studies that used glucose and insulin injections did not look at GLUT expression, the present study sought to investigate the effects of hyperglycaemia and insulin on *glut* mRNA levels in the spiny dogfish (*S. suckleyi*). Thus, dogfish were subjected to a glucose load or bolus insulin injection and the following were determined: 1) how long it takes to reduce plasma glucose, 2) the effect on *glut 1* and *4* and *glycogen synthase* mRNA levels in muscle and liver, and 3) the effect on muscle and liver glycogen content. It was hypothesised that both hyperglycaemia and insulin increase *glut* mRNA levels and cause glycogen deposition in muscle and liver, thus suggesting a conserved mechanism for glucose homeostasis in vertebrates.

3.3 Methods

3.3.1 Animals

Adult male spiny dogfish (*Squalus suckleyi*; 1.5-2.0 kg) were captured by long-line from Barkley Sound (British Columbia, Canada) and transported to the Bamfield Marine Sciences Centre in June 2014 and June 2015 where they were maintained in a 155,000 L circular tank with running seawater and aeration. Dogfish were fed a 2.5% body ration of frozen hake every third day but were then fasted for 7 days prior to the start of the experiments. All experiments were performed in accordance with the Canadian Council of Animal Care guidelines and under approval of the University of Ottawa and the Bamfield Marine Sciences Centre Animal Care Committees.

3.3.2 Glucose-loading Experiment

Following a 7-day fast, a blood sample was taken via caudal puncture with a heparinized syringe and the dogfish were anaesthetised with MS-222 (0.5 g L^{-1}) and weighed. Assuming a plasma glucose concentration of 5 mM as determined previously (Walsh *et al*., 2006; Deck *et al*., 2016), blood volume was estimated as 5% of the total body weight (Good *et al.*, 2008) and the fish were injected (intra-arterially) with enough glucose (dissolved in elasmobranch saline; 400 mM urea, 80 mM TMAO, 257 mM NaCl) to raise their plasma levels to approximately 10 mM. A separate group of fish were injected with saline containing no glucose to determine the effect of the injection on plasma glucose levels. The injected fish were then placed in smaller isolation tanks and at either 6, 24, or 48 h (n=6) post-injection, blood was taken via caudal puncture and the fish sacrificed with an overdose of MS-222 (1 g L^{-1}) and subsequent cervical dislocation. A fourth group of fish were sacrificed at time zero, just following the 7-day fasting period and used as controls. Muscle, liver, intestine, and rectal gland samples were flash frozen in liquid nitrogen and stored at -80°C for further analysis. All blood samples were centrifuged for 2 min at 13 000 x g and the plasma frozen at -80°C.

3.3.3 Insulin Experiment

Following a 7-day fasting period, dogfish were anaesthetised with MS-222 (0.5 g L^{-1}), fitted percutaneously with a caudal artery cannula, and placed in 40 L epoxy-coated wooden boxes with running seawater and aeration where they were allowed to recover for 24 h. To determine the effects on plasma glucose, dogfish were injected via the cannula with either saline only or 10 ng kg^{-1} (dose based on Anderson *et al.*, 2002) of bovine insulin (Sigma-Aldrich) dissolved in 500 μL saline, and 200 μL blood samples taken at times 0, 3, 6, 9, 12, 18, 24, 30, and 36 h (note that haematocrit remained constant throughout the experiment). Blood was

centrifuged to isolate the plasma, flash frozen in liquid nitrogen, and stored at -80°C for further analysis. Groups of fish (n=5-7) were also sacrificed at the 0, 6, 12, and 36 h time points and sampled for muscle, liver, intestine, and rectal gland. Tissues were also frozen in liquid nitrogen and stored at -80°C.

3.3.4 Glucose and Glycogen

Plasma glucose was measured using the Infinity Hexokinase Reagent (Thermo Scientific). Muscle and liver glycogen content were measured using the Anthrone method adapted from Seifter *et al*. (1949) as enzyme digestion with amyloglucosidase was unsuccessful (perhaps due to the high concentration of urea in tissue extracts). For this method, 50 mg of tissue was added to 1 mL of 30% KOH and boiled for 20 min followed by the addition of 1.25 mL 95% ethanol. The samples were again boiled for 5 min, cooled in an ice bath for 5 min, and then centrifuged at 10°C for 15 min at 4000 x g. The pellet was dried and resuspended in 1 mL deionised water and the ethanol precipitation repeated. The pellet was then resuspended in 0.5 mL of deionised water and 1 mL Anthrone reagent (0.2 g Anthrone in 100 mL 95% sulphuric acid) was added to each sample and to the standard (10 mg glucose in 20 mL deionised water). The samples and standard were boiled for 10 min and read in a 96-well microplate at 620 nm.

Glycogen content was calculated as follows and expressed as mg glycogen/50 mg tissue:

$$\text{mg glycogen} = \frac{100 \times \text{Sample Absorbance}}{1.11 \times \text{Standard Absorbance}}$$

3.3.5 qPCR[3]

Total RNA was isolated using Trizol reagent (Invitrogen) and 2 µg (quantified by NanoDrop) was treated with DNase I (Invitrogen) and used for reverse transcription as outlined by Deck *et al*. (2013). Briefly, 375 ng random hexamers (Integrated DNA Technologies; IDT) and 125 ng oligo dTs (IDT) were added to the DNase-treated RNA and incubated at 65°C for 5 min. The mixture was cooled on ice for 5 min and then first-strand reaction buffer (Invitrogen; 1x final concentration), DTT (Invitrogen; 5 mM final concentration), RNase Out (Invitrogen; 0.5 µL), and nuclease-free water (0.5 µL) were added. The reaction was incubated at 42°C for 2 min and then 1 µL Superscript II reverse transcriptase (Invitrogen) was added to a total volume of 20 µL. The reaction was then incubated at 42°C for 50 min and inactivated at 72°C for 15 min. The resultant template was diluted 5x prior to being used for qPCR.

The qPCR reaction combined 6.25 µL of Maxima SYBR Green Master Mix (Thermo Scientific), 200 nM each gene-specific forward (*glut1*- 5'AGTCGGATTGTTCGTCAACC; *glut4*- 5'GGAGGAAAAGAGGAGGATGG; *glycogen synthase*- 5'GCCTCTCTGATCTGCTGGAC) and reverse primers (*glut1*- 5'GCCGGAGTAGATGCCAATAA; *glut4*- 5'GGTGATGTAGACCGGCTGTT; *glycogen synthase* - 5'TGGTAGGGACTGGAATGGAG) (Chapter 2; Deck *et al.*, 2016), 1 µL of diluted cDNA template, and 4.75 µL nuclease free water to a total reaction volume of 12.5 µL. Fluorescence was detected using a Rotor-Gene Q Real-Time PCR cycler (Qiagen). The cycling parameters were 95°C for 10 min followed by 40 cycles of 95°C for 10 s and 60°C for 15 s. All

[3] An attempt to obtain custom dogfish GLUT4 antibodies for protein measurement was made but unfortunately was unsuccessful (see Appendix B).

reactions were run in duplicate and relative mRNA levels were calculated using the delta C_t method and normalised to *S. suckleyi* ubiquitin levels.

3.3.6 Statistics

A linear general effects model with a two-way repeated measures ANOVA and Holm-Sidak post-hoc tests was used to determine significance between the control and experimental groups over time for the plasma glucose data. One-way ANOVAs with a Holm-Sidak post-hoc test were used to determine significance between time points for the tissue glycogen and qPCR data. In cases that lacked equal variance or normality, square root or log transformations were performed.

3.4 Results

3.4.1 Glucose and Glycogen

In the glucose-loading experiment, plasma glucose was successfully elevated to 10 mM (Fig. 3.1). Within 6 h of the glucose injection, plasma glucose was still significantly elevated over the time zero values but had decreased from 10 mM to approximately 7 mM (Fig. 3.1; Two-way RM ANOVA; Treatment:Time $p<0.01$). By 24 h, plasma glucose was not significantly elevated over the time zero values and by 48 h levels had returned to control values. There were no significant changes in plasma glucose within the saline-injected fish, indicating that the injection procedure did not affect our experiment. For the insulin experiment, plasma glucose began to trend downward immediately but was not significantly decreased until 9 h post-injection (Fig. 3.2; Two-way RM ANOVA; Treatment:Time $p<0.01$). Glucose remained

significantly lower through 24 h and then by 30 h had begun to increase back towards control levels (Fig. 3.2).

For the glucose-loading, muscle glycogen content began to trend upward at 6 h in glucose-injected fish but was not significantly elevated until 24 h post-injection (Fig. 3.3A; One-way ANOVA; $p<0.01$). Liver glycogen on the other hand increased 5-fold over the control values by 6 h and remained significantly elevated through 24 h (Fig. 3.3B; One-way ANOVA; $p<0.05$). For insulin, there were no significant changes in either muscle or liver glycogen content.

3.4.2 qPCR

In the muscle following the glucose-loading, both *gluts 1* (Fig. 3.4A; One-way ANOVA; $p<0.01$) and *4* (Fig. 3.4B; One-way ANOVA; $p<0.01$) were significantly elevated over the control values at 24 h post-injection. For *glut1*, the mRNA levels remained elevated through 48 h whereas the *glut4* levels began to decrease back toward control levels. Also in the muscle, *glycogen synthase* mRNA levels were increased at 6 h post-injection but the elevation did not become significant until the 24 h time-point (Fig. 3.4C; One-way ANOVA; $p<0.01$). Levels then remained significantly elevated through 48 h. In the rectal gland, we observed a significant decrease in *glut4* mRNA levels at 24 h post-injection and this remained low through 48 h (Fig. 3.5A; One-way ANOVA; $p<0.01$). In the liver, only *glut4* mRNA levels increased significantly. This was evident by 6 h and the levels continued to increase through 48 h (Fig. 3.5B; One-way ANOVA; $p<0.01$). We could not measure *glycogen synthase* mRNA in the liver as it possesses a separate isoform from the muscle and our attempts to isolate a sequence for the hepatic form were unsuccessful.

In the insulin experiment, the only significant changes in *glut* mRNA levels were in the muscle. *glut4* was significantly elevated at 12 h post-injection but returned to control values by 36 h (Fig. 3.6A; One-way ANOVA; $p<0.01$). No significant changes in *glut1* mRNA levels were observed (Fig. 3.6B). We also observed a significant increase in muscle *glycogen synthase* mRNA levels at 12 h post-injection (Fig. 3.6C; One-way ANOVA; $p<0.01$).

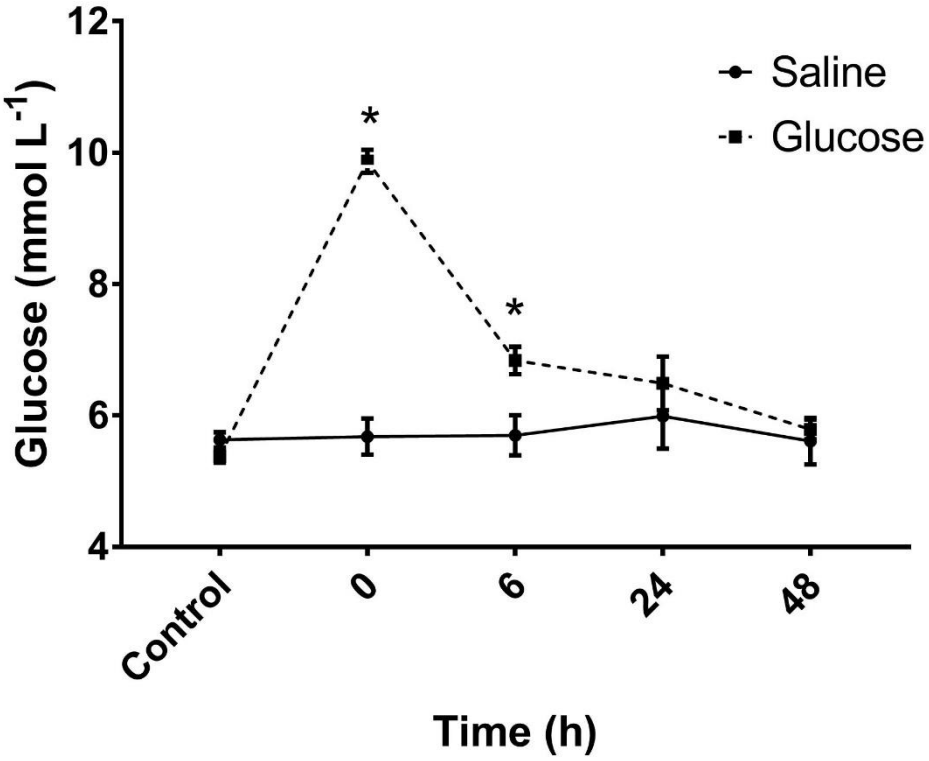

Figure 3.1 Plasma glucose (mmol L^{-1}) in the spiny dogfish (*S. suckleyi*) in response to intra-arterial injections of glucose (---) or saline (—). Control indicates plasma values obtained prior to being anaesthetised and injected and 0 h refers to values obtained shortly after injection. Data are means + SEM. Asterisks indicate significant differences between treatments (Two-way RM ANOVA; n=6; Treatment:Time $p<0.01$).

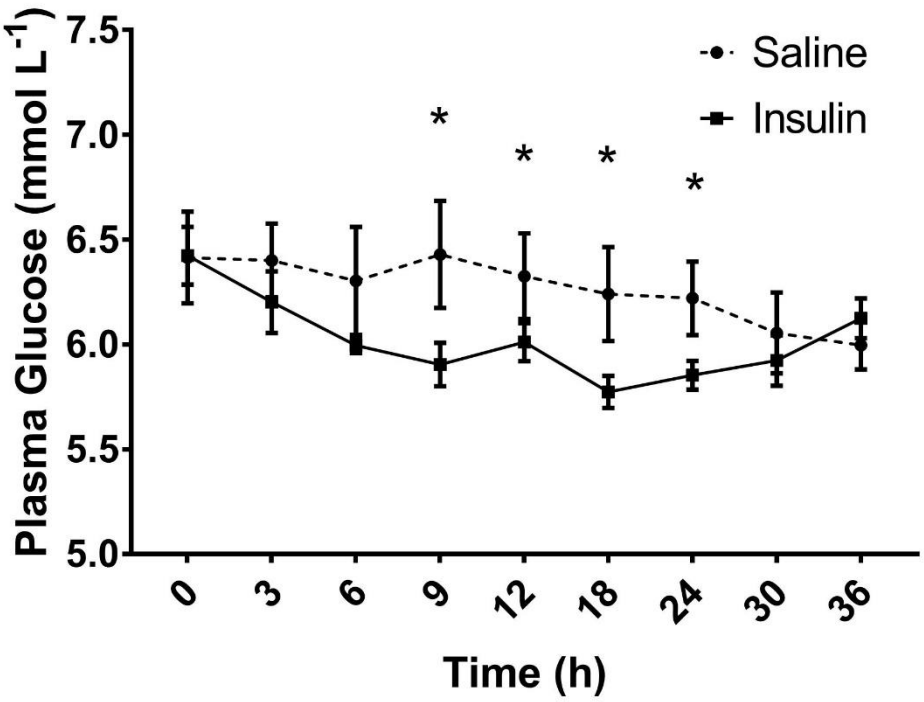

Figure 3.2 Plasma glucose (mmol L^{-1}) in spiny dogfish (*S. suckleyi*) in response to intra-arterial injections of 10 ng kg^{-1} bovine insulin (—) or saline (---). Time 0 h indicates the plasma values prior to injection. Data are means + SEM. Asterisks indicate significant differences between treatments (Two-way RM ANOVA; n=5-7; Treatment:Time $p<0.01$).

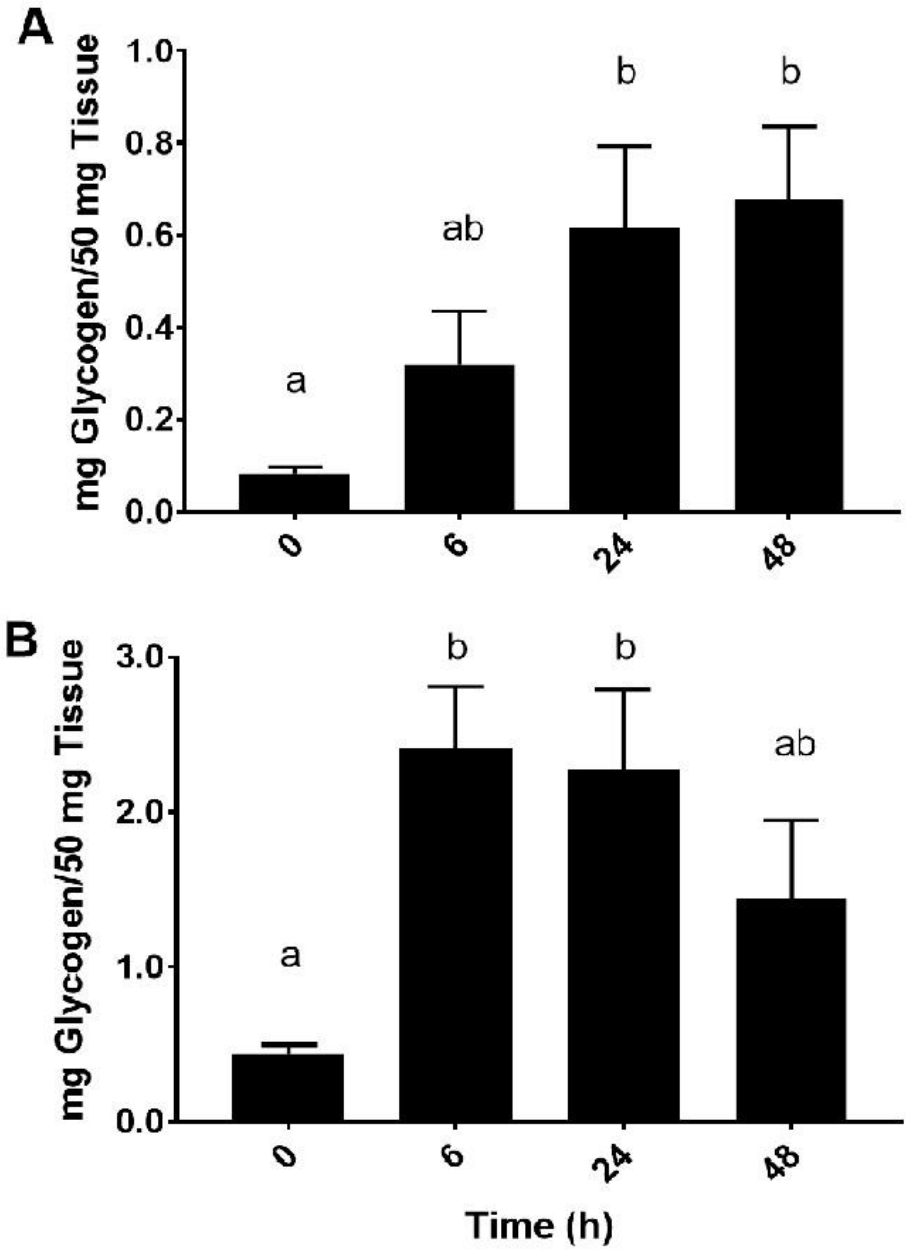

Figure 3.3 Glycogen content in the muscle (A) and liver (B) of the spiny dogfish (*S. suckleyi*) following a glucose injection. Time 0 h represents fish that had been fasted for 7 days but received no glucose injection. Data are means + SEM. Different letters indicate significant differences between treatments (One-way ANOVA; n=6; $p<0.05$).

Figure 3.4 Levels of *glut1* (A), *glut4* (B), and *glycogen synthase* (C) mRNA in the white skeletal muscle of spiny dogfish (*S. suckleyi*) following a glucose load. Time 0 h indicates fish that were starved for 7 days and did not receive any glucose. Data are means + SEM. Different letters indicate significant differences between treatments (One-way ANOVA; n=6; $p<0.01$).

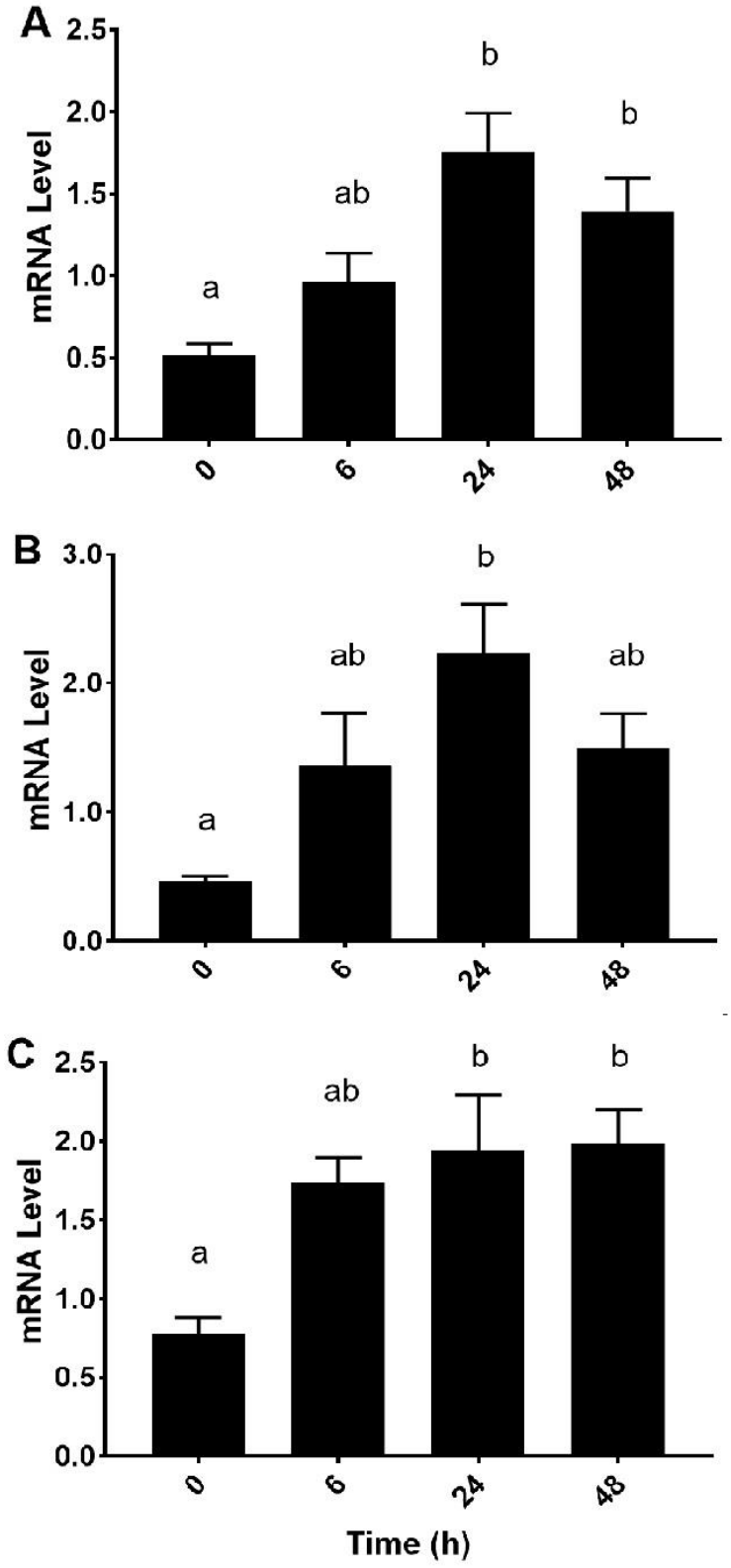
A
mRNA Level
2.5
2.0
1.5
1.0
0.5
0.0
a
ab
b
b
0
6
24
48
B
mRNA Level
3.0
2.0
1.0
0.0
a
ab
b
ab
0
6
24
48
C
mRNA Level
2.5
2.0
1.5
1.0
0.5
0.0
a
ab
b
b
0
6
24
48
Time (h)

Figure 3.4

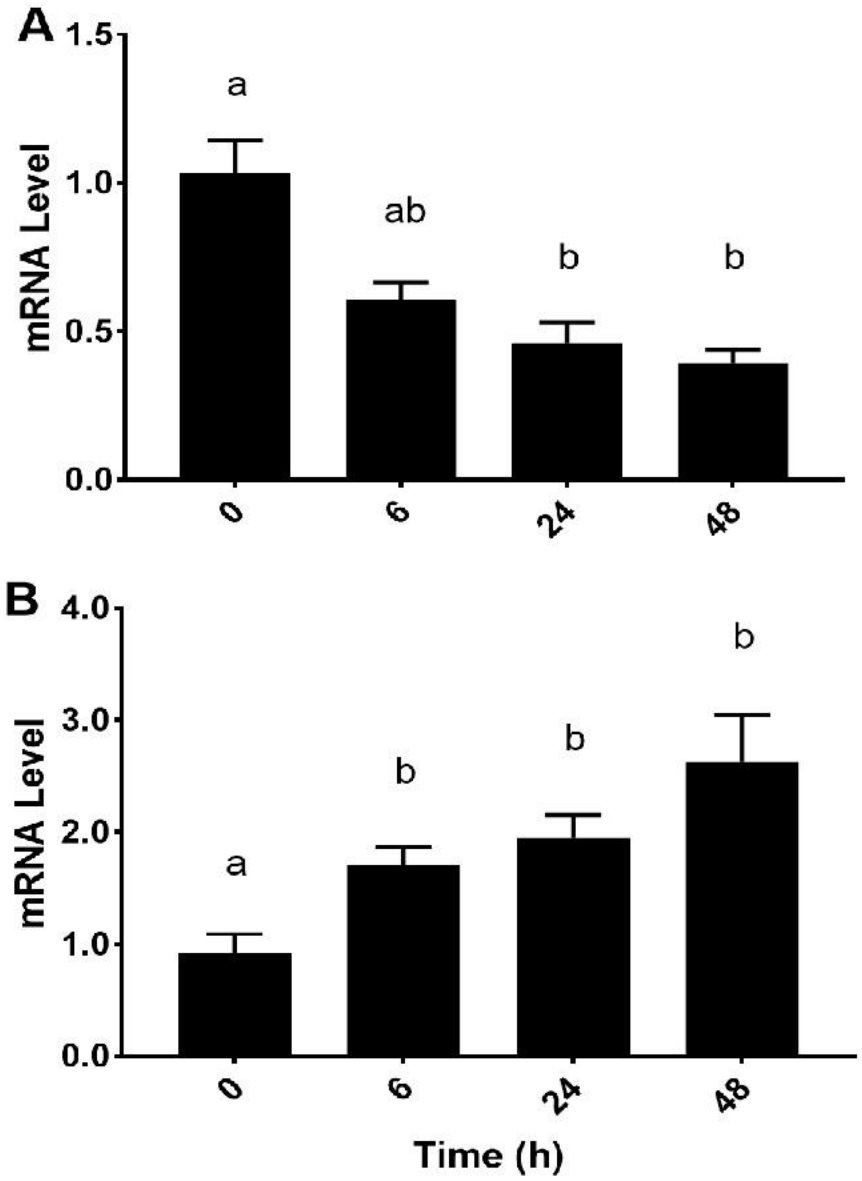

Figure 3.5 Levels of *glut4* mRNA in the rectal gland (A) and liver (B) of spiny dogfish (*S. suckleyi*) in response to a glucose injection. Time 0 h fish were starved for 7 days but this was not followed by a glucose injection. Data are means + SEM. Different letters indicate significant differences between treatments (One-way ANOVA; n=6; $p<0.01$).

