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The rectal gland and euryhalinity in elasmobranch fish.

By

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Doctor of Philosophy

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Declaration

I, Jonathan Good hereby certify that this thesis, which is approximately 53,000 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

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Abstract

1) Both the partially euryhaline *Scyliorhinus canicula* and the fully euryhaline *Carcharhinus leucas* significantly modify plasma concentrations of urea and chloride (Cl^-) (and sodium (Na^+)) in response to changes in environmental salinity, in order to maintain overall plasma osmolality slightly hyper- or isosmotic to the environment. *C. leucas* has a greater capacity for urea retention in dilute environments. In *S. canicula* all of these changes occur within 12 hours of transfer, with the notable exception of increasing plasma urea in response to acute transfer to elevated salinity.

2) A new technique, ^{51}Cr-labelled erythrocytes, was developed to assess blood volume in elasmobranch fish. *S. canicula* displays significant haemodilution and concentration during chronic acclimation to decreased and increased environmental salinity respectively. Significant changes in blood volume were seen within 6 hours of acute salinity transfer.

3) *In vivo* secretion rates were measured in the rectal gland of *S. canicula* during both chronic and acute salinity transfer. Significant changes in Cl^- clearance occur during acute transfer, as plasma Na^+ and Cl^- levels are modified, but do not persist in chronically acclimated animals. This is achieved through modifications in the volume and Cl^- concentration of the secretory fluid.

4) *C. leucas* is able to significantly alter the abundance and/or recruitment of Na^+, K^+-ATPase in both the rectal gland and the kidney during chronic acclimation to salinity transfer. This is presumably in response to increased requirements for NaCl secretion in SW and osmolyte retention in FW respectively. *S. canicula* do not significantly alter abundance and/or recruitment of Na^+, K^+-ATPase in the principle osmoregulatory organs following chronic acclimation to salinity transfer.

5) Chronically SW acclimated *C. leucas* modify the proportion of ouabain-sensitive oxygen consumption in the tissues of the rectal gland in response to the secretory endocrine stimulus C-type natriuretic peptide (CNP). No such modification occurred in the rectal glands of FW acclimated *C. leucas*. This represents a change in the sensitivity and response to endocrine control factors during chronic acclimation to salinity transfer in this species. No such modification was seen the in the proportion of ouabain-sensitive oxygen consumption in the rectal glands of chronically acclimated *S. canicula* in response to CNP.

These results were discussed in relation to the capacity for modification of osmoregulatory organs in partially and fully euryhaline elasmobranchs.

Dedication

If you go through life being the best person you can be
your reward will be that the people you call your friends
are the finest ones you have met.
You can't choose your family, but I am fortunate enough
to be able to say that I consider all of my family
friends of mine.

To my brother, for always giving me something better
to aspire to.

Acknowledgements

I would like to sincerely thank my supervisor Dr. Neil Hazon, quite possibly the man with most demands on his time in history. Despite this you always made time to see me when I needed you. Your guidance was invaluable. You also gave me the opportunity to spend 15 months of my life shark fishing in Australia!

This thesis wouldn't exist without the friendship of three people: Richard Pillans, Peter Kraft, and Alan Wells. Despite the stresses of the last 4 years you have managed to keep me sane and put a smile on my face. Your help and support have been priceless to me and I can only hope to repay that over the coming years.

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Heartfelt thanks go to my housemates from St Andrews: Jane Freel, Teena Johannsen, Benji Heywood, Ryan Saunders, and Martin Cox. Too many good times to do justice to. I'll miss you all.

Thanks also to everyone at BAF, Abstract, Flatball, QUDA, BUDA, Sporting Lesbian, The Love Rats, The Whey Pat Tavern, Funkian, and the crew of the *Strathy* for keeping me hooked on endorphins and reminded of what's really important in life.

Mum and Dad, you've given up so much to give me the opportunities I've had so far, more than I can ever repay. I only hope that I make you proud and that all the sacrifices have been worthwhile. I love you both.

Eireann, you restored my belief that some people can always be honest. Thank you for that and your love, support, and understanding over the time this has taken. They've meant the world to me. All my love.

Jon

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Abbreviations

^{51}Cr	Chromium 51
^{125}I	Iodine 125
ACE	Angiotensin converting enzyme
ACTH	Adrenocorticotrophin
AES	American Elasmobranch Society
Ang II	Angiotensin II
ANOVA	Analysis of variance
ANP	Atrial natriuretic peptide
Asn	Asparagine
AVP	Arginine vasopressin
AVT	Arginine vasotocin
BNP	B-type natriuretic peptide
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CNP	C-type natriuretic peptide
CPM	Counts per minute
CRH	Corticotrophin releasing hormone
Cys	Cysteine
EDT	Early distal tubule
Evans Blue	T-1824
FW	Freshwater
g	Acceleration produced by gravity
GFR	Glomerular filtration rate
GH	Growth hormone
Gln	Glutamine
Gly	Glycine
HES	Hydroxyethyl starch
Ile	Isoleucine
IU	International unit
Leu	Leucine

MCH	Melanin-concentrating hormone
mOsm	Milli-osmole
MRC's	Mitochondria rich cells
MS-222	Ethyl 3-aminobenzoate methanesulphonate salt
MSH	Melanophore-stimulating hormone
Na^+, K^+-ATPase	Sodium/potassium adenosine triphosphatase
NP	Natriuretic peptide
OUC	Ornithine urea cycle
P II	Proximal segment II
Phe	Phenylalanine
Pi	Phosphate
PKA	Protein kinase A
PKC	Protein kinase C
PKG	Protein kinase G
ppm	Parts per million (mass)
Pro	Proline
RAS	Renin-angiotensin system
SEM	Standard error of the mean
Ser	Serine
SkUT	Skate kidney urea transporter
SW	Seawater
TMAO	Trimethylamine oxide
TSH	Thyroid stimulating hormone
Tukey	Tukey-Kramer multiple comparisons
Tyr	Tyrosine
Val	Valine
VIP	Vasoactive intestinal peptide
Welch	Welch standard deviation

Chapter 1: General Introduction

1.1 Elasmobranch taxonomy

1.1.1 Evolution and phylogeny

Extant species of fish with jaws (Gnathostomes) are divided into two classes: Osteichthyes, containing the bony fish in three subclasses (Acanthodii, Actinopterygii, and Sarcopterygii); and Chondrichthyes, which is comprised of the cartilaginous fish. Class Chondrichthyes is divided into two subclasses: Holocephali, containing the ratfish; and Elasmobranchii, containing the sharks, skates, and rays. The extant species of elasmobranchs are believed to have originated in the Early Triassic period (Cunny and Benton 1999; Winchell et al. 2004), with the first appearance of sharks in the fossil record being some 440 million years ago (Martin 2001). The vast majority of species have been marine, and at present, only around 43 species of over 800 within the sub-class elasmobranchii are know to exist in freshwater (FW) environments upstream of tidal river mouths (Compagno and Cook 1995).

There has been much debate over the phylogeny of elasmobranchs because of their importance as a basal position in the vertebrate tree. Winchell and co-workers (2004) highlighted four major problems associated with anatomical cladistics for elasmobranchs: firstly, the poor preservation of cartilaginous endoskeletons; secondly, the divergent features in the musculoskeletal system of the closest extant outgroup, the chimeras; thirdly, the widespread possibility of convergent evolution due to similar ecological niches; and fourthly, the conserved nature of shark morphology and the lack of recognisable synapomorphies.

These reasons have lead to many different phylogenies being suggested for the elasmobranchs based on both morphological and molecular data (Compagno 1973;

Compagno 1977; Maisey 1980; Shirai 1996; Douady et al. 2003; Winchell et al. 2004). Recent studies have suggested an early divergence of Batoids (skates and rays) and other shark species, as well as a grouping of the Squaloid, Squatinoid, Hexanchoid, and Pristiophoroid sharks as "Orbitostylic" sharks due to the presence of an orbital process which projects from the upper-jaw cartilage inside the eye socket (Maisey 1980; Douady et al. 2003; Winchell et al. 2004).

Ongoing research into this area has resulted in many different phylogenies being published at relatively short intervals, with no definitive answer being reached. There are some common patterns among the more compelling studies, such as the "Orbitostylic" grouping noted above. The consensus of these studies gives strong evidence for the following elasmobranch phylogeny (Figure 1.1.1). This represents an amalgamation of the most convincing phylogenies available and is used as a descriptive tool to give an evolutionary background to any comparisons which are made.

There are about 350 extant shark species, with around 55% of these comprising the order Carcharhiniformes (Compagno 1988). These have been divided into 8 families: Scyliorhinidae (catsharks), Proscylliidae (finback catsharks), Pseudotriakidae (false catsharks), Leptochariidae (barbeled houndsharks), Triakidae (houndsharks), Hemigaleidae (weasel and snaggletoothed sharks), Carcharhinidae (requiem sharks), and Sphyrnidae (hammerhead sharks) (Compagno 1988).

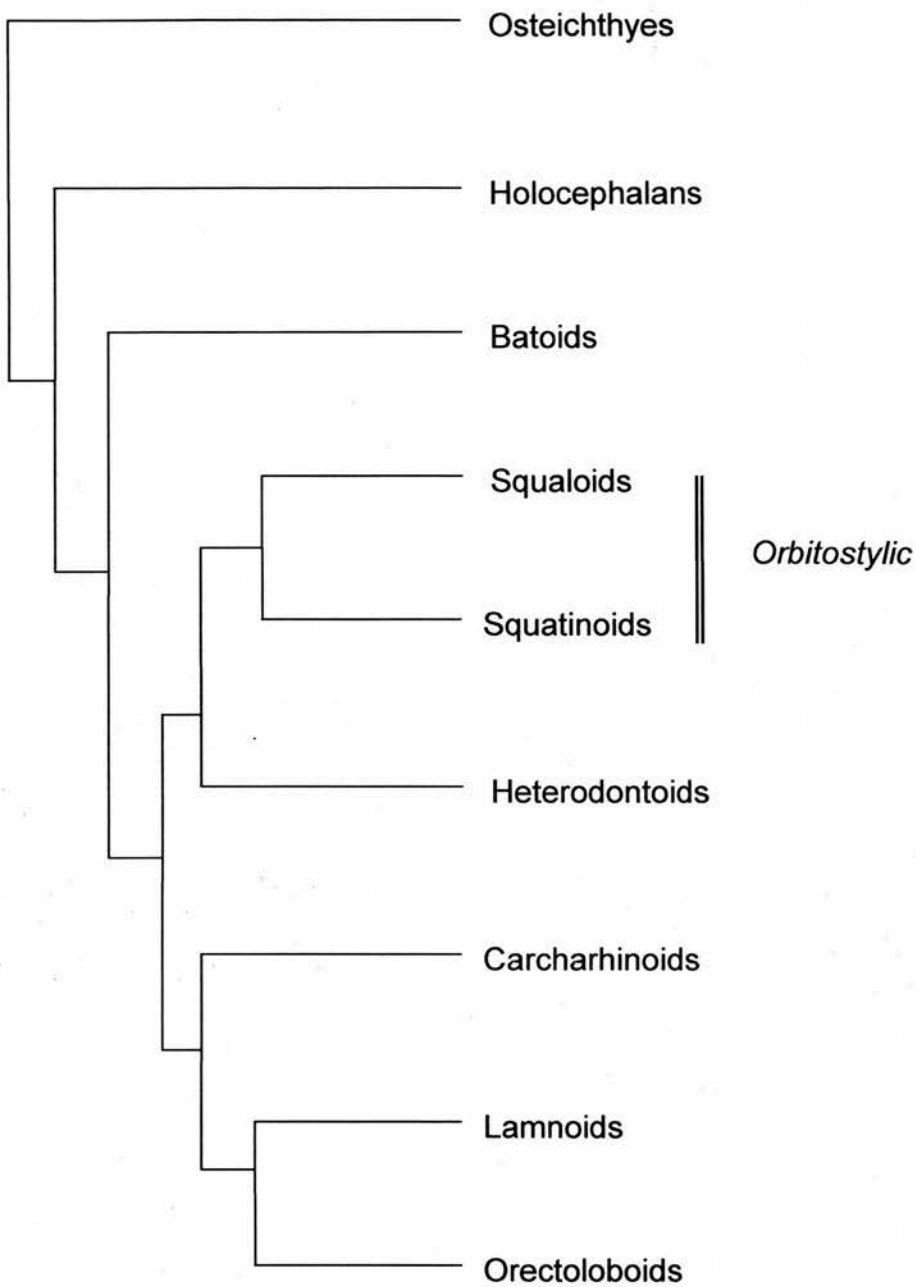

Figure 1.1.1 – Amalgamative phylogeny of the Gnathostomes and the subclass Elasmobranchii (Compagno 1977; Maisey 1980; Douady et al. 2003; Winchell et al. 2004).

Despite the large concentration of species within the order Carcharhiniformes it is morphologically and biologically far less diverse than the other shark orders, with few highly specialised sharks or unusual trophic adaptations. Notable exceptions to this are the bowplane cephalofoils of the hammerheads, and the ability of the swell sharks to gulp air or water to expand their bodies, similar to bony fish such as the puffers (Tetraodontidae) (Compagno 1988). Little work has been conducted on the phylogeny of Carcharhiniform sharks, but it has been suggested that scyliorhinids represent the basal lineage, that triakids branched off second, and that carcharhinids and sphyrnids are the most derived (White 1937; Compagno 1973; Winchell et al. 2004).

The relatively conserved nature of the Carcharhiniformes lends itself to comparative studies for both morphology and physiology; the species chosen for use in this study were taken from this order of sharks: *Scyliorhinus canicula* from the basal scyliorhinid lineage, and *Carcharhinus leucas* from more derived carcharhinids. The two species provided a unique possibility for the comparison of osmoregulation and the factors affecting euryhalinity during this study. The ability to work on animals from fundamentally different environments enabled particular osmoregulatory processes, such as rectal gland oxygen consumption, to be measured at different salinities and directly compared between a fully euryhaline (*C. leucas*) and a partially euryhaline (*S. canicula*) species.

1.1.2 Species information

1.1.2.1 The lesser-spotted dogfish

The lesser-spotted dogfish, *S. canicula*, is a marine elasmobranch which can tolerate moderate changes in salinity, around 60% - 120% seawater (SW). It is found throughout the temperate waters of Europe, reaching a maximum size of around 100cm. *S. canicula* typically gorge feeds on a diet which consists largely of small fish, molluscs and crustaceans.

The majority of physiological research on elasmobranchs has been carried out on *S. canicula* and the spiny dogfish, *Squalus acanthias*. This is due to their manageable average sizes, ability to be maintained in aquaria and relative abundance in the waters of Europe and North America respectively, rather than any particular scientific significance. *S. canicula* in particular is a very robust species; recent studies have shown 98% survival rates in discarded animals from beam trawl fisheries following periods of high stress (Revill et al. 2005). This bias in the fundamental research has resulted in a good depth of understanding concerning the mechanisms involved in the osmoregulation of these species, compared to that of other elasmobranchs. A good illustration of this is the species specific model for stimulation and secretion in the rectal gland of *S. acanthias* suggested by Silva and co-workers (1996) (Section 1.11.1).

1.1.2.2 The bull shark

The bull shark, *C. leucas*, is a fully euryhaline elasmobranch inhabiting SW, estuarine, and FW environments. *C. leucas* is found along many coastlines around the world in tropical and subtropical seas, as well as inland FW systems (Taylor 1997). This is the only species of shark which is known to stay for extended periods in FW. An example of this is the population found in Brisbane, Queensland, Australia: female *C. leucas* give birth to live young in the estuarine reaches of the Brisbane River. The juveniles then migrate upstream into FW for an undetermined length of time, and may then move downstream and finally into SW at Moreton Bay.

The use of different habitats by adults and juveniles is thought to be an adaptation that helps improve the survival of young sharks through a decreased risk of predation from the adults. *C.* leucas is a large species which grows to a length of 3 - 4m. It has an omnivorous diet which includes fishes (including other sharks), dolphins, turtles, birds, molluscs, echinoderms and even terrestrial mammals (Taylor 1997).

Research on *C. leucas* has focused on distribution patterns, population studies, and basic haematic parameters (Thorson et al. 1973; Sosa-Nishizaki et al. 1998; Wintner et al. 2002; Pillans and Franklin 2004). This is largely due to the problems associated with capture, transport, and maintenance of the species in captivity (Sections 2.2.2 and 2.4). Nevertheless, the species is of great scientific importance in terms of osmoregulation due to its fully euryhaline nature.

1.2 Elasmobranch osmoregulation

The majority of extant elasmobranch species inhabit a marine environment and maintain body fluid osmolality slightly hyperosmotic to SW. This is achieved through a combination of organic and inorganic osmolytes, as well as regulating fluid volume. Sodium (Na^+) and chloride (Cl^-) are two of the major osmolytes and in SW elasmobranch plasma concentrations are lower than the surrounding environment, typically around 250 mmol l^{-1} (typical values for the water are around 500 mmol l^{-1}). Plasma osmolality is rendered to a hyperosmotic level via the retention of nitrogenous compounds in the extracellular fluids, the major constituent being urea with a concentration of around 350 mmol l^{-1} (Table 1.2.1) (Ballantyne et al. 1987). A ureosmotic strategy is unusual but has also been studied in other species; notably holocephalans, coelacanths, lungfish, the killifish (*Rivulus marmoratus*), and the crab-eating frog (*Rana cancrivora*) (Griffith 1991; Frick and Wright 2001; Wright et al. 2004).

Urea is formed by the ornithine urea cycle (OUC) (Section 1.7) and retention of such a high concentration would ordinarily have toxic effects via protein denaturation (Yancey and Somero 1978; Yancey and Somero 1980; Yancey et al. 1982). In elasmobranchs some proteins function optimally in elevated urea levels (Yancey and Somero 1978), whilst others require the toxicity of urea to be offset by the action of methylamines such as trimethylamine oxide (TMAO) (Yancey and Somero 1979; Yancey and Somero 1980). TMAO is the major methylamine and the second largest constituent of nitrogenous osmolytes in elasmobranchs.

Fluid	Osmolality (mOsm Kg^{-1})	Na^+ (mmol l^{-1})	Cl^- (mmol l^{-1})	Urea (mmol l^{-1})
Plasma	1018	286	246	351
Urine	780	337	203	14.5
Rectal Gland	1018	540	533	~0
Seawater	930	440	495	~0

Table 1.2.1 - Osmotic activity and principle osmolytes in the fluids of *S. acanthias* (Burger and Hess 1960).

	Osmolality (mOsm Kg^{-1})	Na^+ (mmol l^{-1})	Cl^- (mmol l^{-1})	Urea (mmol l^{-1})
SW	1067	289	296	370
FW	642	208	203	192
Potamotrygon	320	178	146	1.2

Table 1.2.2 - Osmotic activity and principle osmolytes in the blood plasma of *C. leucas* from SW and FW environments (Pillans and Franklin 2004) and a FW *Potamotrygon* stingray (Wood et al. 2002a).

These differences in osmolyte concentrations for marine elasmobranchs result in gradients for the following movements across the semi-permeable surfaces:

- A large efflux of urea
- Influxes of ions, notably Na^+ and Cl^-
- A small influx of water

The relative concentrations of these osmolytes are regulated by the gills, the gut, the rectal gland, and the kidney. The function of these principle osmoregulatory organs is described later (Sections 1.3, 1.4, 1.5, and 1.6). Through the action of these organs elasmobranchs are able to selectively alter the relative concentrations of principle osmolytes in the body fluids in relation to SW. In this way the internal concentrations of individual osmolytes can be maintained at different levels to those in the external environment.

Euryhaline elasmobranchs such as *C. leucas* and the Atlantic stingray, *Dasyatis sabina*, adopt a similar osmoregulatory strategy in SW (Smith 1931b; Smith 1931a; Pillans and Franklin 2004). Through the action of the organs noted above, elasmobranchs in FW maintain reduced levels of urea along with a less severe reduction in Na^+ and Cl^- (Table 1.2.2) (Thorson et al. 1973; Piermarini and Evans 1998; Pillans and Franklin 2004). These concentrations of principle osmolytes in FW lead to the following fluxes:

- A large influx of water
- Effluxes of ions, notably Na^+ and Cl^-
- A large efflux of urea

There are therefore fundamental differences in the osmoregulatory requirements of SW and FW elasmobranchs: FW elasmobranchs experience a far greater influx of water than those in SW, and the gradients for Na^+ and Cl^- are directly opposite in the two environments. Animals in both environments face a continual loss of urea, although this is compounded in FW by the magnitude of the difference between internal and external osmolality. These variations lead to different priorities for osmoregulation in the FW environment, such as the retention of Na^+ and Cl^- and a greater pressure on volume regulation.

There are also a group of stenohaline FW elasmobranchs all of which belong to the family Potamotrygonidae. These stingrays are widespread throughout the river systems of South America draining into the Atlantic Ocean. Some of the Dasyatidae complete their life cycle in FW (Compagno and Roberts 1982), but the potamotrygonid stingrays are the only obligate FW species having lost the ability to survive in waters of salinity greater than 100 mOsm Kg^{-1} (Brooks et al. 1981). Key to this is the inability of the kidneys and gills to retain urea (Thorson 1970), and the absence of salt secretion from a degenerate rectal gland (Thorson et al. 1978). It has been reported that plasma urea concentrations are as low as 1.2 mmol l^{-1}, and that these elasmobranchs are ammoniotelic as opposed to ureotelic (Table 1.2.2) (Wood et al. 2002a).

At the cellular level, free amino acids play a vital role in osmoregulation and regulating cell volume (Forster and Goldstein 1976). In vertebrates, intracellular osmotic parameters are typically isosmotic with those of the extracellular fluid. Changes in environmental conditions are therefore necessarily coupled with changes in intracellular volume and osmolyte concentrations. Urea and TMAO freely diffuse across plasma

membranes (Fenstermacher et al. 1972), therefore the intra- and extracellular concentrations are equivalent. This is not so with free amino acids which constitute 1% of extracellular fluid osmolality and 19% of that of intracellular fluid (Perlman and Goldstein 1988). Acclimation of Batoids to decreases in salinity has been proven to affect free amino acid concentrations. In the little skate, *Raja erinacea*, significant decreases in free amino acid concentrations were measured in wing muscle and erythrocytes upon acclimation to 50% SW, although concentrations in the heart were unaffected (Boyd et al. 1977). Similar effects were also observed in the brain of the *D. sabina* acclimated to 50% SW (Boyd et al. 1977). Clearly free amino acids play an important role in regulating cell volume, particularly during salinity transfer in euryhaline elasmobranchs.

It is therefore evident that osmoregulation is of fundamental importance to euryhalinity in elasmobranchs, at both the cellular and whole animal levels. Through the action of the gills, gut, rectal gland, and kidneys elasmobranch fish have the ability to independently regulate the concentrations of Na^+, Cl^- and urea in both SW and FW environments, as part of their hyperosmoregulatory strategy. The mechanisms by which this osmoregulatory strategy is controlled are poorly understood, particularly during migration between FW and SW. However, the principle organs involved have been reasonably well studied and their modes of action are well described. The principle osmoregulatory organs named above will be described in detail, along with their modes of action and importance in SW and FW. In addition there are a number of other organs that are believed to play an important role in osmoregulation, such as the liver as the main site of urea production, and the pituitary gland, the interrenal gland, and the heart as endocrine organs effecting osmoregulatory control. These too will be discussed.