Figure 3.6 Levels of *glut4* (A), *glut1* (B), and *glycogen synthase* (C) mRNA in the white skeletal muscle of spiny dogfish (*S. suckleyi*) following an injection of 10 ng kg^{-1} of bovine insulin. Time 0 h fish had been fasted for 7 days and cannulated but did not receive insulin injections. Data are means + SEM. Different letters indicate significant differences between treatments (One-way ANOVA; n=5-7; $p<0.01$).

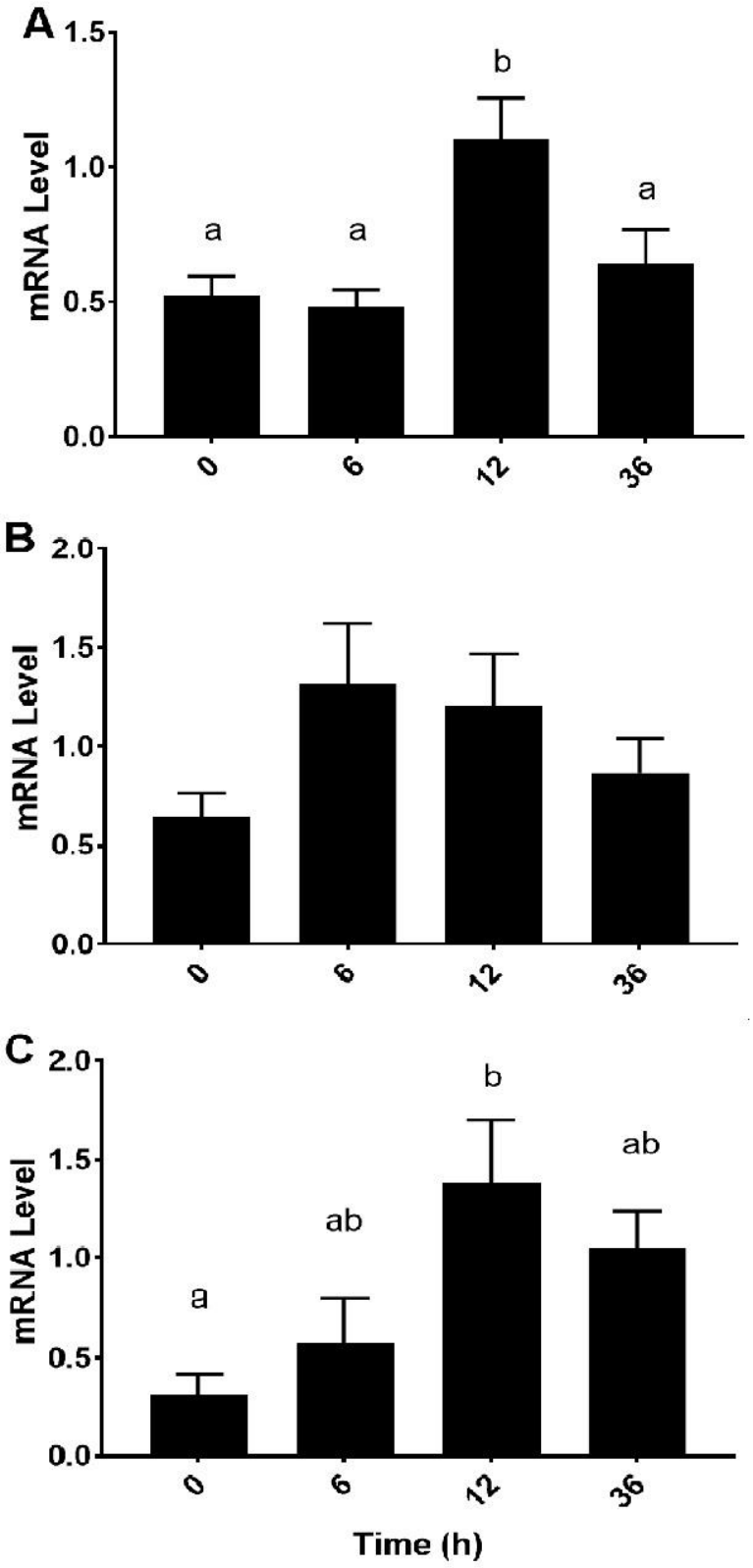
A
mRNA Level
1.5
1.0
0.5
0.0
a
a
b
a
0
6
12
36
B
mRNA Level
2.0
1.5
1.0
0.5
0.0
0
6
12
36
C
mRNA Level
2.0
1.5
1.0
0.5
0.0
a
ab
b
ab
0
6
12
36
Time (h)

Figure 3.6

3.5 Discussion

This study investigated the effects of glucose and insulin administration on plasma glucose levels as well as glucose transporter expression in the spiny dogfish shark (*S. suckleyi*) in an attempt to shed light on the glucoregulatory abilities of this species. Previous work has indicated that elasmobranchs are slow to clear an injected glucose load, with plasma glucose levels remaining elevated until 48 h post-injection (Hartman *et al*., 1944; Patent, 1970) and the delay is similar to what has been observed here, although in the present study the glucose load was cleared by 24 h. Although the restoration of plasma glucose levels took much longer than it would in mammals, the majority of the glucose was cleared within 6 h and factors such as a lower metabolic rate could have played a role in this time discrepancy. What was interesting was that the restoration of plasma glucose levels in this study corresponded to significant increases in both liver and muscle glycogen, as well as increases in *glut* mRNA levels; notably these tissues represent the majority of the mass of the animal. This is the first study to measure these parameters in an elasmobranch in response to glucose-loading, however similar responses occur in teleosts and mammals.

Significant increases in *glut1* and *glut4* mRNA were observed in dogfish white skeletal muscle within 24 h of injection, which appears to be delayed relative to the decreases in plasma glucose, however there are a couple of considerations that need to be taken into account. First, the GLUT4 protein is known to be stored within intracellular vesicles in the muscle and adipose tissue of mammals and teleosts and, when stimulated by insulin binding, it translocates to the cell membrane to enhance glucose uptake (Diaz *et al*., 2007; Huang and Czech, 2007). Once plasma glucose and insulin levels decrease, the transporters are removed from the membrane and recycled. Thus, in the first few hours following a glucose injection, glucose uptake may be

enhanced by the insertion of GLUTs into the muscle cell membrane and then as plasma glucose decreases, the transporters are recycled and transcription increases to help replace the intracellular GLUT4 stores (Pessin and Bell, 1992). Studies in teleosts have indicated that, like in mammals, insulin triggers an increase in *glut4* mRNA levels within skeletal muscle cells (Diaz *et al*., 2007; Diaz *et al*., 2009) and Blasco *et al*. (1996) observed increases in plasma insulin following a glucose injection (albeit using a heterologous assay). It seems possible that insulin may be responsible for increasing *glut4* transcription in elasmobranch muscle as an increase in mRNA levels was observed in response to insulin; however, as there is no antibody for dogfish insulin, we were unable to quantify plasma levels to determine whether the glucose load did in fact stimulate insulin secretion.

The second consideration is that in teleosts and mammals, glucose transporters are more constitutively expressed in the membrane of hepatocytes and as such, increases in glucose uptake can occur immediately as there is less of a need for insulin-stimulated translocation. Indeed, we did observe significant increases in the glycogen content of the dogfish liver in response to a glucose load much earlier than in the muscle suggesting that the liver plays a predominate role in glucose uptake. Although not responsible for GLUT translocation, insulin still acts on the liver by stimulating transcription and glycogen synthesis and inhibiting glycogenolysis and gluconeogenesis (reviewed by Saltiel and Kahn, 2001; Navarro *et al*., 2002). A significant increase in liver *glut4* mRNA as well as the increase in glycogen was observed in response to glucose-loading, although we did not observe any changes in liver glycogen or *glut* mRNA following insulin administration and as plasma insulin could not be measured following the glucose injections, it is difficult to say whether these responses are due to insulin. In this study, however, bovine insulin was used as there is currently no dogfish insulin available and Patent

(1970) showed that bovine insulin had no effect on muscle or liver glycogen content in the dogfish (*Squalus acanthias*). Interestingly, an increase in *glycogen synthase* mRNA levels was observed in the muscle in response to both insulin and glucose-loading. As the decreases in plasma glucose observed during the insulin experiment were small, it is possible that the insulin does affect glycogen metabolism but either the dose or the use of a heterologous insulin did not increase glucose uptake enough to require an increase in glycogen deposition.

As with elasmobranchs, teleosts have also been believed to be glucose intolerant as they also take more time to clear a glucose load relative to mammals (5-36 h; reviewed by Moon, 2001) although some species, such as the American eel (*Anguilla rostrata*) and black bullhead catfish (*Ameiurus melas*), were able to restore plasma glucose levels within 60 min of injection (Legate *et al*., 2001). Species that have been deemed glucose intolerant, however, tend to exhibit mammalian-like responses to hyperglycaemia. For instance, Blasco *et al*. (1996) showed that injecting brown trout (*Salmo trutta*) with glucose caused a 3- and 4-fold increase in glucose uptake by red and white skeletal muscle, respectively. Similarly, rainbow trout (*Oncorhynchus mykiss*) were able to limit the hyperglycaemia experienced during constant glucose infusions through increased uptake and disposal (Choi and Weber, 2015). The authors also determined that the glucose infusions completely inhibited hepatic glycogenolysis and gluconeogenesis which occurs in mammals in response to insulin (Choi and Weber, 2015). These studies suggest that teleosts are capable of glucoregulating despite the time discrepancy observed for the restoration of plasma glucose levels between them and mammals and this also seems to be the case in elasmobranchs.

Overall, it appears that dogfish are capable of glucoregulating and do so in a manner similar to that of mammals and teleosts, suggesting conservation of these mechanisms

throughout the vertebrate lineage. However, a full understanding of the hormonal control of glucose uptake and metabolism in these fish is currently not possible as there are no elasmobranch-specific assays or hormones commercially available.

3.6 Acknowledgements

Many thanks to Dr. Eric Clelland of Bamfield Marine Sciences Centre for ensuring that the experiments ran smoothly; to Alyssa Weinrauch, Alexander Clifford, and Rachel Munger for their assistance with sampling; to Julia Redfern for assistance with the statistical analysis; and to John Planes for providing the dogfish. This work was funded by an NSERC Discovery Grant to PJW, who is also supported by the Canada Research Chair Program.

CHAPTER 4

Rectal gland is glucose-dependent and activated by glucagon-like peptide-1 in the North Pacific spiny dogfish (*Squalus suckleyi*)

Chapter 4 was performed in collaboration with Drs. Gary Anderson and Mike Conlon and is currently being prepped for publication.

4.1 Abstract

Elasmobranchs possess a specialised organ, known as the rectal gland, which is responsible for excreting sodium chloride via the posterior intestine. Previous work has indicated that the gland is activated by a number of hormones as well as by feeding. Further, some evidence exists for the gland being glucose-dependent, a unique trait for an elasmobranch tissue. In this study, the presence and regulation of sodium-glucose cotransporters (SGLTs) in response to feeding feeding in the gland were investigated. Additionally, glucose transport inhibitors were used to further test the notion of glucose-dependence and it was determined whether certain hormones typically involved in glucoregulation (insulin, glucagon, and glucagon-like peptide-1) were capable of activating the gland. The results showed that *sglt1* is present in the gland, and changes in transcript abundance were observed in response to feeding. Further, almost a complete inhibition of chloride secretion was observed when glucose uptake was inhibited, confirming the organ's glucose-dependence. Finally, GLP-1 was shown to activate the gland, significantly increasing chloride secretion rates above baseline. As GLPs are released from the intestine upon feeding and have been shown to regulate SGLTs in mammals, it is possible that GLP-1 acts as a signal for activating the gland post-feeding via the regulation of SGLT activity.

4.2 Introduction

The elasmobranch rectal gland is a specialised organ connected to the posterior intestine that is responsible for the secretion of sodium chloride. Unlike the gills of teleosts and the kidney of mammals, it does not play a role in the excretion of any other major ions and thus it is an ideal model for the study of chloride secretion. The first reports of the gland's function were made by Burger and Hess in 1960. In this study, they determined that the gland secreted a fluid containing sodium and chloride in concentrations that were twice that of the plasma and concluded, based on the volumes secreted, that the rectal gland was capable of removing large amounts of sodium and chloride from the blood. Further investigations showed that plasma chloride steadily increased in dogfish (*Squalus acanthias*) lacking functional rectal glands despite increases in both urine chloride and urine volume (Burger, 1962), and that glandless dogfish were unable to restore normal plasma chloride levels following an injection of sodium chloride (Burger, 1965). Both studies highlighted the importance of the rectal gland as they showed that the kidneys could not fully compensate for the loss of rectal gland function, likely due to their inability to produce urine that is more concentrated than the plasma.

Since then, a number of studies have focused on the rectal gland, putting forth models for chloride secretion and investigating the conditions and mechanisms responsible for activation of the gland. The current model for chloride secretion suggests that basolateral Na^+/K^+-ATPase (NKA) creates a sodium gradient that allows chloride to enter the rectal gland cells via a basolateral $Na^+/K^+/2Cl^-$ transporter (NKCC). This chloride then enters the lumen of the gland via an apical cystic fibrosis transmembrane conductance regulator (CFTR) and is secreted (reviewed by Silva *et al*., 1996). Stoff *et al*. (1977; 1979) determined that activation of this process was due to the accumulation of cyclic AMP as both cAMP itself and compounds that increase

intracellular cAMP, such as theophylline, forskolin, and vasoactive intestinal peptide (VIP), were shown to induce chloride secretion by the gland.

Recently, research on the rectal gland has focused on activation of the gland by feeding. Wood *et al*. (2005) observed an increase in plasma chloride post-feeding in *S. acanthias* and postulated that the rectal gland may be activated mainly under these circumstances. In follow-up studies, they demonstrated significant increases in the activities of a number of enzymes, such as NKA and lactate dehydrogenase (LDH), in the gland following a meal suggesting activation (Walsh *et al*., 2006). It was also shown that rectal glands from starved dogfish display a dormant morphology and physiology relative to their fed counterparts, with reduced tubule and lumen diameters as well as a number of early stage apoptotic cells (Matey *et al*., 2009). These results bring into question whether the rectal gland is important in basal NaCl regulation relative to the salt-load gained directly from the environment (see also Chapter 5). Walsh *et al.* (2006) also investigated fuel usage by the rectal gland and determined that, unlike other dogfish tissues which can rely on ketone bodies, the rectal gland was glucose dependent and could only use ketone bodies to supplement the use of glucose (Walsh *et al*., 2006). This finding led Deck *et al*. (2016) (Chapter 2) to hypothesise that the rectal gland would upregulate glucose transporters (GLUTs; solute carrier family SLC2A) following a meal in order to fuel secretion but instead they observed a decrease in *glut1* and *glut4* mRNA levels post-feeding. An earlier study put forth the notion that the rectal gland stores mRNA to allow for rapid protein synthesis upon activation as the mRNA levels of some key genes such as *nka* decreased with feeding (Deck *et al*., 2013) and Deck *et al*. (2016) suggested that this was also the case for the glucose transporters.

The purpose of the present study was to elucidate the importance of each glucose transporter in glucose uptake by the rectal gland using an *in vitro* preparation and inhibitors

specific to GLUTs 1 and 4. It was hypothesised that the GLUTs were necessary for NaCl secretion by the rectal gland and thus we expected the inhibitors to reduce chloride secretion rates. The presence and importance of sodium glucose cotransporters (SGLTs; solute carrier family SLC5A) in the gland were also investigated, seeing as it has a need for taking up both sodium and glucose.Lastly, it was determined whether peptide hormones that are typically involved in glucoregulation (insulin, glucagon, and glucagon-like peptide-1) can activate chloride secretion by the gland.

4.3 Materials and Methods

4.3.1 Animals

Adult male spiny dogfish (*Squalus suckleyi*; 1.5-2.0 kg) were caught by long-line in Barkley Sound, British Columbia (Canada) in May, 2015 and May, 2016 and transported to the Bamfield Marine Sciences Centre where they were held in a 155, 000 L tank with running seawater and aeration. Dogfish were fed a 2.5% body ration of hake every second day. Prior to experiments, some fish were transferred to a separate tank to be fasted. For qPCR, dogfish were sacrificed at 6 h, 24 h, 48 h, or 7 days post-feeding (n=7). Rectal gland perfusions were carried out on fed fish between 12-24 h post-feeding and on starved fish after at least 7 days of fasting. All experiments were run under the approval of the Animal Care Committees for the University of Ottawa, the University of Manitoba, and the Bamfield Marine Sciences Centre.

4.3.2 SGLTs

To determine the presence of SGLTs in the rectal gland, total RNA was extracted from dogfish rectal glands using Trizol (Invitrogen) and cDNA was synthesised as previously

described (Deck *et al*., 2013). *Sglt1* (SLC5A1) was then isolated by adding 1 μL of diluted cDNA template (1:5) to a 25 μL total volume containing PCR reaction buffer (Denville Scientific; 1x final concentration), dNTPs (Invitrogen; 0.2 mM final concentration), forward (CAGAGCTGGAGTTGTGACCA) and reverse (CAGTGCCTCCCGGATAAATA) primers (IDT; 0.2 μM final concentration), and 0.15 μL Choice Taq (Denville Scientific). The cycling parameters were: 94°C for 30 s, 40 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30s, and concluding with 15 min at 72°C. Products were visualized on a 1.5% agarose gel and sequenced by Genome Quebec. We then performed qPCR to compare mRNA levels in glands from fed and starved dogfish. A total reaction volume of 12.5 μL containing 1 μL diluted cDNA template, 6.25 μL Maxima SYBR Green Master Mix (ThermoFisher), and 200 nm sense and antisense primers was used. Cycling occurred in a Rotor-Gene Q Real Time PCR cycler with the following parameters: 95°C for 10 min, 40 cycles of 95°C for 10 s, and 60°C for 15 s. All reactions were run in duplicate and the C_ts were normalised to *S. suckeyi* ubiquitin levels.

4.3.3 Rectal Gland Perfusions

Dogfish were sacrificed by placing them in a solution of MS-222 (0.5 g L^{-1}) until ventilation ceased. The abdominal cavity was then opened to expose the rectal gland at the posterior end of the intestine. The rectal gland artery and vein were cannulated using PE 50 tubing and flushed with dogfish Ringer's (257 mM NaCl; 400 mM urea; 80 mM trimethylamine oxide (TMAO); 7 mM Na_2SO_4; 3 mM $MgSO_4$; 4 mM KCl; 2 mM $CaCl_2$; 0.1 mM Na_2HPO_4; 6 mM $NaHCO_3$; 5 mM glucose; 5 mM β-hydroxybutyrate (BHB); pH 7.8) with 50 IU heparin that had been run through a 0.45 μm filter. The rectal gland duct was cannulated using PE 90 tubing and flushed with a 500 mM NaCl solution. The gland was then removed, placed on a thermally regulated platform (12 ± 1°C) containing dogfish Ringer's, and connected to a peristaltic pump

as described by Walsh *et al*. (2006). The gland was perfused with thermostatted Ringer's aerated with a mixture of 99.7% O_2 and 0.3% CO_2 at 1 mL min^{-1} until all the blood had been cleared from the gland and then secretion was measured for 30 min. For the first set of experiments, the glands were treated with forskolin (5 μM final concentration) for 15 mins to stimulate secretion (Walsh *et al.*, 2006). Following the forskolin treatment, the gland was perfused with one of the inhibitors (GLUT1- STF 31; GLUT4- Indinavir; SGLT1- Phlorizin; 0.25 μM final concentration) for 15 min followed by Ringer's for 45 min and the duct secretions were collected. Venous flow rates were monitored and the effluent was sampled every 15 min. For the second set of experiments, the glands were perfused with bovine insulin, dogfish glucagon, and dogfish glucagon-like peptide-1 (GLP-1) (the latter two hormones were generously provided by Dr. Michael Conlon, Ulster University) (10 nM final concentration) for 15 min followed by 30 min of saline. Again, duct secretions and venous effluent were sampled every 15 min.

Glucose in the venous effluent was measured using the Infinity Glucose Hexokinase Reagent (Thermo Scientific) and the uptake rates were calculated using the difference in glucose between the arterial and venous saline and expressed as μmol glucose min^{-1} g^{-1}. Chloride in the duct secretions was measured using the mercuric thiocyanate method (Zall *et al*., 1956) and the rates were calculated as μmol Cl^- $hour^{-1}$ g^{-1}. Lactate in the venous effluent was determined enzymatically using hydrazine and lactate dehydrogenase (Brandt *et al.*, 1980) and the production rates were calculated using the amount of lactate that appeared in the venous effluent and expressed as μmol lactate min^{-1} g^{-1}. All standards for venous effluent measurements contained 300 mM urea to account for any effects of dogfish plasma composition.

4.3.4 Statistics

qPCR data were analysed using a One-Way ANOVA and a Holm-Sidak post-hoc test. Glucose and lactate in the venous effluent were analysed with a general linear effects model using a Two-Way RM ANOVA and a Holm-Sidak post-hoc test to determine significance between treatments as well as between fed and starved fish. Chloride in the duct secretions and glucose uptake rates were analysed with a general linear effects model using a Two-Way RM ANOVA and Tukey HSD post-hoc test, again to test for effects of treatment as well as feeding status. The chloride secretion required a square root transformation to normalise the data.

4.4 Results and Discussion

Prior research has shown that the elasmobranch rectal gland is a glucose-dependent tissue and thus in this study we investigated the presence of SGLTs in the dogfish rectal gland and the effect of glucose transport inhibitors on chloride secretion. SGLTs are responsible for the cotransport of sodium and glucose into cells, fueled by the sodium gradient created by NKA. The two best studied isoforms are SGLTs 1 and 2 which are primarily found in the intestine (SGLT1), where they are responsible for glucose uptake, and the kidney (SGLTs 1 and 2), where they facilitate glucose reabsorption (see review by Wright and Turk, 2004). In both cases, the transporters are localised to the apical membrane. The current model of chloride secretion involves the creation of a sodium gradient by basolateral NKA which leads to the uptake of chloride from the blood via the NKCC (Silva *et al*., 1996). Seeing as the rectal gland needs to take up both sodium and glucose and SGLTs are also fueled by NKA, it is reasonable to believe

that these transporters might be present in the rectal gland and indeed, we were able to identify *sglt1* in the gland.

In a previous study (Chapter 2), mRNA levels of *gluts 1* and *4* in the rectal glands of fed and fasted dogfish were investigated and it was determined that the levels decreased following a meal (Deck *et al*., 2016). This finding was on point with the idea of mRNA storage in stress granules for rapid protein synthesis upon activation of the gland (Deck *et al*., 2013). To complement this finding, qPCR for *sglt1* was performed in fed and fasted glands and a significant decrease in mRNA levels by 24 h post-feeding was observed (Fig. 4.1; One-way ANOVA; $p=0.006$). Unlike the *gluts*, whose mRNA levels drop relatively suddenly after 6 h (see Chapter 2), *sglt1* decreases gradually through the first 24 h following a meal which may suggest that this transporter is required earlier on in order to help bring sodium back into the rectal gland cells. The GLUTs would then be recruited to bring in extra glucose to help fuel the increased secretion rates.

The second aspect of this study was to determine the effect of glucose transport inhibitors on rectal gland secretion and it had been hypothesised that glucose transporters are essential to rectal gland function, thus leading to the prediction that inhibiting such transporters would also inhibit chloride secretion. The first aim was to determine that the inhibitors were in fact decreasing glucose uptake by the gland and this was indeed the case. The results show that phlorizin (SGLT inhibitor), indinavir (GLUT4 inhibitor), and STF 31 (GLUT1 inhibitor) all significantly reduced glucose uptake by the gland relative to the forskolin treatment in both fed and starved dogfish, although no differences were observed between the two groups (Fig. 4.2; Two-way RM ANOVA; $n=4$-7; Treatment $p<0.05$). Lactate production by the gland was also assessed as Walsh *et al*. (2006) observed increases in LDH activity but showed that lactate could

not fuel secretion, leading them to suggest that the gland may switch to anaerobic metabolism when the secretion rates outpace oxygen delivery. Thus, it was expected that inhibiting secretion would reduce lactate production by the gland and this is indeed what was found. All three inhibitors significantly reduced lactate production below what was observed for the forskolin treatment (Fig. 4.3; Two-way RM ANOVA; n=4-7; Treatment $p<0.001$). This result indicates that when highly active, the amount of oxygen taken up by the rectal gland is not sufficient to sustain the increased secretion rates and thus the gland must rely on anaerobic metabolism as well.

Chloride secretion rates were also determined following each treatment and, as predicted, all three inhibitors significantly reduced chloride secretion by the gland relative to the forskolin treatment, many times stopping secretion completely (Fig. 4.4; Two-way RM ANOVA; n=4-7; Treatment $p<0.001$). The cessation of secretion occurred even with the presence of BHB (the preferred fuel in most elasmobranch tissues) in the saline with which the gland was being perfused. This result is in agreement with the Walsh *et al*. (2006) study that showed the glucose-dependence of the gland, determining that BHB could only be used to supplement the use of glucose. Although not significant, the chloride secretion rates following addition of the inhibitors were slightly higher in fed glands compared to starved glands. This may indicate that the morphology of those glands reflected the active state as exhibited by Matey *et al*. (2009), and the data presented here suggest that the extent of inhibition of glucose uptake is less in the fed state. One caveat of this study is that the inhibitors used were designed for mammalian transporters and their specificity in elasmobranchs is unknown. All three inhibitors decreased secretion (as well as glucose uptake and lactate production) to an equal extent, suggesting that each one was inhibiting multiple types of glucose transporters rather than the one it is specific for in mammals.

If the inhibitors were specific, we would expect to see differences between them depending on the importance of each individual transporter. However, the reduction in glucose uptake indicates that the inhibitors were performing the desired task and thus the reductions in chloride secretion, glucose uptake, and lactate production are likely a true reflection of the gland's glucose dependence.