1.3 The gills

The gills of elasmobranchs have been the subject of many anatomical studies (Wright 1973; Olson and Kent 1980; DeVries and DeJaeger 1984; Metcalfe and Butler 1986). There are usually five pairs of gills, although six and seven are not uncommon. Each gill arch is made up of lateral rods of cartilage (the gill filaments) supporting a sheet of muscular and connective tissue (the interbranchial septum). The dorsal and the ventral surfaces of each gill filament have a row of secondary lamellae; these are the principal site of gas exchange.

Branchial vasculature is highly complex and varies greatly from species to species. Evans and co-workers (2005) recently published a thorough review of the fish gill, in which detailed descriptions of the vasculature are made. A general model for blood flow through the elasmobranch gills can be drawn. The entire cardiac output enters the afferent branchial arteries (ABAs) via the ventral aorta. Blood flowing through an ABA feeds two hemibranchs of a gill arch where it is oxygenated at the lamellae of the filaments (Evans et al. 2005). The vasculature which supplies the secondary epithelium can be mediated by sphincters located on the efferent primary artery, and on both afferent and efferent secondary arteries (Laurent and Dunel 1980). Oxygenated blood flows into an efferent branchial artery (EBA) which in turn flows into the dorsal aorta for systemic distribution (Evans et al. 2005). There are two distinct but interconnected circulations within the gill filaments: the arterio-arterial pathway which is involved in respiratory gas exchange; and the arteriovenous pathway, a nonrespiratory pathway possibly involved in supplying nutrients to the epithelium and structural tissues (Figure 1.3.1).

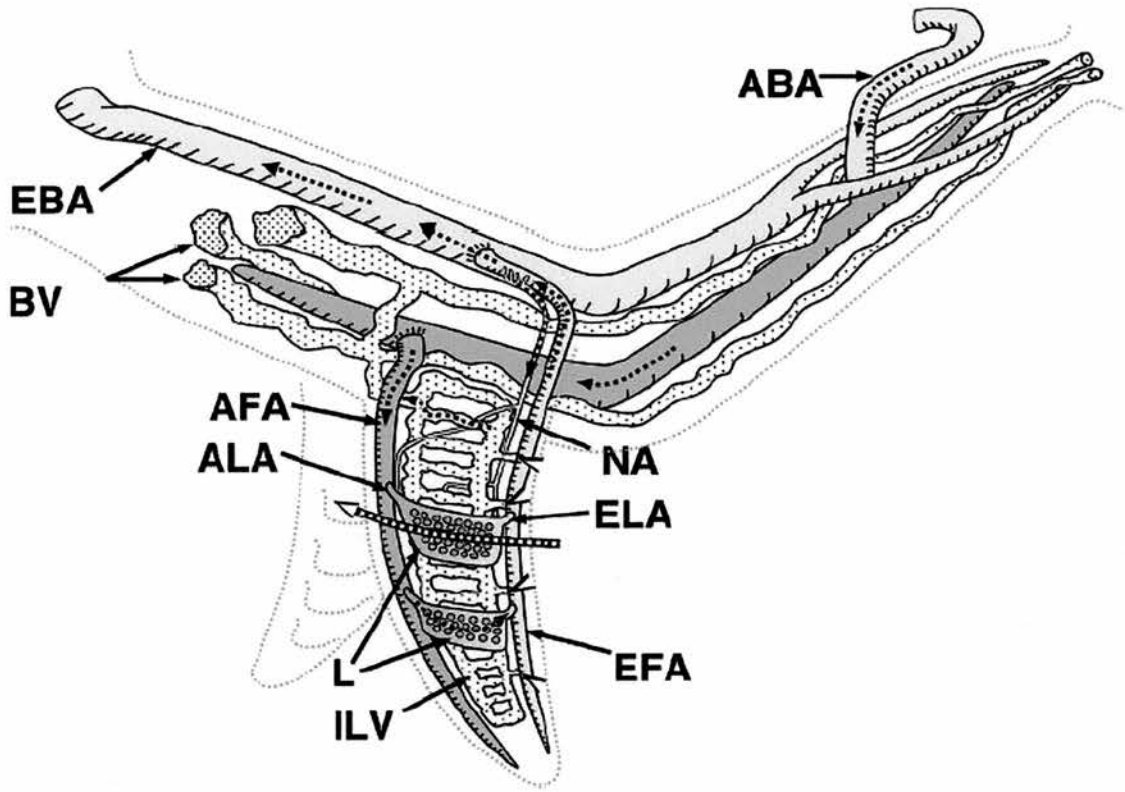

Figure 1.3.1 – Generalised blood flow through an elasmobranch gill arch and filament. Arterio-arterial pathway: blood travels ( ) from the afferent branchial artery (ABA) to an afferent filamental artery (AFA), which runs the length of the filament. This blood is distributed to the lamellae (L) via afferent lamellar arterioles (ALA's). Lamellar blood flows through efferent lamellar arterioles (ELA's) into an efferent filamental artery (EFA). Oxygenated blood then flows to the efferent branchial artery (EBA) and on to the dorsal aorta for systemic distribution. Arteriovenous pathway: blood in the EFA can be distributed to interlamellar vessels (ILV's) via postlamellar arteriovenous anastomoses (>) or nutrient arteries (NA). The ILV's are drained by branchial veins (BV). The direction of water flow ( ) over the gills is also shown (Evans et al. 2005).

The elevated concentrations of urea and TMAO in the blood plasma of elasmobranchs results in a substantial concentration gradient for the diffusive efflux of these osmolytes. The internal concentration of Na^+ and Cl^- result in a gradient for the diffusive influx of these ions across epithelial membranes from the marine environment. Even though the permeability of elasmobranch gill epithelia to urea is the lowest recorded (Boylan 1967) the gills are still the major site of diffusive urea efflux, as well as Na^+ and Cl^- influx in SW. It has been suggested that rates of urea loss are reduced through a combination of structural and active transport mechanisms. The basolateral membranes of *S. acanthias* gill epithelia have the highest cholesterol to phospholipid ratios recorded for a natural membrane (Fines et al. 2001). This could be a means of reducing the diffusion of urea into the cell as cholesterol is know to reduce urea permeability (Mourtisen and Jorgensen 1994). There is also evidence for Na^+ dependent active urea transport by basolateral membrane vesicles (Fines et al. 2001). These findings lead Evans and co–workers (2005) to suggest that the gill epithelium acts as an intermediary compartment where the urea concentration gradient with the environment is lowered below that of blood plasma, thereby reducing diffusive urea loss (Figure 1.3.2).

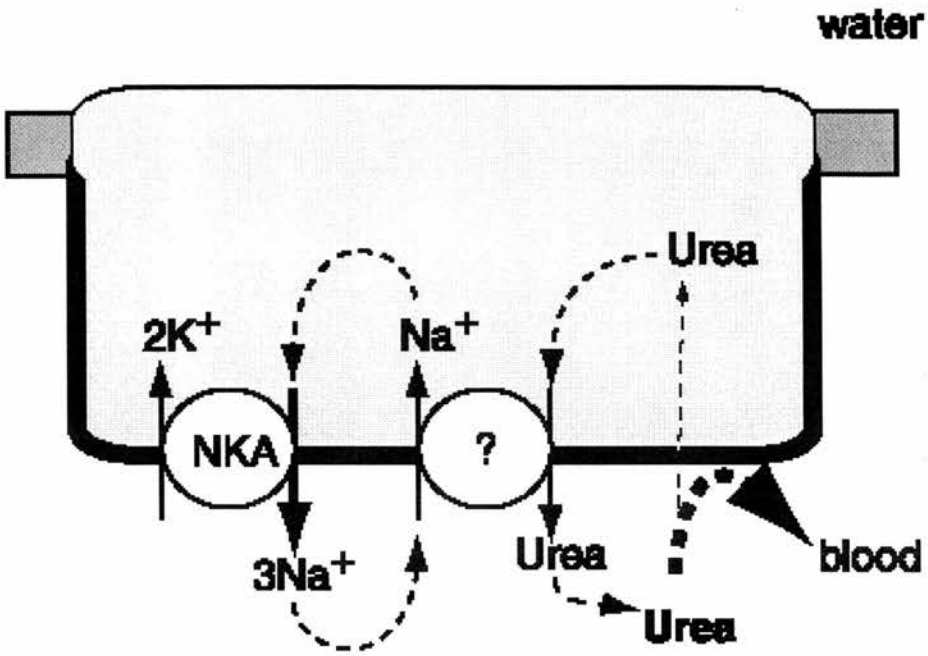

Figure 1.3.2 – Proposed model of urea retention in the elasmobranch gill epithelia. The basolateral membrane has a decreased permeability for urea, in part due to the high cholesterol content (represented by the thick line for the membrane). This greatly reduced the amount of urea in the blood which diffuses into the cell. The concentration of urea which actually enters the cell is then further reduced by an unidentified Na^+-dependent urea transporter in the basolateral membrane. The Na^+ gradient required for the urea transporter is thought to be maintained by the action of Na^+, K^+-ATPase. The relatively low intracellular concentration of urea, as compared to that of the blood plasma, reduces the gradient for the diffusive loss of urea to the external environment (Evans et al. 2005).

Conversely to the situation described for urea there is active accumulation of Na^+ and Cl^- at the gills, despite the osmotic consequences of the salt load. This accumulation is related to the acid-base regulatory system (Bentley et al. 1976) which is involved in the excretion of acidic (e.g. hydrogen, H^+) and basic (e.g. bicarbonate, HCO_3^-) ions (Figure 1.3.3) (Evans 1982; Evans 1984). Studies on teleosts and elasmobranchs have shown consistently that acid secretion is linked to Na^+ absorption, and that base secretion is linked to Cl^- absorption (Evans 1982; Cooper and Morris 2004b; Evans et al. 2005). Faster and more complete compensation for hypercapnia in SW acclimated *D. sabina* (Choe and Evans 2003), and a persistence in alkalosis in *Heterodontus portusjacksoni* acclimated to reduced salinity (Cooper and Morris 2004b), further support the role of Na^+ in branchial acid excretion. It has been suggested that there are two acid secretion mechanisms: an apical V-ATPase which is electrically linked to Na^+ absorption, and an electroneutral exchange of Na^+ and H^+ via the Na^+/H^+ exchange proteins; and two base secretion mechanisms via two apical $Cl^-/HCO3^-$ exchangers: AE1, and pendrin (Evans et al. 2005).

Accumulation of Na^+ and Cl^- and the gills may also act as a means of decreasing the influx of these ions from the external environment. Just as a lower intracellular urea concentration in the gill epithelia decreases the gradient for the diffusional efflux of urea, elevated intracellular Na^+ and Cl^- concentrations would decrease the gradient for the diffusional influx of these ions from the marine environment. However, given the specific evolution of the rectal gland towards secreting excess Na^+ and Cl^- (Section 1.5) the necessity for decreasing the influxes of these ions is not as great as that for the retention of urea.

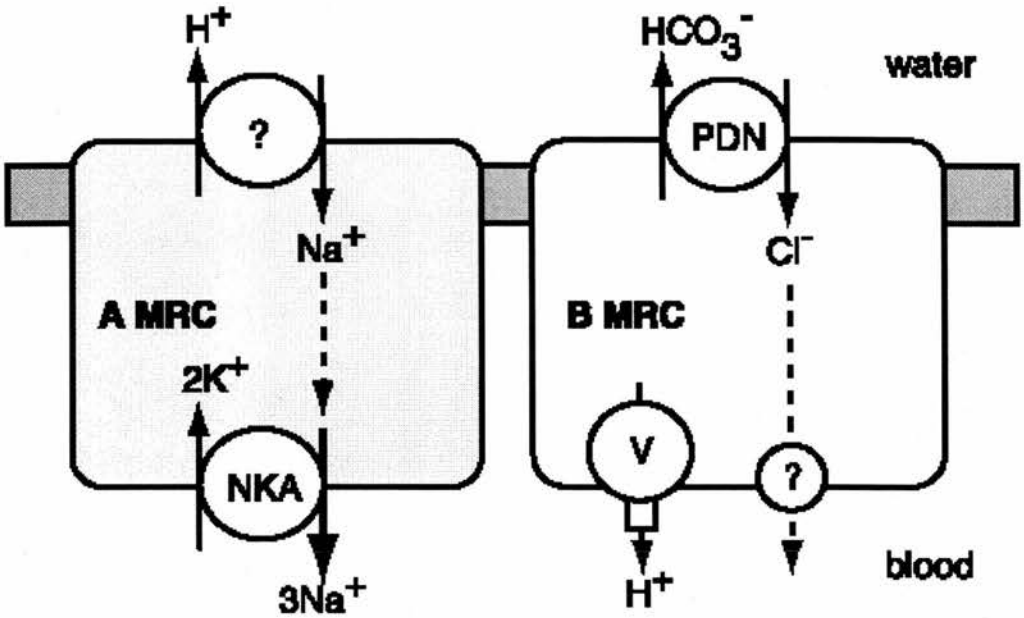

Figure 1.3.3 – A working model of NaCl-linked acid base extrusion in the chloride cells of *D. sabina*. One type of chloride cell (A MRC) expresses Na^+, K^+-ATPase (NKA) on its basolateral membrane and is hypothesized to draw in Na^+ across the apical surface in exchange for cytoplasmic H^+. The other type of chloride cell (B MRC) expresses V-H^+-ATPase (V) on its basolateral membrane and draws Cl^- into the cell via pendrin (PDN) in exchange for HCO_3^-. The pathway for basolateral Cl^- movement is unknown (Evans et al. 2005).

There is also substantial efflux of Na^+ and Cl^- across the gills by the chloride cells or mitochondria-rich cells (MRC's) (Figures 1.3.3 and 4). The rate of Na^+ and Cl^- efflux by the chloride cells is still less than the rate of influx. Branchial activity of Na^+, K^+-ATPase, the active protein in Na^+ and Cl^- transport (Section 1.5), is ten to fifteen times below that of marine teleosts, and hence there is net accumulation of Na^+ and Cl^- at the gills and no net efflux (Jampol and Epstein 1970; Shuttleworth 1988). Chloride cells are pear-shaped secretory cells in the epithelia of the gills. As well as being rich in mitochondria, there is an extensive network of smooth endoplasmic reticulum, and copious basolateral infoldings of the plasma membrane so as to increase surface area (Wright 1973).

Comparative studies of *Raja clavata* and *S. canicula* revealed two types of chloride cells. In one cell type the apical membrane is buried deep in a *cul-de-sac* and connects to the external milieu by a narrow opening; conversely, the other cell type has a protruding apical membrane (Laurent and Dunel 1980). Both of these cell types lack the tubular system which is found in teleost chloride cells. In elasmobranchs these are functionally replaced by copious infoldings of the basolateral membrane.

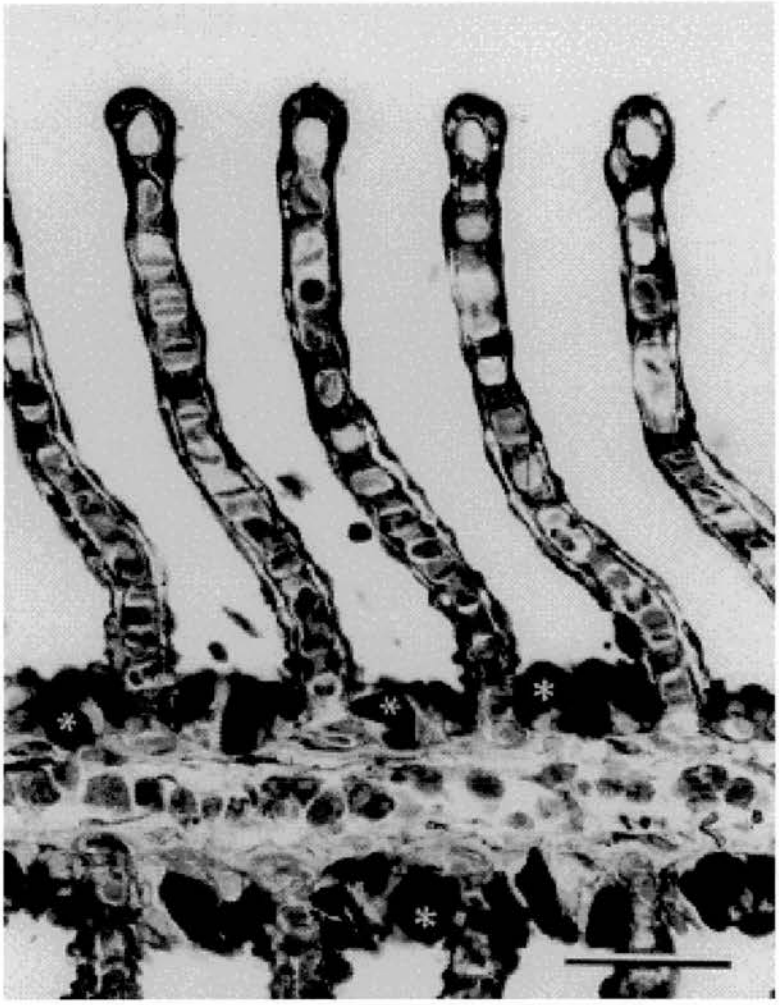

Figure 1.3.4 – Light micrograph of *S. acanthias* gill lamellae showing the darkly stained chloride cells (*). Scale bar of 50 μm (Wilson et al. 2002).

Wilson and co-workers (2002) showed strong Na^+, K^+-ATPase immunoreactivity associated with the basolateral membrane in *S. acanthias*. High abundance of Na^+, K^+-ATPase in the gills of SW elasmobranchs not only confirms their role in acid-base regulation and possible Na^+ dependent urea transport, it also presents a possible role for the gills in excretion of Na^+ and Cl^-. Indeed, *S. acanthias* which had the rectal glands (Section 1.5) removed were able to maintain ionic balance, although the chloride cells showed no change in number, structure, or Na^+, K^+-ATPase activity (Wilson et al. 2002). This suggests that the gills and the kidney (Section 1.6) are able to maintain ionic balance in elasmobranchs during stable environmental conditions. However, given the specific evolution of the rectal gland in elasmobranchs towards the secretion of excess Na^+ and Cl^-, it is unlikely that animals undergoing acute salt loading from feeding or salinity transfer could adequately regulate solely through the action of the gills.

It has been shown that levels and abundance of Na^+, K^+-ATPase change in relation to external salinity in certain species. In experiments carried out on the euryhaline species *D. sabina* the highest activity and relative abundance of Na^+, K^+-ATPase in the gills was seen in long term acclimated FW animals. These animals showed a reduction in both activity and abundance of Na^+, K^+-ATPase after a 7 day period at SW. Long term acclimated SW animals had the lowest activity and abundance of Na^+, K^+-ATPase of all three groups (Piermarini and Evans 2000). Na^+, K^+-ATPase and the effects of salinity are discussed in detail below (Sections 5.1 and 4).

In the instance of FW elasmobranchs the gills are possibly acting like those of teleosts and are a site of active Na^+ and Cl^- uptake. This elevation in Na^+, K^+-ATPase abundance and activity in low salinities is presumably due to the fact that as external salinity

increases the requirement for active Na^+ and Cl^- uptake across the gills will decrease as the ion flux gradient is reversed. These results also demonstrate the capacity for modification of gill physiology and morphology to changing environmental conditions in a euryhaline elasmobranch. The discrepancy between *S. acanthias* (SW) and *D. sabina* (euryhaline) suggests that plasticity in chloride cell structure and/or abundance, and associated branchial Na^+, K^+-ATPase may therefore be a key factor in elasmobranch euryhalinity.

1.4 The gut

The oesophagus, stomach, spiral intestine, and rectum comprise the elasmobranch gut (Figure 1.4.1). The oesophagus of most elasmobranchs is relatively short and lined with finger-like extensions which prevent food escaping from the mouth. Elasmobranch stomachs are generally J-shaped organs, some of which have longitudinal folds (rugae) which allow expansion to accommodate gorge feeding. The stomach is comprised of two histologically distinct sections: the cardiac stomach and the pyloric stomach. The cardiac stomach can be subdivided into the proximal section with a striated muscle wall, and the distal section with a smooth muscle wall (Nilsson and Holmgren 1988).

The valvular intestine is also relatively short, having a greatly increased surface area due to the valves. There are three basic types of intestinal valve in sharks, termed spiral, scroll, and ring. The spiral valve is found in Squalidae and Scyliorhinidae, the scroll valve is found in Carcharhinidae, and the ring valve is found in all extant lamnoids (Martin 2003b). These increase nutrient absorption in the intestine not only by increasing surface area, but also by increasing the length of time taken for material to pass through. Despite the relative compact nature of the elasmobranch gut, absorption efficiencies are as high as those of carnivorous teleosts: 62-83% for energy (quantifying energy lost through non-assimilated food), 76-88% for organic matter, and 76-87% for dry matter (Wetherbee and Gruber 1993). Gross conversion efficiency for ingested food can be calculated by dividing annual production (growth, metabolism, excretion, and egestion) by annual consumption. This varies greatly depending on species and dietary composition (Wetherbee and Cortes 2004). Estimates for *C. leucas* range between 5 and 12% (Schmid and Murru 1994), estimates are not available for *S. canicula*.

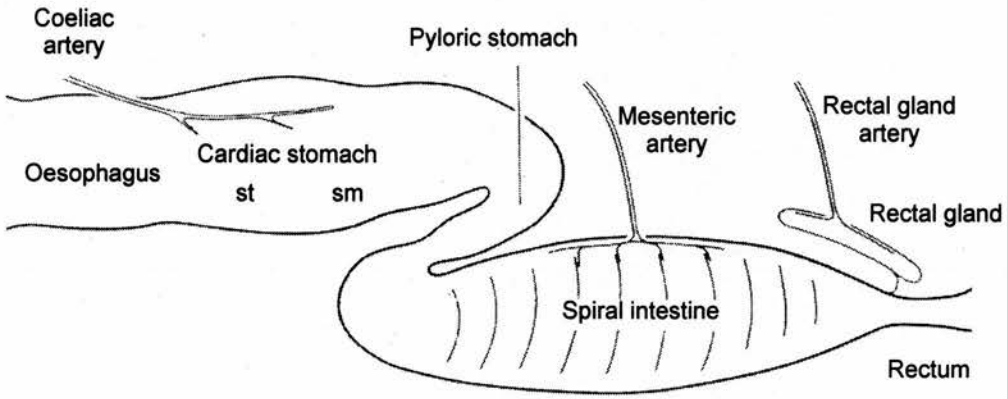

Figure 1.4.1 – Diagram of the gut of *S. acanthias* (Nilsson and Holmgren 1988). The Cardiac stomach is comprised of the striated muscle cardiac stomach (*st*) and the smooth muscle cardiac stomach (*sm*).

There are two major factors which influence the role of the gut in osmoregulation: diet and drinking rate. Due to the nature of aquatic environments the surrounding media is necessarily imbibed during a feeding event. Elasmobranchs were not thought to actively drink their environmental media, because a hyperosmotic strategy means the osmotic gradient is for water to enter the animal, hence there is no requirement to imbibe water. However Hazon and co-workers (1989) demonstrated that *S. canicula* does indeed drink and pharmacological manipulation of the endogenous renin angiotensin system (RAS) resulted in an increase in drinking rate. A detailed description of the RAS is provided below (Section 1.10). Basal drinking rates are considerably lower in SW elasmobranchs than in teleosts, even when compared to *Anguilla anguilla* which has one of the lowest recorded teleost drinking rates (Table 1.4.1). This is due to the fact that marine teleosts are hyposmotic and face a continual loss of water to the environment across semi-permeable surfaces. The basal rate of drinking in *S. canicula* increases with environmental salinity and the ingested Na^+ does enter the blood stream (Hazon et al. 1989). Drinking rate also increases during acute transfer to increased salinity in both *S. canicula* and *Triakis scyllia* (Anderson et al. 2002b). Drinking rate may therefore be a key factor in elasmobranch euryhalinity, by elevating plasma osmolality during transfer to increased salinity.