The final aspect of this study involved determining whether peptide hormones that are typically involved in glucoregulation (insulin, glucagon, and glucagon-like peptide-1) would activate the rectal gland. As insulin is typically responsible for stimulating the uptake of glucose into cells, it was believed that it may play a role in rectal gland activation. However, it was determined that neither insulin nor glucagon stimulated the gland as the chloride secretion rates were lower than the baseline saline values (Fig. 4.5). The lack of stimulation by glucagon in this species concurs with previous observations (Stoff *et al*., 1979) although this phenomenon appears to be species-specific as glucagon stimulated chloride secretion in both *Scyliorhinus canicula* (Anderson, 2015) and *Leucoraja erinacea* (Kelley *et al*., 2014). In contrast to insulin and glucagon, however, GLP-1 significantly increased chloride secretion, rivaling the rates observed for forskolin during the first experiment (Fig. 4.5; Two-way RM ANOVA; n=6; Treatment $p<0.001$). Interestingly, there was only a small, non-significant increase in glucose uptake by the gland following treatment with GLP-1 (Fig. 4.6), although there was a significant increase in lactate production (Fig 4.7; Two-way RM ANOVA; n=6; Treatment $p<0.001$).

GLPs are peptide hormones that come about from cleavage of the proglucagon gene within intestinal cells. In mammals, they are released in response to the appearance of nutrients in the lumen of the intestine and GLP-1 acts to stimulate insulin release and inhibit glucagon release from the pancreas while GLP-2 stimulates morphological changes within the intestine

itself (Brubaker and Anini, 2003). In most fish species however, the proglucagon gene encodes only GLP-1, not GLP-2 (Mommsen, 2001), and this also appears to be the case in the one elasmobranch fish studied (*Scyliorhinus canicula*; Conlon *et al*., 1994). Thus, it is possible that GLP-1 in fishes also has roles similar to those of GLP-2 in mammals, one notable effect being the stimulation of glucose uptake by SGLT1 (Cheeseman, 1997). We believe that SGLT1 may be an important aspect of chloride secretion in the elasmobranch rectal gland as it is responsible for the uptake of both sodium and glucose and have proposed its addition to the model in Fig. 4.8. The localization of an SGLT to the basolateral membrane would be a novel finding as these transporters are typically associated with the apical membrane. Should the proposed model hold true, however, GLP-1 in the dogfish may be acting to stimulate both glucose and sodium uptake via SGLT1 in the rectal gland, leading to increased chloride secretion. Further, as GLP-1 is typically released from the intestine in response to the presence of nutrients, it could serve as one of the signals for activating the gland post-feeding.

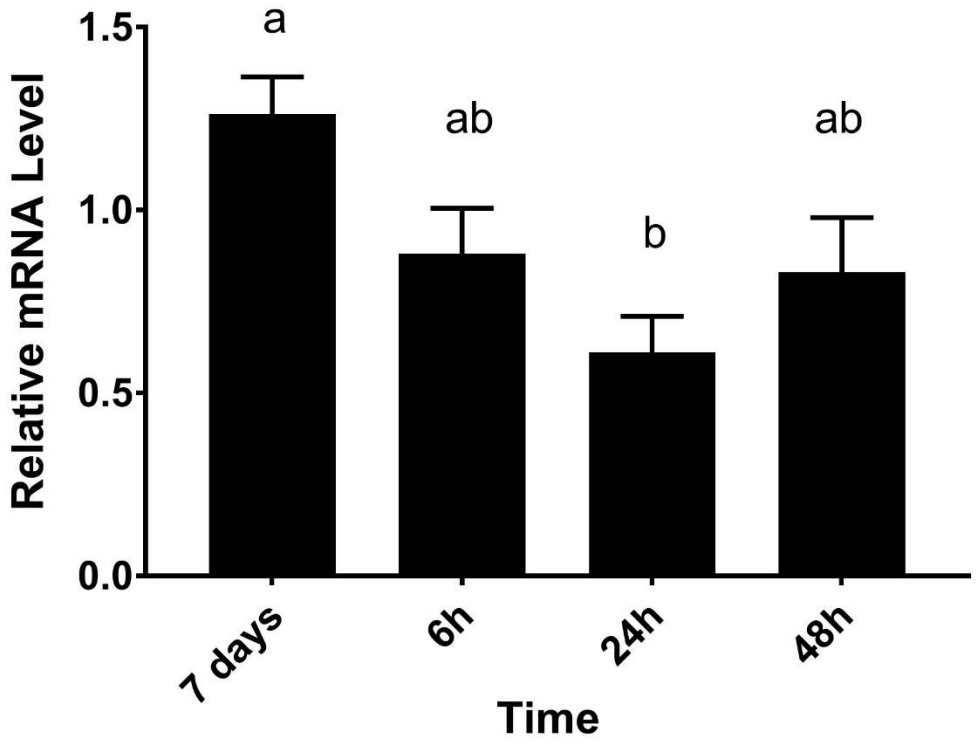

Figure 4.1 Relative mRNA levels of sodium glucose cotransporter 1 (*sglt1*) in the rectal gland of the spiny dogfish (*S. suckleyi*) in fed and 7-day fasted fish. Data represented are means + SEM. Different letters indicate statistical significance (One-Way ANOVA; n=7; $p<0.01$).

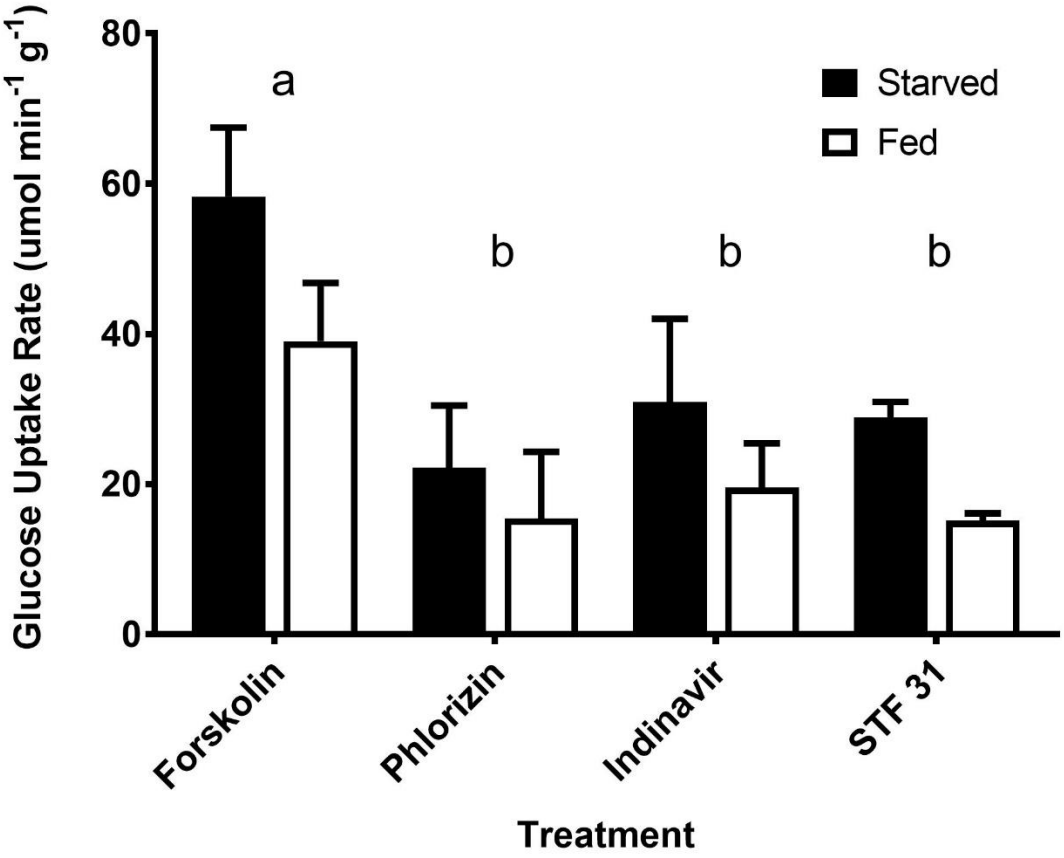

Figure 4.2 Glucose uptake rates (μmol min^{-1} g^{-1}) of a perfused rectal gland preparation in fed and 7-day starved spiny dogfish (*S. suckleyi*) following treatment with glucose transport inhibitors. Data represented are means + SEM and different letters indicate statistical significance (Two-way RM ANOVA; n=4-7; Treatment $p<0.05$). The starved and fed groups were not significantly different from one another.

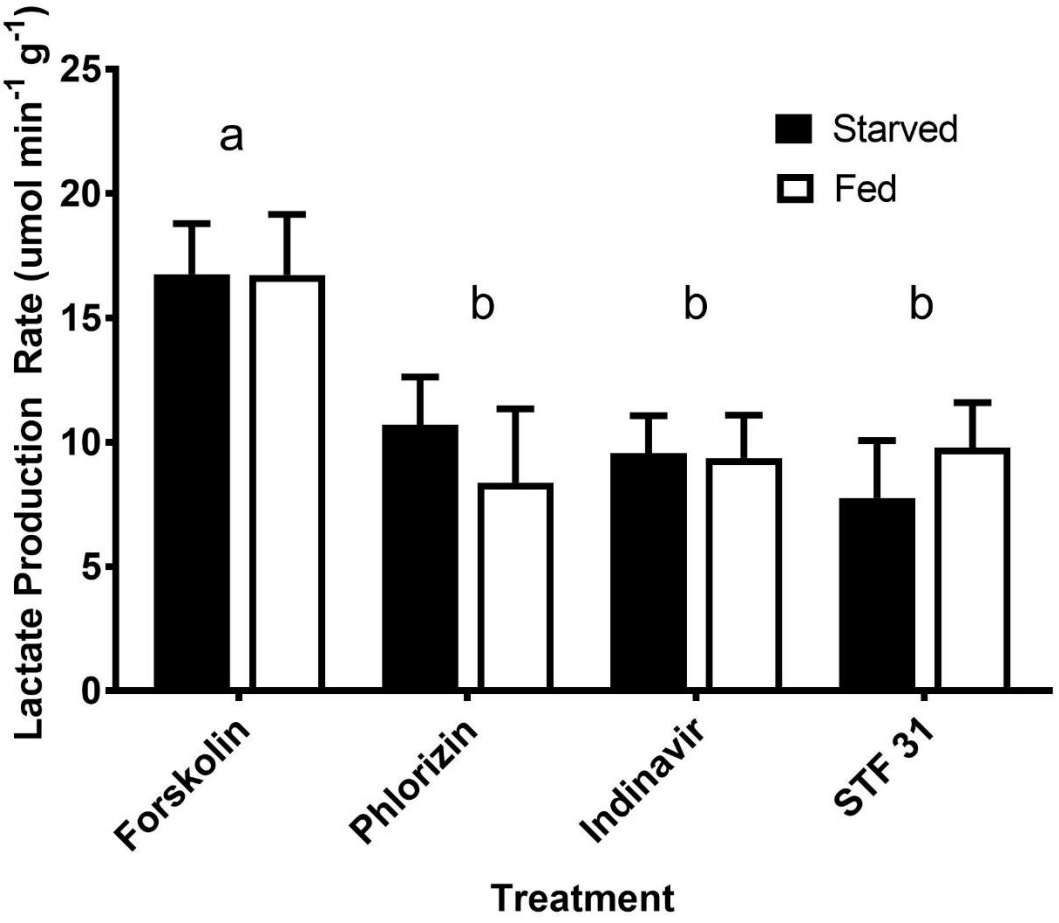

Figure 4.3 Lactate production rates (μmol min^{-1} g^{-1}) in a perfused rectal gland preparation from fed and 7-day starved spiny dogfish (*S. suckleyi*) following treatment with various glucose transport inhibitors. Data are means + SEM and different letters indicate statistical significance (Two-way RM ANOVA; n=4-7; Treatment $p<0.01$). There are no statistical differences between the fed and starved groups.

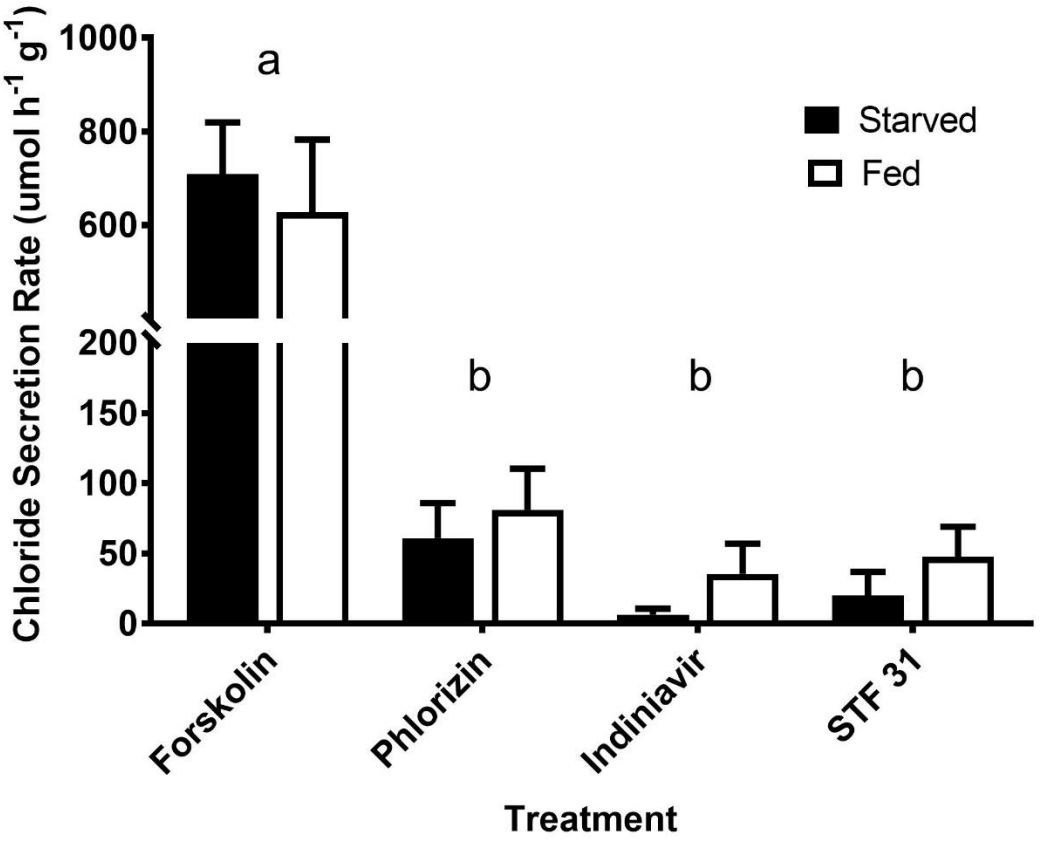

Figure 4.4 Chloride secretion for an *in vitro* rectal gland preparation from fed and 7-day starved spiny dogfish (*S. suckleyi*) following treatment with glucose transport inhibitors. Data are means + SEM and different letters indicate statistical significance (Two-way RM ANOVA; n=4-7; Treatment $p<0.001$). There are no statistical differences between the fed and starved groups.

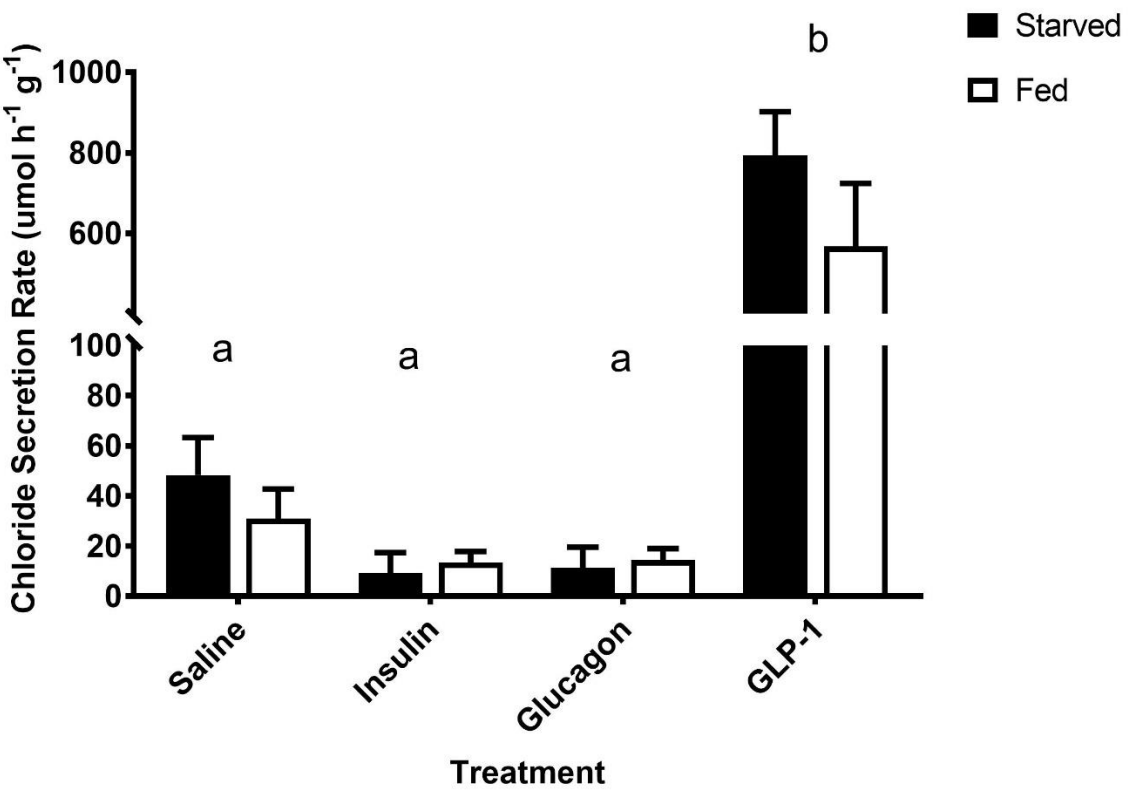

Figure 4.5 Chloride secretion for an *in vitro* rectal gland preparation from fed and 7-day starved spiny dogfish (*S. suckleyi*) following treatment with peptide hormones. Data are means + SEM and different letters indicate statistical significance (Two-way RM ANOVA; n=6; Treatment $p<0.001$). There are no statistical differences between the fed and starved groups.

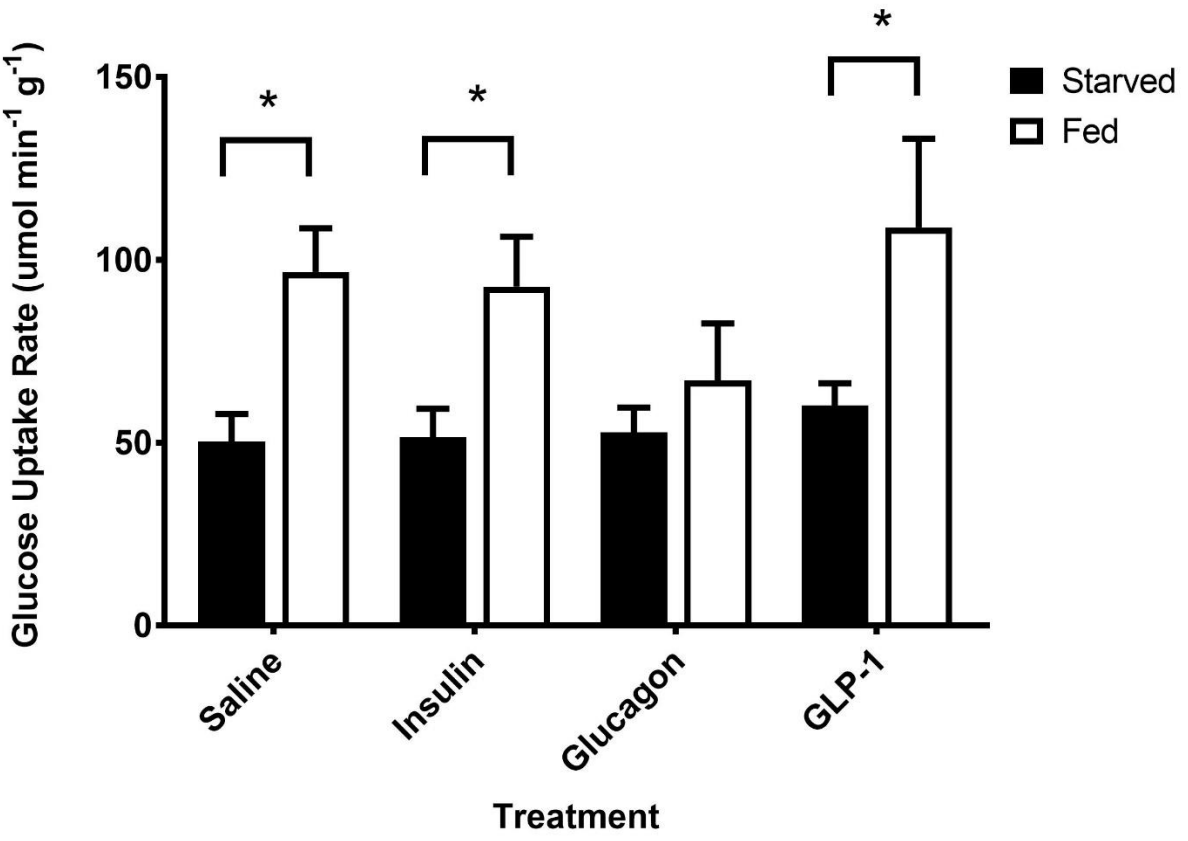

Figure 4.6 Glucose uptake rates (μmol min^{-1} g^{-1}) of a perfused rectal gland preparation in fed and 7-day starved spiny dogfish (*S. suckleyi*) following treatment with peptide hormones. Data represented are means + SEM and asterisks indicate statistical significance between the fed and starved groups (Two-way ANOVA; n=6; $p<0.001$).

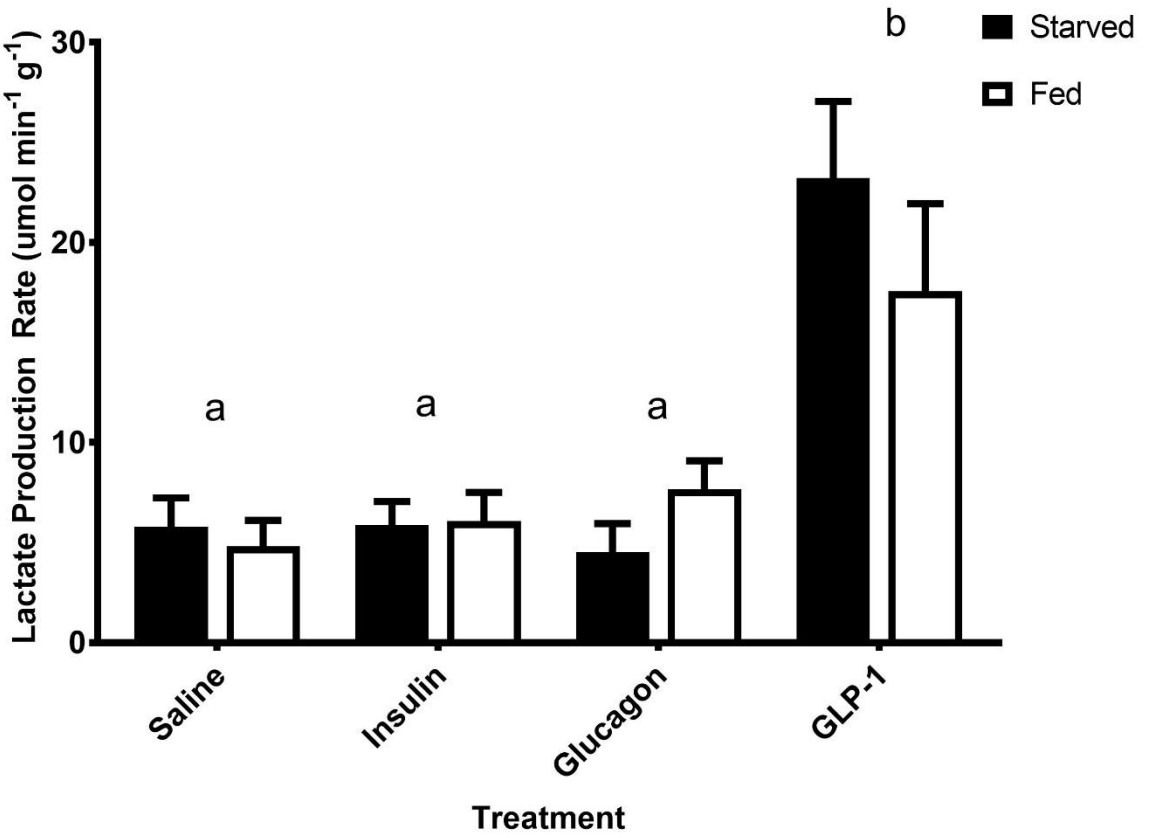

Figure 4.7 Lactate production rates (μmol min^{-1} g^{-1}) in a perfused rectal gland preparation from fed and 7-day starved spiny dogfish (*S. suckleyi*) following treatment with peptide hormones. Data are means + SEM and different letters indicate statistical significance (Two-way ANOVA; n=6; $p<0.001$). There are no statistical differences between the fed and starved groups.

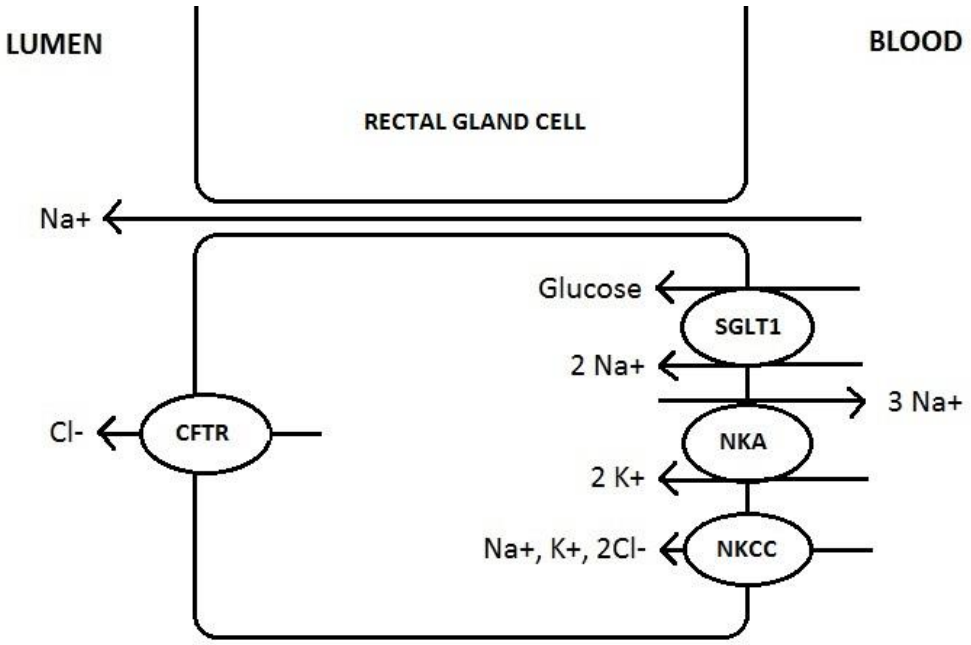

Figure 4.8 Modified chloride secretion model for the elasmobranch rectal gland. SGLT1- sodium glucose cotransporter 1; NKA- Na^+/K^+-ATPase; NKCC- $Na^+/K^+/2Cl^-$ transporter; CFTR- cystic fibrosis transmembrane conductance regulator.