Species	Drinking rate ($ml\ Kg^{-1}\ h^{-1}$)	Reference
S. canicula	0.3	(Hazon et al. 1997b)
T. scyllia	0.4	(Anderson et al. 2001)
*Anguilla anguilla**	1.0	(Perrott et al. 1992)
*Pleuronectes platessa**	2.5	(Carroll et al. 1995)
*Ammodytes lanceolatus**	3.0	(Perrott et al. 1992)
*Limanda limanda**	3.6	(Perrott et al. 1992)
*Myxocephalus scorpius**	7.8	(Perrott et al. 1992)

Table 1.4.1 – Drinking rates in SW elasmobranch and teleost (*) fish.

Captivity period	Osmolality ($mOsm\ Kg^{-1}$)
Wild	681
1-7 days	638
+ 12 days	558

Table 1.4.2 – Mean blood plasma osmolality in wild, short term, and long term captive FW *C. leucas* (n = 9, 13, and 14 respectively). Captive animals were not fed whereas wild animals had unrestricted access to natural prey species.

When examining the role of the gut in elasmobranch osmoregulation a large consideration must go to dietary composition. By definition the effects of this will vary greatly between species, and also between populations. Not only will the diet itself vary, but the requirements from that diet will vary depending on the environment in which the elasmobranch inhabits, whether the species is an active or ambush predator, and whether or not the species is ram ventilating. Many marine elasmobranchs, including *S. canicula*, are typically gorge feeders. One of the consequences of this is that the animal is subjected to large and infrequent salt loading during feeding events. This situation is exaggerated if the diet is also particularly rich in salts, such as one comprised largely of invertebrates as in *S. canicula*.

Dietary intake may also be a key source of salts for FW elasmobranchs. Potamotrygonid rays experienced negative salt balance with their native ion-poor waters during periods of starvation (Wood et al. 2002a). FW elasmobranchs may therefore require dietary salts to maintain osmotic stasis.

Metabolic urea is also important for osmoregulation, and this is directly related to food availability. Infrequently fed *Poroderma africanum* could not adequately osmoregulate during acclimation to changes in salinity. Reduced metabolic urea production resulted in decreases in plasma osmolality and hyposmotic regulation (Haywood 1973). Similar effects of starvation were seen in *C. leucas* during captivity trials in this study (Table 1.4.2). This highly active species displayed a visible loss in body condition prior to a sharp decrease in plasma osmolality during periods of starvation.

Armour and co-workers (1993a) showed that *S. canicula* fed on a low protein diet showed an impaired osmoregulatory ability when acclimating to hypersaline water. Animals adopted a strategy utilising increased plasma Na^+ and Cl^- concentrations to compensate for the lack of metabolic urea. This further supports the idea of the gut being an important source for elevating osmolyte levels during salinity transfer.

There is therefore large scope for the gut to be involved in the overall osmoregulatory mechanisms in elasmobranchs given that imbibed Na^+ does enter the blood, the possibility of large salt loads entering the interstitial fluid during feeding, and the importance of dietary derived salts and urea. Clearly more research is required into this area to discover the specific role of the gut for both osmolyte and water exchange, and possible humoral effects on other osmoregulatory organs.

1.5 The rectal gland

The rectal gland is the only organ in elasmobranchs which is capable of producing a NaCl solution more concentrated than blood plasma levels, and has evolved specifically for this purpose. The cost of NaCl secretion by the rectal gland has been estimated at 0.5% of the standard metabolic rate (Morgan et al. 1997). The gland itself is a blind-ending, usually bullet-shaped tube in the dorsal mesentery, which is suspended above the valvular intestine. It is attached to the intestine postvalvularly. Rectal glands vary in size and shape depending on the species of elasmobranch, and its life history. Glands may be smaller in euryhaline, and particularly in freshwater, than in marine animals (Oguri 1976). This is presumably due to the lower influxes and variations of Na^+ and Cl^- in a more dilute environment. The structure and vasculature of the rectal gland are highly complex and a detailed description is provided later (Section 4.1).

The mechanisms involved in ion transport in the tissues of the rectal gland have been well documented (Shuttleworth 1988; Silva et al. 1997; Olson 1999). Localised on the basolateral membrane of the epithelial cells of the secretory tubules is the protein Na^+, K^+-ATPase (Dubinsky and Monti 1986). This actively pumps Na^+ into the extracellular space as well as transporting K^+ into the secretory cell. Also located on the basolateral membrane is the Na^+K^+-$2Cl^-$ cotransporter. The action of this protein is passive as it is driven by the inward Na^+ gradient set up by the action of Na^+, K^+-ATPase. Along this concentration gradient Na^+ enters the cell facilitating the coupled translocation of K^+ and Cl^- into the intracellular space. Na^+, K^+-ATPase then actively pumps Na^+ back out of the cell (Haas and Forbush 1998). The internal accumulation of excess K^+ is prevented by passive flow through the basolateral potassium specific channel, thereby maintaining equilibrium (Riordan et al. 1994).

These processes result in a high concentration of Cl^- in the secretory cells and a high Na^+ concentration in the intercellular space. Located on the apical membrane of the secretory cells are chloride-selective channels. Through these channels Cl^- ions move passively into the lumen of the secretory tubule so as to restore the intercellular electrochemical equilibrium. The Na^+K^+-$2Cl^-$ cotransporter is stimulated by a fall in intracellular Cl^- concentration pursuant to increased Cl^- efflux across the apical membrane. Na^+ then passively moves paracellularly through the Na^+-selective tight junctions into the lumen to balance the electrical potential created by the movement of Cl^- ions (Fig 1.5.1) (Olson 1999).

In contrast to the situation described in the gills (Section 1.3), activity and abundance of Na^+, K^+-ATPase in the rectal gland is lowest in long term acclimated FW animals. Levels in acclimated and wild caught SW animals are relatively constant (Piermarini and Evans 2000; Pillans et al. 2005). This is due to a relative influx of Na^+ and Cl^- across semi-permeable membranes in SW and an efflux in FW. Hence there is a reduced requirement for rectal gland secretion of Na^+ and Cl^- in more dilute environments.

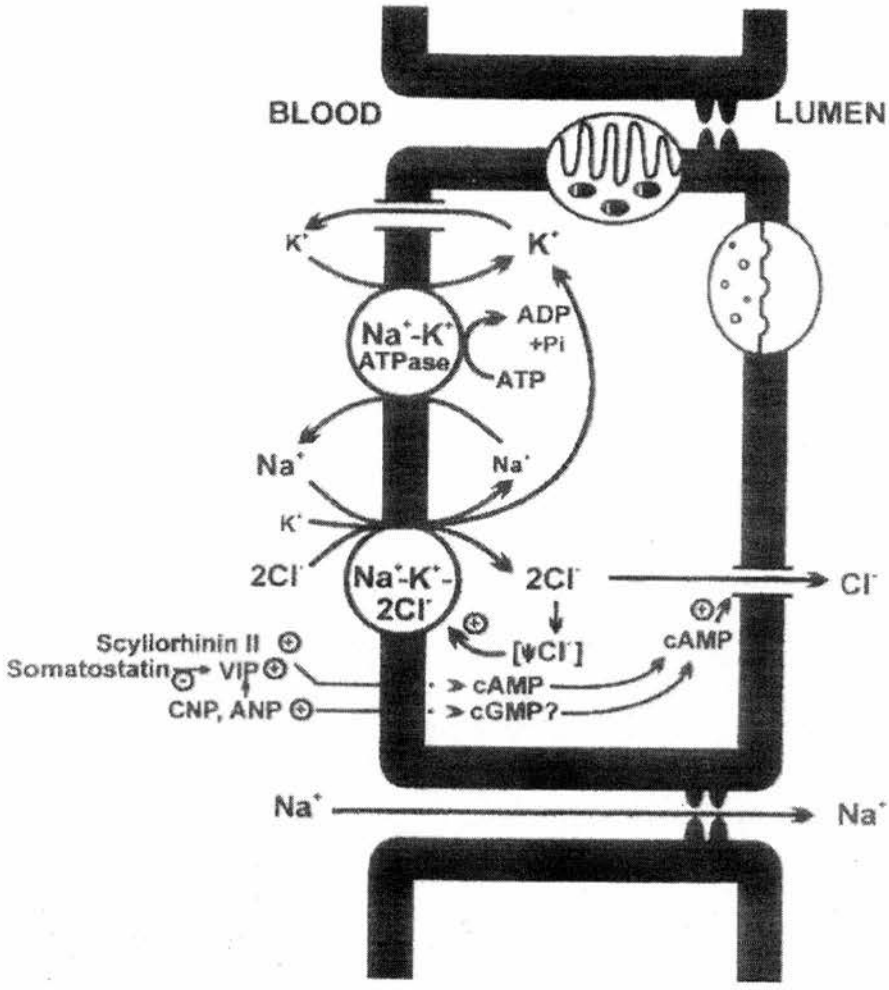

Figure 1.5.1 - Mechanism of Na^+ and Cl^- ion secretion by secretory tubule cells and their control. ADP = adenosine diphosphate; ANP = atrial natriuretic peptide; ATP = adenosine triphosphate; cAMP = cyclic adenosine monophosphate; cGMP = cyclic guanosine monophosphate; Cl^- = chloride ion; K^+ = potassium ion; Na^+ = sodium ion; Pi = phosphatidylinositol; VIP = vasoactive intestinal peptide (Olson 1999). Also shown are some of the hormones affecting the mechanism: the stimulatory actions of scyliorhinin II, VIP, and two natriuretic peptides (Section 1.11.1), and the inhibitory action of somatostatin.

The hormonal control of rectal gland secretion is detailed below (Sections 1.11.1 and 6.1), but secretion rates can also be affected by neurotransmitters present in the nerves of the rectal gland. Vasoactive intestinal peptide (VIP) is found in the rectal gland nerves of *S. acanthias* (Holmgren and Nilsson 1983; Chipkin et al. 1988) and stimulates Cl^- secretion by activating adenylate cyclase (Stoff et al. 1979). The species specific model of rectal gland activation involving VIP is detailed below (Section 1.11.1). The rectal gland of *S. acanthias* also contains inhibitory neuropeptides including somatostatin, bombesin, cholecystokinin and neuropeptide Y (Holmgren and Nilsson 1983; Bjenning and Holmgren 1988; Silva et al. 1993). Somatostatin has a direct inhibitory effect on rectal gland cells both proximally and distally to the release of cAMP (Stoff et al. 1979; Silva et al. 1985) (Figure 1.5.1), while bombesin inhibits indirectly through the release of somatostatin (Silva et al. 1990). The method of inhibition by cholecystokinin has yet to be defined. The inhibitory action of neuropeptide Y does not affect adenylate cyclase activity, having a direct effect on Cl^- secretion at a site distal to the generation of cAMP. Neuropeptide Y also inhibits VIP-stimulated transport related oxygen consumption by Na^+, K^+-ATPase (Silva et al. 1993). There is therefore much scope for mediating the activity of the elasmobranch rectal gland with many factors having stimulatory (Sections 1.11.1 and 6.1) and inhibitory effects.

Given the highly specialised nature of the rectal gland as a means of NaCl secretion, and the depth of factors which influence its function, it must be of key osmoregulatory importance during acclimation to salinity changes. Expectations would be for high levels of activity and secretion during acclimation to reduced salinity in order to rapidly decrease plasma osmolality and minimise the osmotic influx of water. This is of

paramount importance as excess water is excreted via the kidneys (Section 1.6) and increases in urine volume may increase the loss of urea. The importance of urea retention is discussed in detail elsewhere (Sections 1.2, 2.1, 2.4, 7.1.2 and 3). Low levels of activity and secretion would be expected during acclimation to increased salinity as a means of increasing plasma osmolality.

1.6 The Kidney:

Elasmobranch kidneys consist of a pair of elongate structures found on either side of the dorsal aorta. In sharks they have a thread-like appearance at the anterior end, midway along the dorsal surface of the abdominal cavity. They gradually widen posteriorly and fuse below the cloaca (Shuttleworth 1988). Elasmobranchs posses a renal portal system in which portal veins are formed from the bifurcation of the caudal vein. Upon entering the kidney these divide to form a matrix of smaller vessels. Blood from the portal system mixes freely with that from the glomerular vasa efferentia before exiting the kidney through the renal vein (Hentschel 1988). There is also evidence of a glomerular bypass vessel which permits blood to flow from the afferent to the efferent vessel, thereby avoiding filtration (Brown and Green 1992).

The functional unit of the kidney, the nephron, is a complex tubular system. It has been extensively reviewed by Lacy and Reale (1995) and Hentschel and co-workers (1993). There is strong evidence of a counter current exchange system involving specialised epithelial transport (Hentschel and Zierold 1993). There are two regions of renal tissue in *S. canicula*: firstly, a dorsal 'bundle' region which is contained in a urea impermeable sheath (Figure 1.6.1) and the tubules are closely packed into discrete bundles. It is hypothesised that the counter current exchange system operates in this region (Stolte et al. 1977). The second region of renal tissue is the ventral 'sinus' which lies outside of the sheath but has two further loops with the potential for counter current exchange. In this region the tubules are loosely arranged and segregated by blood sinuses (Lacy and Reale 1995). The division between the two zones is also marked by large renal corpuscles.

Each individual nephron forms two loops in the bundle zone and two long convolutions in the sinus region (Hentschel 1988). There is much diversity and specialisation of epithelial tissue throughout the length of the nephron (Hazon et al. 1997b).

Due to the osmolyte concentrations described above, marine elasmobranchs face a slight continual influx of water across their semi-permeable surfaces. This excess water is excreted by the kidneys by an increase in renal clearance, primarily through increased glomerular filtration rate (GFR) and urine flow rate (Goldstein and Forster 1971; Forster et al. 1972). Upon exposure to reduced salinity and the associated increase in water influx, *H. portusjacksoni* displays a doubling of GFR (Cooper and Morris 2004b).

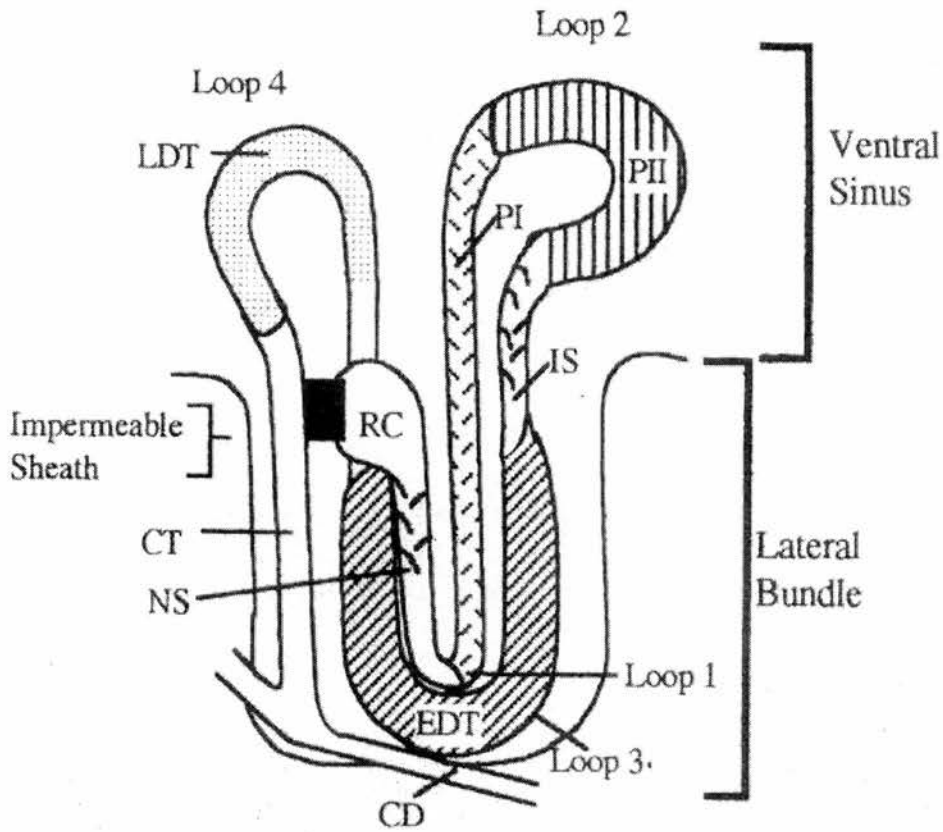

Figure 1.6.1 - Schematic diagram of a single nephron from *S. canicula*. Filtrate from the renal corpuscle/glomerulus (**RC**) flows through the neck segment (**NS**) and into loop 1 in the bundle region. Filtrate then passes through proximal segments I and II (**PI** and **PII**) of loop 2 in the sinus region. Then the filtrate passes through the intermediate segment (**IS**) into the early distal segment (**EDT**) and loop 3 in the bundle region. Filtrate then flows into the late distal segment (**LDT**) and loop 4 in the sinus region before entering the collecting tubule (**CT**). Filtrate then passes into the collecting duct (**CD**) (Hazon et al. 1997b).

Elasmobranch kidneys cannot produce hyperosmotic urine, typically urine is hyposmotic relative to blood plasma (Henderson et al. 1988). This fact, coupled with the use of urea as a plasma osmolyte means that the major roles of the elasmobranch kidney are urea retention and volume regulation. *S. canicula* acclimating to reduced salinity show a marked diuresis along with a reduction of plasma osmolality (Wells et al. 2002). However, FW acclimated euryhaline elasmobranchs appear to be able to selectively reduce the urinary concentration of Na^+ and Cl^- (Shuttleworth 1988; Janech et al. 1998). The kidney in FW is therefore also capable of regulating the concentration of Na^+ and Cl^- in the blood plasma. Lacy and Reale (1991b; 1991a) discovered that tubular cells in the early distal tubule (EDT) have similar characteristics to cells which are known to actively transport Na^+.

The major role of the elasmobranch kidney is urea retention. There is active urea transport and reabsorption in the elasmobranch nephron, micropuncture studies have implicated the second proximal segment (PII) (Figure 1.6.1) as a possible site of Na^+-linked urea reabsorption (Stolte et al. 1977). Levels of skate kidney urea transporter (SkUT) significantly decreased in response to a decrease in salinity in the marine elasmobranch *Raja erinacea* (Morgan et al. 2003). This suggests there is scope for physiological modification within the kidney to changes in environmental salinity.

The kidney is also the site of the elasmobranch RAS, a key osmoregulatory enzyme cascade. A detailed description of the RAS is offered below (Section 1.10).

1.7 The liver

The Elasmobranch liver performs a number of functions for hydrodynamics and metabolism. The liver is the main store for energy reserves in the form of fatty acids although these do perform another function. To elaborate, elasmobranchs lack the swim bladder of teleost species and are heavier than the surrounding environment. Dynamic lift is generated from the pectoral fins whilst the animal is in motion. This imposes hydrodynamic constraints on shark size as a doubling of body length equates to a square of fin surface area but a cube of body mass. This reduction in relative lift is offset by an increase in proportional liver size in larger animals which increases the relative amount of body fatty acids which are less dense than SW. An example of this can be found in the basking shark, *Cetorhinus maximus*: the liver from an 8.8 m, 5.9 tonne specimen accounted for nearly 25% of total body mass yielding 2270 l of oil (Martin 2003a).

With the exception of the FW Potamotrygonid stingrays, elasmobranch fish are ureotelic with urea production largely occurring in the liver via the OUC (Figure 1.7.1). This has been extensively reviewed by Goldstein (1967), Anderson (1995; 2001), and Walsh and Mommsen (2001). A synopsis of the OUC in *S. acanthias* has been produced: a mitochondrial glutamine synthase converts a CO_2^- group of glutamic acid into an amide group of glutamine; a glutamine-dependent carbamoyl phosphate synthase (CPS III) and an ornithine carbamoyl transferase make citrulline, then arginine; and finally a mitochondrial arginase splits arginine into urea and ornithine (Perlman and Goldstein 1988; Acher 1996).

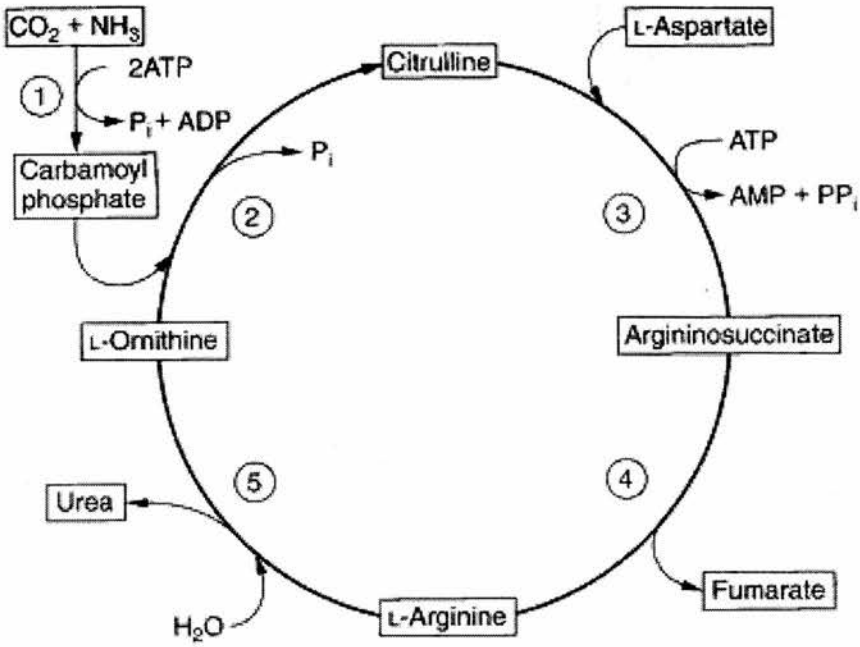

Figure 1.7.1 – The OUC. Numbered circles represent the following enzymes: 1) Carbamoyl phosphate synthase, 2) Ornithine carbamoyl transferase, 3) Argininosuccinate synthase, 4) Argininosuccinate lyase, 5) Arginase (Saunders 2002). Elasmobranchs posses an OUC in the liver which utilises CPS III as enzyme number 1. This is located in the mitochondria and preferentially uses glutamine (to NH_3) as a nitrogen donor (Tam et al. 2003).

Potamotrygonid rays do possess an OUC, but have been demonstrated as being ammoniotelic (Wood et al. 2002a). Marine elasmobranchs acclimating to reduced salinity show a reduction in plasma urea levels which can be due to increased renal clearance of urea, as seen in *Negaprion brevirostris* (Goldstein et al. 1968); or a combination of increased clearance and decreased biosynthesis, as seen in *R. erinacea* (Goldstein and Forster 1971), *S. canicula* (Hazon and Henderson 1984), and the FW stingray *Himantura signifer* (Tam et al. 2003).

Recent studies have demonstrated a functional OUC in the stomach of *H. signifer*, with 70% capacity of that of the liver (based on CPS III activity) (Tam et al. 2003). This is also found in SW *Taeniura lymma*, although the capacity of this was only around 1% of that of the liver. Furthermore, ammonia excretion via this route decreases in response to elevated salinity. It has been suggested that this localised urea production provides a means of preventing loss of ingested nitrogen as ammonia and amino acids (Tam et al. 2003). This could be of vital importance in FW elasmobranchs when acclimating to increases in salinity given that they may have a reduced capacity for renal urea retention (Section 1.6).

The liver also secretes angiotensinogen, the first protein in the RAS protein cascade. The process and osmoregulatory effects of the RAS are detailed below (Section 1.10).