4.5 Acknowledgements

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CHAPTER 5

Effects of short-term hyper- and hypo-osmotic exposure on the osmoregulatory strategy of unfed North Pacific spiny dogfish (*Squalus suckleyi*)

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5.1 Abstract

The North Pacific spiny dogfish (*Squalus suckleyi*) is a partially euryhaline species of elasmobranchs that often enters estuaries where they experience fluctuations in environmental salinity that can affect plasma osmolality. Previous studies have investigated the effects of altered salinity on elasmobranchs over the long term, but there are very few reports on how rapidly they can adapt to such changes. In this study, we exposed unfed (no exogenous source of nitrogen or TMAO) spiny dogfish to hyper- and hypo-osmotic conditions and measured plasma and tissue osmolytes, nitrogen excretion, and changes in enzyme activity and mRNA levels in the rectal gland over 24 h. It was determined that plasma osmolality was altered to approximately match the ambient seawater within 18-24 h. In the hypersaline environment, significant increases in urea, sodium, and chloride were observed whereas in the hyposaline environment, only significant decreases in TMAO and sodium were observed. Both urea and ammonia excretion increased at low salinities suggesting a reduction in urea retention and possibly urea production. qPCR and enzyme data for Na^+/K^+-ATPase and *gluts* did not support the idea of rectal gland activation following exposure to increased salinities. Therefore, it was suggested that the rectal gland may not be a quantitatively important aspect of the dogfish osmoregulatory strategy during changes in environmental salinity, or it may be active only in the very early stages (i.e., less than 6 h) of responses to altered salinity.

5.2 Introduction

A number of elasmobranch species are fully or partially euryhaline and thus, one area of research interest has been the effects of salinity, particularly low salinity, on plasma and tissue composition, and on nitrogen excretion. Elasmobranchs are ureosmotic, converting ammonia into urea and retaining this urea such that their plasma is isosmotic or slightly hyperosmotic to the ambient seawater (see review by Hazon *et al*., 2003). This strategy results in a slight influx of water and thus reduces the need to gain water via drinking (the strategy used by marine teleosts), which, in turn, reduces the intake of salts. Unfortunately, urea can destabilize proteins at these high concentrations (Rajagopalan *et al*., 1961) and thus must be counteracted, a feat elasmobranchs accomplish by maintaining relatively high plasma levels of trimethylamine oxide (TMAO; Yancey and Somero, 1979). TMAO is present in a wide variety of marine organisms (Norris and Benoit, 1945; Seibel and Walsh, 2002), however not all species have the ability to synthesize TMAO from choline or trimethylamine precursors (Baker and Chaykin, 1962; Goldstein and Funkhouser, 1972; Goldstein and Dewitt-Harley, 1973) and thus they must obtain TMAO from their diet, a strategy believed to be used by the North Pacific spiny dogfish (*Squalus suckleyi*; Baker *et al*., 1963).

Although retaining urea reduces the salt intake that marine teleosts experience from drinking seawater, influxes of salt still occur in elasmobranchs due to the ion gradients that exist across the gills. Marine teleosts use branchial Na^+/K^+-ATPase (NKA) to eliminate excess sodium from the body but elasmobranchs have a specialized organ called the rectal gland that is responsible for excreting excess Na^+ and Cl^- (Burger and Hess, 1960). A number of studies have examined different aspects of the rectal gland and observed changes in response to the salt load experienced during feeding. Walsh *et al*. (2006) observed large increases in the activity of a

number of enzymes such as NKA and lactate dehydrogenase (LDH) following a meal. A second study by Deck *et al*. (2013) used suppression subtractive hybridization to investigate changes in the mRNA expression of relevant genes and observed changes in the transcript levels of genes associated with metabolism and transport such as *nka* and *ldh*. It was also revealed that the gland may be storing RNA for rapid protein synthesis upon activation as a number of genes, most notably *nka*, had higher mRNA levels in the glands of fasted relative to fed fish which does not coincide with the increases seen in protein or enzyme activities, but would be consistent with a rapid subsequent increase in protein synthesis leading to increased enzyme activities. In regards to salinity, Piermarini and Evans (2000) observed that NKA activity and abundance were greater in rectal glands of seawater acclimated stingrays (*Dasyatis sabina*) relative to freshwater acclimated rays. Cramp *et al*. (2015), however, observed no such changes in rectal gland NKA activity in *Chiloscyllium punctatum* exposed to hyper-saline conditions.

Since their osmoregulatory strategy involves the production of urea, elasmobranchs excrete the majority of their nitrogenous waste in this form; however, this is primarily through diffusion at the gills rather than active excretion (Wood *et al*., 1995). In response to hypo-osmotic conditions, however, plasma osmolality would be expected to decrease in order to match the surrounding seawater. Indeed, Guffey and Goss (2014) showed decreases in plasma osmolality in the spiny dogfish (*S. suckleyi*) following exposure to 60% seawater that could be accounted for by decreases in plasma sodium, chloride, and urea concentrations. The latter corresponded with a significant increase in urea excretion (Guffey and Goss, 2014), suggesting a lessening of the urea retention mechanisms. Steele *et al*. (2005) also observed an increase in urea excretion but no change in ammonia excretion when little skates (*Raja erinacea*) were exposed to 75% seawater. Two studies have exposed the small-spotted catshark (*Scyliorhinus canicula*) to

both high and low salinities. The first showed that plasma osmolality, urea, sodium, and chloride all increased or decreased with salinity (Hazon and Henderson, 1984). This was also the case in a more recent study that exposed the brown-banded bamboo shark (*C. punctatum*) to both hypo- and hyper-saline conditions (Cramp *et al*., 2015). In a second study on catsharks, fish that were fed a high protein diet and subsequently exposed to high salinities had higher plasma concentrations of urea, sodium, and chloride than control fish (Armour *et al*., 1993). However, fish that were fed a low protein diet to hinder urea synthesis showed no ability to increase urea upon exposure to the increased salinity and thus sodium and chloride were increased to a greater extent (Armour *et al*., 1993).

In the literature, there is a bias towards low salinity exposures for marine elasmobranchs because it is more common to encounter a hyposaline environment than a hypersaline one, although the latter can occur in estuaries during times of low water flow. In the three studies that included exposures to both hyper- and hypo-saline environments (Hazon and Henderson, 1984; Armour *et al*., 1993; Cramp *et al*., 2015), the salinity was altered gradually over days to weeks and the animals were held at the desired salinity for a number of days before measurements were taken. In addition, these studies did not measure TMAO or total nitrogen excretion, both of which are important aspects of the elasmobranch osmoregulatory strategy (albeit Hazon and Henderson, 1984 and Armour *et al*., 1993 were more focused on hormonal control of osmoregulation). Thus, in the present study, the North Pacific spiny dogfish (*S. suckleyi*), a species that has been shown to be at least partially euryhaline (Burger, 1965; Guffey and Goss, 2014), was used to investigate the effects of short-term high and low salinity exposures on plasma and tissue osmolytes, nitrogen balance, and rectal gland activity. In contrast to most other studies, unfed fish were used because 1) this approach would allow us to determine whether the

dogfish are capable of increasing TMAO (which can only be synthesised *de novo* by select species) and urea as they were not receiving an exogenous source of nitrogen (an element many elasmobranchs appear to be limited in due to their sporadic feeding; Wood *et al*., 2005) and 2) the rectal gland is inactive under these conditions so we hoped that any changes in enzyme activity would be due solely to our salinity treatments. Plasma osmolality, sodium, chloride, urea, and TMAO were measured as well as ammonia and urea excretion in order to determine how dogfish alter their osmoregulatory strategy to adapt to various salinities. All plasma parameters were expected to either increase or decrease with salinity as has been shown in the studies mentioned previously. In addition, it was hypothesized that less urea would be produced at low salinities due to the predicted reduction in plasma osmolality and thus an increase in ammonia excretion was also predicted. Furthermore, mRNA levels and enzyme activities of NKA and LDH were measured in the rectal gland because it was postulated that a hypersaline environment would activate the gland, mimicking the salt load experienced following a meal. Lastly, glucose (*glut*) and monocarboxylate (*mct*) transporter mRNA levels were investigated since it is known that the gland requires glucose as a fuel and may be producing excess lactate during peak activity (Walsh *et al*., 2006). *Glut1* mRNA has been shown to increase in response to increases in salinity in the gill and brain of the sea bream (Balmaceda-Aguilera *et al*., 2012), something that has also been observed for hyperosmolarity in mammalian cell lines (Hwang *et al*., 2001) and thus it was believed that this may also be the case in the elasmobranch rectal gland at high salinities.

5.3 Materials and Methods

5.3.1 Animals and Salinity Exposures

Adult male North Pacific spiny dogfish (*Squalus suckleyi*; 1.5-2.5 kg) were caught by angling in Barkley Sound, British Columbia (Canada) in June 2014 and transported to Bamfield Marine Sciences Centre where they were held in a 155,000 L circular tank with running seawater and aeration at a maximum stocking density of less than 150 animals in the tank. Animals were allowed to acclimate to captivity for 7 days prior to experimentation during which time they were not fed. Following this period, the dogfish were anaesthetised using MS-222 (0.5 g L^{-1}) and fitted with a cannula of PE 50 tubing in the caudal artery (similar to methods published by DeBoeck *et al*., 2001) before being placed in 40 L epoxy coated boxes with running seawater and aeration to recover for 24 h. The dogfish were then exposed to 70% (by addition of freshwater), 100%, or 130% (by addition of Instant Ocean) seawater (N=6) for 24 h and blood (500 μL) and water samples were taken every 6 h. The blood was centrifuged at 13 000 x g for 2 min to obtain the plasma which was then stored at -80°C. During the experimental period, the boxes were not supplied with flow through seawater but the water was changed following each sample (every 6 h) by siphoning water from a header tank into the box as the old water was drained. This was done multiple times to ensure full replacement of the water. At the end of the 24 h period, dogfish were sacrificed by an overdose of MS-222 (1 g L^{-1}) and subsequent cervical dislocation. Muscle, liver, and rectal gland samples were taken and flash frozen in liquid nitrogen before being stored at -80°C.

5.3.2 Water Analysis

The osmolarity of the water was measured using a Vapro vapour pressure osmometer. Ammonia was measured using the salicylate-hypochlorite method (Verdouw *et al*., 1978) and urea was measured using the diacetylmonoxime method (Rahmatullah and Boyd, 1980). For both assays, we used 100 μL of sample in each well and all samples were run in duplicate. Excretion rates were calculated as μmol of nitrogen per kilogram body weight per hour for each 6 h sampling period.

5.3.3 Plasma and Tissue Analysis

Plasma osmolality was measured using a Vapro vapour pressure osmometer. Muscle and liver samples (250 mg) were homogenised in 5 volumes of 5% trichloroacetic acid (TCA) using a glass homogeniser and mortar and pestle, respectively. They were then centrifuged at 13 000 x g for 5 minutes to obtain the supernatant. Plasma samples (250 μL) were also combined with 5 volumes of TCA and centrifuged for 5 min. Urea was then measured using the diacetylmonoxime method (Rahmatullah and Boyd, 1980) while TMAO was measured using the ferrous sulphate/EDTA method (Wekell and Barnett, 1991). An Atomic Absorption Spectrometer was used to measure plasma sodium and plasma chloride was measured using the mercuric thiocyanate method (Zall *et al*., 1956).

5.3.4 Enzyme Activity

For measurements of lactate dehydrogenase activity, rectal glands were homogenised on ice in 20 mM Hepes, 1 mM EDTA, and 0.1% Triton X-100 at pH 7.0 and centrifuged at 8000 x g for 5 min at 4°C. The supernatant was then assayed spectrophotometrically in 50 mM imidazole (pH 7.0), 0.15 mM NADH, and 0.2 mM pyruvate, following the change in absorbance at 340 nm

wavelength. Na^+/K^+-ATPase activity was measured using the methods of McCormick (1993) and a Bradford assay was used to determine protein content.

5.3.5 qPCR

Total RNA was extracted from each rectal gland using Trizol reagent (Invitrogen) according to the manufacturer's instructions and treated with DNase I (Invitrogen). The RNA (2 μg) was then combined with 375 ng random hexamers (Integrated DNA Technologies), 125 ng oligo dTs (IDT), and dNTPs (Invitrogen; 0.5 mM final concentration) and incubated for 5 min at 65°C. The reaction was then chilled on ice for 5 min and first-strand reaction buffer (Invitrogen; 1x final concentration), dithiothreitol (5 mM final concentration), RNase Out (Invitrogen; 20 U), and nuclease-free water (0.5 μL) were added. The mixture was incubated at 42°C for 2 min before Superscript II reverse transcriptase (Invitrogen; 1 μL) was added (total reaction volume of 20 μL). Lastly, the reaction was incubated at 42°C for 50 min and then 72°C for 15 min.

The cDNA template was diluted 5x and 1 μL was added to a 12.5 μL reaction containing 6.25 μL Rotor-Gene SYBR Green PCR master mix (Qiagen) and 200 nM of each gene specific sense and antisense primers (Table 5.1). Fluorescence was detected using a Rotor-Gene Q Real Time PCR cycler with the following cycling parameters: 95°C for 5 min followed by 40 cycles of 95°C for 5 s and 60°C for 10 s. All reactions were run in duplicate. For the rectal gland, genes were normalised using the NORMA-Gene method (Heckmann *et al*., 2011). Relative mRNA levels were then calculated using the comparative delta C_t method (Schmittgen and Livak, 2008).

5.3.6 Statistics

Two-way repeated measure ANOVAs were used to analyse plasma osmolality, sodium, chloride, urea, and TMAO as well as ammonia and urea excretion while one-way ANOVAs were

used to analyse tissue urea and TMAO as well as the qPCR data. The Holm-Sidak post-hoc test was used when significance was observed. A log transformation was used when the assumption of equal variance failed.

Table 5.1 qPCR primer sequences.

Gene	Forward Primer	Reverse Primer
Na+/K+-ATPase	TACTGCTCGAGGTGTTGTCG	GCTTCAAGCCAGCTGTATCC
Lactate dehydrogenase	AAAATGGTGGTGGAAAGTGC	TCAGACCATCAGCACTCAGG
Glut1	AGTCGGATTGTTCGTCAACC	GCCGGAGTAGATGCCAATAA
Glut4	GTGGATGTGCGATTCTGATG	GATGAAATTTGCGGTCCAGT
Mct1	CAGCAGTCTCCTGGTGAACA	AGTTGAAGGCGAGTCCAAGA
Mct2	GTGAGGCCAAGGATCCAGTA	TCCCAGAAGTACAGGGCAAC

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5.4 Results

5.4.1 Plasma

In all three treatment groups, mean plasma osmolality at the beginning of the experiment was between 950-960 mmol kg^{-1} and the seawater was approximately 910 mmol kg^{-1}. By 6 h, osmolality had decreased significantly to 923 mmol kg^{-1} ± 7 SEM in the low salinity group and it continued to decrease throughout the experiment, ending at around 831 mmol kg^{-1} ± 7 SEM (Two-way RM ANOVA; n=6; $p<0.001$; Fig. 5.1). The mean osmolality of the water for the low salinity group was 636 mmol kg^{-1} ± 25 SEM. In the high salinity group, osmolality increased significantly to 996 mmol kg^{-1} ± 8 SEM by 6 h and continued to increase to a value of 1083 mmol kg^{-1} ± 8 SEM at 24 h (Fig. 5.1). The mean osmolality of the water for this group was 1182 mmol kg^{-1} ± 25 SEM. Plasma osmolality in the control group remained constant throughout the experiment and the mean water osmolality was 933 mmol kg^{-1} ± 7 SEM.

Plasma urea did not differ significantly from the time zero value at any of the time points within each treatment group. However, there was a trend towards an increase in the 130% seawater group such that at 24 h, this group had significantly higher urea concentrations relative to the 100% and 70% seawater groups (Two-way RM ANOVA; n=6; $p<0.01$; Fig. 5.2A). There were also no significant differences within treatment groups for plasma TMAO. However, plasma TMAO in the 70% seawater group decreased enough that at 24 h, it was significantly lower than in the other two treatment groups (Two-way RM ANOVA; n=6; $p<0.01$; Fig. 5.2B). By 24 h, plasma sodium was significantly lower than the time zero value within the 70% seawater group and significantly higher than the time zero value within the 130% seawater group. However, only the 70% seawater group was significantly different from the 100% seawater group at this same time point (Two-way RM ANOVA; n=6; $p<0.001$; Fig. 5.2C). As

with urea and TMAO, plasma chloride did not change within treatment groups. However, at 24 h, the 130% seawater group had significantly higher plasma chloride relative to the 70% and 100% seawater groups (Two-way RM ANOVA; n=6; $p<0.01$; Fig. 5.2D). We also calculated the proportion of the osmolality that each osmolyte accounted for. In the 70% and 100% seawater groups, the percent composition for sodium, chloride, urea, and TMAO remained constant over the 24 h period. In the 130% seawater group, the proportions of urea and TMAO remained constant (32% and 8% respectively) but those of sodium and chloride increased. At time zero, sodium composed 26% and chloride 19% of the osmolality whereas at 24 h, these increased to 31% and 22%, respectively.

5.4.2 Muscle and Liver

In the liver, there were no significant differences in urea concentrations between the three groups (data not shown), but TMAO was significantly higher in the high salinity group (28 mmol L^{-1} ± 3 SEM) relative to the other two groups (both 19 mmol L^{-1}; One-way ANOVA; n=6; $p<0.05$). The high salinity group had significantly greater muscle urea concentrations (463 mmol L^{-1} ± 19 SEM) than both the control (404 mmol L^{-1} ± 14 SEM) and low salinity (361 mmol L^{-1} ± 15 SEM; One-way ANOVA; n=6; $p<0.01$) groups whereas muscle TMAO did not change.

5.4.3 Nitrogen Excretion

Urea excretion rates for the low salinity group increased significantly from the control value of 327 μmol N kg^{-1} h^{-1} ± 40 SEM to 612 μmol N kg^{-1} h^{-1} ± 53 SEM at the end of the experiment, but significant differences could be seen as early as 6 h (Fig. 5.3A). For the control and high salinity groups, urea excretion also increased gradually such that the rates were significantly higher than the time zero values at 18 h but by 24 h the rates had begun to decrease

again, at which point the low salinity group had significantly higher excretion rates than the control and high salinity groups (Fig. 5.3A). Mean ammonia excretion rate increased significantly from 57 μmol N kg^{-1} h^{-1} ± 2 SEM to 123 μmol N kg^{-1} h^{-1} ± 18 SEM in the low salinity group and by 18 h was significantly higher than the ammonia excretion rate of the control and high salinity groups whose rates remained relatively constant throughout the experiment (Fig. 5.3B).

5.4.4 Enzyme Activity and qPCR

In the rectal gland, *nka* mRNA levels were significantly higher in the low salinity group compared to the other two groups but there was no change in activity levels (Fig. 5.4). In contrast, we observed no changes in *ldh* mRNA levels but LDH activity was significantly lower in the low salinity group (122 μmol min^{-1} g^{-1} ± 8.55 SEM) compared to the control (155 μmol min^{-1} g^{-1} ± 9.29 SEM) and high salinity (157 μmol min^{-1} g^{-1} ± 9.65 SEM) groups (Fig. 5.5). For *gluts 1* and *4*, there was a decrease in mRNA levels with increasing salinity but this was only significant for *glut4* (Fig. 5.6 A,B). There were significantly higher levels of both *mcts 1* and *2* in the low salinity group relative to the control and high salinity groups (Fig. 5.6 C,D).

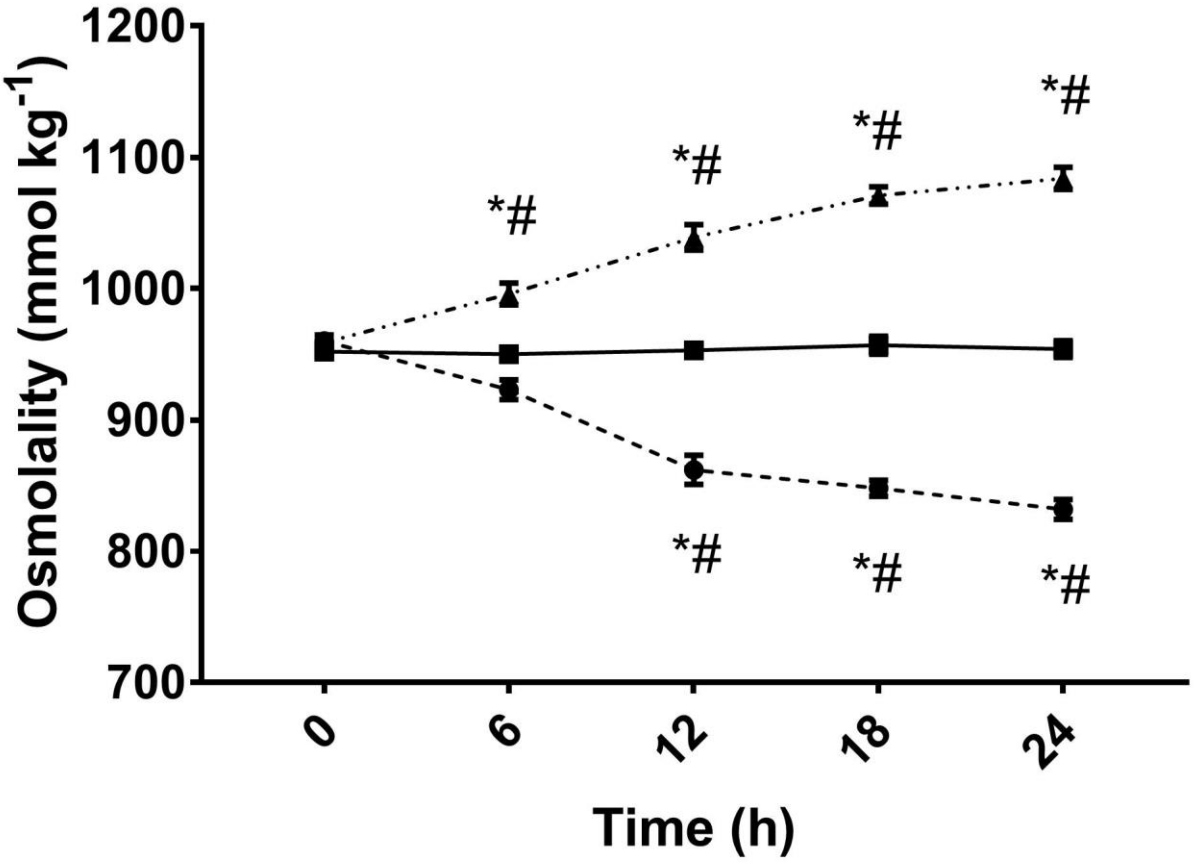

Figure 5.1 Plasma osmolality (mmol kg^{-1}) for *Squalus suckleyi* in 70% seawater (●), 100% seawater (■), and 130% seawater (▲). Data presented is means ± SEM. * indicates significant difference from the 100% seawater group and # indicates significant difference from the 0 h time point within a treatment (Two-way RM ANOVA; n=6; $p<0.001$).

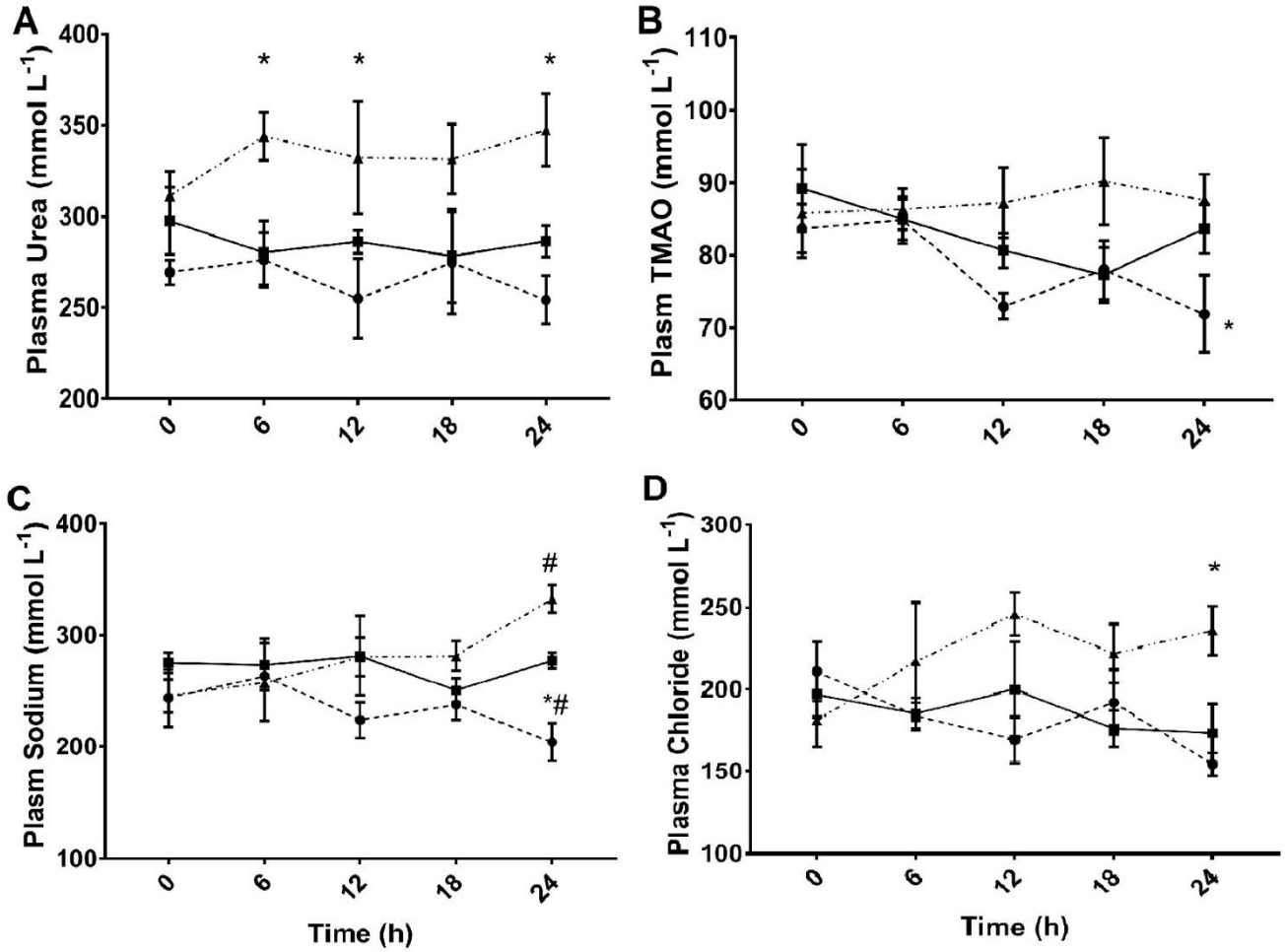

Figure 5.2 Plasma urea (A), TMAO (B), sodium (C), and chloride (D) for *Squalus suckleyi* in 70% seawater (●), 100% seawater (■), and 130% seawater (▲). Data presented is means ± SEM. * indicates significant difference within a time point and # indicates significant difference from the 0 h time point within a treatment (Two-way RM ANOVA; n=6; $p<0.01$).

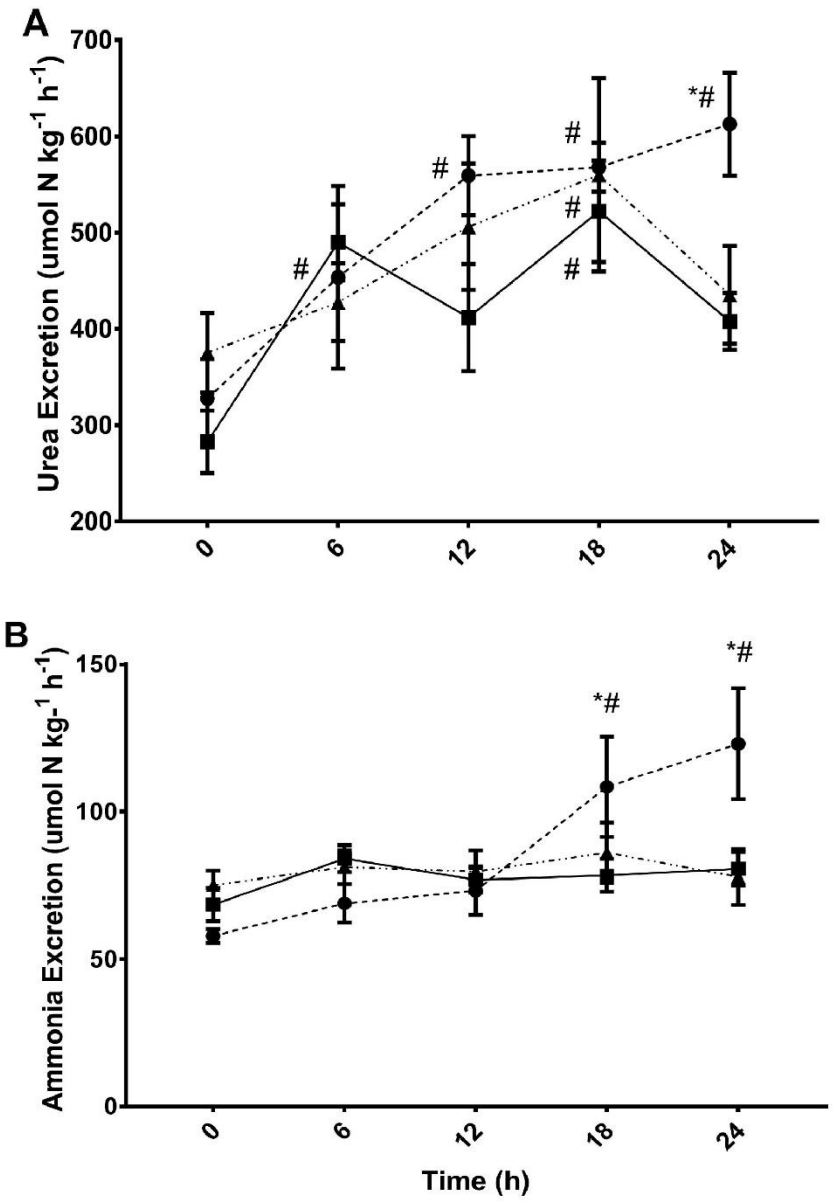

Figure 5.3 Urea (A) and ammonia (B) excretion rates for *Squalus suckleyi* in 70% seawater (●), 100% seawater (■), and 130% seawater (▲). Data are means ± SEM. * indicates significant difference within a time point and # indicates significant difference from the 0 h time point within a treatment (Two-way RM ANOVA; n=6; $p<0.05$).

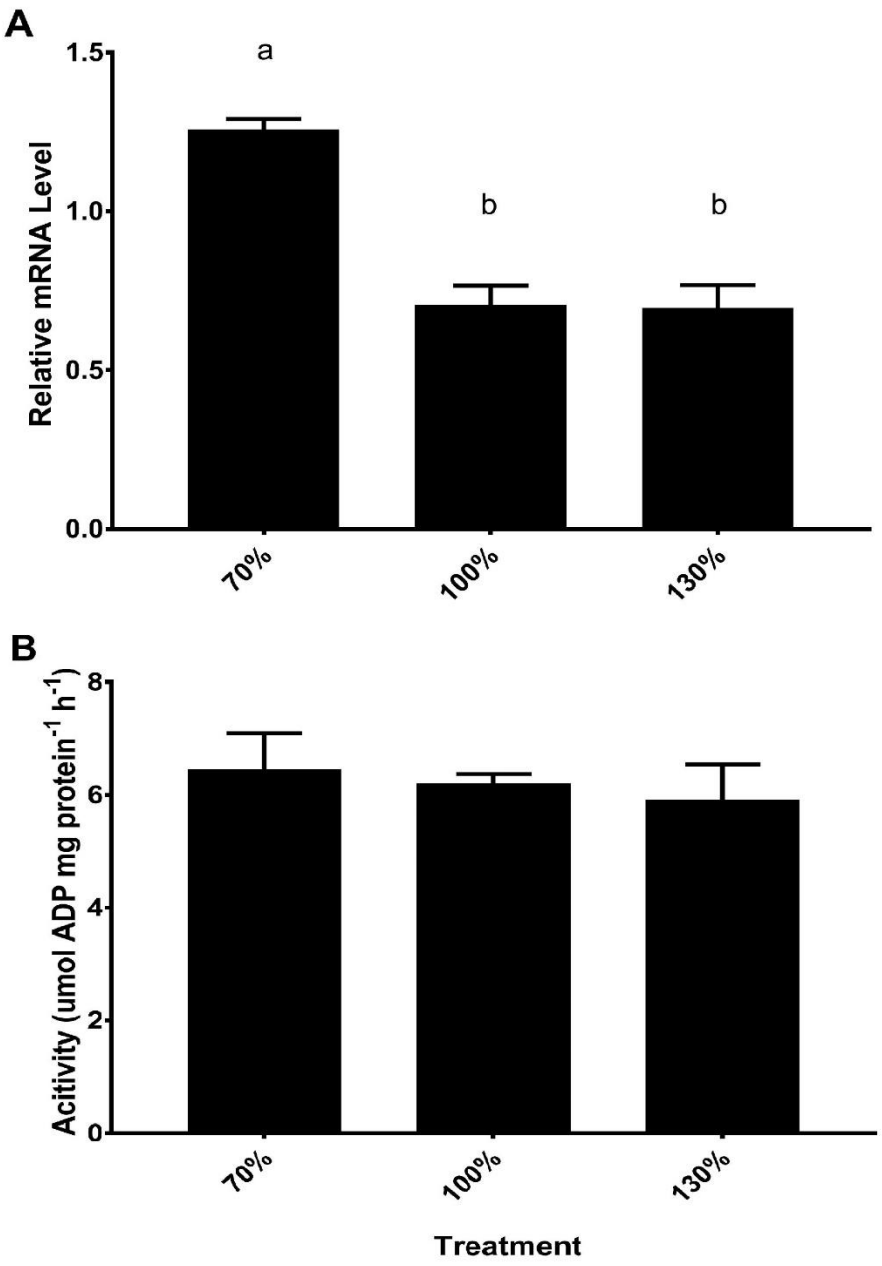

Figure 5.4 Na^+/K^+-ATPase mRNA level (A) and enzyme activity (B) for rectal glands of *Squalus suckleyi* in 70%, 100%, and 130% seawater. Data are means + SEM. Different letters indicate significant difference (One-way ANOVA; n=6; $p<0.001$).

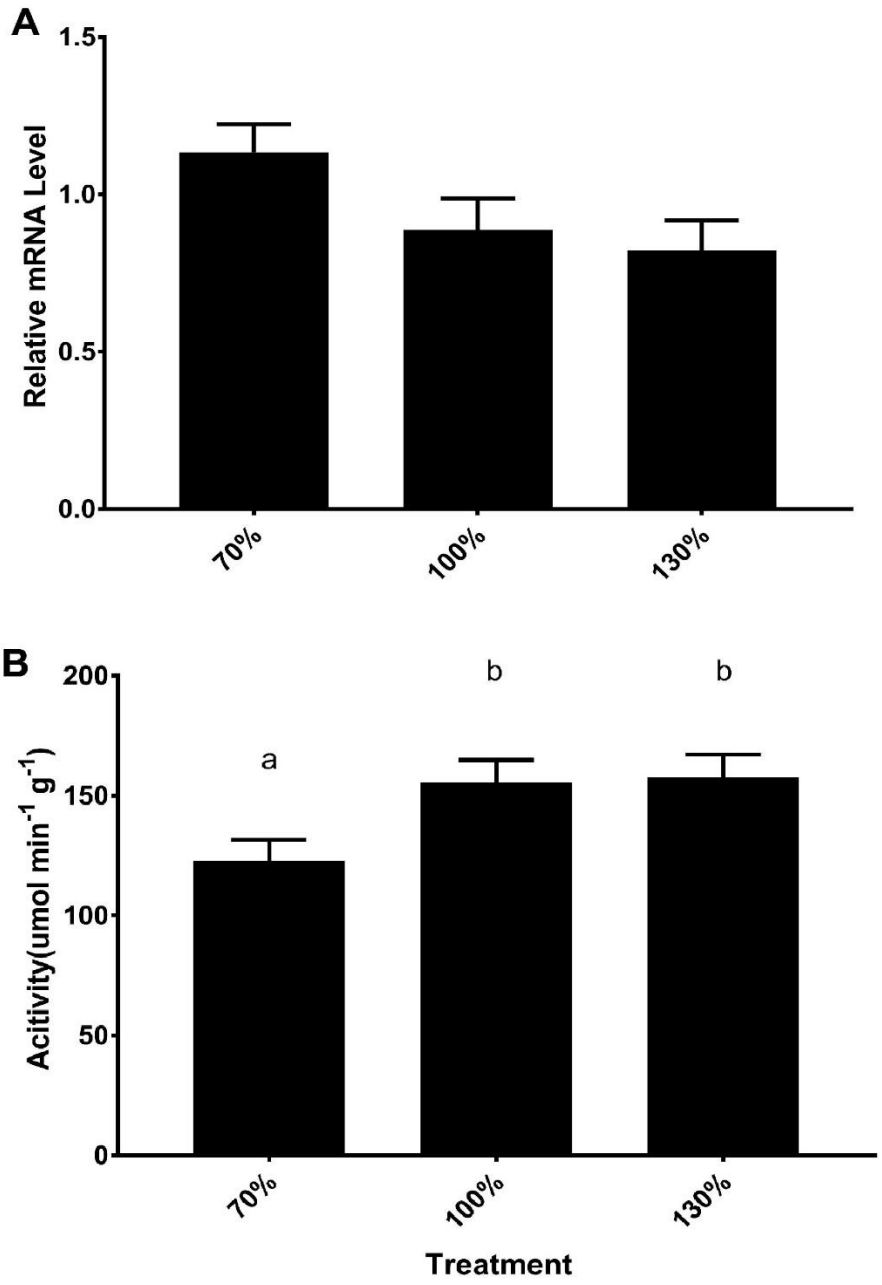

Figure 5.5 Lactate dehydrogenase mRNA level (A) and enzyme activity (B) for rectal glands of *Squalus suckleyi* in 70%, 100%, and 130% seawater. Data are means + SEM. Different letters indicate significant difference (One-way ANOVA; n=6; $p<0.05$).

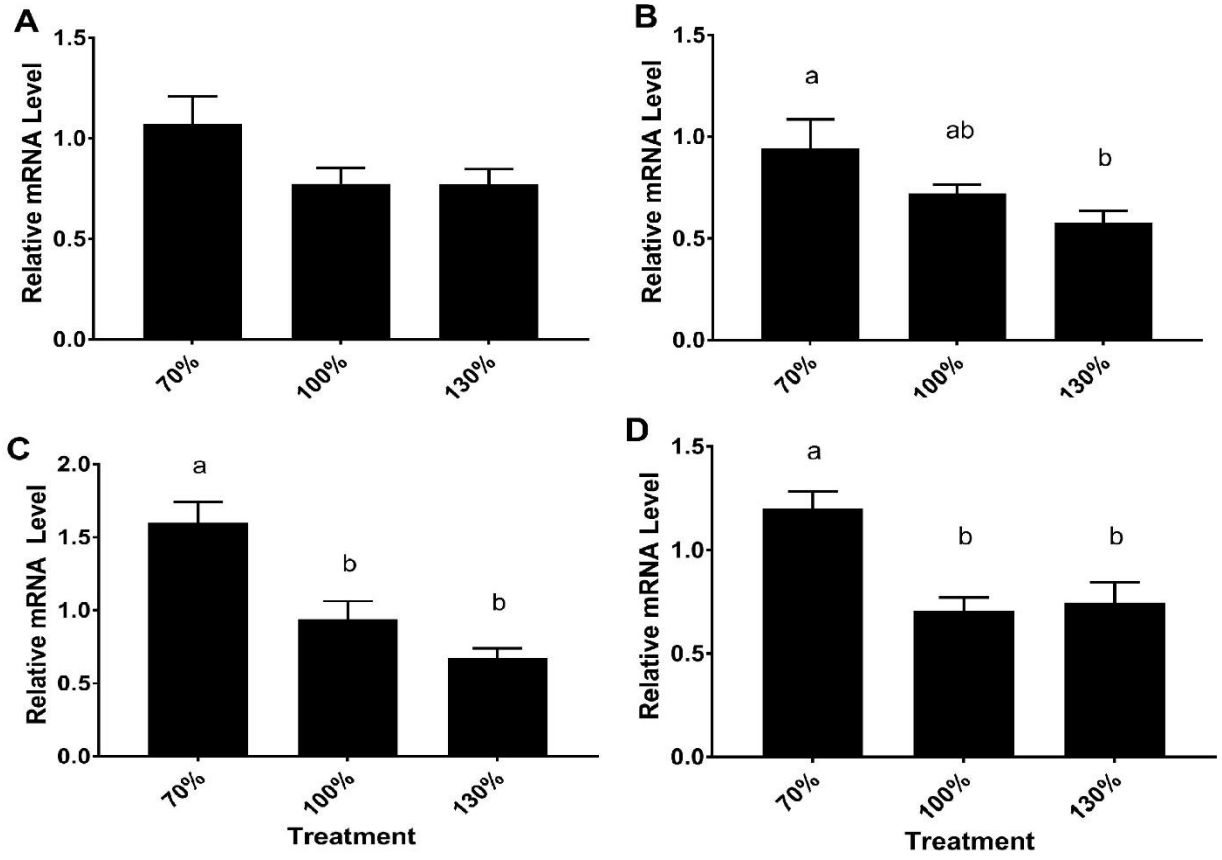

Figure 5.6 mRNA levels of *glut1* (A), *glut4* (B), *mct1* (C), and *mct2* (D) for rectal glands of *Squalus suckleyi* in 70%, 100%, and 130% seawater. Data are means + SEM. Different letters indicate significant difference (One-way ANOVA; n=6; $p<0.05$).

5.5 Discussion

As previously mentioned, there has been a bias towards low salinity exposures for marine elasmobranchs because it is more common for them to experience such environments in the wild. Previous studies investigating the effects of hyper-saline conditions used long-term exposures and thus were unable to show a time course for adapting to such environments. For our study, we investigated the effects of both elevated and reduced salinity on plasma and tissue osmolytes, nitrogen excretion, and the rectal gland in *S. suckleyi* over a 24 h period to assess the short-term response to osmotic challenges.

It was first noted that plasma osmolality increased in the high salinity group and decreased in the low salinity group. It is well known that the osmoregulatory strategy of marine elasmobranchs involves retaining high concentrations of plasma urea to maintain a slight hyper-osmolality relative to that of seawater. This causes a slight influx of water that reduces the need to drink. When the external salinity increases however, the plasma is no longer hyperosmotic to the environment, leading to larger salt influxes as well as water efflux. Thus, an increase in plasma osmolality must occur to match or exceed the ambient seawater so as to avoid these problems, which is what was observed here, and these findings concur with studies in *S. canicula* (Hazon and Henderson, 1984; Armour *et al*., 1993) and *C. punctatum* (Cramp *et al*., 2015). In contrast, the dogfish lowered their plasma osmolality at low salinities to prevent the influx of water, something that has been observed previously in *S. suckleyi* (Guffey and Goss, 2014), *S. canicula* (Hazon and Henderson, 1984; Armour *et al*., 1993), *Carcharhinus leucas* (Pillans *et al*., 2005), and *Dasyatis sabina* (De Vlaming and Sage, 1973; Piermarini and Evans, 1998; Janech *et al*., 2006). With the exception of Guffey and Goss (2014), samples were only taken after the 5-7 day acclimation period, not during, so no time course for the changes in osmolality was

available. This study shows that the dogfish significantly alter their plasma osmolality within 6 h of being exposed to a new salinity and that these changes begin to taper off by 18 h.

Altering plasma osmolality involves changing the concentrations of a number of solutes in the plasma. A significant increase in plasma urea was observed in the high salinity group, which has also been shown in *S. canicula* (Hazon and Henderson, 1984), but no changes were detected in the low salinity group. Hazon and Henderson (1984) showed that at low salinities, sodium is preferentially retained over urea, which would suggest that a significant decrease in plasma urea would occur, as was the case in most of the studies mentioned previously. Janech *et al*. (2006), however, did not observe a decrease in plasma urea. In their study, *D. sabina* were only exposed to low salinity for 24 h rather than days or weeks, as was the case in the present study. This, along with the lower control urea concentrations observed in the low salinity group (relative to the control and high salinity groups) could explain why a significant decrease in plasma urea was not observed. Another possibility is that urea is being released from the tissues into the plasma as muscle urea was lower in the low salinity group. Furthermore, urea is not the only solute that can change with salinity; plasma TMAO, sodium, and chloride are also of importance. Indeed, a significant decrease in plasma sodium in the low salinity group was observed.

It is interesting that an almost two-fold increase in urea excretion in the low salinity group was observed because there was not a large reduction in plasma urea levels. Furthermore, significant increase in ammonia excretion was seen in the low salinity group, which leads to the belief that urea production had been reduced. In elasmobranchs, most of the ammonia produced from protein catabolism is converted to glutamine and used to produce urea. Therefore, only a very small proportion of nitrogenous wastes are excreted in the form of ammonia (Wood *et al*.,

1995). A two-fold increase in ammonia excretion then, suggests that less ammonia is being shuttled into the urea cycle and since large concentrations of ammonia are toxic, it is being excreted. Thus, although the increase in urea excretion was expected and it agrees with previous studies conducted on *S. suckleyi* (Guffey and Goss, 2014) and *R. erinacea* (Steele *et al*., 2005), it was not associated with a decrease in plasma urea as predicted.

Gradual but significant increases in urea excretion were also observed in the high salinity group, which was unexpected. Elasmobranchs that are exposed to higher salinities increase their plasma osmolality as was discussed previously and part of this increase comes from urea. Therefore, it would be logical to reduce urea excretion in order to maintain a higher plasma osmolality. The gills, however, have an outwardly directed gradient by which urea passively flows and it is possible that the increase in plasma urea has led to greater outward diffusion simply based on the increased size of the gradient from the plasma to the ambient seawater. Furthermore, the greater the urea concentration in the plasma, the more inefficient the renal urea transporter is (Kempton, 1953), which may help to account for the increases in urea excretion observed. In addition, elasmobranchs have been shown to excrete large amounts of urea in response to stress (Evans and Kormanik, 1985), and as increases in urea excretion were also observed in the control group, it is possible that the stress imposed by the water changes may have been a factor (although no changes in plasma glucose were observed; data not shown). Regardless, it is interesting that the dogfish significantly increased plasma urea concentrations under hyper-saline conditions despite these increases in excretion as they were not provided with an external source of nitrogen.

A noteworthy point, however, is the difference in the proportion of the osmolality that is made up by each osmolyte between the different salinities. Hazon and Henderson (1984) showed

that at low salinities the percent osmolality that is accounted for by both sodium and chloride is increased compared to higher salinities and vice versa for urea and this seems to hold true for the Cramp *et al*. (2015) study as well, although the urea levels in the latter study were exceptionally high. In contrast, we observed no changes in percent composition of any of the osmolytes measured between the 0 and 24 h time points of the 70% and 100% seawater groups. We also did not observe any changes in urea or TMAO for the 130% seawater group. However, the proportions of sodium and chloride (particularly sodium) did increase in the 130% seawater group relative to both the time zero value and the 24 h values for the 70% and 100% seawater groups. This, along with the lack of increase in urea, may indicate that the dogfish were limited in their ability to increase their plasma urea to the levels required at the salinity tested, possibly due to the lack of a dietary nitrogen source, and thus sodium and chloride had to be increased to a greater extent in order to generate plasma that is at least iso-osmotic to the ambient seawater. This concurs with the study conducted by Armour *et al*. (1993) in which catsharks (*S. canicula*) fed a low protein diet to hinder urea synthesis showed no ability to increase plasma urea in response to increases in salinity. The percent composition of TMAO did not differ widely between treatments, and no increase in plasma TMAO levels was observed at the high salinity. It is believed that the spiny dogfish lacks the enzyme required to synthesise TMAO and must obtain it from their diet (Baker *et al*., 1963). Since they had been held without food for some time prior to experimentation, this finding may provide additional evidence for the inability of *S. suckleyi* to synthesise TMAO.

In regards to the rectal gland, the mRNA levels of *nka* were significantly higher in the low salinity group compared to the other two groups. This could be similar to the findings of Deck *et al*. (2013) (see Appendix A) where starved fish had higher levels of *nka* than fed fish

even though gland activation occurs with feeding (eg. Walsh *et al*., 2006). Deck *et al*. (2013) put forth the notion that the rectal gland stores mRNA for proteins that would be required immediately upon activation. In the wild, exposure to hyposaline conditions is relatively short-lived as behavioural modifications allow a return to full strength seawater and thus the higher levels of mRNA in the low salinity group could signify the rectal gland preparing for this return. We did not, however, see an even greater reduction in *nka* mRNA levels in the high salinity group relative to the controls and there were no differences in NKA activity (which is much more indicative of activation) across the three groups, suggesting that the rectal gland may not have been further activated during our experiment. Notably, the rectal gland NKA activity in the control group was approximately as high as the highest NKA activity observed by Walsh *et al*. (2006) at 6 h post-feeding, and higher than their unfed group (which displayed NKA activities of about 4 μmol ADP h^{-1} mg $protein^{-1}$). If NKA activity can be used as a proxy for gland activity, it is clear that the gland is 'active' in our experimental treatments. It is possible that the short time frame of our experiment, however, did not necessitate an increase in enzyme activity as only modest (although significant) increases in plasma sodium and chloride were observed. On the other hand, since our results suggest that the dogfish are capable of rapidly adapting to a change in salinity, the relevant time frame for increases in NKA activity could have occurred earlier on in the experiment, leading to reduced increases in plasma sodium and chloride. In a study by Piermarini and Evans (2000), higher NKA activity in the rectal glands of seawater acclimated *D. sabina* relative to freshwater rays was reported. This is a fully euryhaline species however, compared to the partially euryhaline dogfish so the osmoregulatory strategies likely vary. In contrast to NKA, higher activity levels of LDH were observed in the rectal glands of control and high salinity dogfish suggesting that either the gland is more active at these salinities, or that

blood or oxygen supply to the gland has diminished. However, since the activity level did not increase between the control and high salinity groups, the salinity may not have been high enough to require rectal gland activation or the relevant time point occurs prior to our first measurement.

Levels of glucose and monocarboxylate transporters (which transport lactate and ketone bodies) were also measured in the rectal gland. Walsh *et al*. (2006) determined that the gland was glucose-dependent, but could use ketone bodies to augment secretion rates, and that it may be producing excess lactate since LDH activity increased with feeding but there was an inability for the gland to use lactate as a fuel. Since it was believed that hypersaline conditions would activate the gland, an upregulation of both types of transporters was expected at this time and a significant decrease in *glut4* mRNA levels was observed. Additionally, the mRNA levels of both *mct 1* and *2* were significantly higher in the low salinity group. These findings oppose our predictions but as was previously mentioned for *nka* in both this study and the Deck *et al*. (2013) study, the higher mRNA levels that appear when the gland is expected to be inactive likely indicate mRNA storage in preparation for reactivation of the gland. Indeed, MCT1 is responsible for transporting ketone bodies into cells and higher protein levels are associated with greater oxidative capacity of the tissue, while MCT2 is responsible for transporting lactate out of cells and GLUT4 is responsible for the uptake of glucose into the cells. Decreases in all three of these at high salinities suggest that the gland may be activated and requires higher levels of these proteins.

Overall, this study has shown that *S. suckleyi* are capable of rapidly adapting to changes in salinity (within 24 h) as indicated by the positive relationships between salinity and plasma osmolality. They can also increase plasma urea in response to a high salinity exposure, but the

increase appears to be limited, possibly due to the lack of dietary nitrogen. On the other hand, the dogfish did not elevate plasma TMAO in response to elevated salinity, suggesting that they lack the enzyme for TMAO synthesis. Furthermore, mRNA data for the rectal gland reiterates the idea of mRNA storage in order to prepare for reactivation of the gland and LDH activity suggests that the gland is less active at low salinities. However, the lack of difference between the control and high salinity groups for both LDH and NKA may indicate that activation of the gland is just not necessary at the salinities tested. Another possibility is that due to the apparent limited ability to increase plasma urea, the fish were retaining any salt that entered in order to help match their plasma osmolality to the ambient seawater. It would be interesting to conduct a similar study using recently fed dogfish to identify whether rectal gland activation is required under those circumstances.