The organs described above are therefore the major sites of osmoregulatory processes in elasmobranch fish, and it has been shown that they have modified functions and priorities in SW and FW. It is through the control of these organs that fully euryhaline species are able to move between these two environments and maintain their

hyperosmotic state. Therefore in order to fully assess the roles these glands have in elasmobranch osmoregulation it is necessary to detail the endocrine systems which influence them.

1.8 The pituitary gland

The elasmobranch pituitary gland consists of the pars distalis (anterior), pars intermedia, and neurohypophysis with a large pituitary cleft, similar to other vertebrates. The elasmobranch gland differs from that of tetrapods through the presence of a partially separated ventral lobe as opposed to the pars tuberalis seen in the pituitary gland of other vertebrates. This unique structure contains both a gonadotropin and a thyrotropin (Fig 1.8.1) (Young 1981).

The hypothalamus is directly linked to the pituitary gland through a portal system which passes through the median eminence (Fig. 1.8.1). Some neurons within the hypothalamus secrete hormones, carried via this route, which strictly control secretion of hormones from the anterior pituitary.

The neurointermedia is permeated by numerous nerve fibres from the neurohypophysial tract. In elasmobranchs, this contains melanophore-stimulating hormone (MSH) whereas the teleost equivalent, melanin-concentrating hormone (MCH) is mostly found in the pars lateralis (Kawauchi 1992). The neurointermedia also contains some arginine vasotocin (AVT) the action of which is described below (Section 1.8.2), as well as various neutral octapeptides whose functions remain unknown (Young 1981).

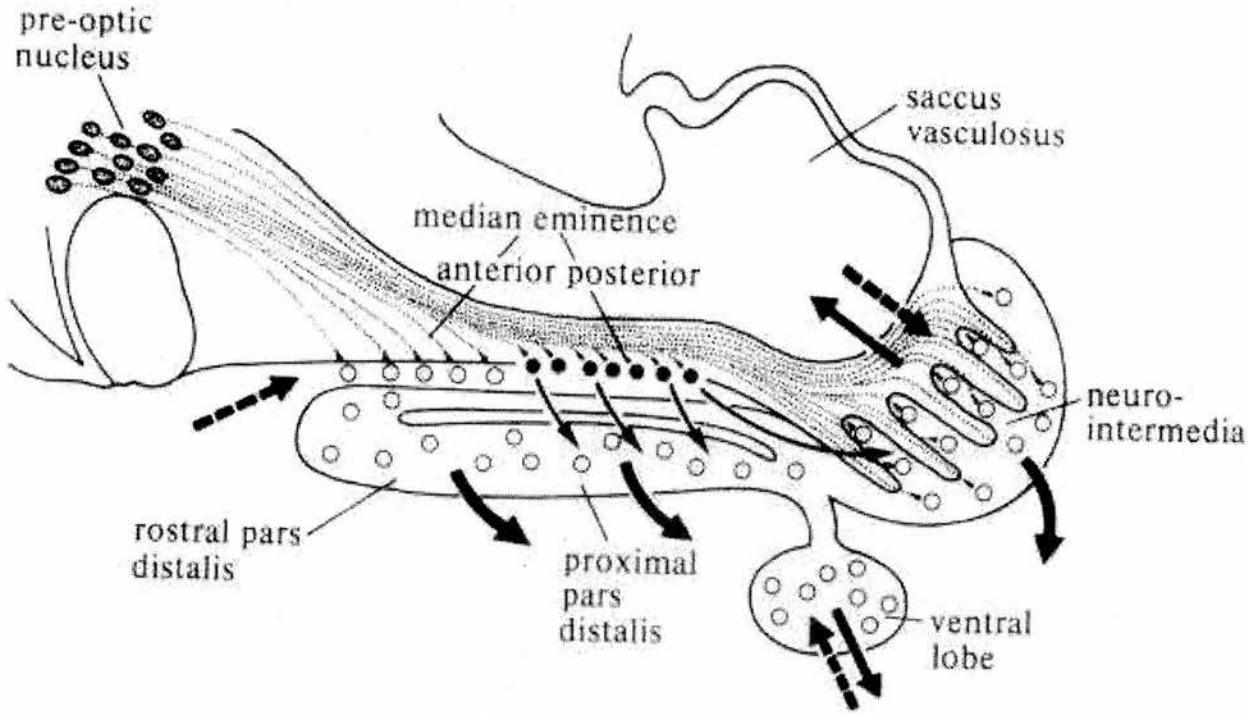

Figure 1.8.1 - Generalised elasmobranch pituitary gland. **Solid arrows** = veins; **Broken arrows** = arteries; **Thin arrows** = portal veins (Young 1981).

Very little experimental work has been carried out on the elasmobranch pituitary itself, but the actions of many hormones which it secretes have well documented affects on osmoregulatory systems. For ease of description these have been separated into those emanating from the anterior pituitary and the posterior pituitary. Elasmobranch specific studies have been utilised where possible.

1.8.1 The anterior pituitary

De Vlaming and co-workers (1975) have investigated the effects of hypophysectomy on *D. sabina*, resulting in a decrease in plasma osmolality, mostly through a decrease in urea concentration. Removal of the rostral lobe of the pars distalis resulted in an increase in plasma osmolality through greater concentrations of Na^+ and urea, although this could be reversed through an injection of mammalian prolactin. Injection of adrenocorticotrophin (ACTH) alone into these animals had no effect, but when coupled with the injection of prolactin, ACTH did negate the effects of prolactin. ACTH is released from the anterior pituitary in response to corticotropin releasing hormone (CRH) from the hypothalamus. ACTH stimulates the release of corticosteroids; for example, 1α-hydroxycorticosterone from the interrenal gland (Klesch and Sage 1975; Hazon and Henderson 1985) (Section 1.9).

Also released from the anterior pituitary is thyroid stimulating hormone (TSH) which acts as a stimulus for the secretion of thyroid hormones. One such hormone is thyroxine which has been shown to reduce renal Na^+, K^+-ATPase activity, and the intracellular concentrations of cAMP and cGMP in *Ginglymostoma cirratum* (Honn and Chavin 1976). Removal of the thyroid gland caused increases in plasma urea concentrations and osmolality in *D. sabina* (De Vlaming et al. 1975). Replacement therapy with thyroxine returned plasma urea concentrations to normal levels in these animals (De Vlaming et al. 1975). This is illustrative of the importance of the pituitary as a means of stimulating other organs which have important osmoregulatory roles.

Other hormones are also released from the anterior pituitary such, as growth hormone (GH) and prolactin. Prolactin cells are located in the pars distalis and transfer from SW

to FW has been shown to activate prolactin release from these cells in teleosts (Olivereau and Ball 1970). The role of these hormones in elasmobranch osmoregulation is largely unknown.

1.8.2 The posterior pituitary

The posterior pituitary is the site of neurohypophysial hormone secretion. These can be divided into the vasotocin-vasopressin lineage and oxytocin-like hormones, both of which have been well reviewed for elasmobranchs (Acher 1996; Acher et al. 1999). From the former AVT is one of the key osmoregulatory hormones secreted by the posterior section of the pituitary gland. All elasmobranchs studied thus far posses AVT, a homologue of mammalian arginine vasopressin (AVP) (Acher 1996; Acher et al. 1999). AVT is the major neurohypophysial peptide in lower vertebrates. In teleosts, dose-dependent decreases in urine flow rates, GFR, and tubular transport maxima for glucose have been seen in trout, *Oncorhynchus mykiss*, in response to AVT (Amer and Brown 1995). AVT was thought to have similar antidiuretic effects in elasmobranchs and this has recently been demonstrated in *S. canicula* (Wells et al. 2002). In *Triakis scyllium* vasotocin levels in the hypothalamus and blood plasma significantly increased in response to elevated salinity (Hyodo et al. 2004). Clearly neurohypophysial hormones such as AVT are important endocrine signals for osmoregulation, particularly during salinity transfer.

Hormones from the oxytocin lineage display much structural variation within the Chondrichthyes (Table 1.8.2.1). Examination of the effects of these hormones could therefore become species specific. From the oxytocin lineage both asvatocin and phasvatocin have been identified in the posterior pituitary of *S. canicula* (Chauvet et al. 1994). Despite high concentrations of oxytocin-like hormones in the neurohypophysis no clear function of these peptides has been discovered (Acher et al. 1999), perhaps because of the high degree of structural variation.

	Structure								
Classification/hormone	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Holocephali									
Oxytocin	Cys	Tyr	Ile	Gln	Asn	Cys	Pro	Leu	Gly (NH_2)
Elasmobranchii									
Sharks									
Aspargtocin	Cys	Tyr	Ile	Asn	Asn	Cys	Pro	Leu	Gly (NH_2)
Valitocin	Cys	Tyr	Ile	Gln	Asn	Cys	Pro	Val	Gly (NH_2)
Asvatocin	Cys	Tyr	Ile	Asn	Asn	Cys	Pro	Val	Gly (NH_2)
Phasvatocin	Cys	Tyr	Phe	Asn	Asn	Cys	Pro	Val	Gly (NH_2)
Rays									
Glumitocin	Cys	Tyr	Ile	Ser	Asn	Cys	Pro	Gln	Gly (NH_2)

Table 1.8.2.1 – Structure of the oxytocin-like hormones of cartilaginous fish (Acher et al. 1999).

1.9 The interrenal gland

Separate from the kidney is the interrenal gland (Fig 1.9.1), the site of 1α-hydroxycorticosterone secretion. 1α-hydroxycorticosterone is synthesised in the tissue from corticosterone (Kime 1987). This steroid was first isolated in the blood plasma of *Raja radiata* (Idler and Truscott 1966). Plasma concentration of 1α-hydroxycorticosterone increases at low salinities, corresponding to the point at which Na^+ becomes regulated at a lower level and urea concentrations continue to decrease (Armour et al. 1993a). It is likely therefore that 1α-hydroxycorticosterone acts to minimise Na^+ (and Cl^-) excretion from the rectal gland, kidney, and gills (Armour et al. 1993a).

Homologous renal extract and heterologous angiotensin II (Ang II) cause *in vivo* increases in the plasma concentration of 1α-hydroxycorticosterone (Hazon and Henderson 1985), as well as increasing secretion from isolated perfused interrenal glands (O'Toole et al. 1990; Armour et al. 1993b). This suggests the RAS (Section 1.10) may have a regulatory effect on the interrenal gland. However, the pituitary gland has been proposed as a major site of regulation for 1α-hydroxycorticosterone secretion from the interrenal gland (Section 1.8.1) (Hazon and Henderson 1985). Secretion of 1α-hydroxycorticosterone is stimulated by ACTH, through synergistic action of intracellular Ca^{2+} and cAMP.

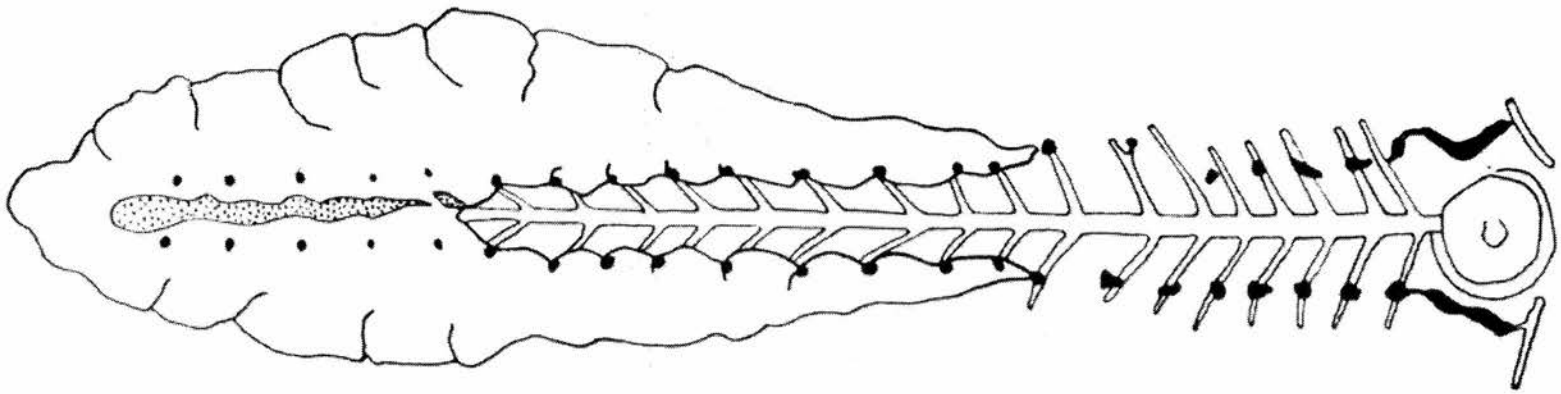

Figure 1.9.1 - Ventral view of the aorta and paired circulatory branches, kidneys, and interrenal gland of *Scyllium catulus*. **White** = kidney; **Stippled** = interrenal tissue; **Black** = chromaffin bodies (Chester-Jones 1957).

Ang II also stimulates secretion via the action of both intracellular and extracellular Ca^{2+} (Armour et al. 1993b). Alterations in Na^{+} concentration to perifused sections of glands have inconsistent effects on secretion rates. An increase in urea concentration in the perifusate increased 1α-hydroxycorticosterone secretion, however a decrease of urea had no affect (O'Toole et al. 1990). Therefore the factors affecting the interrenal gland have documented effects on osmoregulation but, although clearly influential, the exact osmoregulatory role of 1α-hydroxycorticosterone remains to be established.

The chromaffin tissue of elasmobranchs is also discrete from the renal and interrenal tissue, again contrasting with teleosts. Chromaffin tissue is a site of catecholamine secretion. The physiological effects of catecholamines are detailed below (Section 1.12).

1.10 The RAS

The RAS is a peptide cascade beginning with angiotensinogen which is released from the liver. This is acted upon by renin which occurs in the kidneys to initiate the cascade (Fig 1.10.1) (Hazon et al. 1999). The juxtaglomerular apparatus, the site of renin in other vertebrate species, is located at the vascular pole of the renal corpuscle in granulated peripolar cells and has been found in a number of elasmobranchs (Lacy et al. 1987; Lacy and Reale 1989; Lacy and Reale 1990). The macula densa, an important part of the juxtaglomerular apparatus has also been identified (Lacy and Reale 1990). The presence of the juxtaglomerular apparatus suggest that the RAS may be involved in the control of GFR, as is the case in teleosts (Brown et al. 1980). In addition to a systemic RAS, this is strong evidence for the presence of an intrarenal RAS in the elasmobranch kidney.

There is also a possible presence of Ang II receptors in the elasmobranch kidney (Tierney et al. 1997). Only two studies have examined the physiological actions of Ang II in the elasmobranch kidney. Wells and co-workers (2003) demonstrated that inhibition of angiotensin-converting enzyme resulted in a glomerular diuresis, an increase in urea and Cl^- clearance, and an increase in transport maxima for glucose. Later work showed that Ang II caused a glomerular antidiuresis and decreases in perfusion flow rate, transport maxima for glucose, and the proportion of filtering glomeruli in *S. canicula*. In addition to this renal urea, Na^+, and Cl^- clearance were all significantly reduced by Ang II (Wells et al. *In Press*).

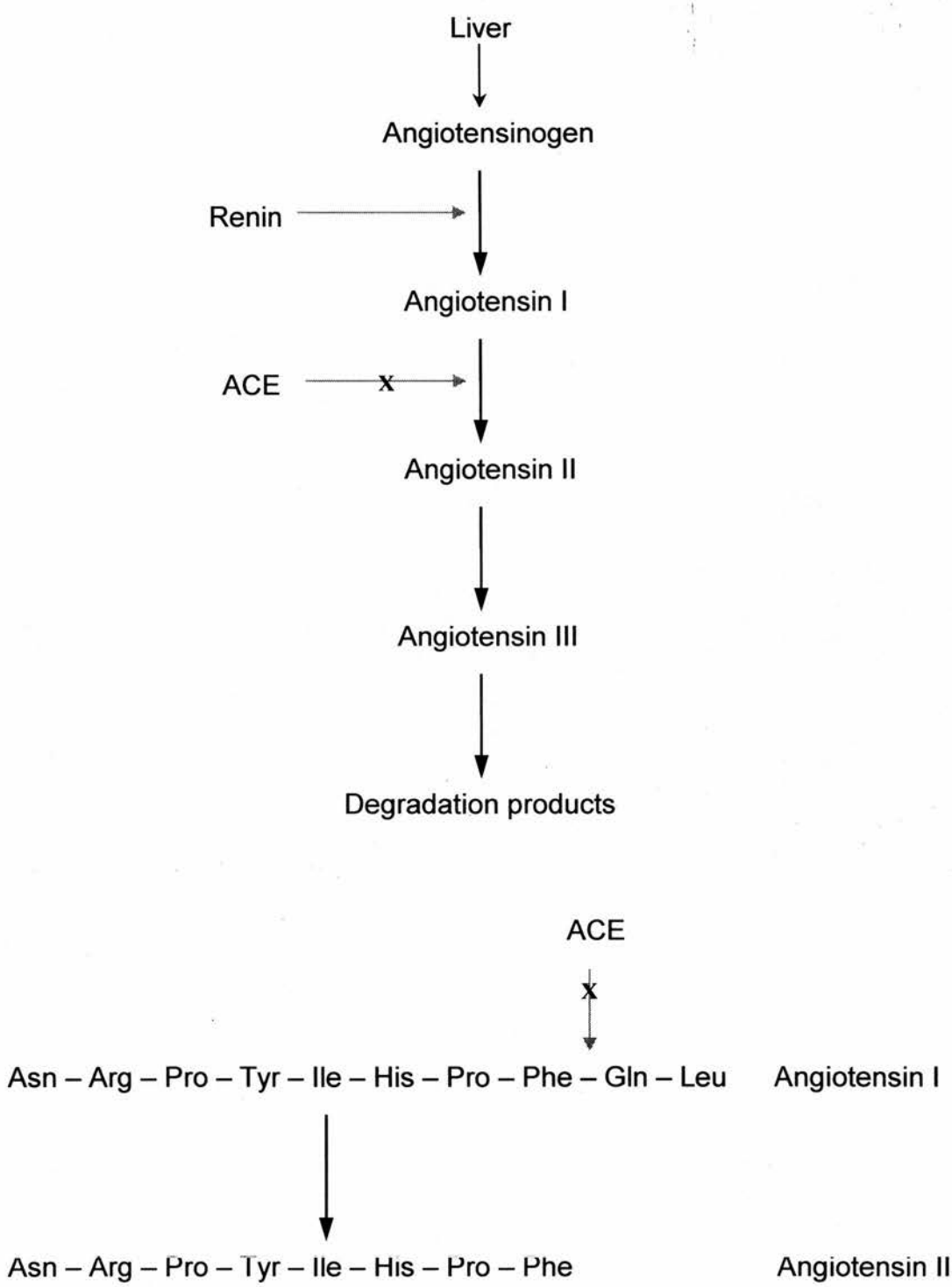

Figure 1.10.1 - The vertebrate RAS showing the action of renin and angiotensin converting enzyme (ACE), and inhibition by captopril (**X**) (Hazon et al. 1999). Also shown in detail are the structures for elasmobranch angiotensin I and II, mammalian Ang II has Val at position 3 (Takei et al. 1993).

The structure of elasmobranch Ang I was first deduced in *T. scyllia* (Takei et al. 1993). Elasmobranch Ang II has an asparagine residue at position 1, like teleosts, an isoleucine residue at position 5, like mammals, and a unique proline residue at position 3 (Kobayashi and Takei 1996).

Circulating levels of Ang II in *S. canicula* have been calculated at 100 - 150 pg/ml (Tierney et al. 1998). Receptors for Ang II have been suggested in the heart (high and low affinity receptors) (Cerra et al. 2001), interrenal gland, gills and intestine (Tierney et al. 1997), as well as in the rectal gland (Masini et al. 1993; Tierney et al. 1997), with most binding occurring in the subcapsular region (Hazon et al. 1997a).

Ang II has been shown to have a variety of effects in elasmobranchs. Homologous Ang II causes a dose-dependent increase in drinking rate in both *S. canicula* and *T. scyllia* (Anderson et al. 2001). Furthermore, inhibition of ACE significantly reduces the dipsogenic effect of the smooth muscle relaxant papaverine in the same two species (Anderson et al. 2001).

Heterologous Ang II shows vasopressor activity in *S. acanthias* (Opdyke and Holcombe 1976), *S. canicula* (Hazon et al. 1989), and *T. scyllia* (Hazon et al. 1995). Homologous Ang II shows a response almost 23 times greater than heterologous peptides in *T. scyllia* (Takei et al. 1993). This pressor response seems to be mediated by catecholamines (Section 1.12) (Opdyke and Holcombe 1976; Opdyke et al. 1981). Possible receptors for Ang II have been identified in the tissues of the rectal gland (Tierney et al. 1997), although Ang II had no effect on the vascular perfusion of the secretory parenchyma nor on Cl^- secretion rates (Anderson et al. 2002a).

The heart of elasmobranchs (Section 1.11) may also be affected by angiotensin. Angiotensin II binding sites have been found in tissues of the hearts of *S. canicula* (Cerra et al. 2001). The action of Ang II is potentially complex with distinct receptor subtypes, high and low affinity, each having different distributions (Cerra et al. 2001).

These findings suggest that the RAS is an important endocrine control system in elasmobranch osmoregulation which affects many of the organs outlined above, although its role in the control of the rectal gland remains unclear.

1.11 The Heart

The elasmobranch cardiovascular system consists of a single circulation of a closed circuit. The heart consists of four contractile chambers which are arranged in the order of sinus venosus, atrium, ventricle, and conus arteriosus running posterior to anterior (Fig 1.11.1). The walls of all four chambers contain myocardial tissue, lacking the smooth muscle found in the heart of teleosts (Tota 1999; Ramos 2004). Venous blood flows to the atrium via the sinus venosus. Connecting these two chambers is the sinoatrial orifice where a sinoatrial valve prevents the back flow of blood during atrial systole. The atrium lies ventral to the sinus venosus and dorsal to the ventricle. The atrioventricular orifice lies on the ventral side of the boundary between the two chambers. The opening is circular and surrounded by two ellipsoid flaps, the atrioventricular valves. The ventricle contains the largest amount of myocardial tissue of all the chambers. The ventricle is composed of two layers of tissue: the compacta and the spongiosa (Fig 1.11.1). The compacta is a dense layer of tissue and lies exterior to the spongiosa which is more diffuse in structure. In *S. canicula* which have been acclimated to 120% SW there is a decrease in the tissue of the spongiosa and an increase in the tissue of the compacta as compared to fish acclimated to 100% and 70% SW. This is coupled with an increase in the amount of collagen in the tissue (Anderson and Good, unpublished). This change in tissue ratios could be a modification to the more viscous blood of the volume depleted animals acclimated to increased salinity. The conus arteriosus is the most anterior chamber and is confluent with the ventral aorta (Tota 1999).