5.6 Acknowledgements

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CHAPTER 6

General Discussion

6.1 Summary and Significance

Elasmobranchs are a unique group of vertebrates that, in most respects, have been vastly understudied. Part of this paucity of information stems from conservation concerns and the difficulty in maintaining most species in captivity, but also from a lack of available tools such as homologous antibodies and hormones or full genomes. However, since elasmobranchs hold an important position on the vertebrate tree, they can provide important insights into the evolution of physiological systems. For this thesis, the non-threatened North Pacific spiny dogfish *Squalus suckleyi* was studied to provide one of the first, in-depth examinations of the regulation of glucose transport and metabolism in an elasmobranch. Despite the generally carnivorous nature of elasmobranchs, some species do consume meals with relatively high carbohydrate contents and thus, in addition to the evolutionary aspects, an understanding of glucoregulation in these fish is warranted.

The main findings of this thesis, which are detailed throughout this chapter, are as follows:

1. Contrary to prior speculation, elasmobranchs possess members of the GLUT family of transporters, most notably a putative GLUT4 transporter.

2. Dogfish do not appear to be as glucose or insulin intolerant as previously thought and can in fact regulate glucose concentrations through uptake via tissues. They respond to both in a manner similar to that observed for mammals and teleosts, suggesting a conserved mechanism for glucoregulation.

3. The rectal gland is indeed glucose-dependent and appears to store mRNA for many genes when inactive, notably when exposed to lowered salinities.

6.1.1 Glucose Transporter Evolution

The first aspect of this thesis was a phylogenetic analysis of Class I facilitative glucose transporters (GLUTs) in vertebrates, which determined that elasmobranchs do possess these transporters, most notably GLUT4 (generally considered to be the insulin-responsive transporter; reviewed by Huang and Czech, 2007), which was believed to be absent in these fish as postulated by Anderson *et al*. (2002). The presence of a putative GLUT4 is a significant finding as it brings into question the proclaimed glucose- and insulin-intolerance of elasmobranch fish. Further, PCR analysis revealed that the tissue distribution of *glut* mRNA follows patterns observed in other vertebrate groups, suggesting a potential conservation of function (Table 6.1). Unfortunately, protein expression could not be measured in any of the studies presented in this thesis as the mammalian antibodies were not specific to elasmobranch GLUTs and the attempt to produce a homologous antibody was not successful (see Appendix B).

A second interesting finding was that, although these fish appear to possess a putative GLUT4 transporter, sequences for a GLUT2 transporter could not be identified. Kuraku *et al*. (2009) showed that GLUTs 1 and 3 are phylogenetically similar and GLUTs 2 and 4 are phylogenetically similar, suggesting that these pairs are likely duplications of one another and the addition of elasmobranch sequences in the phylogeny presented in Chapter 2 appears to concur with these findings. Thus, as stated in Chapter 2, GLUT2 may have been lost in elasmobranchs due to a lack of necessity, possibly stemming from their carnivorous diets (although other carnivores do not appear to have lost this transporter). Similarly, the Elephantfish (a holocephalan) was also revealed to only possess three of the four Class 1 GLUTs but in this species it was a GLUT4 sequence that was absent. GLUT2 has been shown to also be insulin-responsive and the Elephantfish sequence contains similar functional motifs to those found in the

GLUT4 sequences from other vertebrates suggesting that it may take on the role of GLUT4 in holocephalans. It is interesting both that these two sister taxa appear to have lost an insulin-responsive transporter, and that the 'lost' transporter is not consistent between them. This suggests that both the elasmobranchs and the holocephalans may have been susceptible to similar selection pressures based on either their lifestyle or unique physiology. However, limited genomic information exists for either elasmobranchs or holocephalans and as such the data for this analysis was acquired from the transcriptomes of only four species, using cDNA from single tissues. Thus, although this thesis has provided important insights into the evolution of GLUTs and put to rest uncertainties about the presence of GLUT4 in elasmobranchs, the absence of GLUT2 (and GLUT4 in holocephalans) may remain an open question until genomic data becomes available for more species.

Table 6.1 Distribution of Class I *glut* mRNA in elasmobranchs relative to other vertebrates.

Transporter	Common Name	Functional Information	*Elasmobranchs*	Teleosts	Birds	Mammals	References
SLC2A1	GLUT1	Erythrocyte-type	*All Tissues Examined*	All Tissues Examined	All Tissues Examined	All Tissues Examined	Welch et al. (2013); Sweazea and Braun (2006); Kono et al. (2005); Balmaceda-Aguilera et al. (2012); Bell et al. (1990); Hall et al. (2014)
SLC2A2	GLUT2	Low-affinity; pancreatic glucose sensor	*?*	Kidney, Liver, Muscle, Brain, Intestine	Kidney Liver	Kidney, Liver, Intestine	Welch et al. (2013); Terova et al. (2009); Hall et al. (2006); Panserat et al. (2001); Bell et al. (1990); Kono et al. (2005); Hall et al. (2014)
SLC2A3	GLUT3	High affinity	*All Tissues Examined*	All Tissues Examined	All Tissues Examined	All Tissues Examined	Welch et al. (2013); Bell et al. (1990); Hall et al. (2005); Zhang et al. (2003); Kono et al. (2005); Hall et al. (2014)
SLC2A4	GLUT4	Insulin-responsive	*Brain, Muscle, Heart, Gill, Liver, Intestine*	Kidney, Heart, Gill, Muscle, Intestine, Adipose Tissue	Not Present	Heart, Muscle, Brain, Kidney, Adipose Tissue	Welch et al. (2013); Bell et al. (1990); Kono et al. (2005); Capilla et al. (2004); Planas et al. (2000); Hall et al. (2014)

6.1.2 Glucoregulation in Elasmobranchs

A large part of this thesis was the examination of potential glucoregulatory mechanisms in elasmobranchs, something that had yet to be studied. The final aspect of Chapter 2 provided an investigation into the effects of feeding on *glut* expression in various tissues. It was determined that significant increases in mRNA levels (particularly *glut4*) were observed in the liver, intestine, and muscle. This is the first report of GLUT regulation in elasmobranchs and although the meal consumed consisted of very little glucose, amino acids are potent insulinotropins in teleost fish (Mommsen and Plisetskaya, 1991), and thus it was postulated that changes in *glut* levels in elasmobranchs may be hormonally induced, as is the case in other vertebrates. The regulation of *glut* expression was further investigated through the administration of glucose (Chapter 3). Here, it was determined that glucose-loading results in similar changes in *glut* mRNA levels to what was observed following feeding, as well as significant increases in both liver and muscle glycogen content and muscle *glycogen synthase* mRNA. Previous studies (Hartman *et al*., 1944; Patent, 1970) have investigated the ability of dogfish to clear a glucose load from their plasma, and the time frame observed in this study concurs with their findings. However, this is the first examination of how elasmobranchs are able to restore plasma glucose levels and the mechanisms (increases in mRNA levels of *gluts* and *glycogen synthase*, as well as tissue glycogen) appear to be similar to what is observed in other vertebrates. For instance, in mammals, an increase in plasma glucose induces an increase in insulin secretion, which then acts to increase glucose uptake into the liver and muscle by either translocating or upregulating GLUTs, and activates glycogen synthase to induce glycogen synthesis (Saltiel and Kahn, 2001). In teleosts, Choi and Weber (2015) showed that hyperglycaemia leads to an increase in glucose uptake by the liver, a known action of insulin in these fish (Mommsen and Plisetskaya, 1991).

Further, Blasco *et al*. (1996) indicated that glucose stimulates insulin release in teleosts and two studies by Diaz *et al*. (2007; 2009) showed that insulin increases levels of *glut4* mRNA in skeletal muscle. Such similarities between elasmobranchs and other vertebrates supports the conservation of glucoregulatory mechanisms throughout the vertebrate lineage. There is an interesting parallel between GLUT expression in elasmobranchs and that of the genes for the Ornithine-Urea Cycle and urea transporter family. Much as glucose tolerance and clearance were viewed as a 'mammalian invention', it was originally believed that urea synthesis evolved multiple times in vertebrate evolution and was re-invented by mammals. Indeed, urea synthesis and transport occur in the elasmobranchs by much the same mechanisms as in mammals (see LeMoine and Walsh, 2015 for review). Combined with the results of this thesis, it is likely that many physiological adaptations of the vertebrate group were in place very early in the lineage.

The results of the glucose-loading study reinforced the idea that insulin may be regulating *glut* levels and thus insulin injections were performed to determine whether similar responses would be observed (Chapter 3). The only effects of the insulin injection were a slight decrease in plasma glucose and increases in *glut4* and *glycogen synthase* mRNA in the muscle. It should be noted that heterologous insulin was used for this study which could have dampened the effect[4]. On the other hand, muscle is typically the most insulin-responsive tissue and thus it could have been expected that this was the only tissue in which effects would be observed. In any case, the observed increase in *glut4* is a significant finding as this occurs in other vertebrates (Bourey *et al*., 1990; Diaz *et al*., 2009) and suggests that, not only do elasmobranchs possess a putative GLUT4 transporter, but that it likely functions in a similar manner to mammals and teleosts;

[4] Note that a study by deRoos and deRoos (1979) showed that bovine insulin induced a drastic increase in dogfish plasma glucose levels, however, their doses were much higher than the ones used in Chapter 3.

specifically, translocating to the plasma membrane in response to insulin (Diaz *et al*., 2007; Huang and Czech, 2007). Relative to GLUTs 1 and 3, GLUT4 is less conserved in vertebrates, however all sequences used in the phylogeny from Chapter 2 possess specific motifs that regulate both its storage in intracellular vesicles, as well as its insulin-responsiveness, and these amino acids were also highly conserved in the elasmobranch sequences.

6.1.3 Rectal Gland

Rectal gland function and fuel usage was a prominent theme in this thesis. Chapter 4 investigated this directly, using an *in vitro* perfusion protocol along with glucose transport inhibitors and glucoregulatory hormones to investigate the glucose-dependence of the gland. In this study, it was determined that the prediction was correct and preventing the gland from taking up glucose from the perfusate significantly diminished its capacity to secrete chloride. Further, it was shown that GLP-1 is a potent activator of the gland, in contrast to insulin and glucagon. One of the most prominent activators of the rectal gland is the act of feeding, and in mammals GLP-1 is released by the intestine in response to the presence of nutrients in the lumen (Brubaker and Amini, 2003). Thus, it is possible that GLP-1 could be one of the signals that leads to rectal gland activation post-feeding, perhaps through the activation of SGLTs. This chapter also highlighted the use of anaerobic metabolism by the gland during times of high secretory activity, something that had been postulated by Walsh *et al*. (2006) following the observation that LDH activity significantly increased post-feeding. Forskolin and GLP-1 both activated the gland and, in turn, caused a significant increase in lactate production, supporting the notion that, during times of peak activity, fuel usage by the gland outpaces oxygen delivery.

In Chapter 2, the presence of GLUTs in the gland and their regulation by feeding was investigated. Interestingly, the results opposed the initial hypothesis, indicating that transcript

abundance decreases rather than increases following a meal. However, similar trends were observed in a study by Deck *et al*. (2013) (see Appendix A) and it was believed that the rectal gland was storing mRNA of genes that are essential for chloride secretion to allow for rapid protein synthesis upon activation. Although it may not seem as though GLUTs themselves would play an essential role in secretion, both Walsh *et al*. (2006) and Chapter 4 of this thesis provided evidence for the gland's glucose-dependence and thus, taking up glucose from the blood would indeed be essential for activation.

Both Chapters 2 and 4 highlighted gland activation via biotic factors (feeding, hormones) but the abiotic factor of salinity was also investigated. In Chapter 5, dogfish were exposed to hyper- and hypo-osmotic environments and changes in transcript abundances and enzyme activities were used as a proxy for gland activation. As a general trend, mRNA levels were higher in rectal glands from dogfish exposed to low salinities, namely *nka* and *gluts*. This was not entirely unexpected as it was predicted that the gland would not be secreting under such conditions and it was suggested in both Chapter 2 and the Appendix that the gland may store mRNA when inactive. Interestingly, there were no differences in mRNA levels between the control and high salinity fish. This could indicate that either abiotic factors are less likely to activate the gland than biotic ones or the percent change in salinity was not great enough to pose a significant osmoregulatory challenge. Perfused rectal glands from *Scyliorhinus canicula* exposed to hyper- and hypo-osmotic conditions did have higher and lower rates of secretion relative to control fish, respectively (Anderson *et al*., 2005), although this species is thought to be less tolerant to changes in salinity than *S. suckleyi* and thus lifestyle is likely an important factor in determining under which conditions the gland would be active.

6.2 Future Directions

As relayed a number of times throughout this thesis, the lack of tools available for elasmobranchs greatly diminishes our ability to study various aspects of their physiology. In order to truly understand glucoregulation in these fish, we need to acquire homologous antibodies for GLUTs, produce assays that can reliably measure plasma insulin and glucagon levels, have homologous hormones synthesised, and have elasmobranch genomes sequenced. This would allow us to determine which substances are insulinotropic in elasmobranchs as well as investigate whether GLUT4 is stored in intracellular vesicles and translocated to the plasma membrane in response to insulin as it is in mammals and teleosts. Further, we would be able say with more certainty whether these fish have lost the GLUT2 transporter.

In the absence of such tools however, mammalian glucagon and GLP-1 could be used to investigate the effects on *glut* expression and glucose mobilisation. In addition, oocyte studies should be performed to ascertain the functional ability of elasmobranch GLUTs to transport glucose into the cell, as well as determine the kinetics of each transporter. Although difficult in elasmobranchs, the use of hepatocytes and myocytes to examine the effects of insulin and other hormones on glucose transport would also be beneficial. Additionally, in terms of the rectal gland, it would be interesting to measure glucose uptake and lactate production in glands isolated from fish exposed to different salinities.

Additional studies on the mechanisms of action for GLP-1 in elasmobranchs are also warranted. Should homologous assays become available, it would of interest to determine under what conditions this peptide is released as in mammals it is secreted from the intestine in response to feeding. *In vitro* pancreatic perfusions as well as intestinal preparations using Ussing chambers could help to elucidate this as well as tell us the predominate tissue from which the

peptide is released. Further, investigations into its effect on the rectal gland and whether it does act as a regulator of SGLT activity should also be performed.

6.3 Concluding Remarks

Overall, this thesis has provided a framework for understanding the evolution of glucose transport and homeostasis in vertebrates by highlighting the glucoregulatory abilities of elasmobranchs, an evolutionarily important group of fishes. A full examination of Class I GLUTs was conducted for the first time and a number of factors regulating their expression were investigated, showing that indeed, elasmobranchs are capable of regulating plasma glucose levels in a manner similar to that observed for mammals and teleosts. Further, insights were also provided into the function of the rectal gland in these fish, confirming the notion of glucose-dependence (a unique trait for an elasmobranch tissue) and showing the ability of GLP-1, an intestinal peptide involved in feeding and glucoregulation, to activate the gland. The role of the rectal gland in osmotic challenges was also investigated but it was determined that abiotic factors may be less likely to activate the gland relative to biotic factors such as feeding or hormones, at least in the species examined who regularly experience shifts in ambient salinity.

APPENDIX

Appendix A

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Transcriptome responses in the rectal gland of fed and fasted spiny dogfish shark (*Squalus acanthias*) determined by suppression subtractive hybridization

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Abstract

Prior studies of the elasmobranch rectal gland have demonstrated that feeding induces profound and rapid up regulation of the gland's ability to secrete concentrated NaCl solutions and the metabolic capacity to support this highly ATP consuming process. We undertook the current study to attempt to determine the degree to which up regulation of mRNA transcription was involved in the gland's activation. cDNA libraries were created from mRNA isolated from rectal glands of fasted (7 days post-feeding) and fed (6 h and 22 h post-feeding) spiny dogfish sharks (Squalus acanthias), and the libraries were subjected to suppression subtractive hybridiza-tion (SSH) analysis. Quantitative real time PCR (qPCR) was also used to ascertain the mRNA expression of several genes revealed by the SSH analysis. In total the treatments changed the abundance of 170 transcripts, with 103 up regulated by feeding, and 67 up regulated by fasting. While many of the changes took place in 'expected' Gene Ontology (GO) categories (e.g., metabolism, transport, structural proteins, DNA and RNA turnover, etc.), KEGG analysis revealed a number of categories which identify oxidative stress as a topic of interest for the gland. GO analysis also revealed that branched chain essential amino acids (e.g., valine, leucine, isoleucine) are potential metabolic fuels for the rectal gland. In addition, up regulation of transcripts for many genes in the anticipated GO categories did not agree (i.e., fasting down regulated in feeding treatments) with previously observed increases in their respective proteins/enzyme activities. These results suggest an 'anticipatory' storage of selected mRNAs which presumably supports the rapid translation of proteins upon feeding activation of the gland.

1. Introduction

The elasmobranch rectal gland has fascinated physiologists for de-cades. Capable of secreting a nearly 0.5 M NaCl solution to its lumen (for eventual voiding via the lower intestine), it plays an important role in osmoregulation and has served as a key model for understanding trans-epithelial transport of ions. Especially in the spiny dogfish shark (*Squalus acanthias*), its utility has no doubt been aided by the relatively simple architecture of the gland and the ease with which both afferent and efferent blood vessels, and the gland's duct itself, can be cannulated for in vitro experimental manipulation. Indeed, studies have mapped out a now classic model where several plasma membrane-bound proteins, including Na^+K^+ ATPase, the 'CFTR' Cl^- channel, and $Na^+K^+2Cl^-$ transporters, are coordinated to excrete salt (Silva et al., 1997; Olson, 1999; Anderson et al., 2007). Previous findings also included the demonstration of several potential natural and artificial signals for activation of the gland (e.g., C-type natriuretic peptide (CNP); vasoactive intestinal peptide (VIP), forskolin, etc.) (Epstein et al., 1983; Schofield et al., 1991). While originally it was believed that the gland functioned continuously to aid in overall osmoregulatory balance, more recently it has been shown that the gland is relatively inactive during frequent bouts of fasting, but becomes highly active very shortly after feeding; notably dogfish feed opportunistically, and therefore sporadically, in the wild (see references contained within Wood et al., 2010 for review).

The activation of the gland by feeding has been studied from a number of perspectives: morphological, physiological, metabolic, bio-chemical, and proteomic (for review, see Wood et al., 2010). These studies lead to an integrated view that: (i) feeding and digestion trigger activation of the gland, with at least one of the key signals being the increase in plasma pH associated with acid-secretion to the stomach (the so-called 'alkaline tide') (Wood et al., 2005; Shuttleworth et al., 2006; Wood et al., 2007a,b), while another may be the plasma volume expansion that accompanies the ingestion of salty food and accompanying seawater (Solomon et al., 1984; Silva et al., 1997); (ii) morphologically, the gland transitions from a 'dormant' (somewhat apoptotic) state, to a highly active secretory morphology (Matey et al., 2009), accompanied by increases in amounts of key cytoskeletal/muscular proteins (e.g., transgelin, tropomyosin) (Dowd et al., 2008); (iii) likewise, amounts and activities of key proteins of the ion transport pathway are up regulated, including Na^+K^+-ATPase and voltage-dependent anion channels (MacKenzie et al., 2002; Walsh et al., 2006; Dowd et al., 2008); (iv) the metabolic rate of the gland increases markedly, with a relatively complex pattern of metabolic pathway dependence (Walsh et al., 2006; Dowd et al., 2008). While glucose appears to be the main fuel source, evidence exists that β-hydroxybutyrate can serve as an auxiliary fuel (at least in the early stages of activation). Furthermore, al-though the gland appears to be a highly aerobic tissue with high densities of mitochondria, and high activities of tricarboxylic acid (TCA) cycle and energy production enzymes (e.g., isocitrate dehyrogenase, citrate synthase,

ATP synthase) that increase in activity upon feeding, it is likely that at least some of its energy comes from anaerobic glycolysis as evidenced by large increases in lactate dehydrogenase activity when the gland is activated. The inability of lactate to fuel the gland at least in vitro (Walsh et al., 2006) argues against the increase in LDH being for lactate oxidation.

The above picture has been developed using several tools, and leads to a relatively 'straightforward' view of the gland's activation involving changes in morphology, ion secretion, and supporting metabolic path-ways. The time course of changes (especially protein concentrations and enzyme activities), many of which were elevated after only 6 h post-feeding, was consistent with either a coordinated increase in both transcription and translation for appropriate genes, or rapid translation of pre-existing message (Dowd et al., 2008). With this background in mind, we undertook a study of the response of the transcriptome of the gland to feeding/fasting in order to test the hypothesis that wide-spread transcriptomic responses occur rapidly and agree with the physiological activation of the rectal gland upon feeding. Our null hypothesis is that a transcriptomic response was not a large part of the gland's activation in the short-term. Furthermore, assuming at least some role for transcriptional activation, we wished to gain insight into aspects of the activation of the gland that might have been overlooked by prior approaches.

For this examination, we used the approach of suppression subtractive hybridization (SSH), in which the mRNA (converted to cDNA) population of a 'tester' sample (e.g., rectal gland tissue from a fed dog-fish) is hybridized with an excess of mRNA from a 'driver' sample (e.g., rectal gland tissue from a fasted dogfish), such that the mRNA transcripts in common are subtracted from the tester, enriching the tester sample for its unique transcripts. The SSH approach (Diatchenko et al., 1996), although not without its biases, has proven to be a useful tool in complementary studies to traditional physiological and metabolic biochemistry approaches, including many studies in comparative bi-ology (Gracey et al., 2001; Fiol et al., 2006).

2. Materials and methods

2.1. Animals and feeding protocols

Males of the spiny dogfish shark (*Squalus acanthias suckleyi*) were captured by angling or trawl in Barkley Sound, BC (Canada) offshore from Bamfield Marine Sciences Centre in June, 2007 and 2009 and August 2012 under various permits from the Canadian Department of Fisheries and Oceans. Note that Ebert et al. (2010) have recently pro-posed that these north-east Pacific dogfish may be a separate species (*Squalus suckleyi*) rather than a subspecies of *S. acanthias*. Consistent with our prior feeding experiments (see Wood et al., 2010), fish were maintained in a 155,000 L circular tank supplied with running seawater and aeration. After acclimation to the tank, dogfish fed voraciously on a diet of frozen hake supplied every 4th day to a ration size of approximately 2.5% body weight. At 6 h,

22 h (48 h for qPCR only), and 7 days post-feeding (this latter group was removed to a separate tank to ensure that additional feeding did not take place, and is referred to as 'Fasted' below), dogfish were sacrificed by overdose of MS-222 (1 g/L) and the rectal glands removed, frozen in liquid nitrogen and stored at −80 °C. Feeding status was confirmed by examining the gut for the presence (or absence) of food. All experiments were conducted in accordance with guidelines of the Canadian Council of Animal Care, and under approvals from the Animal Care Committees of the University of Ottawa, McMaster University and the Bamfield Marine Sciences Centre.

2.2. Isolation of RNA and construction of a normalized cDNA library

A normalized cDNA library from dogfish rectal gland was created in order to form a potential basis for comparison to the distribution of genes identified (e.g., Gene Ontology (GO) analysis categorization) from more specific treatments by the SSH approach. Total RNA was isolated by standard Trizol (Invitrogen, Grand Island, NY, USA) extraction methods from individual rectal gland samples from 6 fish each from the 6 h, 22 h and 'Fasted' treatments and the RNA integrity was verified with a BioAnalyzer 2100 (Agilent, Mississauga, ON, Canada). Equal amounts of total RNA from these 18 fish were pooled to yield a single sample from which polyA mRNA was further purified by a magnetic bead-based method (Ambion Poly(A)Purist MAG, Ambion, Austin, TX, USA). A normalized cDNA library was created using the Creator SMART cDNA Construction Kit (Clontech, Mountain View, CA, USA) and CDS-3M Adaptors in the Trimmer Direct cDNA Normalization Kit (Evrogen, Moscow, Russia); following normalization, SfiI digestion, and size fractionation, cDNA was directionally inserted into a pTB vector, and transformed into Escherichia coli via electroporation. Bacteria were grown on LB agar plates with carbenicillin (50 μg/mL), and individual colonies were hand-picked at random and grown over-night at 37 °C in LB medium with carbenicillin. PCR was performed directly on 1 μL aliquots of this broth (using primers against the arms of the pTB vector) and PCR products cleaned and sequenced on an ABI 3730xl sequencer (using slight modifications of the methods from Oleksiak et al., 2001).

2.3. Construction of SSH libraries

Equal quantities of total RNA from the above samples of 18 glands were pooled (6 fish per pool) into three samples each representing 6 h, 22 h and 'Fasted' treatments and mRNA was purified as above. Tester and Driver cDNA was synthesized and adaptor-ligated, and subtractions performed as per instructions in the PCR-Select cDNA Subtraction Kit (Clontech). Six libraries were created representing forward and re-verse subtractions for the three treatments (letters correspond to those in tables in Results and discussion): (A) fasted minus 6 h; (C) 6 h minus fasted; (E) fasted minus 22 h; (G) 22 h minus fasted; (I) 6 h minus 22 h; and (K) 22 h minus 6 h. PCR products from second

amplifications were cloned into pCR2.1 TOPO vector (Invitrogen), transformed into E. coli, and colonies were hand-picked, grown in liquid broth overnight at 37 °C and PCR and sequencing was performed as above, except that primers were to the M13R and M13F(-20) sites of this vector.

2.4. Sequence analysis and annotation

Much of the analysis was similar to that conducted in a prior study by our group on transcriptome variation in Aplysia sp. (Fiedler et al., 2010). We have defined quality ESTs as sequence reads with length ≥100 bp after trimming of low quality (phred score b 20) ends, and vector sequence removal using cross_match (Ewing and Green, 1998; Ewing et al., 1998). ESTs were clustered separately for each of the seven rectal gland libraries using CAP3 (Huang and Madan, 1999). We refer to the clustered ESTs as contigs, after the definition of Staden (1980), which comprised both clustered EST and un-clustered singletons. Contigs were subjected to BLAST analysis (NCBI BLAST; Altschul et al., 1997) against protein and sequence databases using ad hoc Perl scripts based on the BioPerl programming interface (Stajich et al., 2002) to run BLAST analysis.