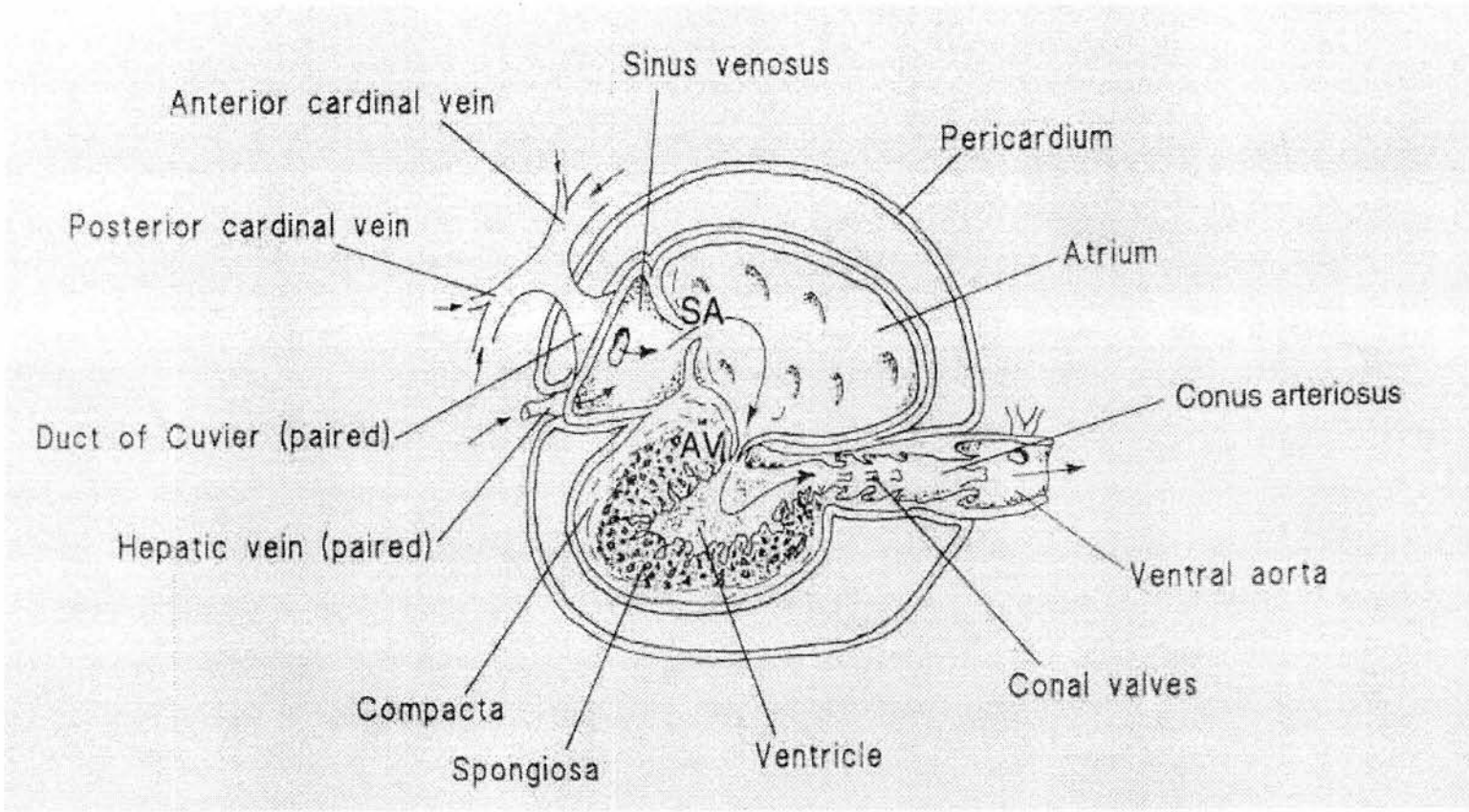

Figure 1.11.1 - Schematic illustration showing the four chambers of the elasmobranch heart. The S-shaped arrangement of the chambers means atrial contraction may assist ventricle filling. **SA** = sinoatrial valve; **AV** = atrioventricular valve (Tota 1999).

Changes in environmental salinity can also affect the heart in other ways. Heart mitochondria from a variety of species of pelagic sharks have been shown to exhibit decreased respiratory control ratios (a marker for functional integrity of the isolated mitochondria) at osmolalities above and below 1000 mOsm (Lewiston et al. 1979). Increases in extracellular concentrations of organic osmolytes can also have a profound effect on the tissues of the heart. With increasing concentrations of mannitol, sucrose, and betaine *S. acanthias* showed an increase in respiratory control ratio and the rate of oxygen uptake. However at high concentrations a decrease below basal levels was demonstrated. This pattern was not seen with increases in extracellular urea concentration, but was also observed with increases in concentration of TMAO. Unlike the other osmolytes tested, urea and TMAO readily permeate the mitochondria of the heart and therefore the cellular osmotic gradient is not present (Lea and Hillman 1990). TMAO may therefore affect respiratory control ratios by other means. These findings illustrate the potential consequences when animals fail to completely osmoregulate or acclimate slowly to external salinity. It is also important to note for the planning of experimental procedures.

The heart has been shown to affect the kidneys of elasmobranchs. The action of the heart over the kidneys appears to be in two methods: neural reflex regulation and endocrine regulation. These two pathways combine to show an increase in renal clearance with increased blood volume (Peterson and Benjamin 1992).

1.11.1 Natriuretic peptides

The natriuretic peptide (NP) system in vertebrates is antagonistic to the RAS and consists of three types of hormones (Figure 1.11.2). Atrial natriuretic peptides (ANP) are secreted by mammalian atria in response to an increase in central venous volume or pressure (Olson 1999). Brain or B-type natriuretic peptide is also found in most vertebrates, although is replaced by ventricular NP in teleosts (Takei 1999). However, in elasmobranch fish C-type natriuretic peptide (CNP) is the only circulating natriuretic peptide (Schofield et al. 1991; Suzuki et al. 1991b; Suzuki et al. 1992; Suzuki et al. 1994). Circulating levels of CNP in *T. scyllia* far exceed those recorded for any other species (1.97 pmol ml^{-1}) (Suzuki et al. 1994). CNP is a brain peptide in most vertebrate species and is therefore present at very low levels in the blood plasma of other species. This circulating CNP is believed to originate from the heart. A high molecular weight form of CNP has been isolated from both the atrium and ventricle of the heart of *S. canicula* (Suzuki et al. 1991b). The highest concentration of CNP was found in the atrium, then the ventricle, brain, and pituitary gland respectively (Suzuki et al. 1994). In the heart the majority of CNP is the pro-hormone CNP-115, whereas in the brain the majority is the mature peptide CNP-22 (Figure 1.11.2). Contrasting to teleost and mammals, most CNP in elasmobranch blood is the pro-hormone, not a processed form (Suzuki et al. 1994). This suggests that the heart is the main storage organ for pro-CNP which is the major circulating hormone. It is only at specific sites within tissues that the pro-hormone is processed into other forms.

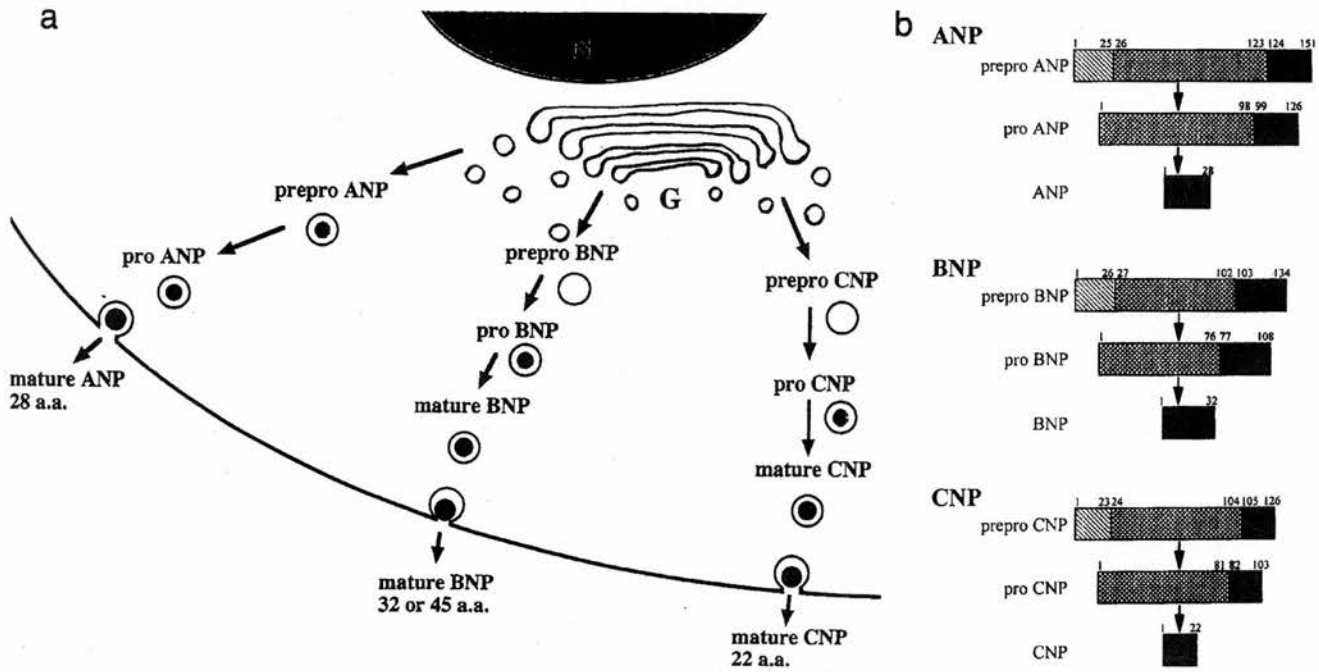

Figure 1.11.2 – (a) A schematic drawing of the generalised processing of natriuretic peptides in vertebrates. The numbers of amino acids in the mature peptides are noted. (b) Sequential processing of natriuretic peptides in vertebrates. The different patterns in the boxes of preprohormones denote the signal peptide (striped), N-terminal prohormone (hatched), and mature peptide (solid). The number of amino acid residues are noted (Takei 1999).

As the only natriuretic peptide in elasmobranchs CNP has wide scope for effecting osmoregulatory processes. Recently CNP has been shown to have effects on the elasmobranch kidney. Wells and co-workers (*In Press*) illustrated that CNP caused a glomerular diuresis, an increase in transport maxima for glucose, but no change in the proportion of filtering glomeruli. CNP did cause significant increases in renal clearance of urea, Na^+, and Cl^-. These effects are antagonistic to those reported for Ang II (Section 1.10).

It has been proposed that CNP may also influence the vasculature of the rectal gland (Hazon et al. 1997b). Recently it was illustrated experimentally that CNP significantly increased blood flow to the secretory parenchyma (Anderson et al. 2002a). CNP has also been shown to dilate the smooth muscle of the capsular region in the rectal gland of *S. acanthias* (Evans and Piermarini 2001). This could affect the degree of perfusion seen in the inner regions of the gland by reducing pressure in the capillaries, and therefore potentially affect secretion rate.

CNP also has direct effects on the secretory tubules of the rectal gland (Solomon et al. 1992b) (Figure 1.5.1). Endogenous CNP has been shown to stimulate rectal gland secretion 7 to 8 times above basal levels in isolated perfused glands of *S. acanthias* (Solomon et al. 1992a) via guanylyl cyclase-linked receptors (Gunning et al. 1997). Similar stimulation with CNP was also seen in *S. canicula* (Anderson et al. 1995b). CNP released from the heart can therefore have a direct influence on the secretory activity of the rectal gland.

A slightly different model of stimulation has been suggested for the rectal gland of *S. acanthias*. In response to a volume stimulus the tissues of the heart release CNP. This circulates to the rectal gland where it stimulates the nerves to release VIP (Section 1.5). CNP and VIP then both induce effects on the secretory tissues of the rectal gland and activate NaCl secretion (Silva et al. 1996) (Figure 1.5.1). Experimental evidence for the presence of a receptor mechanism in the atrial and cardiac region which triggers the sequence to activate glandular secretion has been gathered (Erlij and Rubio 1986).

It is important to note here that VIP does not stimulate the rectal glands of all elasmobranch species; this is a species specific model of activation although the outlying principles on the direct effects of CNP may be extrapolated to elasmobranchs in general. Unlike *S. acanthias*, the rectal glands of *S. canicula* and *R. clavata* have been shown to be unaffected by VIP (Anderson et al. 1995a).

The rectal glands of both *S. canicula* and *R. clavata* are stimulated by an intestinal factor which was first termed rectin, but has been identified as scyliorhinin II (Anderson et al. 1995a) (Figure 1.5.1). Scyliorhinin II has been isolated from the intestine of *S. canicula* and *Torpedo marmorata* (Conlon and Thim 1988; Anderson et al. 1995a).

The different intestinal peptides which stimulate the rectal glands of *S. canicula* and *S. acanthias* are perhaps a reflection of the difference in feeding behaviour and therefore the resulting salt loads: *S. canicula* is typically a gorge feeder, whereas *S. acanthias* does not gorge feed.

Regardless of which intestinal factor they react to, elasmobranch rectal glands are stimulated to secrete NaCl by CNP, both directly and indirectly. The heart must therefore have a role to play in the overall osmoregulatory strategy of elasmobranchs, principally as a major source of one of the main osmoregulatory hormones, CNP, but also exerting neural control over other organs. CNP is possibly the most important endocrine factor with regards to the control of rectal gland secretion (Section 6.1).

1.12 Catecholamines

Catecholamines are secreted by the chromaffin tissue in the elasmobranch interrenal gland. They have been shown to increase blood flow to the gills of *S. canicula* (Davies and Rankin 1973), and decrease blood flow to the gut of *S. acanthias* (Holmgren et al. 1992). The vasculature of the rectal gland has also been shown to be constricted by catecholamines (Shuttleworth 1983).

The principle catecholamines in elasmobranchs are adrenaline and noradrenaline. Both are known to have major effects on blood pressure: increasing dorsal aortic blood pressure and decreasing coeliac arterial blood flow in *S. acanthias* (Holmgren et al. 1992). Branchial vasculature can be manipulated by the action of adrenaline and noradrenaline. Both of these hormones appear to act via β–adrenoceptor-mediated vasodilation (Davies and Rankin 1973; Capra and Satchell 1977). This vasodilatory response masks a smaller α–adrenoceptor-mediated vasoconstriction (Davies and Rankin 1973; Capra and Satchell 1977). Adrenaline has been shown to have effects on the kidney, reducing the proportion of filtering glomeruli but causing an overall diuresis in *S. canicula* (Brown and Green 1987).

Two of the major osmoregulatory peptides have been shown to affect catecholamine release. Circulating levels of noradrenaline increase 15-fold in *S. acanthias* in response to CNP (McKendry et al. 1999). Ang II has been shown to increase plasma concentrations of adrenaline and noradrenaline in *S. acanthias* (Bernier et al. 1999). Given that two of the major osmoregulatory hormones affect catecholamine release from chromaffin tissue, and that catecholamines affect blood flow and function of different organs, it is likely that they play a role in mediating osmoregulatory responses.

1.13 Objectives

Elasmobranchs require the combined actions of the gills, gut, rectal gland and kidney in order to maintain osmotic stasis and alter osmolality during changes in salinity. There appears to be scope for interspecific differences in the processes behind this. The aim of this study was to examine the differences between a partially and a fully euryhaline species in terms of their osmotic profiles at different salinities, and the processes by which these are achieved and maintained. Specific interest was given to the rectal gland due to its role in Na^+ and Cl^- balance, and the difference in activity between SW and FW. The specific aims are:

1) To produce replicable chronic salinity transfer protocols for *S. canicula* to 80, 100, and 120% SW; and for *C. leucas* to FW and SW in captivity. Also to produce experimental protocols for the acute transfer of both species to 100% SW from all chronic acclimation conditions.

2) To produce plasma osmotic profiles for *S. canicula* chronically acclimated to 80, 100, and 120% SW conditions; and for *C. leucas* in FW and SW. Also, to gain equivalent data for both species during acute salinity transfer to 100% SW.

3) To assess blood volume in *S. canicula* during chronic and acute salinity transfers.

4) To measure *in vivo* rectal gland secretion rates of *S. canicula* during chronic and acute salinity transfer.

5) To examine the histology of the rectal gland from both species chronically acclimated to the different salinities.

6) To measure maximal Na^+, K^+-ATPase activity in the gills, gut, rectal gland, and kidney of both species chronically acclimated to the different salinities.

7) To measure oxygen consumption of the rectal gland in both species chronically acclimated to the different salinities, measure what proportion of that was attributable to Na^+, K^+-ATPase, and to asses the effects of CNP on both parameters.

Chapter 2: Haematic parameters

2.1 Introduction

As outlined above, elasmobranch fish are able to selectively alter the concentrations of key osmolytes in relation to the surrounding environment (Section 1.2). Therefore, in order to accurately assess the roles of specific organs in different environments it is necessary to produce plasma osmotic profiles of elasmobranchs from different salinities and, importantly, during acute transfer. For only by quantifying the relative concentrations of the plasma osmolytes and highlighting the differences between animals from different salinities can the relative role of the osmoregulatory organs be deduced. Hazon and Henderson (1984) published an osmotic profile for *S. canicula* following 14 day acclimations to final conditions for a series of environmental salinities (Table 2.1.1). This clearly illustrates the modifications in concentrations of the major plasma osmolytes in response to salinity change.

As previously stated, the reduction in overall plasma osmolality associated with acclimation to reduced salinities is principally achieved through reductions in the concentrations of urea, Na^+, and Cl^-. Concentrations of these osmolytes are increased during acclimation to increased salinity. Interestingly, the data presented for *S. canicula* shows that the proportional content of Na^+ and Cl^- in the plasma is relatively constant between SW and hypersaline conditions, and also at hyposaline conditions down to 50% SW, when their relative abundance is increased. This suggests there is a critical point at which further reductions in salinity cause a shift in the relative abundance of these ions in the plasma. The relative abundance of urea decreases with salinity (Table 2.1.2). Similar trends were described in the more euryhaline species *H. portusjacksoni* during acclimation to 50% SW (Cooper and Morris, 1998).

Percentage SW	Osmolality (mOsm Kg^{-1})	Na^+ (mmol l^{-1})	Cl^- (mmol l^{-1})	Urea (mmol l^{-1})
140%	1341	378	383	468
120%	1168	353	363	376
100%	970	279	298	311
90%	846	223	239	280
80%	754	211	213	209
70%	684	199	202	160
60%	600	197	199	120
50%	503	184	186	82

Table 2.1.1 – Plasma osmotic profile of *S. canicula* following 14 day acclimation to final salinities. Acclimation was via 10% increments in salinity every 10 days (Hazon and Henderson, 1984).

Species and salinity	Environmental osmolality (mOsm Kg^{-1})	Plasma osmolality (mOsm Kg^{-1})	Percentage contribution to overall osmolality (%)		
			Na^+	Cl^-	Urea
S. canicula					
140% SW	**1380**	1341	28.2%	28.6%	34.9%
120% SW	**1184**	1168	30.2%	31.1%	32.2%
100% SW	**975**	939	28.7%	30.7%	32.1%
80% SW	**795**	754	28.0%	28.2%	27.7%
50% SW	**500**	503	36.6%	37.0%	16.3%
C. leucas					
SW	**1024**	1068	27.0%	27.7%	34.6%
FW	**≈ 60**	641	32.4%	31.7%	30.0%
H. signifer					
≈ 60% SW	**≈ 600**	571	40.5%	38.5%	0.1%
FW	**≈ 20**	416	40.1%	39.4%	0.1%
P. motoro					
≈ 40% SW	**≈ 380**	378	43.9%	47.6%	0.003%
FW	**≈ 20**	349	45.0%	46.7%	0.002%

Table 2.1.2 – Proportional content of major plasma osmolytes in *S. canicula* and *C. leucas* from different salinities (Hazon and Henderson, 1984; Pillans and Franklin, 2004). Also shown are equivalent values in a ureotelic (*H. signifer*) and an ammoniotelic (*Potamotrygon motoro*) species of FW stingray (Tam *et al.*, 2003).

This trend has also been reported for the fully euryhaline species *C. leucas* caught in the wild (Pillans and Franklin, 2004). FW animals also show increases in the proportion of Na^+ and Cl^-, and a reduction in the proportion of urea in blood plasma relative to SW animals (Table 2.1.2). These differences, particularly in the relative abundance of urea, are smaller than those described for the marine species *S. canicula*. Regulation of the levels of these osmolytes is therefore of great importance in elasmobranch euryhalinity. The inability of FW elasmobranchs to retain elevated concentrations of urea in the plasma is again highlighted here (Table 2.1.2), and may be the key factor which limits their capacity for osmotic acclimation to increased salinity.

In this study osmotic profiles were produced for *S. canicula* chronically acclimated to both hypo- (80% SW) and hypersaline (120% SW) conditions, as well as those of 100% SW animals. Furthermore, profiles were produced for *S. canicula* from all three environmental conditions during acute transfer to 100% SW. Profiles were also produced for captive *C. leucas* acclimated to FW and SW.

Upon exposure to reduced salinities elasmobranchs necessarily encounter an increase in the gradient for the osmotic influx of water across semi-permeable surfaces. Despite the regulatory mechanisms described above (Sections 1.2 and 6) this can result in a dilution of intra- and extracellular fluids, especially in more stenohaline species. This is well illustrated by a doubling of plasma volume and an 11% increase in body mass within 24 hours after transfer from 100 to 75% SW in the reasonably euryhaline species *H. portusjacksoni* (Cooper and Morris, 2004a). Furthermore, significant decreases in the haematocrit and whole blood haemoglobin concentration of *H. portusjacksoni*

acclimated to 100% SW were seen within 24 hours of transfer to both 50 and 75% SW, although a return to control values was seen within 72 hours (Cooper and Morris, 1998).

Decreases in haematocrit following chronic (3/4 day) acclimation to 50% SW have also been described in *R. erinacea* (Goldstein and Forster, 1971), although no decrease was seen throughout 7 day transfer in the estuarine species *Trygonoptera testacea* (Cooper and Morris, 1998). Marine *S. canicula* showed no changes in blood haematocrit after long term (14 day) acclimation to reduced salinity (Hazon and Henderson, 1984). Neither were any significant differences noted in the haematocrits of *C. leucas* populations from SW and FW environments (Thorson *et al.*, 1973). This suggests that any persistent effect salinity transfer has on haematocrit may be highly species specific. Furthermore any variation in blood haematocrit is likely to occur within the early stages of acute transfer, with levels possibly returning to basal as animals fully acclimate to the new environmental conditions. In order to assess these changes accurately it is necessary to have an understanding of the blood volume of elasmobranchs, how it is influenced by salinity, and over what timescale changes occur. This topic is discussed in detail below (Section 3.1). Quantification of blood volume at different salinities would allow an assessment of the degree of concentration and dilution of extracellular body fluids elasmobranchs incur during salinity transfer.

Salinity transfer affects intracellular as well as extracellular fluid levels, as previously discussed (Section 1.2). Changes in plasma and erythrocyte fluid ionic composition during acute transfer have been found to be quite different (Table 2.1.3) (Forster and Goldstein, 1976; Boyd *et al.*, 1977). This is to be expected as the plasma membranes of different cells in different species will permit varying levels of diffusion for individual

osmolytes. The maintenance of erythrocyte osmolyte concentrations is crucial in determining haemoglobin function and thus respiratory gas transport (Nikinmaa, 1990; Scholnick and Magnum, 1991). For example, concentrations of urea and TMAO are typically greater in erythrocytes than in blood plasma, and elasmobranch haemoglobin is dependent on these high urea concentrations for optimal efficiency (Yancey and Somero, 1979; Yancey and Somero, 1980; Nikinmaa, 1990). However in *H. portusjacksoni* transferred to reduced salinities, despite the plasma dilutions described above, there was no change in O_2 consumption rate, blood O_2 partial pressure, cardiac output, or the arterial-venous O_2 content difference (Cooper and Morris, 2004b). Cooper and Morris (2004b) concluded that O_2 delivery to the tissues was facilitated by decreased blood O_2 affinity that could not be simply ascribed to changes in the osmolyte concentration, as whole blood haemoglobin concentration *in vitro* was unaffected by changes in intra-erythrocyte fluid urea or TMAO level. This shows that changes in elasmobranch haematic parameters during salinity transfer are not only due to changes in extra- and intracellular osmolyte concentration. It also highlights the species specific nature of response to salinity transfer and must be borne in mind when extrapolating findings from individual species of elasmobranch.