In order to identify matches with previously published EST sequences, individual ESTs were BLASTed against the subset of dbEST (Boguski et al., 1993) that excluded human and mouse ESTs. The tBLASTx (translated nucleotide against translated nucleotide) program was used to identify more distantly related ESTs. An expect-value cutoff of 1e−8 was used as the retention criterion for BLAST hits (see below for discussion of this cutoff). GO and KEGG annotations were performed with the annot8r software package (Schmid and Blaxter, 2008). As part of this analysis, contigs from all libraries were BLASTed against subsets of UniProt (The UniProt Consortium, 2008) databases optimized for identification of Gene Ontology (GO, The Gene Ontology Consortium, 2000) or KEGG pathways (Kanehisa et al., 2008) using the program BLASTx (translated nucleotide against protein). As suggested in the pro-gram documentation, the expect-value cutoff of 1e−8 was used for retention of BLAST matches. The top 10 best hits for each EST were used to assign GO_slim terms and KEGG pathways.

Contigs were also used for BLAST analysis against the complete Uniprot Knowledgebase (Swissprot and TREMBL) protein databases with the BLASTx program. In order to get a general sense of which EST clusters matched annotated proteins, we retained up to the top 10 BLAST hits for each contig that met the expect-value cutoff of 1e−8 as recommended in Schmid and Blaxter (2008). The higher quality annotations in Swissprot were preferentially retained, and TREMBL was consulted only where 10 Swissprot hits were not found. The taxonomic ranks Kingdom, Phylum and Class (or nearest equivalent) of these BLAST hits were identified by recursion through a local copy of the NCBI taxonomy database (Wheeler et al., 2000).

2.5. Real time quantitative PCR

In order to determine the validity of the SSH approach in this system, real time PCR (qPCR) was conducted. We quantified the relative amounts of transcripts for selected genes that appeared to change with treatment in the SSH libraries, notably lactate dehydrogenase, cytochrome C oxidase, glutamine synthetase, and Na^+K^+ ATPase. Since RNA from the original SSH libraries created in 2007–2009 had degraded by this point in our study, we obtained new samples in 2012. Due to permit restrictions, and the need to coordinate sampling with other investigators of ongoing studies to reduce fish use, the time points of some of these new samples do not exactly coincide with those obtained for the SSH libraries (i.e., in some cases 6 h points were not available and 48 h points were added). Nonetheless, they represent key pre- and post-feeding junctures identified in prior research (Walsh et al., 2006; Dowd et al., Walsh et al., 2006; Dowd et al., 2008). Total RNA was extracted from the glands using Trizol reagent (Invitrogen) according to the manufacturer's instructions, treated with DNase I (Invitrogen), and used as a template for reverse transcription (RT). The RT reactions combined 2 mg RNA (quantified spectrometrically by NanoDrop) with 375 ng random hexamers (Integrated DNA Technol-ogies), 125 ng oligo dTs (IDT), and dNTPs (Invitrogen; 0.5 mM final con-centration) and incubating the mixture at 65 °C for 5 min. The reaction was then chilled on ice for 5 min and first-strand reaction buffer (Invitrogen; 1× final concentration), dithiothreitol (5 mM final concentration), RNase Out (Invitrogen; 0.5 μL), and nuclease-free water (0.5 μL) were added. The mixture was incubated at 42 °C for 2 min and then combined with Superscript II reverse transcriptase (Invitrogen; 1 μL) to a total volume of 20 μL. Lastly, the reaction was incubated at 42 °C for 50 min and then 72 °C for 15 min.

Following RT, the cDNA encoding each of the chosen genes was cloned to sequence verify the amplicons by adding 1 μL of diluted template (1:5) to a 25 μL total volume containing PCR reaction buffer (Denville Scientific; 1× final concentration), dNTPs (0.2 mM final con-centration), 0.15 μL Choice Taq (Denville Scientific), and sense and anti-sense primers (0.2 mM final concentrations). Primers for each gene were derived from existing *S. acanthias* sequences where applicable (Supplementary Table 1). The cycling parameters were 94 °C for 30 s followed by 40 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s and concluding with 15 min at 72 °C. The products were visualized on a 2% agarose gel and the amplicons gel purified and spliced into a pDrive Cloning Vector (Qiagen). The vectors were subsequently cloned using Subcloning Efficiency DH5α Competent Cells (Invitrogen), purified, and sequenced using the M13F primer.

For qPCR, 1 μL of the diluted cDNA template was added to a 12.5 μL total volume containing 6.25 μL Rotor-Gene SYBR Green PCR master mix (Qiagen) and 200 nM each gene specific sense and antisense primer. Relative mRNA levels for each gene of interest were normalized to *S. acanthias* EF1α levels. Fluorescence was detected using a Rotor-Gene Q Real Time

PCR cycler with the following cycling parameters: 95 °C for 5 min followed by 40 cycles of 95 °C for 5 s and 60 °C for 10 s. All reactions were run in duplicate and relative mRNA levels were calculated using the δ Ct method. Statistics on these values were by one-way ANOVA and Holm-Sidak post-hoc test.

3. Results and discussion

In this study, we produced Normalized and SSH libraries in order to investigate mRNA transcript responses to feeding and fasting in the rectal gland of *S. acanthias*. Information on aspects of sequencing and contig creation appear in Supplementary Tables 2 and 3, and unedited results of blast hits from these seven libraries appear in Supplementary Table 4. Sequences for these Contigs are available in Supplementary File A.

Since one potential bias of the SSH approach is that highly abundant transcripts may 'leak' through the subtraction process, we transformed the data to remove potential false positives from the SSH libraries (Supplementary Table 5); a false positive was suspected and removed when a given gene appeared as up regulated in both the forward and reverse library subtractions. These edited lists were then utilized for GO and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analyses, the results of which appear in Table 1 and Fig. 1, and Table 2, respectively. Notable biological processes being represented include metabolic processes and regulation, transport, and cell death, while the KEGG analysis revealed pathways involved in the progression of neurodegenerative diseases. In addition to these analyses, we further condensed the number of genes of interest from the SSH lists by removing duplicately named genes (e.g., different EST hits for the same basic gene) (Table 3a, b). We also conducted qPCR on four genes representing various biological pathways in order to complement the SSH approach (Figs. 2 and 3).

3.1. General overview

A total of 170 transcripts were up regulated in the various treatments, 67 of which were up regulated by feeding treatments (Libraries C, G, I and K), and 63 of which were up regulated by fasting treatments (Libraries A and E) (Supplementary Table 5). Of the GO categories, those of 'Biological Process' are the most interesting. The largest changes in transcript abundances relative to both fasting and the normalized library are in the sub-categories of 'Metabolic Process' and 'Transport' (Table 1 and Fig. 1). This result is not surprising given the main function of the rectal gland is to secrete NaCl and that it is an energetically expensive process. This result is also consistent in general with prior observations at the enzymatic/proteomic level (Walsh et al., 2006; Dowd et al., 2008). Furthermore, the role of structural proteins during activation of the gland as initially revealed in proteomic studies (Dowd et al., 2008) is confirmed and expanded by the current results (Table 3a,b), as well as the

Table 1. Total number of EST identifications that are up regulated between time points in each Gene Ontology category in the rectal glands of fasted (F) and fed (6 h, 22 h) dogfish sharks (*Squalus acanthias*). Letters refer to the SSH libraries listed in Materials and methods.

GO_slim term	Total	Norm	A (F-6 h)	C (6 h-F)	E (F-22 h)	G (22 h-F)	I (6 h-22 h)	K (22 h-6 h)
Cellular component								
Intracellular (GO:0005622)	568	239	89	72	21	68	12	67
Membrane (GO:0016020)	373	148	52	39	15	32	10	77
Cell (GO:0005623)	40	9	6	4	2	3	1	15
Extracellular region (GO:0005576)	33	9	8	1	10	2	0	3
Biological process								
Metabolic process (GO:0008152)	311	101	57	51	16	44	6	36
Transport (GO:0006810)	196	92	26	17	6	18	5	32
Regulation of biological process (GO:0050789)	190	43	36	31	11	7	0	62
Nucleobase-containing compound metabolic process (GO:0006139)	126	59	20	8	8	14	7	10
Multicellular organismal development (GO:0007275)	104	24	3	4	14	3	1	55
Response to stimulus (GO:0050896)	71	32	8	6	9	7	0	9
Cellular process (GO:0009987)	70	32	14	4	1	7	1	11
Cell differentiation (GO:0030154)	31	7	1	1	1	2	0	19
Cell death (GO:0008219)	12	7	1	2	0	0	0	2
Cell communication (GO:0007154)	3	0	1	0	0	0	0	2
Extracellular structure organization (GO:0043062)	2	0	0	0	1	0	0	1
Cellular membrane fusion (GO:0006944)	2	2	0	0	0	0	0	0
Cellular component movement (GO:0006928)	2	0	1	0	0	0	1	0
Molecular function								
Binding (GO:0005488)	533	211	114	50	29	49	10	70
Catalytic activity (GO:0003824)	134	61	30	6	2	17	4	14
Oxidoreductase activity (GO:0016491)	88	24	13	13	5	20	0	13
Transporter activity (GO:0005215)	70	31	10	10	0	3	6	10
Transferase activity (GO:0016740)	45	22	12	7	0	1	0	3
Structural molecule activity (GO:0005198)	31	13	3	6	2	2	1	4
Enzyme regulator activity (GO:0030234)	18	2	8	4	3	0	0	1
Ligase activity (GO:0016874)	17	5	2	0	4	6	0	0
Regulation of biological process (GO:0050789)	15	5	4	1	1	0	0	4
Isomerase activity (GO:0016853)	12	2	2	2	0	4	0	2
Nucleobase-containing compound metabolic process (GO:0006139)	10	2	3	2	1	0	0	2
Motor activity (GO:0003774)	4	2	1	0	0	0	0	1
Lyase activity (GO:0016829)	3	0	0	2	0	1	0	0
Signal transducer activity (GO:0004871)	1	0	0	0	0	1	0	0

role of apoptosis/cell death (Table 3a,b) as initially hypothesized from morphological studies (Matey et al., 2009). There were also changes in transcripts from a number of other sub-categories that fit well with a dynamically coordinated regulation of gene transcription/ translation (e.g., Process Regulation, RNA Processing, Transcription, Translation, etc.) (Table 3a,b). However, there were a number of other categories of interest, as well as incidences of unanticipated genes up regulated in treatments. These interesting points, as well as further details on some of the anticipated changes, will be discussed in more detail in the following sections that are based on some of the above sub-categories.

3.2. Cellular metabolism

The GO term analysis revealed that a large percentage of the genes from the SSH libraries are involved in metabolic processes and there appears to be a general up regulation of such genes with feeding (Fig. 1). Due to the aerobic nature of the rectal gland and the hypothesis that it may resort to anaerobic metabolism during times of peak activity (Walsh et al., 2006), we selected a gene involved in each of these pathways with which to conduct qPCR. Cytochrome C oxidase (CCO) is an enzyme involved in aerobic metabolism that typically correlates with mitochondrial oxidative capacity. The SSH libraries developed in this study indicate an up regulation of the mRNA for this gene with feeding (Table 3a), which correlates well with previous observations that feeding promotes increased development of mitochondria in the rectal gland (Matey et al., 2009) and increases in mitochondrial enzyme activities (Walsh et al., 2006). qPCR for this gene confirmed a significant up regulation of CCO mRNA by 22 h post-feeding as seen in Fig. 2B.

LDH is an indicator of anaerobic pathway capacity and the activity of this enzyme at ~130 units/g wet mass is reasonably high even in fasted dogfish rectal glands (Walsh et al., 2006). This activity also in-creased significantly at 6 h and 20 h post-feeding (Walsh et al., 2006). The increase in activity appears to be at least partly supported by increased transcription as we observed an up regulation of transcripts for this enzyme in the SSH libraries of both 6 and 22 h minus fasted, although no difference between 6 and 22 h (Table 3). A trend toward up regulation of transcripts at 22 and 48 h was further supported by qPCR methods (Fig. 3B), although the time points do not directly correspond to the SSH samples.

SSH also revealed a number of genes up regulated at 22 h post feeding associated with branch chain amino acid catabolism (Table 3a). In particular, the transcripts of enzymes involved in the catabolism of valine (methylmalonate-semialdehyde dehydrogenase, propionyl-CoA carboxylase beta chain and isobutyryl-CoA dehydrogenase), isoleucine and leucine (methylmalonate-semialdehyde dehydrogenase) were enriched in the 22 h library (Table 3a). Interestingly, the plasma con-centration of these three essential amino acids rapidly rise following a meal in dogfish, but start declining 30 h post feeding (Wood et al., 2010). Thus, this increase in transcripts for genes coding for amino acid catabolic enzymes detected by the SSH at 22

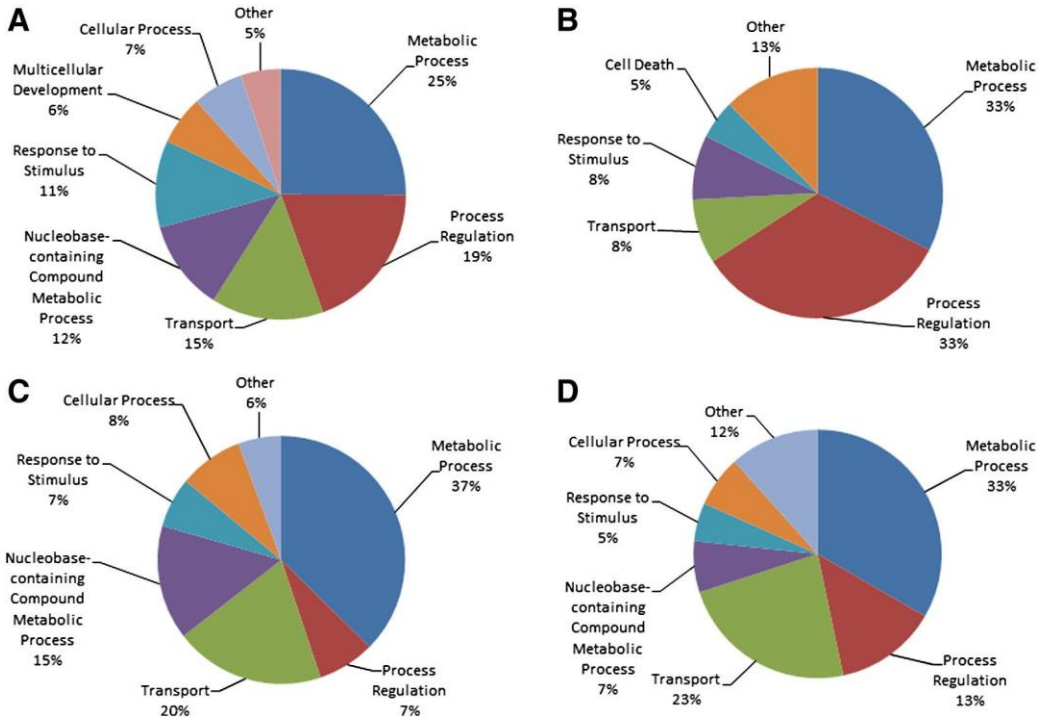

Fig. 1. Pie charts of categories of genes identified from rectal glands of fed and fasted spiny dogfish (*Squalus acanthias*) from the 'Biological Function' heading in GO analysis for (A) the NORM library, and SSH libraries from the following treatments: (B) 6 h minus 7 day fasted, (C) 22 h minus 7 day fasted, and (D) 22 h minus 6 h.

h concomitant with high circulating levels of these amino acids in the blood suggest that the gland increases its capacity to metabolically rely on these amino acids as a secondary metabolic response to a feeding event. Walsh et al. (2006) had previously concluded a small role for amino acids and fuels for the rectal gland, but this was based on the inability of alanine and glutamine to stimulate salt secretion. It would be of interest to test the ability of these essential amino acids to fuel rectal gland metabolism and salt secretion.

Another metabolic enzyme that was of interest to us was glutamine synthetase (GS) as it is responsible for trapping ammonia nitrogen to be used in urea (osmolyte) synthesis. Changes in activities of GS in the rectal gland are similar to those observed for LDH in that there is more than a doubling of GS activity at 6 and 20 h compared to fasted or 30 and 48 h post feeding (Walsh et al., 2006). This increase in GS activity at times of peak gland activity makes sense in terms of the need to scavenge ammonia for urea synthesis, especially in light of the above proposed role for essential amino acids as metabolic fuels. Therefore it was surprising that we observed an up regulation of transcripts for this enzyme with fasting in the SSH libraries, a result that was confirmed by qPCR (Fig. 2A). Matey et al. (2009) noted that it was not uncommon to observe cells in the early stages of apoptosis in fasted rectal glands, so this tissue may be

releasing ammonia by protein degradation. Since during fasting, dogfish are extremely limited in the amount of nitrogen they can devote to urea synthesis, procuring nitrogen from degrading tissues may be what allows them to maintain their plasma urea levels during these periods, as observed in prior studies (Haywood, 1973; Armour et al., 1993; Kajimura et al., 2008). Glutamine supply from an active tissue like the rectal gland would be one source for urea synthesis, thus creating the need for modest GS activity even as the gland is deactivated (notably an enzyme of the Ornithine-Urea Cycle, ornithine carbamoyltransferase, is up regulated by feeding (Table 3a) indicating that at least a partial cycle might be operating in the rectal gland). There may be enhanced degradation of protein in general in the fasted rectal gland, and a higher standing stock of GS mRNA relative to fed fish may be required to sustain even this modest level of GS activity. The above results serve to reinforce the cautionary note that transcript abundances are not always reflected in enzyme activity, even in an enzyme like GS that is not believed to be subjected to post-translational modification of activity.

The above results for GS transcripts are mirrored in a more general way for many metabolic genes in that transcripts for 12 metabolic genes, many of them mitochondrial, are up regulated during fasting (Table 3b), a point we will return to below.

3.3. Ion transport

Secretion by the rectal gland is largely dependent on the ability to transport various ions across both the apical and basolateral mem-branes, thus it was not surprising to see that this process was heavily represented in the SSH libraries (Fig. 1). One gene of particular interest was Na^+K^+-ATPase because: i) its activity is required to create gradients necessary for the entry of chloride into the cell and paracellular transport of Na^+; and ii) previous studies have shown increases in its activity post-feeding (Mackenzie et al., 2002; Walsh et al., 2006). In light of these observed increases in enzyme activity, it was surprising to find that the mRNA for this gene was down regulated with feeding according to the SSH libraries (Table 3b). In order to investigate this further, we conducted qPCR and the results indicated no significant differences between the rectal glands from fed and fasted dogfish (Fig. 3A). This investigation of Na^+K^+-ATPase mRNA levels was focused on the alpha subunit as this is what appeared in the SSH libraries. Little is known about the presence of different isoforms of this gene in elasmobranchs, or of the abundance of other subunits. In the teleost gill however, numerous studies have investigated the abundance of various alpha subunit isoforms and their relation to overall enzyme activity. Richards et al. (2003) determined that, upon transfer to sea water, there was a decrease in Na^+K^+-ATPase alpha 1a mRNA and an increase in alpha 1b mRNA in the gills of *Oncorhynchus mykiss* and these changes were accompanied with an increase in overall Na^+K^+-ATPase enzyme activity. Similar results were found by Bystriansky et al. (2006) in the gills of three salmonid species (*Salmo salar, O. mykiss, Salvelinus alpinus*). It is possible that *S. acanthias* also possesses multiple isoforms for the alpha subunit which could

Table 1. Total number of EST identifications that are up regulated between time points in each KEGG pathway in the rectal glands of fasted (F) and fed (6 h, 22 h) dogfish sharks (*Squalus acanthias*). Letters refer to the SSH libraries listed in Materials and methods.

KEGG pathway	Total	Norm	A (F-6 h)	C (6 h-F)	E (F-22 h)	G (22 h-F)	I (6 h-22 h)	K (22 h-6 h)
Parkinson's disease [ko:05012]	49	26	4	5	1	4	1	8
Huntington's disease [ko:05016]	48	28	2	5	1	3	1	8
Oxidative phosphorylation [ko:00190]	46	24	4	4	1	4	1	8
Alzheimer's disease [ko:05010]	42	24	2	5	1	3	1	6
Cardiac muscle contraction [ko:04260]	26	16	3	2	0	1	0	4
Ribosome [ko:03010]	21	11	1	4	1	2	1	1
RNA transport [ko:03013]	10	4	3	1	1	0	0	1
Pyruvate metabolism [ko:00620]	9	0	2	2	1	3	0	1
Propanoate metabolism [ko:00640]	8	1	0	2	0	5	0	0
Mineral absorption [ko:04978]	7	4	3	0	0	0	0	0
Viral myocarditis [ko:05416]	7	2	1	1	0	2	0	1
Glycolysis/gluconeogenesis [ko:00010]	7	0	2	2	0	2	0	1
Protein processing in endoplasmic reticulum [ko:04141]	6	4	0	0	1	0	0	1
Toxoplasmosis [ko:05145]	6	3	0	2	0	0	0	1
Pathways in cancer [ko:05200]	6	3	1	1	0	0	0	1
Valine [ko:00280]	6	1	0	1	0	3	0	1
Citrate cycle (TCA cycle) [ko:00020]	6	0	1	1	1	2	0	1
Carbon fixation in photosynthetic organisms [ko:00710]	6	0	2	1	1	1	0	1
Purine metabolism [ko:00230]	5	3	2	0	0	0	0	0
Endocytosis [ko:04144]	5	1	2	0	0	0	0	2
Spliceosome [ko:03040]	5	2	0	1	0	1	0	1
Focal adhesion [ko:04510]	5	0	2	0	1	1	0	1
Phagosome [ko:04145]	5	2	1	0	0	1	0	1
Glyoxylate and dicarboxylate metabolism [ko:00630]	5	0	0	1	1	2	0	1
Arginine and proline metabolism [ko:00330]	5	2	1	1	0	1	0	0

help explain the disconnect between Na^+K^+-ATPase activity and the mRNA levels observed in this study. However, Mahmmoud et al. (2000) previously discovered an FXYD domain-containing protein in the rectal gland of *S. acanthias*. These proteins are substrates for protein kinase C and have been shown to dissociate from the α-subunit of Na^+K^+-ATPase when phosphorylated, activating the enzyme (Mahmmoud et al., 2000). Thus, these proteins act as tissue- specific regulators of Na^+K^+-ATPase and may provide an alternate explanation for the aforementioned disconnect.

3.4. Morphology

It has previously been determined that the rectal gland undergoes significant changes in morphology when switching to its active state. During fasting, the gland's muscle and epithelial layers thicken and many cells enter the early stages of apoptosis (Matey et al., 2009). Upon feeding, this thickening is reversed in order to increase the lumen diameter and thus a significant amount of tissue remodeling is expected to occur. The SSH libraries indicate an increase in cell death at 6 h post-feeding, as seen in Fig. 1. This result appears to be counter-intuitive, however it is likely that allowing cells in the early stages of apoptosis to die off and subsequently increasing cell proliferation to allow tissue remodeling is more efficient than trying to reverse apoptosis as is the case in Burmese pythons, another species known to go for extended periods without feeding (Lignot et al., 2005; Helmstetter et al., 2009). These studies on the python gastrointestinal tract have shown that feeding induces cellular replication and extensive remodeling of the intestinal mucosa. This increase in cell death observed in the dogfish post-feeding could also explain the increases in GS activity observed by Walsh et al. (2006) as there will be a greater need to scavenge nitrogen from the degrading proteins at this time.

The SSH libraries revealed a few specific genes that are involved in apoptotic and cell proliferation pathways, one of which is the voltage-dependent anion channel (VDAC). Dowd et al. (2008) found an increase in VDAC protein levels at both 6 and 20 h post-feeding and in this study the SSH libraries revealed an increase in mRNA levels at 22 h (Table 3a). This protein forms a pore in the outer mitochondrial membrane and is said to be involved in allowing the mitochondrial contents that initiate apoptosis to leak out into the cytoplasm. A programmed cell death protein also appears at 22 h (Table 3a) suggesting that the rectal gland begins preparing to re-enter its dormant state shortly following a meal, possibly to minimize extra energy expenditures. Another interesting finding was the presence of a butyrate response factor in the fasted glands (Table 3b). This protein is a transcription factor that regulates the cell's response to growth factors, playing a role in cell proliferation. Its appearance in the fasted glands likely has to do with the extensive remodeling that is required to produce the dormant state, acting to prepare the gland for its next feeding period.

Another notable gene whose mRNA was up regulated in fasting was the iron storage protein ferritin (Table 3b). Perhaps some of

Table 3. Lists of EST identities for Contigs from 7 SSH libraries grouped by Gene Ontology 570 category for rectal glands of (A) fed and (B) fasted dogfish sharks (*Squalus acanthias*). Letters 571 refer to the SSH libraries listed in Materials and methods.