Sample	Percentage water (%)	K^+ (mmol l^{-1})	Na^+ (mmol l^{-1})	Amino acids (mmol l^{-1})	Urea (mmol l^{-1})
Plasma					
100% SW	92.7	4.96	299	11	361
50% SW	94.9	4.25	217	12	264
Erythrocytes					
100% SW	68.1	120.8	51	280	413
50% SW	74.3	135.7	33	150	283

Table 2.1.3 – Osmolyte concentrations in the blood plasma and erythrocyte fluid of the marine species *R. erinacea* acclimated to 100 and 50% SW (Forster and Goldstein, 1976).

It is clear therefore that the effects of the haemodilution and changes in concentration gradients associated with salinity transfer in elasmobranchs are wide reaching. The magnitude of change in individual parameters may be highly dependent on the species and its degree of euryhalinity. For this reason osmotic profiles of both a partially (*S. canicula*) and a fully euryhaline (*C. leucas*) species at different salinities were produced. Furthermore, as the early stages of salinity transfer have been shown to be the periods where the majority of modifications occur, profiles were made during these stages, where species characteristics permitted.

In order to achieve this, protocols were developed for maintaining both species in captivity. *S. canicula* is relatively easy to keep in captivity with a large proportion of laboratory studies on elasmobranchs being conducted on this species (Wright, 1973; Bentley *et al.*, 1976; Gutierrez *et al.*, 1988; O'Toole *et al.*, 1990; Tort *et al.*, 1991; Brown and Green, 1992; Armour *et al.*, 1993b; Hentschel and Zierold, 1993; Anderson *et al.*, 1995a; Bernier *et al.*, 1999; Ramos, 2004). However, despite the relative ease of capture and maintenance in captivity of this species few studies have been conducted on the effects of captivity on *S. canicula*. Gutierrez and co-workers (1988) examined plasma levels of insulin and some important metabolites during a 1 year period of captivity, relating fluctuations to different periods of the breeding season. This study also demonstrated the longevity of *S. canicula* in captivity.

One of the major influences of captivity on elasmobranchs is the possible effect on feeding rates. This is of great importance for studies of osmoregulation due to the influence of dietary intake on plasma osmolality (Section 1.4). Furthermore, the nature of the diet can influence the morphology of (MacKenzie, 1996), and Na^+, K^+-ATPase

activity and expression in the rectal gland (MacKenzie *et al.*, 2002). The nature of these changes is discussed below (Sections 5.1 and 4). *S. canicula* is a gorge feeding species and as such will endure periods of starvation as well as periods of intense feeding. The active feeding period of the annual cycle appears to peak in September and October for European populations, and increases in plasma insulin levels coincide with this (Gutierrez *et al.*, 1988). Outside of this stage of the annual cycle periods of starvation and relative inactivity are not uncommon (Lyle, 1983). This ability to cope with periods of starvation and gorge feeding makes the species ideal for captivity due to the low maintenance required.

In stark contrast to *S. canicula*, very little is known on the biology of *C. leucas* and the effects of captivity. The methods used for maintaining elasmobranchs in captivity have evolved greatly over the last 40 years, particularly for larger species such as *C. leucas*. This is reflected by the dramatic increase in the diversity of species held by major aquaria. In 1963, *Eugomphodus Taurus* and *G. cirratum* were listed as the only large species of elasmobranch maintained in captivity for over five years (Clark, 1963). In 1980, Sea World in Florida was successfully maintaining six large species of sharks (Schmid *et al.*, 1999). Today, most public aquaria house a sizeable elasmobranch population, and in many this represents the major attraction. In 2005, 2176 individuals from 44 different species of elasmobranch were held in public aquaria in the USA (AES, 2005). This increase in success is largely due to improvements in clinical treatments, advances in tank and filtration designs, and a greater depth of experience and knowledge of elasmobranch biology. However, despite these advances some species (notably large carcharhinids) remain difficult to maintain in captivity for extended periods.

Although protocols for keeping *S. canicula* in captivity and for salinity transfer are well established (Hazon and Henderson, 1984; Tierney *et al.*, 1998; Anderson *et al.*, 2002a; Wells *et al.*, 2002), similar protocols for *C. leucas* have not been produced. One of the main objectives of this study was therefore to produce protocols for the maintenance of *C. leucas* in captivity and for chronic salinity transfer from FW to SW. Once these had been established plasma osmotic profiles could be produced for both species following chronic acclimation to salinity transfer. In *S. canicula* similar profiles could also be produced during acute transfer to salinity change. Blood haematocrit was also measured for both species during these transfers in order to assess the volaemic effects of salinity transfer.

2.2 Materials and methods

All experiments on both *S. canicula* and *C. leucas* were conducted in accordance with UK Home Office regulations on the use of animals in scientific procedures (1986). Data for all parameters were collected from each individual within the experimental groups.

2.2.1 Protocol for *S. canicula*

S. canicula were captured by trawler from the English Channel, Irish Sea, and North Sea areas off of the British coastline. Animals were held in recirculating aquaria of varying size prior to transportation. All animals were transported in custom built water tight transportation tanks bubbled with oxygen (O_2) or 95% O_2 and 5% Carbon dioxide (CO_2) (BOC Gases, Windlesham, Surrey) to the Gatty Marine Laboratory, St Andrews, Fife.

The holding tank in St Andrews was a 2000 l flow through tank bubbled with air. SW was pumped from St Andrews Bay and held on site prior to flow into tanks. Flow rate into the tank was 90 -100 l h^{-1}. Animals were fed with *Loligo forbesi*, approximately 4 g 100 g^{-1} body mass, twice a week. *S. canicula* is a largely sedentary species, particularly in captivity, which typically gorge feeds and displays no aggression to conspecifics. For these reasons animals can be kept at higher stocking densities and fed less frequently than *C. leucas*. Animals were held in the holding tank for between 1 and 3 weeks prior to experimentation with a maximum stocking density of 40 animals per tank.

SW from St Andrews Bay has a variable osmolality depending on local weather conditions, more so than in Moreton Bay (QLD, Australia). This is due to the topography of the areas, as well as the frequency of rainfall. For the purpose of this

study the osmolalities used in all transfers were manipulated, and tanks were set up as recirculating in order to maintain constant environmental conditions. Water of 980 mOsm Kg^{-1} was therefore defined as 100% SW for St Andrews, with increases in salinity being made via the addition of Red Sea Salt (Interpet, Dorking, Surrey). Decreases in salinity were made via the addition of tap water. In this manner absolute values of osmolality were given to percentage dilutions of SW for the entirety of the study (Table 2.2.1.1).

Final SW concentrations of 80 and 120% were taken to represent hypo- and hypersaline conditions as previous work on *S. canicula* had shown these salinities to have significant effects on osmoregulation without causing animal mortality (Anderson *et al.*, 2002a; Wells *et al.*, 2002).

Percentage SW	Osmolality (mOsm Kg^{-1})
120%	1176
110%	1078
100%	980
90%	882
80%	784

Table 2.2.1.1 – Osmolalities used for percentage dilutions of seawater during studies on *S. canicula*.

2.2.1.1 Chronic transfer

A Fluval 403 filtration pump (Rolf C. Hagen Incorporated, Montreal, Canada) filtering at 1200 l h^{-1} was attached to 300 l tanks which had been manipulated to 110 or 90% SW. *S. canicula* were then transferred to the tanks at a stocking density of 8 animals per tank. After 7 days animals were moved to identical tanks containing 120 or 80% SW where they were held for 14 days prior to experimentation. Animals were not fed during transfer to avoid the endocrine effects of gorge feeding (Section 1.4), and to avoid overloading the filtration system.

2.2.1.2 Acute transfer

A Fluval 103 (Rolf C. Hagen Incorporated) filtering at 390 l h^{-1} was attached to 2 x 40 l tanks connected in series (Figure 2.2.1.1). Both tanks were filled with water of the appropriate salinity, with air bubbled into the top tank which housed the animal. This produced an isolated 80 l recirculating system for individual experimental animals.

To initiate the acute transfer the control tap was opened flowing 100% SW into the top tank at 18 – 22 l h^{-1}. Excess volume was removed from the system via an overflow pipe attached to the bottom tank. This produced an isolated 80 l flow through system for individual experimental animals. The time taken to turnover the entire tank volume was therefore between 3.6 and 4.4 hours; although the environmental osmolality took between 4 and 8 hours to reach that of 100% SW (Figure 2.3.2.1).

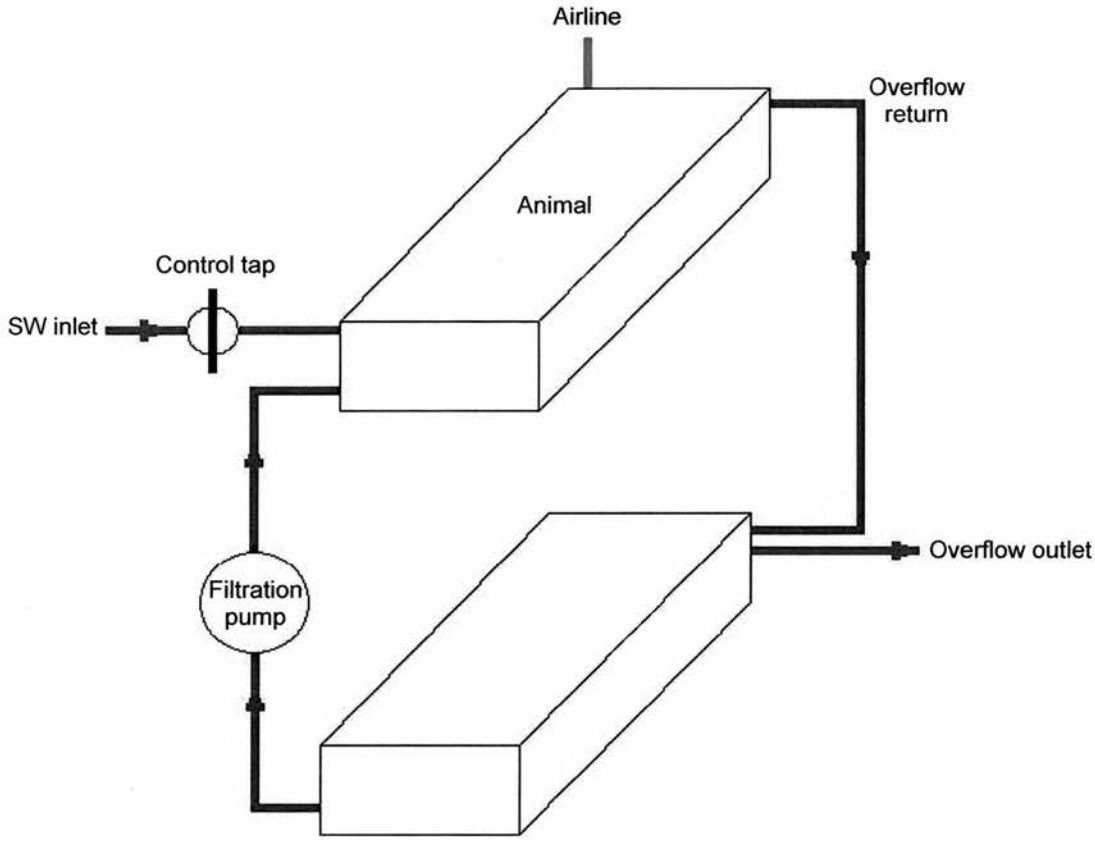

Figure 2.2.1.1 – Tank setup for acute transfer experiments on *S. canicula*. 2 x 40 l tanks in series which could be used as both a recirculating and a flow through system. Opening the control tap initiated the flow of 100% SW into the top tank thereby converting environmental salinity to that of 100% SW throughout the system. The osmotic profile of the flow through system during acute transfer is detailed below (Figure 2.3.2.1).

2.2.2 Protocol for *C. leucas*

Juvenile *C. leucas* between 60 and 90 cm total length (TL) (mean = 83.4, SE = 12.9, n = 28) were captured with rod and line in the FW (3 mOsm Kg^{-1}) reaches of the Brisbane River, Queensland, Australia (Pillans *et al.*, 2005). Captured animals were placed in a 400 l tank, filled with water from the site of capture, and bubbled with a mixture of 95% oxygen (O_2) and 5% carbon dioxide (CO_2) (BOC Gases Australia, North Ryde, NSW, Australia). Animals were then transferred to an identical tank in a vehicle and immediately transported to the holding aquarium at the University of Queensland.

The holding aquarium contained three identical recirculating 10000 l tanks, each with a 200 l submerged coral rubble filter, a 200 l trickle *BioBall* (Polytech, Brisbane, Qld, Australia) filter, and a venturi action foam fractionator (Aquasonic, Wauchope, NSW, Australia) running in parallel, all powered by LZS4-6 pumps (Aquasonic). In addition to the flow provided by the filtration, a circulating current was produced in the tanks by a hose attached to one of the pumps (Figure 2.2.2.1). Water was filtered at a rate of 1,800 – 2,000 l h^{-1}, and tested daily for temperature and levels of dissolved O_2 (YSI 55 DO, YSI Incorporated, Yellow Springs, OH, USA), nitrites, nitrates, and ammonia (Aquasonic, Wauchope, NSW, Australia).

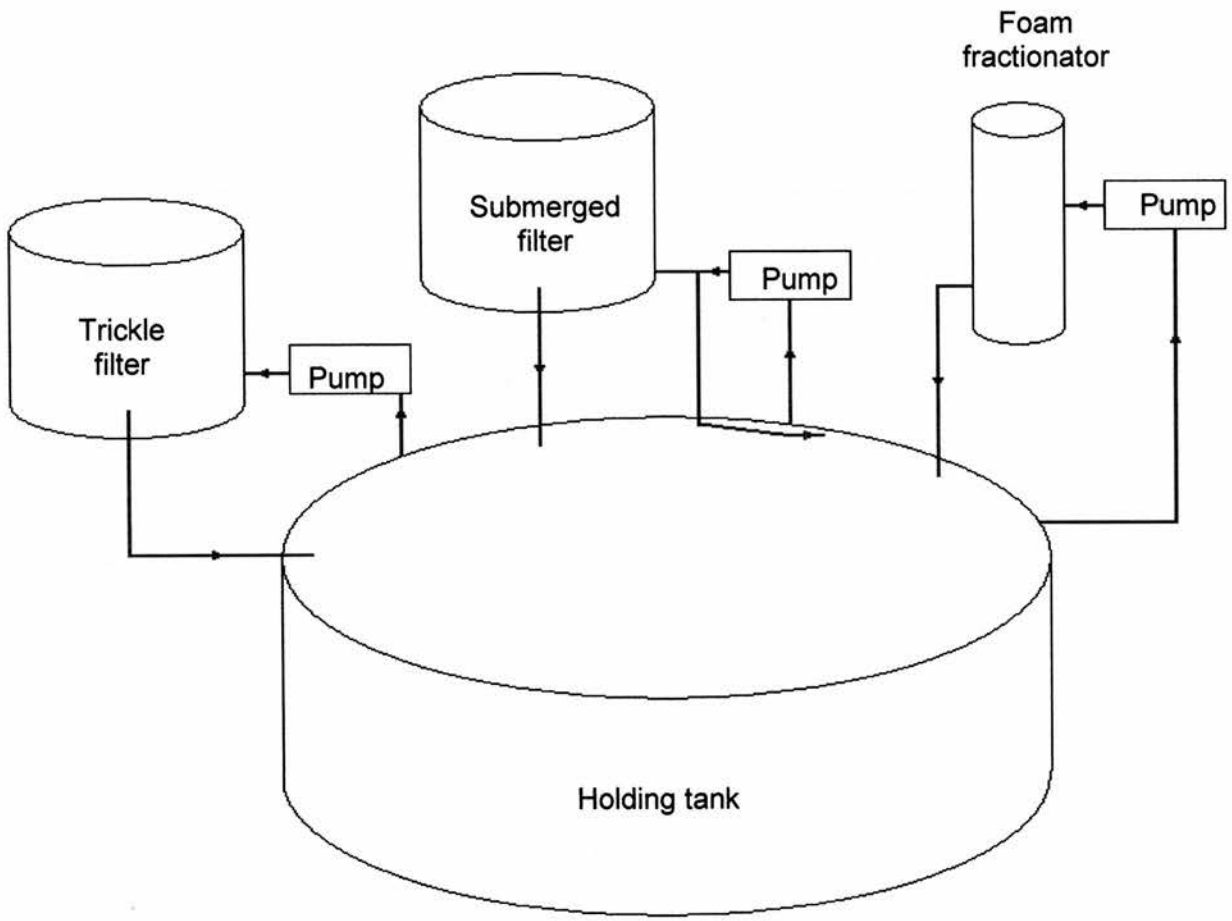

Figure 2.2.2.1 – Recirculating tank setup for holding *C. leucas*, showing the action of all filtration mechanisms. The aquarium comprised 3 x 10000 l holding tanks, each with the above set up.

All animals were fed *ad libitum* with *Mugil cephalus* and/or *Nematalosa erebi* from the Brisbane River or Wyndham Creek (Qld, Australia) every 4 days. Animals in FW were fed either FW *M. cephalus* or *N. erebi*; animals undergoing transfer were fed with estuarine *M. cephalus*; animals in SW were fed with SW *M. cephalus*. *C. leucas* is a ram ventilating species, as well as an active predator. Animals which were not fed *ad libitum* suffered noticeable decreases in body condition and reduced plasma osmolality (Section 1.4), and for these reasons animals were fed throughout the period of captivity. Stocking densities were kept to a maximum of 6 animals per tank to ensure the animals were fed *ad libitum* and to minimise inter-individual aggression in the tanks.

During this study comparisons are drawn between the populations of *C. leucas* in the Brisbane River and that in Lake Nicaragua, Rio San Juan. The Brisbane River has a length of approximately 70 Km which is accessible to *C. leucas*, with sizeable fluctuations in salinity (113 – 231 mOsm Kg^{-1}) occurring beyond 45 Km upstream from the river mouth (Pillans and Franklin, 2004). At the site of capture tidal influence was over 1.5 m during spring tides with an average salinity of 3 mOsm Kg^{-1}. However, given the high degree of tidal influence and relatively short length of the river system, individual animals may have had markedly different levels of exposure to more saline conditions. Conversely, Lake Nicaragua is over 180 Km from the mouth of the Rio San Juan and is a body of completely FW over 165 Km in length (Thorson *et al.*, 1973). The two river systems therefore represent completely different FW environments for *C. leucas*.

2.2.2.1 Chronic transfer

One of the three 10000 l tanks contained FW collected from the Brisbane River (3 mOsm Kg^{-1}, 0.5 mmol l^{-1} Na^+, 0.1 mmol l^{-1} Cl^-), the other two contained diluted SW (400 mOsm Kg^{-1}). SW was collected from Moreton Bay and diluted with water from the Brisbane River to obtain the desired salinity. All animals were left to acclimate for 2 days in FW in the aquarium prior to experimentation.

For acclimation to SW, animals were then transferred to the tanks containing dilute SW (around 400 mOsm Kg^{-1}) and left for 24 hours. After this period the osmolality of the water was raised 100 mOsm Kg^{-1} every 24 hours via the addition of SW until the tank water had an osmolality of 600 mOsm Kg^{-1}. The osmolality of the tank water was then increased by 50 mOsm Kg^{-1} every 24 hours until 800 mOsm Kg^{-1}. This was believed to be the most sensitive period of the transfer as the external media neared the iso-osmotic point of the fish. The water was then increased to 1000 mOsm Kg^{-1} in increments of 100 mOsm Kg^{-1} every 24 hours. Animals were left to acclimate in SW (410 – 440 mmol l^{-1} Na^+, 540 – 560 mmol l^{-1} Cl^-, 7.4 mmol l^{-1} K^+,) for a period of 7 days prior to sampling (Pillans *et al.*, 2005).

For FW acclimation, *C. leucas* were held in the FW tank, under identical conditions for the same period of 16 days. Records of dates of capture and transfer for individual animals were kept, with animals distinguished by individual tags (Suntag, Department of Primary Industries and Fisheries, Qld, Australia) at the base of the dorsal fin.

2.2.3 Chemicals and equipment

Unless otherwise stated all chemicals were obtained from Sigma (Sigma Chemical Company, Poole, Dorset) and all solutions and buffers were made using deionised water (Milli Q reagent water system, Millipore (UK) Ltd., Watford, Herefordshire). Ringer solution for *S. canicula* at 100% SW contained (in mM): 240 NaCl, 7 KCl, 4.9 $MgCl_2$, 0.5 Na_2HPO_4 (BDH Chemicals (UK) Ltd., Poole, Dorset), 0.5 Na_2SO_4, 360 urea, 60 TMAO, 10 $CaCl_2$, and 2.3 $NaHCO_3$; pH 7.6. Ringer for *S. canicula* at 80 and 120% SW was adjusted through the following alterations (in mM): 212 NaCl and 210 urea for 80% SW; 358 NaCl and 376 urea for 120% SW (Hazon and Henderson, 1984). Body mass was measured accurate to 0.5 g on a QBW-1500 digital balance (Adam Equipment Co. Ltd., Bletchley, Milton Keynes), rectal gland mass was measured accurate to 0.001 g on a B154 digital balance (Mettler Toledo UK, Beaumont Leys, Leicester).

Ringer solution for FW *C. leucas* contained (in mM): 213 NaCl, 3 KCl, 2.5 $MgCl_2$, 1 Na_2HPO_4, 0.5 Na_2SO_4, 181.1 urea, 30 TMAO, 2.5 $CaCl_2$, 10 $NaHCO_3$, and 55.5 glucose; pH 7.6. Ringer for SW *C. leucas* was adjusted through the following alterations (in mM): 279.9 NaCl, 6 KCl, 350 urea, and 58.8 TMAO (Pillans and Franklin, 2004). Body mass was recorded accurate to 100g on a 235 6S mechanical scale (Salter Brecknell, Fairmont, MN, USA), rectal gland mass was not recorded due to a lack of suitable equipment on site (subsequent measurement of slices for respirometry was conducted accurate to 0.001 g on a SA 210 digital balance (Scientech Inc. Boulder, CO, USA).

2.2.4 Surgical procedures

S. canicula were anaesthetised in a 5 l induction bath containing 120 ppm (by mass) ethyl 3-aminobenzoate methanesulphonate salt (MS-222) with an equal mass of $NaHCO_3$ dissolved in the appropriate salinity of SW. Upon induction, opercular rate slowed, equilibrium was lost, and a surgical level of anaesthesia was deemed to have been reached when there was no reflex to a firm pinch on the dorsal fin. The animals were then placed on a surgical tray with a 5 l recirculating volume of anaesthetic (50 ppm MS-222 and 50 ppm $NaHCO_3$ dissolved in the appropriate salinity SW) washing over the gills.

An incision was made in the flank of the animal from posterior of the pectoral fin to the pelvic girdle. This incision was cauterised (RB 708, Rimmer Brothers, London) to prevent blood loss during the experiment. The stomach and valvular intestine were retracted from the body cavity and the coeliac artery was then located, this runs parallel to the splenic vein along the stomach wall to the spleen. A Mersilk tie (Genusxpress, Bridge of Don, Aberdeen) was placed around the artery, as close as possible to the stomach wall, and fastened with three alternating half-hitches. A second tie was placed around the artery close to the gonad tissue and left unfastened. An incision was then made between the two ties and a 60cm cannula with an obliquely cut tip was passed into the artery. All cannulae were Portex polythene tubing of 0.96 mm outer diameter (SIMS Portex Ltd, Hythe, Kent) and had been filled with Ringer solution of the appropriate salinity and 200 IU ml^{-1} of heparin. The cannula was fed into the artery until the tip was in the dorsal aorta, or close to it. The second tie was then fastened in a similar fashion to the first, with a third tie fastened around the cannula as close to the dorsal aorta as

possible. Once pressure was established within the cannula the protruding end was sealed with a coloured pin.

The mesenteric artery was then located and cannulated in a similar fashion to the coeliac, using a different coloured pin to block the protruding end. The mesenteric artery branches from the dorsal aorta posteriorly to the coeliac, and supplies the valvular intestine.

Animals used in haematic parameter studies were also used to assess rectal gland secretion rate (Chapter 4). The surgical procedure for this is outlined below (Section 4.2.2). After all cannulations animals were sutured with polyamide thread (Genusxpress) using an appropriate number of stitches and left in the salinity transfer tanks (Figure 2.2.1.1) for 24 hours after surgery under a 12 hour photoperiod.

2.2.5 Analysis and collection

For *S. canicula*, 200 µl of blood was withdrawn via the coeliac arterial cannula (mesenteric was used if no sample could be obtained) for osmolyte analysis. Blood and water samples were collected after 0, 2, 6, 8, and 24 hours for basal levels and 0, 2, 4, 6, 8, 10, and 12 hours for acute transfer; the 24 hour basal and 0 hour transfer samples being the same sample. An equivalent volume of the appropriate salinity Ringer solution was then injected via the same cannula. Blood samples were centrifuged at 10490 *g* for 3 minutes and the plasma portion was retained. Osmolality and Cl^- concentration were assessed immediately, samples were then stored at -70 °C until subsequent urea analysis. Osmolalities were measured via a freezing point osmometer (Roebling, Messtechnik, Berlin, Germany), Cl^- concentration via titration (Corning 925 Chloride Analyser, Halstead, Essex), and urea concentration via assay kit UR107 (Randox, Crumlin, Co. Antrim). Haematocrit was measured as the proportional volume of erythrocytes within a blood sample via centrifugation for 3 minutes (Micro Haematocrit MK IV, Hawksley and Sons Ltd, Sussex).

For *C. leucas*, blood samples (approximately 10 ml) were taken from the caudal vein and haematocrit was immediately determined. Blood was then centrifuged at 9000 *g* for 3 minutes with the plasma portion being retained and stored at -80 °C until subsequent osmolyte analysis. Osmolalities were measured via a semi-micro freezing point osmometer (Knauer ADI, Berlin, Germany), whilst concentrations of Na^+, Cl^-, and urea were measured via a modular multiple biochemistry analyser (Roche Diagnostics Australia Pty. Ltd., Castle Hill, NSW, Australia). Haematocrit was measured in an identical manner to *S. canicula*.

2.2.6 Statistical analysis

All data are presented as means ± the standard error of the mean (SEM). For the osmotic data gathered on *S. canicula* statistical analysis was performed via one-way analysis of variance (ANOVA) and a Tukey-Kramer multiple comparisons (Tukey) post hoc test for basal levels (InStat, GraphPad Software, San Diego, CA) (significance was denoted as * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.005$). Data gathered during the acute transfer studies was analysed in two ways: differences between the two experimental groups and the control group were analysed via one-way ANOVA and a Tukey post hoc test (significance was denoted as * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.005$); differences between values during transfer and at time 0 within each group were analysed via a one-tailed unpaired students t-test with a Welch standard deviation (Welch) correction factor (significance was denoted as † $P < 0.05$, †† $P < 0.01$, and ††† $P < 0.005$) (InStat). Once differences had occurred they persisted throughout the transfer, although for clarity differences are only noted at the first and last instances.

Osmotic data on *C. leucas* was statistically analysed via a two-tailed student's t-test with Welch correction factor (InStat). Significance was denoted as $P < 0.05$, ** $P < 0.01$, and *** $P < 0.005$.

2.3 Results

The results for haematic parameters are presented in two sections: a comparison of basal levels in both *S. canicula* and *C. leucas* acclimated to different salinities; and a comparison of acute transfer in *S. canicula* from all salinities to 100% SW.

2.3.1 Basal levels

Haematic parameters of *S. canicula* acclimated to 80, 100, and 120% SW are presented below (Table 2.3.1.1). Blood plasma osmolality was found to be highly significantly decreased upon acclimation to 80% SW, and highly significantly increased upon acclimation to 120% SW. Similar trends are seen in the values for plasma Cl^- and urea concentrations at the three salinities Acclimation to 80% SW resulted in a highly significant decrease in blood haematocrit, acclimation to 120% SW resulted in an extremely significant increase in blood haematocrit.

Haematic parameters of captive *C. leucas* acclimated to FW and SW are presented below (Table 2.3.1.2). Captive animals acclimated to SW show a significant increase in blood plasma osmolality as compared to those in FW. Plasma concentrations of Na^+ and Cl^- are both significantly higher in SW acclimated animals. Plasma urea levels were also significantly higher in SW acclimated animals when compared to those in FW. No significant difference was seen between the blood haematocrit of animals from FW and SW.

The percentage contribution of major osmolytes to overall osmotic pressure was then calculated for both species and compared to previously reported values (Table 2.3.1.3).

Salinity (SW)	Osmolality (mOsm Kg-1)	Cl^- (mmol l^{-1})	Urea (mmol l^{-1})	Haematocrit (RBC %)
80%	776 ± 2 ***	231 ± 2 ***	212 ± 4 ***	13.1 ± 0.5 **
100%	1003 ± 5	308 ± 2	302 ± 7	16.6 ± 0.5
120%	1137 ± 11 ***	338 ± 5 ***	357 ± 9 ***	21.5 ± 1.2 ***

Table 2.3.1.1 – Haematic parameters of *S. canicula* after >14 day acclimations to 80, 100, and 120% SW. All values are presented as means ± SEM (n = 42, 22, and 22 respectively for osmolality, Cl^-, urea, and haematocrit). Haematocrit was deduced as percentage red blood cells (RBC %). Statistical analysis was performed via one-way ANOVA and a Tukey post hoc test. Significant differences from values for 100% SW were denoted as * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.005$).

Salinity	Osmolality (mOsm Kg^{-1})	Na^+ (mmol l^{-1})	Cl^- (mmol l^{-1})	Urea (mmol l^{-1})	Haematocrit (RBC %)
FW	588 ± 13	192 ± 5	216 ± 5	151 ± 6	20.7 ± 1.0
SW	940 ± 10 ***	304 ± 4 ***	315 ± 3 ***	293 ± 9 ***	18.3 ± 1.2

Table 2.3.1.2 – Haematic parameters of captive *C. leucas* acclimated to FW and SW. All values are presented as means ± SEM (n = 13 and 11 respectively, with the exception of SW haematocrit, n = 9). Haematocrit was deduced as percentage red blood cells (RBC %). Statistical analysis was performed via a two tailed unpaired student's t-test with Welch correction factor. Significant differences from values for FW were denoted as * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.005$).

Species and salinity	Environmental osmolality (mOsm Kg^{-1})	Plasma osmolality (mOsm Kg^{-1})	Percentage contribution to overall osmolality (%)		
			Na^+	Cl^-	Urea
S. canicula					
80% SW	784	776	_	29.8	27.3
100% SW	980	1003	_	30.7	30.1
120% SW	1176	1137	_	29.7	31.4
C. leucas					
FW	≈ 0	588	32.6	36.8	25.7
SW	980	940	32.4	33.5	31.2

Table 2.3.1.3 – Percentage contributions of major plasma osmolytes in chronically acclimated captive *S. canicula* and *C. leucas* from different salinities. Acclimation periods were 14 and 7 days respectively at the final salinities.

2.3.2 Acute transfer levels

All figures for acute transfer have had the values for animals acclimating from 80 and 120% offset on the time axis for clarity; the measurements are all taken at equivalent time periods. It was necessary to analyse the environmental conditions associated with the acute transfer studies in order to put the results for haematic parameters into context (Figures 2.3.2.1 and 2). During acute transfer the environmental osmolality increased for animals acclimating from 80% SW and decreased for those acclimating from 120% SW. Environmental osmolality for animals acclimating from 100% SW did not vary significantly throughout acute transfer. Environmental osmolality had significantly changed for both experimental groups during the first 2 hours of transfer. The osmolality of the 120% SW environment was not significantly different to 100% SW after 4 hours, and the 80% SW environment was of a similar osmolality after 8 hours. For 120% SW a similar trend was recorded for Cl^- concentration as for overall osmolality. However, the 80% SW environment remained significantly lower in Cl^- concentration than the 100% SW until 12 hours into the transfer. Therefore the environment starting at 120% SW was altered to 100% SW in a shorter time than that starting at 80% SW, despite having similar flows of SW into the tanks.

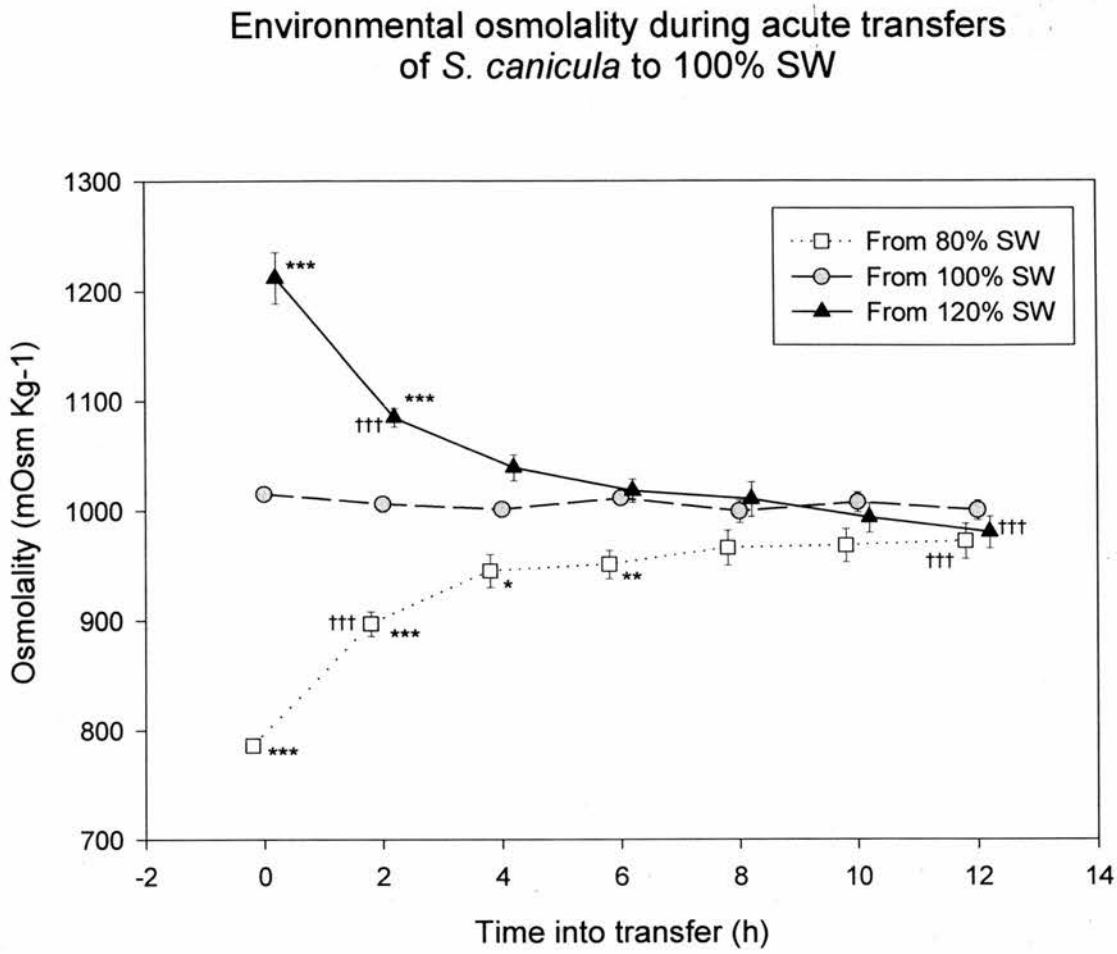

Figure 2.3.2.1 – Osmolality of the 80, 100, and 120% SW environments during acute transfer to 100% SW. Values are presented as means ± SEM (n = 14, 7, and 12 respectively). Statistically significant differences from the control transfer were assessed via one-way ANOVA and a Tukey post hoc test (significance was denoted as * (P < 0.05), ** (P < 0.01), and *** (P < 0.005)); statistically significant differences from values at time 0 were assessed via a one tailed unpaired students t-tests with Welch correction factor (significance was denoted as † (P < 0.05), †† (P < 0.01), and ††† (P < 0.005)). Once differences had occurred they persisted throughout the transfer, although for clarity differences are only noted at the first and last instances.

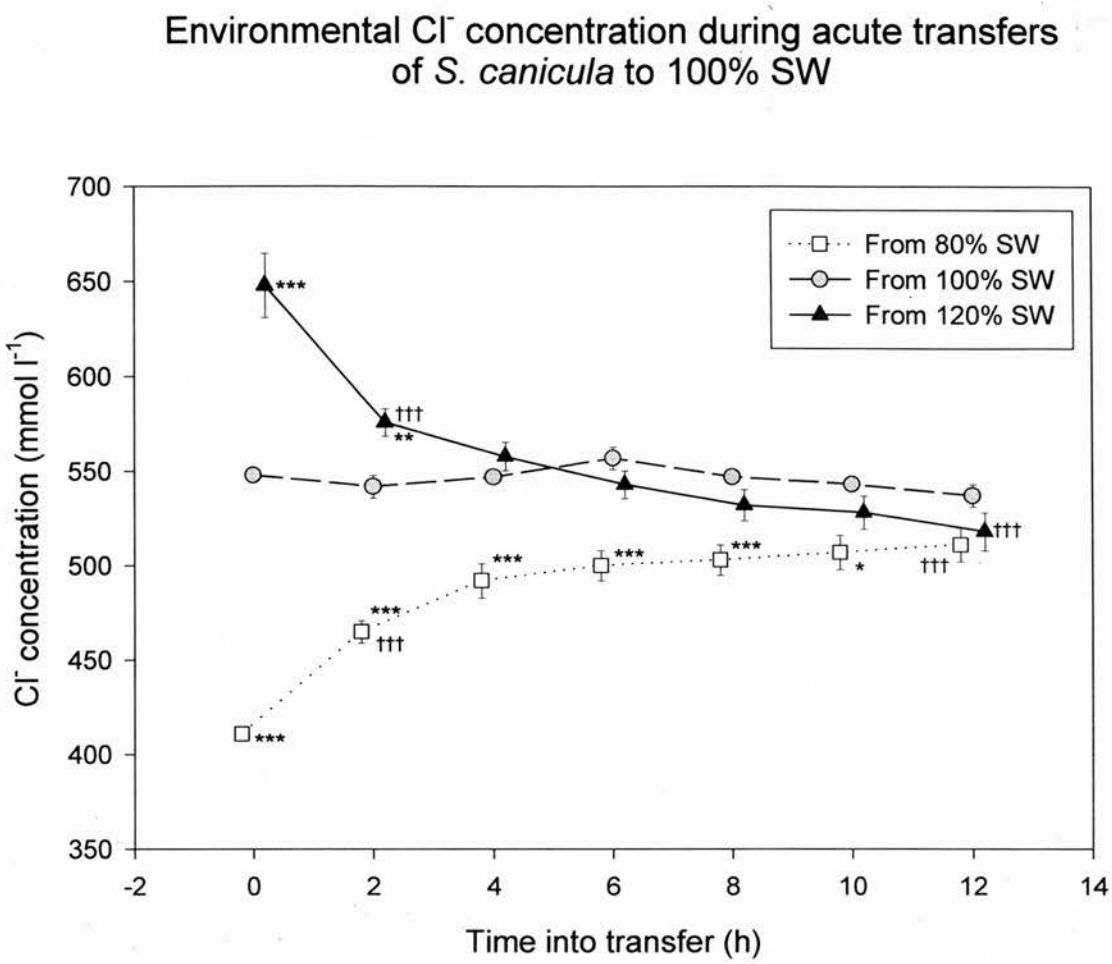

Figure 2.3.2.2 – Cl^- concentration of the 80, 100, and 120% SW environments during acute transfer to 100% SW. Values are presented as means ± SEM (n = 14, 7, and 12 respectively). Statistically significant differences from the control transfer were assessed via one-way ANOVA and a Tukey post hoc test (significance was denoted as * (P < 0.05), ** (P < 0.01), and *** (P < 0.005)); statistically significant differences from values at time 0 were assessed via a one tailed unpaired students t-tests with Welch correction factor (significance was denoted as † (P < 0.05), †† (P < 0.01), and ††† (P < 0.005)). Once differences had occurred they persisted throughout the transfer, although for clarity differences are only noted at the first and last instances.

The osmotic parameters of *S. canicula* measured during acute transfer to 100% SW showed the trends expected for the changing environmental conditions (Figures 2.3.2.3 – 6). Overall plasma osmolality was significantly different from starting values 4 hours into the transfer, and remained so for the rest of the transfer, in both experimental groups. Plasma osmolality increased in animals undergoing acute transfer from 80% SW, and decreased in animals undergoing acute transfer from 120% SW. Plasma osmolality in the control transfer group did not change significantly over the time period. At the end of the transfer the plasma osmolality of animals from 120% SW was not significantly different from the control animals, whilst animals from 80% SW had plasma osmolalities which remained lower than the control animals throughout the transfer period.

Plasma Cl^- concentration followed a similar pattern to overall osmolality in the three groups. After 6 hours Cl^- concentrations were significantly different from values at the start of the transfer, and remained so for the rest of the transfer, in both experimental groups. Plasma Cl^- concentration did not vary significantly in the control group during the transfer. Plasma Cl^- levels in animals from 80% SW remained significantly lower than those of the control animals throughout the transfer period. After 6 hours plasma Cl^- concentration in animals from 120% SW was not significantly different to that of the control animals.

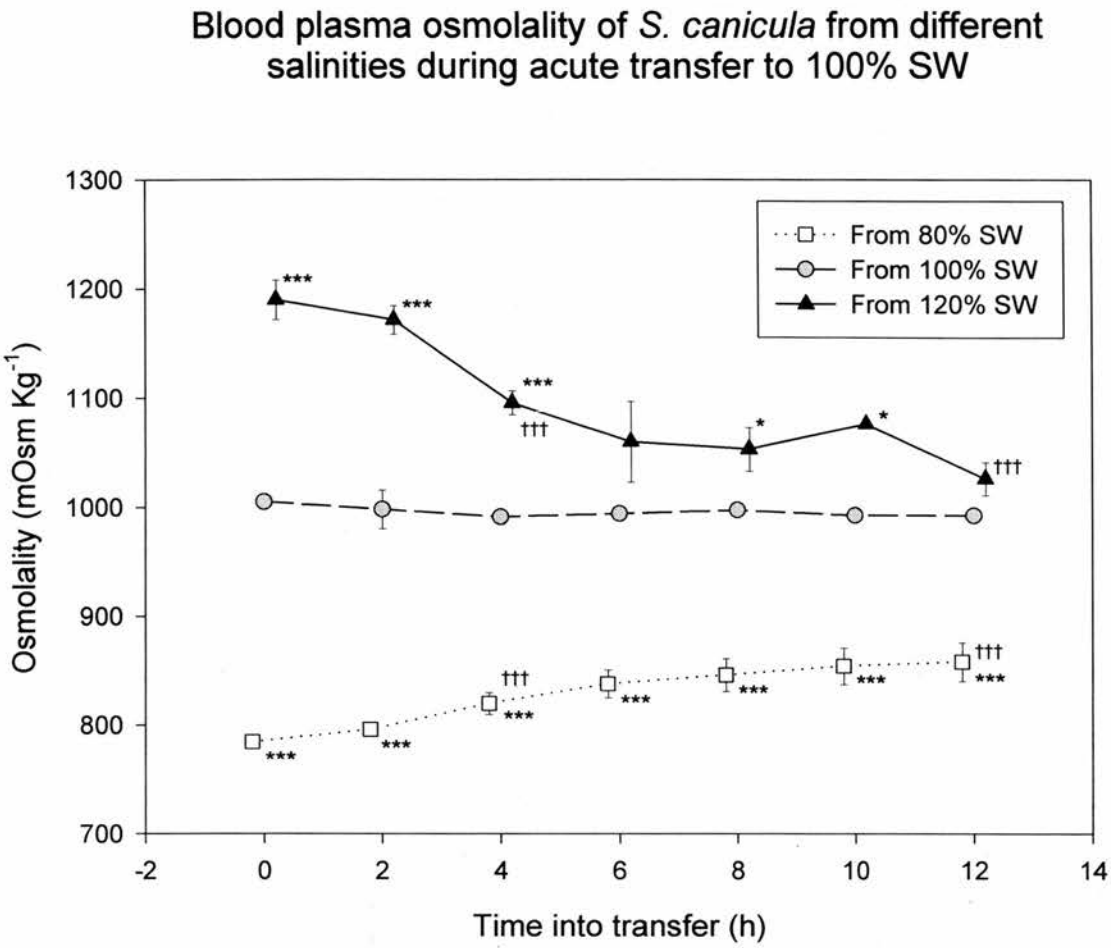

Figure 2.3.2.3 – Plasma osmolality of *S. canicula* acclimated to 80, 100, and 120% SW during acute transfer to 100% SW. Values are presented as means ± SEM (n = 11, 4, and 4 respectively). Statistically significant differences from the control transfer were assessed via one-way ANOVA and a Tukey post hoc test (significance was denoted as * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.005$)); statistically significant differences from values at time 0 were assessed via a one tailed unpaired students t-tests with Welch correction factor (significance was denoted as † ($P < 0.05$), †† ($P < 0.01$), and ††† ($P < 0.005$)). Once differences had occurred they persisted throughout the transfer, although for clarity differences are only noted at the first and last instances.