Contig	Gene Ontology	Accession number	Description
a			
RG_G_Contig23	Metabolic	E1BFG0	Alpha-aminoadipic semialdehyde dehydrogenase OS = Bos taurus GN = ALDH7A1 PE = 2 SV = 3
RG_G_Contig9	Metabolic	Q5TI65	Brain protein 44 OS = Homo sapiens GN = BRP44 PE = 1 SV = 1
RG_G_Contig31	Metabolic	P21158	C-factor OS = Myxococcus xanthus GN = csgA PE = 1 SV = 1
RG_G_Contig21	Metabolic	Q9ZZ52	Cytochrome c oxidase subunit 1 OS = Squalus acanthias GN = MT-CO1 PE = 3 SV = 1
RG_G_Contig26	Metabolic	Q9ZZ48	Cytochrome c oxidase subunit 3 OS = S. acanthias GN = MT-CO3 PE = 3 SV = 1
RG_G_Contig18	Metabolic	Q96KP4	Cytosolic non-specific dipeptidase OS = H. sapiens GN = CNDP2 PE = 1 SV = 2
RG_G_Contig53	Metabolic	P79345	Epididymal secretory protein E1 OS = B taurus GN = NPC2 PE = 1 SV = 1
RG_G_Contig6	Metabolic	Q4R4W5	Isopentenyl-diphosphate delta-isomerase 1 OS = Macaca fascicularis GN = IDI1 PE = 2 SV = 2
RG_G_Contig76	Metabolic	Q9YI05	L-lactate dehydrogenase B chain OS = S. acanthias GN = ldhb PE = 2 SV = 1
RG_G_Contig19	Metabolic	Q02252	Methylmalonate-semialdehyde dehydrogenase [acylating]; mitochondrial OS = H. sapiens GN = ALDH6A1 PE = 1 SV = 2
RG_G_Contig16	Metabolic	Q2TBR0	Propionyl-CoA carboxylase beta chain; mitochondrial OS = B. taurus GN = PCCB PE = 2 SV = 1
RG_G_Contig63	Metabolic	P21670	Proteasome subunit alpha type-4 OS = Rattus norvegicus GN = Psma4 PE = 1 SV = 1
RG_G_Contig12	Metabolic	Q9Z2I9	Succinyl-CoA ligase [ADP-forming] subunit beta; mitochondrial OS = Mus musculus GN = Sucla2 PE = 1 SV = 2
RG_C_Contig3	Metabolic	Q63569	26S protease regulatory subunit 6A OS = R. norvegicus GN = Psmc3 PE = 2 SV = 1
RG_C_Contig1	Metabolic	P08428	ATP synthase subunit alpha; mitochondrial OS = Xenopus laevis GN = atp5a PE = 2 SV = 1
RG_C_Contig2	Metabolic	P13184	Cytochrome c oxidase polypeptide 7A2; mitochondrial OS = B. taurus GN = COX7A2 PE = 1 SV = 3
RG_C_Contig81	Metabolic	P00430	Cytochrome c oxidase subunit 7C; mitochondrial OS = B. taurus GN = COX7C PE = 1 SV = 3
RG_C_Contig20	Metabolic	Q58DM8	Enoyl-CoA hydratase; mitochondrial OS = B. taurus GN = ECHS1 PE = 2 SV = 1
RG_C_Contig84	Metabolic	Q5XJ10	Glyceraldehyde 3-phosphate dehydrogenase; testis-specific OS = Danio rerio GN = gapdhs PE = 2 SV = 1
RG_C_Contig8	Metabolic	Q5NVR2	Malate dehydrogenase; mitochondrial OS = Pongo abelii GN = MDH2 PE = 2 SV = 1
RG_C_Contig48	Metabolic	Q4R4E0	NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 5; mitochondrial OS = M. fascicularis GN = NDUFB5 PE = 2 SV = 1
RG_C_Contig28	Metabolic	P00480	Ornithine carbamoyltransferase; mitochondrial OS = H. sapiens GN = OTC PE = 1 SV = 3
RG_C_Contig26	Metabolic	Q5PQ01	Phosphatidylinositol-5-phosphate 4-kinase type-2 gamma OS = X. laevis GN = pip4k2c PE = 2 SV = 1
RG_C_Contig5	Metabolic	Q96IV6	Uncharacterized protein C5orf4 OS = H. sapiens GN = C5orf4 PE = 1 SV = 1
RG_K_Contig59	Metabolic	Q03248	Beta-ureidopropionase OS = R. norvegicus GN = Upb1 PE = 1 SV = 1
RG_K_Contig55	Metabolic	Q9N1E2	Glucose-6-phosphate isomerase OS = Oryctolagus cuniculus GN = GPI PE = 1 SV = 3
RG_K_Contig77	Metabolic	Q9UKU7	Isobutyryl-CoA dehydrogenase; mitochondrial OS = H. sapiens GN = ACAD8 PE = 1 SV = 1
RG_I_Contig19	Metabolic	Q9ER34	Aconitate hydratase; mitochondrial OS = R. norvegicus GN = Aco2 PE = 1 SV = 2
RG_I_Contig6	Metabolic	P06576	ATP synthase subunit beta; mitochondrial OS = H. sapiens GN = ATP5B PE = 1 SV = 3
RG_G_Contig5	Transport	Q9CZ13	Cytochrome b-c1 complex subunit 1; mitochondrial OS = M. musculus GN = Uqcrc1 PE = 1 SV = 1
RG_G_Contig20	Transport	Q9ZZ54	NADH-ubiquinone oxidoreductase chain 1 OS = S. acanthias GN = MT-ND1 PE = 3 SV = 1
RG_G_Contig70	Transport	Q6NRB0	Protein Hook homolog 2 OS = X. laevis GN = hook2 PE = 2 SV = 1
RG_C_Contig6	Transport	Q5E9B7	Chloride intracellular channel protein 1 OS = B. taurus GN = CLIC1 PE = 2 SV = 3
RG_C_Contig35	Transport	P00027	Cytochrome c OS = Squalus suckleyi GN = cyc PE = 1 SV = 2
RG_C_Contig45	Transport	Q62760	Mitochondrial import receptor subunit TOM20 homolog OS = R. norvegicus GN = Tomm20 PE = 1 SV = 2
RG_K_Contig15	Transport	P11442	Clathrin heavy chain 1 OS = R. norvegicus GN = Cltc PE = 1 SV = 3
RG_K_Contig2	Transport	Q8TAG9	Exocyst complex component 6 OS = H. sapiens GN = EXOC6 PE = 1 SV = 3
RG_G_Contig58	Process Regulation	P07737	Profilin-1 OS = H. sapiens GN = PFN1 PE = 1 SV = 2
RG_G_Contig29	Process Regulation	P35467	Protein S100-A1 OS = R. norvegicus GN = S100a1 PE = 1 SV = 3
RG_G_Contig39	Process Regulation	P46939	Utrophin OS = H. sapiens GN = UTRN PE = 1 SV = 2
RG_C_Contig25	Process Regulation	P63245	Guanine nucleotide-binding protein subunit beta-2-like 1 OS = R. norvegicus GN = Gnb2l1 PE = 1 SV = 3
RG_C_Contig14	Process Regulation	O42148	Ornithine decarboxylase antizyme OS = Gallus gallus GN = OAZ PE = 2 SV = 3
RG_C_Contig67	Process Regulation	P37805	Transgelin-3 OS = R. norvegicus GN = Tagln3 PE = 1 SV = 2
RG_K_Contig33	Process Regulation	Q9NPA3	Mid1-interacting protein 1 OS = H. sapiens GN = MID1IP1 PE = 1 SV = 1
RG_C_Contig4	Cell Death	P30405	Peptidyl-prolyl cis-trans isomerase; mitochondrial OS = H. sapiens GN = PPIF PE = 1 SV = 1
RG_K_Contig24	Cell Death	Q6DF07	Programmed cell death protein 10 OS = Xenopus tropicalis GN = pdcd10 PE = 2 SV = 1
RG_K_Contig52	Cell Death	Q9TT15	Voltage-dependent anion-selective channel protein 1 OS = O. cuniculus GN = VDAC1 PE = 2 SV = 3
RG_G_Contig35	Translation	Q6P8D1	40S ribosomal protein SA OS = X. tropicalis GN = rpsa PE = 2 SV = 1
RG_G_Contig22	Translation	P50878	60S ribosomal protein L4 OS = R. norvegicus GN = Rpl4 PE = 2 SV = 3
RG_C_Contig77	Translation	Q9BYC8	39S ribosomal protein L32; mitochondrial OS = H. sapiens GN = MRPL32 PE = 1 SV = 1
RG_C_Contig61	Translation	P41105	60S ribosomal protein L28 OS = M. musculus GN = Rpl28 PE = 1 SV = 2
RG_C_Contig69	Translation	P04646	60S ribosomal protein L35a OS = R. norvegicus GN = Rpl35a PE = 3 SV = 1
RG_C_Contig29	Translation	A5A6I3	Eukaryotic translation initiation factor 3 subunit F OS = Pan troglodytes GN = EIF3F PE = 2 SV = 1
RG_I_Contig15	Metabolic	Q90705	Elongation factor 2 OS = G. gallus GN = EEF2 PE = 1 SV = 3
RG_G_Contig25	RNA Processing	Q9ESX5	H/ACA ribonucleoprotein complex subunit 4 OS = M. musculus GN = Dkc1 PE = 1 SV = 3
RG_G_Contig54	RNA Processing	Q5XH16	NHP2-like protein 1 OS = X. laevis GN = nhp2l1 PE = 2 SV = 1
RG_C_Contig10	RNA Processing	P51991	Heterogeneous nuclear ribonucleoprotein A3 OS = H. sapiens GN = HNRNPA3 PE = 1 SV = 2
RG_C_Contig75	RNA Processing	Q06066	Nuclease-sensitive element-binding protein 1 OS = G. gallus GN = YBX1 PE = 2 SV = 1
RG_C_Contig36	RNA Processing	Q09167	Splicing factor; arginine/serine-rich 5 OS = R. norvegicus GN = Sfrs5 PE = 2 SV = 1
RG_G_Contig3	DNA Replication	Q4QQE9	Endonuclease-reverse transcriptase OS = Schistosoma mansoni PE = 2 SV = 1
RG_K_Contig40	DNA Replication	Q95SX7	Probable RNA-directed DNA polymerase from transposon BS OS = Drosophila melanogaster GN = RTase PE = 2 SV = 1
RG_K_Contig8	DNA Replication	P04323	Retrovirus-related Pol polyprotein from transposon 17.6 OS = D. melanogaster GN = pol PE = 4 SV = 1
RG_K_Contig75	Stress Response	Q58DR2	DnaJ homolog subfamily B member 12 OS = B. taurus GN = DNAJB12 PE = 2 SV = 1
RG_K_Contig5	Stress Response	P19120	Heat shock cognate 71 kDa protein OS = B. taurus GN = HSPA8 PE = 1 SV = 2
RG_C_Contig16	Calcium Binding	Q8VC88	Grancalcin OS = M. musculus GN = Gca PE = 2 SV = 1
RG_C_Contig78	Calcium Binding	Q5E984	Translationally-controlled tumor protein OS = B. taurus GN = TPT1 PE = 2 SV = 1
RG_G_Contig30	Structural	Q6P378	Actin; cytoplasmic 2 OS = X. tropicalis GN = actg1 PE = 2 SV = 1
RG_G_Contig27	Structural	O88342	WD repeat-containing protein 1 OS = M. musculus GN = Wdr1 PE = 1 SV = 3
RG_C_Contig43	Structural	P48649	Gamma-crystallin M2 OS = Chiloscyllium colax GN = GM2 PE = 2 SV = 2
RG_K_Contig47	Structural	Q90617	Lysosome-associated membrane glycoprotein 2 OS = G. gallus GN = LAMP2 PE = 2 SV = 1
RG_K_Contig22	Structural	Q64119	Myosin light polypeptide 6 OS = R. norvegicus GN = Myl6 PE = 1 SV = 3

Table 3 (continued).

Contig	Gene Ontology	Accession number	Description
a			
RG_K_Contig39	Structural	Q9QXS1	Plectin-1 OS = M. musculus GN = Plec1 PE = 1 SV = 2
RG_G_Contig68	Extracellular	Q8R3W7	Anterior gradient protein 3 homolog OS = M. musculus GN = Agr3 PE = 2 SV = 1
RG_G_Contig67	Cell Differentiation	Q6DEL2	Cleft lip and palate transmembrane protein 1 homolog OS = D. rerio GN = clptm1 PE = 2 SV = 1
RG_K_Contig41	Mitochondrial	A8KB87	Coiled-coil domain-containing protein 56 OS = D. rerio GN = ccdc56 PE = 3 SV = 1
RG_K_Contig1	Membrane	Q5R3Z8	F-box/LRR-repeat protein 2 OS = P. abelii GN = FBXL2 PE = 2 SV = 1
b			
RG_A_Contig24	Metabolic	P52209	6-Phosphogluconate dehydrogenase; decarboxylating OS = H. sapiens GN = PGD PE = 1 SV = 3
RG_A_Contig58	Metabolic	Q8QGP3	Carboxypeptidase Z OS = G. gallus GN = CPZ PE = 1 SV = 1
RG_A_Contig104	Metabolic	Q5RDE7	Cysteine desulfurase; mitochondrial OS = P. abelii GN = NFS1 PE = 2 SV = 1
RG_A_Contig115	Metabolic	P41320	Glutamine synthetase; mitochondrial OS = S. acanthias PE = 2 SV = 1
RG_A_Contig31	Metabolic	Q9ZZ53	NADH-ubiquinone oxidoreductase chain 2 OS = S. acanthias GN = MT-ND2 PE = 3 SV = 1
RG_A_Contig6	Metabolic	Q9ZZ54	NADH-ubiquinone oxidoreductase chain 3 OS = S. acanthias GN = MT-ND3 PE = 3 SV = 1
RG_A_Contig66	Metabolic	Q6AZB8	Putative nuclease HARBI1 OS = D. rerio GN = harbi1 PE = 2 SV = 1
RG_A_Contig8	Metabolic	Q92122	Pyruvate kinase muscle isozyme OS = X. laevis GN = pkm PE = 2 SV = 1
RG_A_Contig47	Metabolic	Q9YHT1	Succinate dehydrogenase [ubiquinone] flavoprotein subunit; mitochondrial OS = G. gallus GN = SDHA PE = 1 SV = 2
RG_E_Contig11	Metabolic	Q6PAB3	Malate dehydrogenase; cytoplasmic OS = X. laevis GN = mdh1 PE = 2 SV = 1
RG_E_Contig14	Metabolic	Q0MQ87	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 12 OS = Pan troglodytes GN = NDUFA12 PE = 2 SV = 1
RG_E_Contig7	Metabolic	Q9HAB8	Phosphopantothenate-cysteine ligase OS = H. sapiens GN = PPCS PE = 1 SV = 2
RG_A_Contig62	Transport	Q5TZ80	Protein Hook homolog 1 OS = D. rerio GN = hook1 PE = 2 SV = 1
RG_A_Contig22	Transport	P05025	Sodium/potassium-transporting ATPase subunit alpha OS = Torpedo californica PE = 1 SV = 1
RG_A_Contig46	Transport	Q92030	Sodium/potassium-transporting ATPase subunit alpha-1 OS = A. anguilla GN = atp1a1 PE = 2 SV = 1
RG_A_Contig11	Transport	B5DEN9	Vacuolar protein sorting-associated protein 28 homolog OS = R. norvegicus GN = Vps28 PE = 2 SV = 1
RG_E_Contig4	Transport	Q9D0F3	Protein ERGIC-53 OS = M. musculus GN = Lman1 PE = 2 SV = 1
RG_E_Contig13	Transport	Q4QQS3	Protein OSCP1 OS = R. norvegicus GN = Oscp1 PE = 2 SV = 1
RG_A_Contig35	Process Regulation	A6QNS3	Active breakpoint cluster region-related protein OS = B. taurus GN = ABR PE = 2 SV = 1
RG_A_Contig40	Process Regulation	P20111	Alpha-actinin-2 OS = G. gallus GN = ACTN2 PE = 2 SV = 1
RG_A_Contig2	Process Regulation	Q9QXT1	Dual adapter for phosphotyrosine and 3-phosphotyrosine and 3-phosphoinositide OS = M. musculus GN = Dapp1 PE = 1 SV = 1
RG_A_Contig36	Process Regulation	Q5RB37	Inter-alpha-trypsin inhibitor heavy chain H3 OS = P. abelii GN = ITIH3 PE = 2 SV = 1
RG_A_Contig102	Process Regulation	Q13526	Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 OS = H. sapiens GN = PIN1 PE = 1 SV = 1
RG_A_Contig26	Process Regulation	P69735	Rab3 GTPase-activating protein catalytic subunit (Fragments) OS = R. norvegicus GN = Rab3gap1 PE = 1 SV = 2
RG_A_Contig12	Process Regulation	O00560	Syntenin-1 OS = H. sapiens GN = SDCBP PE = 1 SV = 1
RG_E_Contig6	Process Regulation	P06238	Alpha-2-macroglobulin OS = R. norvegicus GN = A2m PE = 2 SV = 2
RG_A_Contig94	Cell Death	Q95M17	Acidic mammalian chitinase OS = B. taurus GN = CHIA PE = 1 SV = 1
RG_A_Contig59	Cell Death	B2GV05	RNA-binding protein 5 OS = R. norvegicus GN = Rbm5 PE = 2 SV = 1
RG_A_Contig97	Translation	P12749	60S ribosomal protein L26 OS = R. norvegicus GN = Rpl26 PE = 1 SV = 1
RG_A_Contig57	Translation	P81795	Eukaryotic translation initiation factor 2 subunit 3 OS = R. norvegicus GN = Eif2s3 PE = 1 SV = 2
RG_A_Contig7	Translation	Q641X8	Eukaryotic translation initiation factor 3 subunit E OS = R. norvegicus GN = Eif3e PE = 2 SV = 1
RG_A_Contig54	RNA Processing	Q9CWY4	Gem-associated protein 7 OS = M. musculus GN = Gemin7 PE = 2 SV = 1
RG_A_Contig92	DNA Replication	Q4QQE9	Endonuclease-reverse transcriptase OS = S. mansoni PE = 2 SV = 1
RG_A_Contig98	DNA Replication	P21328	RNA-directed DNA polymerase from mobile element jockey OS = D. melanogaster GN = pol PE = 1 SV = 1
RG_A_Contig69	Transcription	A0JN61	DNA-directed RNA polymerase III subunit RPC9 OS = B. taurus GN = CRCP PE = 2 SV = 1
RG_A_Contig48	Transcription	Q2T9L9	General transcription factor IIF subunit 2 OS = B. taurus GN = GTF2F2 PE = 2 SV = 1
RG_A_Contig89	Transcription	Q1KKY3	Homeobox protein Hox-B13a OS = T. rubripes GN = hoxb13a PE = 3 SV = 1
RG_A_Contig109	Transcription	Q28C74	Leucine-rich PPR motif-containing protein; mitochondrial OS = X. tropicalis GN = lrpprc PE = 2 SV = 1
RG_A_Contig28	Transcription	Q64152	Transcription factor BTF3 OS = M. musculus GN = Btf3 PE = 2 SV = 3
RG_A_Contig85	Structural	Q5ZJ81	Endophilin-B2 OS = G. gallus GN = SH3GLB2 PE = 2 SV = 1
RG_A_Contig110	Structural	Q5ZLA6	Myosin-Ic OS = G. gallus GN = MYO1C PE = 2 SV = 1
RG_A_Contig78	Structural	Q28IX8	Tubulin alpha chain OS = X. tropicalis GN = tuba PE = 2 SV = 1
RG_E_Contig2	Structural	P02460	Collagen alpha-1(II) chain (Fragment) OS = G. gallus GN = COL2A1 PE = 2 SV = 1
RG_A_Contig1	Iron Storage	Q95MP7	Ferritin heavy chain OS = Canis familiaris GN = FTH1 PE = 2 SV = 3
RG_A_Contig14	Calcium Binding	Q641Z8	Peflin OS = R. norvegicus GN = Pef1 PE = 2 SV = 1
RG_A_Contig9	Ubiquitination	Q8R3P2	Protein deltex-2 OS = M. musculus GN = Dtx2 PE = 1 SV = 2
RG_A_Contig15	Cell Communication	A8WCF8	Tumor protein p63-regulated gene 1-like protein OS = R. norvegicus GN = Tprg1l PE = 1 SV = 1
RG_A_Contig82	Platelet Adhesion	Q28833	von Willebrand factor (Fragment) OS = Sus scrofa GN = VWF PE = 2 SV = 2
RG_E_Contig16	Cell Proliferation	P47974	Butyrate response factor 2 OS = H. sapiens GN = ZFP36L2 PE = 1 SV = 3
RG_E_Contig15	DNA Ligation	P12682	High mobility group protein B1 OS = S. scrofa GN = HMGB1 PE = 2 SV = 3
RG_A_Contig61	Nucleus	Q96A49	Synapse-associated protein 1 OS = H. sapiens GN = SYAP1 PE = 1 SV = 1
RG_A_Contig18	Membrane	O95857	Tetraspanin-13 OS = H. sapiens GN = TSPAN13 PE = 2 SV = 1
RG_A_Contig5	Membrane	Q9NRQ5	UPF0443 protein C11orf75 OS = H. sapiens GN = C11orf75 PE = 2 SV = 1

the iron generated from the degradation of heme-containing respiratory chain proteins is being sequestered within the rectal gland so as to enable rapid resynthesis of these proteins as needed upon feeding activation.

3.5. Oxidative stress

The rectal gland is a highly aerobic tissue, taking up 95% of the oxy-gen from the blood it is perfused with (

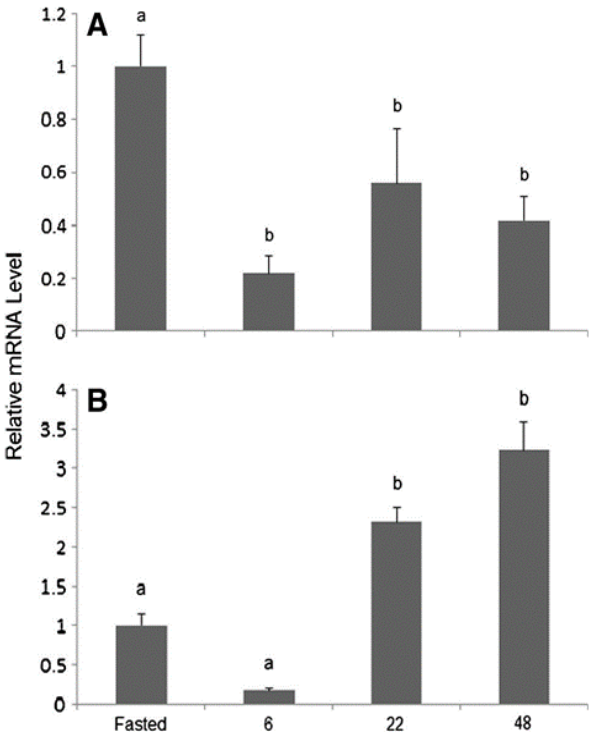

Fig. 2. Real time quantitative PCR analysis of relative transcript abundance in rectal glands of fed (6, 22 and 48 h) relative to 7 day fasted (ratio set at 1.0) spiny dogfish (*Squalus acanthias*) for (A) glutamine synthetase and (B) cytochrome c oxidase compared to ex-pression of elongation factor 1 alpha as the reference gene. Values are means ± 1 S.E.M., and those with different letters are statistically different (p b 0.05 by ANOVA).

Solomon et al., 1984). Thus, one would expect the production of large amounts of reactive oxygen species (ROS), particularly during peak levels of activity when blood (and thus oxygen) supply to the gland is expected to increase. An interesting finding that resulted from our KEGG pathway analysis (Table 2) was the number of genes associated with neurodegenerative diseases, which are often characterized by the inability of the neurons to neutralize ROS. One interesting gene in particular to show up regulation during feeding was dna-J (Table 3a). This gene is a molecular chaperone that is believed to play a role in neutralizing ROS and it is one of the genes that are known to be affected in Parkinson's disease. Dna-J shows up in the SSH libraries at 22 h (Table 3a), which is after the gland's activity levels (and likely ROS production) have peaked. Thus, it is possible that this gene is playing a role in the gland's response to oxidative stress. Notably, another gene typically associated with stress (HSP 71) was also up regulated by feeding (Table 3a).

It was previously mentioned that LDH activity in the rectal gland is already high in fasted fish and yet this still increases post-feeding, along with mRNA levels. Although one explanation is that this increase is responsible for maintaining activity levels when the gland's activity is outpacing oxygen delivery, another possible explanation is that it is serving to reduce the production of ROS by anaerobically converting pyruvate to lactate rather than oxidizing it. Thus, the enzyme may be responsible for helping to regulate the amount of oxidative stress to which the gland is exposed.

4. Final perspectives

Many of the categories of genes (e.g., metabolism, transport, morphological/structural), and many genes in particular, whose transcripts were up regulated by feeding and activation of the rectal gland were in fact expected. Strikingly though, transcripts for many genes in these same categories were up regulated during

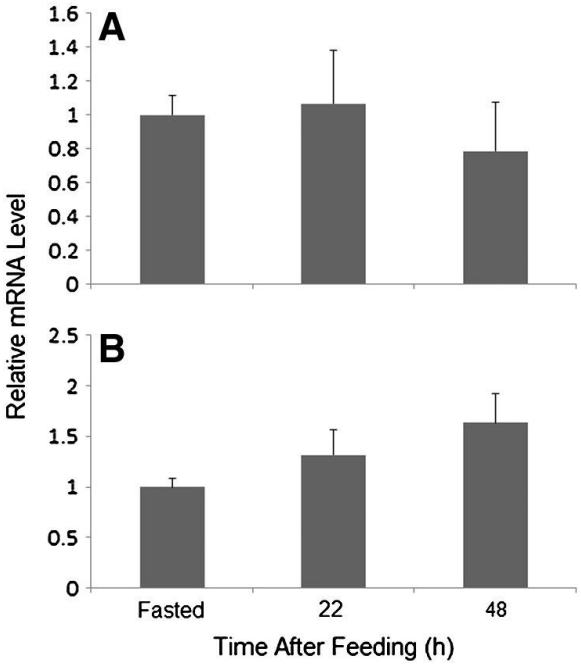

Fig. 3. Real time quantitative PCR analysis of relative transcript abundance in rectal glands of fed (22 and 48 h) relative to fasted (ratio set at 1.0) spiny dogfish (*Squalus acanthias*) for (A) Na^+/K^+-ATPase and (B) lactate dehydrogenase compared to expression of elongation factor 1 alpha as the reference gene. Values are means ± 1 S.E.M., and there were no significant differences among treatments.

fasting, and in several cases appear to be out of phase with previously reported increases in enzyme activities and protein contents during activation of the gland during feeding. A reasonable explanation for this phase variance is that the gland stores mRNA during fasting for selected genes in 'anticipation' of the need for rapid synthesis of selected proteins immediately upon feeding. Given the growing body of evidence for the role of microRNA regulation of mRNA availability for translation (Cai et al., 2009), it would be of interest to examine their role in the regulation of rectal gland function in elasmobranchs.

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Appendix B

For the study in Chapter 3, custom dogfish GLUT4 antibodies were synthesised by 21st Century Biochemicals with the following epitopes: CGFTYFKVPETRGKTFDQI-amide (Ab1); CPESPRYLYIIRNEESKA-amide (Ab2). A 1:1000 dilution was used for the primary antibodies and a 1:5000 dilution for the secondary anti-rabbit antibody in PBST + 2% milk. Unfortunately, I was unable to get the antibodies to work prior to the completion of this thesis. Firstly, there was binding to multiple products by both antibodies, and secondly, I was unable to achieve band disappearance by pre-incubation of the membrane with the peptides to which the antibodies were designed for. Thus I was unable to ascertain which band (if any) corresponded to my desired protein.

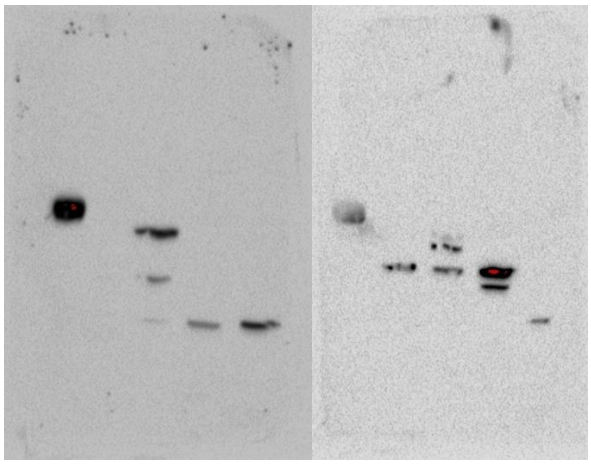

Figure i.i Western blots using custom dogfish GLUT4 antibodies. The image on the left is Ab1 and the image on the right is Ab2. The lanes for both are as follows: Ladder, Liver, Gill, Intestine, Muscle.

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