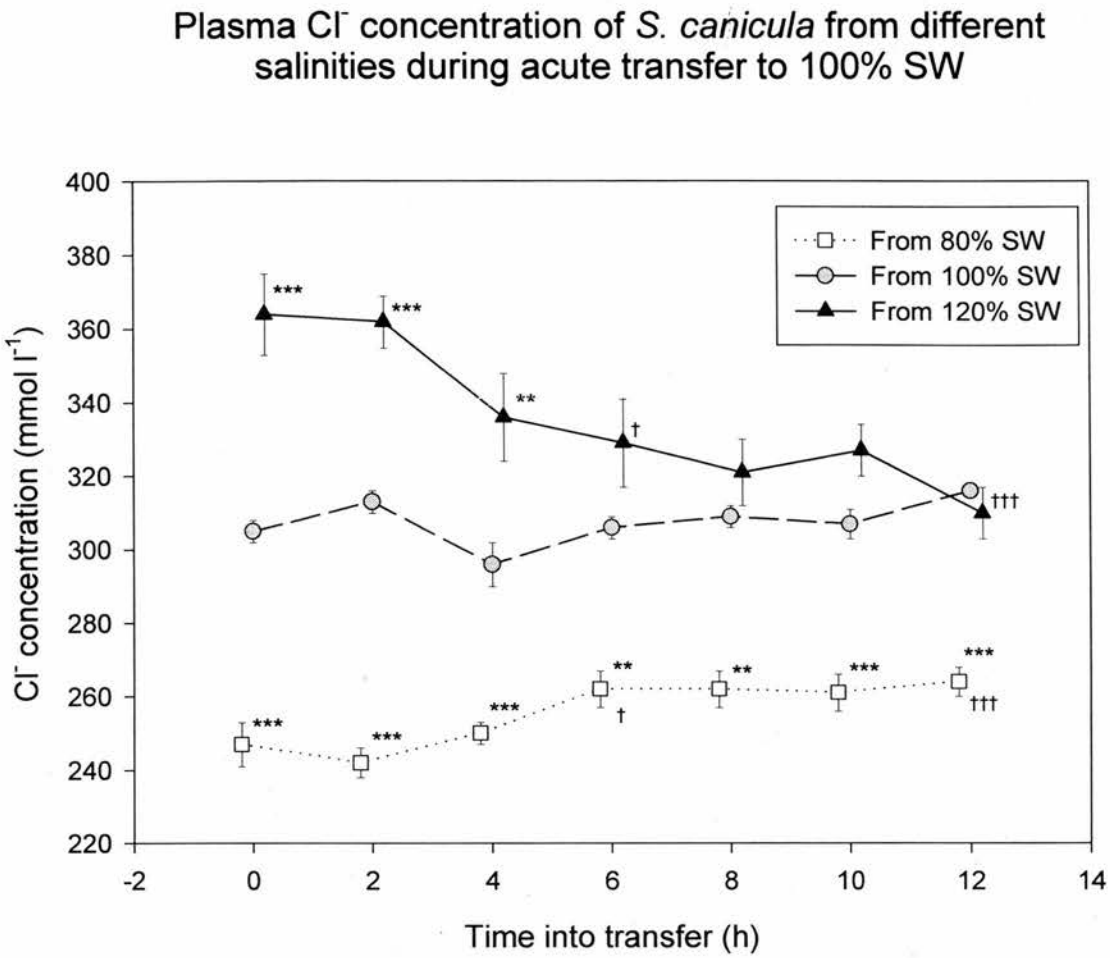

Figure 2.3.2.4 – Plasma Cl^- concentrations of *S. canicula* acclimated to 80, 100, and 120% SW during acute transfer to 100% SW. Values are presented as means ± SEM (n = 11, 4, and 4 respectively). Statistically significant differences from the control transfer were assessed via one-way ANOVA and a Tukey post hoc test (significance was denoted as * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.005$)); statistically significant differences from values at time 0 were assessed via a one tailed unpaired students t-tests with Welch correction factor (significance was denoted as † ($P < 0.05$), †† ($P < 0.01$), and ††† ($P < 0.005$)). Once differences had occurred they persisted throughout the transfer, although for clarity differences are only noted at the first and last instances.

Plasma urea levels in both the control group and that from 80% SW remained unchanged throughout the transfer, whilst concentration was significantly reduced after 12 hours in animals from 120% SW. Plasma urea concentration in animals from 120% SW was not significantly different from that in the control group after 2 hours of acute transfer. With the exception of the 4 hour sample, plasma urea levels in animals from 80% SW remained significantly lower than those in the control group.

At the start of the acute transfer to 100% SW, only animals from 120% SW had a blood haematocrit which was significantly different from the control group. This difference did not persist after the first 2 hours of the transfer. Blood haematocrit did not change significantly during acute transfer in the group from 80% SW. Blood haematocrit in the group from 120% SW was significantly reduced after 4 hours of the acute transfer, and remained so for the rest of the study period. Blood haematocrit was significantly reduced in the control group after 10 and 12 hours of the transfer.

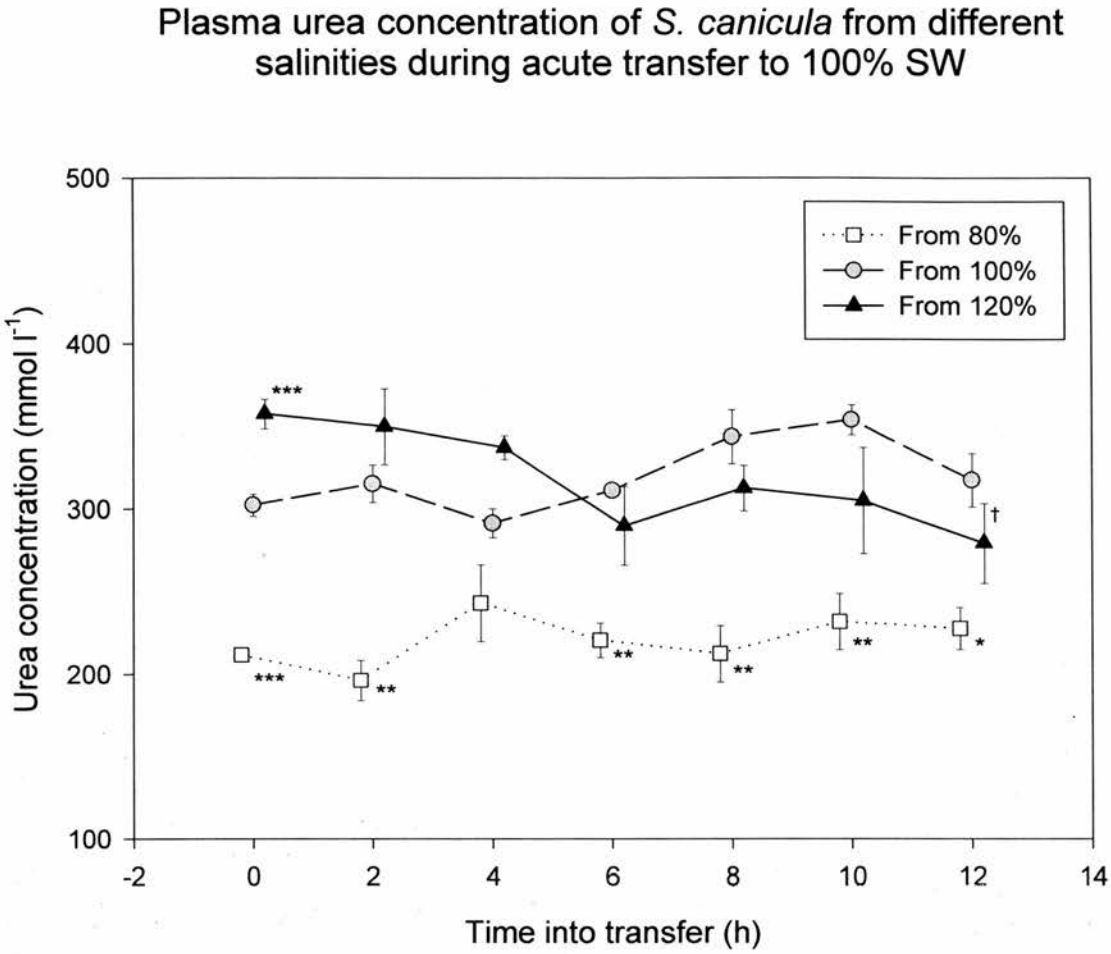

Figure 2.3.2.5 – Plasma urea concentrations of *S. canicula* acclimated to 80, 100, and 120% SW during acute transfer to 100% SW. Values are presented as means ± SEM (n = 11, 4, and 4 respectively). Statistically significant differences from the control transfer were assessed via one-way ANOVA and a Tukey post hoc test (significance was denoted as * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.005$)); statistically significant differences from values at time 0 were assessed via a one tailed unpaired students t-tests with Welch correction factor (significance was denoted as † ($P < 0.05$), †† ($P < 0.01$), and ††† ($P < 0.005$)). Once differences had occurred they persisted throughout the transfer, although for clarity differences are only noted at the first and last instances.

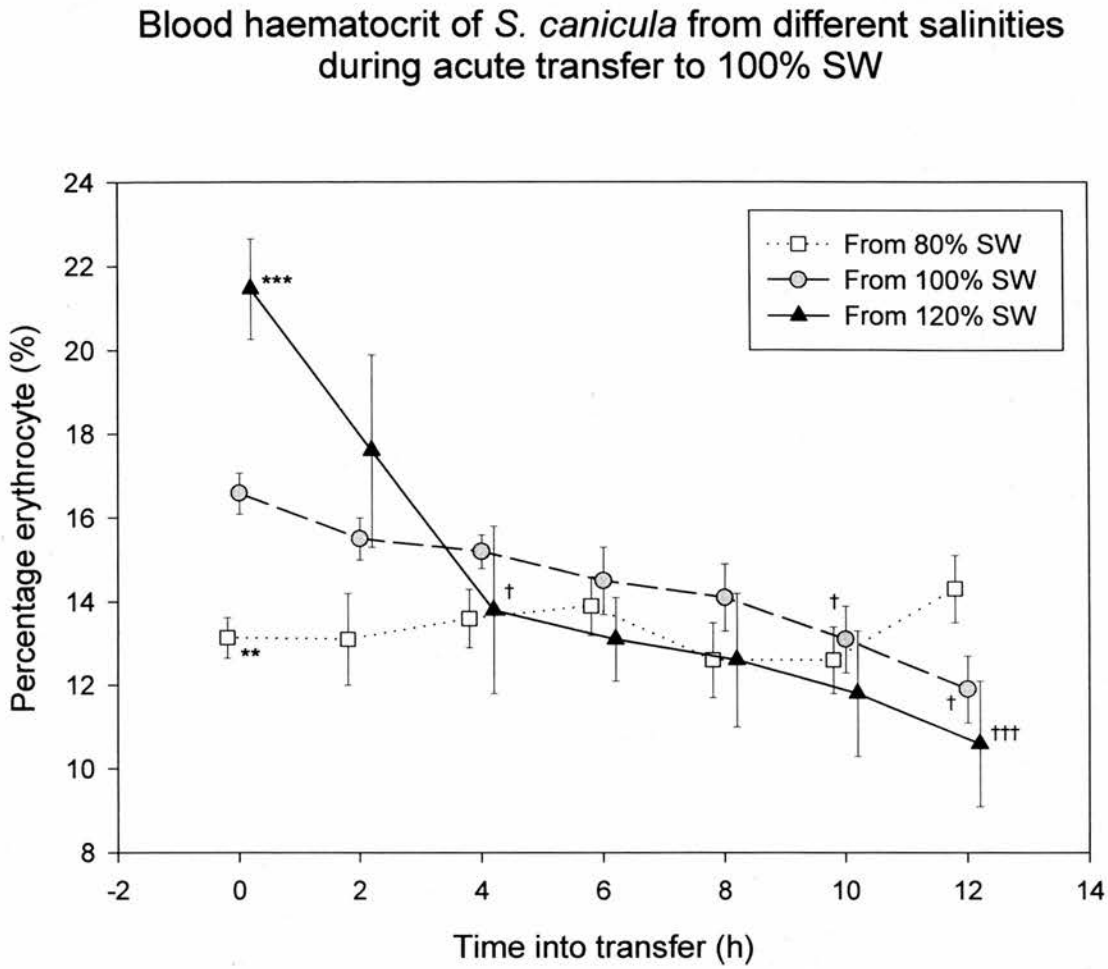

Figure 2.3.2.6 – Blood haematocrit of *S. canicula* acclimated to 80, 100, and 120% SW during acute transfer to 100% SW. Values are presented as means ± SEM (n = 11, 4, and 4 respectively). Statistically significant differences from the control transfer were assessed via one-way ANOVA and a Tukey post hoc test (significance was denoted as * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.005$)); statistically significant differences from values at time 0 were assessed via a one tailed unpaired students t-tests with Welch correction factor (significance was denoted as † ($P < 0.05$), †† ($P < 0.01$), and ††† ($P < 0.005$)). Once differences had occurred they persisted throughout the transfer, although for clarity differences are only noted at the first and last instances.

2.4 Discussion

Osmotic profiles for captive *S. canicula* and *C. leucas* acclimated to different salinities were successfully produced. Despite the previous reporting of an osmotic profile for wild *C. leucas* along a salinity gradient by Pillans and Franklin (2004), because of the effects of captivity on the species osmotic state (Table 1.4.2) it was necessary to produce osmotic profiles for both SW and FW acclimated animals in captivity. Osmotic profiles for *S. canicula* from 80, 100, and 120% SW environments during acute transfer to 100% SW were also produced. No acute transfer studies were conducted on *C. leucas* for a number of reasons. Firstly, because the species is ram ventilating and active, attempting to confine individuals in small tanks would have been highly stressful for the animals with an increase in breaching. Acute transfers using the 10000 l tanks would not have been cost effective and would have logistical problems regarding the volume of water required to turnover 10000 l in a flow through system. Serial sampling of *C. leucas* is also highly problematic as the species did not react well with attempted anaesthesia using MS-222, thus prohibiting cannulation of major arteries. Alternative means were not used on *C. leucas*, as despite being well reviewed by Dunn and Koester (1990), little is known about the physiological effects of different anaesthetics on elasmobranchs. Obtaining serial samples without cannulation would have required netting and removal of the animals, restraint, and blood sampling via syringe and needle. The stress associated with this, and the resultant handling to return the animals to the tank, would have affected plasma osmolality, blood glucose and lactate concentrations, as well as blood pH (Hoffmayer and Parsons, 2001).

Both the partially euryhaline *S. canicula* and the fully euryhaline *C. leucas* are able to significantly alter the plasma concentrations of Na^+, Cl^-, and urea in response to salinity

change (Tables 2.3.1.1 and 2). Values for *S. canicula* acclimated to 100% SW were comparable to those reported by Hazon and Henderson (1984), considering the different osmolalities of 100% SW. Interestingly, the values recorded for 80 and 120% SW were higher and lower respectively than the values previously reported; even though the environmental osmolalities were lower and higher respectively. Similar trends were seen in plasma Cl^- concentration (Tables 2.2.1.1 and 2.1.2). Plasma urea levels were consistent between the two studies. These differences are discussed below as percentage contributions to overall osmotic pressure.

Captive *C. leucas* had significantly lower plasma osmolyte concentrations and overall osmolalities as compared to wild caught animals (Tables 2.2.1.2 and 2.1.2) (Pillans and Franklin, 2004). These are discussed below as percentage contributions to overall osmotic pressure.

The percentage contribution of Na^+, Cl^-, and urea to overall osmotic pressure can vary with salinity (Table 2.3.1.3), suggesting modifications in their rates of influx and efflux. For example, the percentage contribution of Cl^- to overall osmotic pressure in *S. canicula* is relatively consistent between 80, 100, and 120% SW conditions; whereas the percentage contribution of urea increases with salinity. This suggests that there are modifications in the relative rates of urea production and/or retention between animals from the three environments which result in this. The consistency in the percentage contribution of Cl^- to overall osmotic pressure in *S. canicula* suggests either that the rate of influx is the same from all three environments (this is unlikely given that the increase in environmental osmolality is largely due to increases in the concentrations of Na^+ and Cl^-), or that any change in influx is balanced by a corresponding change in efflux. Given

that animals from 100% SW were hyperosmotic and those from 80 and 120% were slightly hyposmotic to their environments there are changes in the concentration gradients and relative rates of influx for Cl^- (and Na^+) between the three salinities. As the percentage contributions of Cl^- are relatively consistent despite this relative change in influx, there must be modifications in the rate of efflux to achieve this. Elasmobranchs have evolved a rectal gland which is the only organ capable of secreting a NaCl solution which is hyperionic to blood plasma. Changes in the rate of efflux of Na^+ and Cl^- are therefore almost certain to be the result of changes in the activity of the rectal gland. This highlights the importance of the gland in maintaining homeostatic balance in marine elasmobranchs.

The osmolyte data collected for captive *C. leucas* shows similar trends to that reported for wild individuals, although there are some noticeable differences (Pillans and Franklin, 2004). Captive FW *C. leucas* have a lower concentration of urea in the blood plasma, by around 40 mmol l^{-1}, whilst concentrations of Na^+ and Cl^- are relatively consistent with those caught in the wild. This is the primary source of the reduced plasma osmolality recorded in captive animals. In response to SW acclimation captive *C. leucas* do increase plasma urea levels, although these are again much lower than values reported for wild SW animals (around 65 mmol l^{-1} less urea). This relatively low concentration of urea in both FW and SW captive animals results in lower plasma osmolalities as compared to wild animals. This increases the percentage contribution of Na^+ and Cl^- in the overall osmolalities of captive *C. leucas*.

The low urea levels in captive animals from both salinities are most likely a reflection in dietary intake of protein. Related studies on *S. canicula* have demonstrated that low

dietary protein intake significantly decreases urea production, and there is an associated decrease in urea clearance in an attempt to compensate for this. Furthermore, upon acclimation to 130% SW low protein fed fish were unable to increase plasma urea concentrations and elevated plasma Na^+ and Cl^- levels to compensate for this (Armour *et al.*, 1993a).

Despite being fed *ad libitum* every 4 days captive *C. leucas* were still unable to synthesise and/or retain urea as well as wild individuals. Being a ram ventilating species *C. leucas* requires a much larger amount of energy per day than a similarly sized sedentary species. This energy demand may therefore reduce the proportion of energy which can be utilised to convert ammonia (NH_3) into urea via the OUC (Section 1.7) during periods of reduced feeding. It therefore appears that wild *C. leucas* have a higher dietary intake than can be match by gorge feeding every 4 days in captivity, particularly in FW acclimated animals. This is important to note for future studies on the species. These results not only show the importance of dietary protein intake for elevating plasma urea concentrations, but also the capacity of elasmobranchs to acclimate to increased environmental salinity through increasing plasma Na^+ and Cl^- concentrations. This is a reflection of the availability of these three plasma osmolytes to elasmobranchs in a saline environment.

Due to the large size and aggressive nature of adult *C. leucas* it was only feasible to hold juvenile animals in the aquarium. This may have had consequences for the developmental state of the osmoregulatory organs, although work on other species suggests early development of osmoregulatory mechanisms (Kormanik, 1992; Kormanik, 1993; Steele *et al.*, 2004).

The partially euryhaline *S. canicula* showed significant differences in blood haematocrit upon acclimation to both 80 and 120% SW (Table 2.2.1.1). It is likely that these reflect the differences in the osmotic influx of water which is associated with the three environmental salinities. The increased influx of water associated with acclimation to more dilute environments leads to an associated degree of haemodilution within *S. canicula*. Conversely, during acclimation to elevated environmental salinity there is likely to be an osmotic efflux of water and an associated degree of haemoconcentration. These significant changes in blood haematocrit are suggestive of, but not evidence for, changes in blood volume of the same animals. Unlike *S. canicula* the blood haematocrit of *C. leucas* was unaffected by acclimation to salinity change (Table 2.2.1.2). This is highly suggestive of a greater degree of control over osmotic water fluxes in the latter species.

Therefore the degree of haemodilution and concentration associated with acclimating to salinity change may be considerably less in the fully euryhaline *C. leucas* than in the partially euryhaline *S. canicula*. This would in turn have effects on plasma osmolyte concentration and therefore on the actions of the principle osmoregulatory organs. Practical assessment of blood volume in both a partially and a fully euryhaline elasmobranch would therefore give key insight into the volaemic changes experienced during salinity transfer. Given the central stimulatory role of blood volume change on osmoregulatory organs such as the rectal gland (Solomon *et al.*, 1984; Silva *et al.*, 1996; Silva *et al.*, 1999; Anderson *et al.*, 2002a), insight into elasmobranch blood volume is of pivotal importance in understanding euryhalinity in elasmobranch fish. Blood volume in elasmobranchs is discussed in detail below (Chapter 3).

The acute transfer studies on *S. canicula* showed some interesting results, not only for the haematic parameters, but also for the environmental conditions. Converting a 120% SW environment into 100% SW took less time than converting an 80% SW environment, particularly with regard to Cl^- concentration. This is probably a reflection of changes in rainfall between the study periods on the two experimental groups. The salinity of SW in St Andrews bay is highly dependent on the degree of rainfall due to the topography of the shore line, and the currents of SW in the bay. The amount of run off from the crescent of land around the bay, and the inlet from the Kiness Burn and Eden rivers, coupled with the low flow of fresh SW into the bay all lead to large changes in the osmolality of SW drawn into the aquarium. Such a fall in salinity of SW would account for the longer turnover time for the 80% SW environment. This difference in environmental conditions must be borne in mind when comparing the acute transfer results for the two experimental groups. In terms of environmental osmolality the 120% SW environment was converted to 100% SW after 4 hours, whilst the 80% SW environment took 8 hours.

For *S. canicula* acclimating from 80% SW a significant increase in plasma osmolality was recorded after 4 hours of acute transfer to 100% SW (Figure 2.3.2.3). These animals did not increase plasma osmolality to levels equivalent to animals acclimated to 100% SW. At least in part this may be a reflection of the greater length of time taken to change the 80% SW environment to that of the control animals at 100% SW. Nevertheless plasma osmolality was significantly increased, primarily through an increase in plasma Cl^- (and Na^+) concentration (Figure 2.3.2.4). Plasma Cl^- concentration was significantly increased after 6 hours of the acute transfer. There was no significant increase in plasma urea concentration associated with acute transfer from

80 to 100% SW (Figure 2.3.2.5). The disparity between the ability of these animals to increase plasma Cl^- (and Na^+) and urea levels is largely due to the availability of these osmolytes in the environment. Na^+ and Cl^- are readily available in a marine environment and increasing drinking rate will facilitate increases in the intake of these ions. Indeed, both *S. canicula* and *T. scyllia* increase drinking rate during acclimation to increased salinity (Anderson *et al.*, 2002b). Conversely, increasing plasma urea levels requires not only an increase in urea synthesis and a decrease in urea clearance, but also an increase in substrate for urea synthesis. This would require the breakdown of protein within the tissues of these animals during transfer. As such any increase in plasma urea concentration is liable to take longer than 12 hours due to the availability of substrate for, and the metabolic costs associated with the increased synthesis of urea. This lack of increase in plasma urea during the first 12 hours of acute transfer to elevated salinities also gives further evidence for the importance of dietary protein intake in elevating plasma urea levels.

Blood haematocrit was not significantly altered during transfer from 80 to 100% SW (Figure 2.3.2.6). The fact that there was a significant decrease in the blood haematocrit of the control group during the transfer suggests that repeated sampling over the basal and transfer studies may well have the effect of decreasing blood haematocrit. Therefore, whilst a decrease was seen in the control group, the lack of any effect in the group acclimating from 80% SW may reflect the fact that any decrease caused by repeated sampling was offset by the increase expected with haemoconcentration. This could also be a reflection of the technique used to analyse haematocrit in this study. Haematocrit was measured as the volume taken up by erythrocytes within a blood sample and given the increase in blood plasma osmolality in these animals (Figure

2.3.2.3) there may be a decrease in erythrocyte cell volume during acute transfer from 80% to 100% SW. It is possible therefore that any increase in the concentration of erythrocytes resulting from the haemoconcentration associated with acute transfer to increased salinity is offset by a decrease in erythrocyte cell volume. Hence their proportional volume within a blood sample is not significantly altered during the transfer. However, these two factors also oppose each other in animals undergoing acute transfer from 120% to 100% SW, although their affects on proportional erythrocyte volume in the blood are reversed as compared to animals acutely transferring from 80% to 100% SW. The fact that there is a significant change in the haematocrit of animals undergoing acute salinity transfer from 120% to 100% SW, but not in those from 80% to 100% SW, suggests that changes in erythrocyte cell volume are not the cause for this discrepancy.

One final explanation of the unchanged haematocrit in *S. canicula* undergoing acute transfer from 80% to 100% SW is the formation of blood clots due to internal bleeding following the surgical procedures conducted on these animals (Section 2.2.4). *Post mortem* analysis of the animals used in this group revealed that the incidence of clot formation was greater in this experimental group, due to a lack of refinement in surgical technique. Animals chronically acclimated to 100% and 120% SW were studied after those from 80% SW and surgical techniques had been refined from experience. The formation of blood clots would remove erythrocytes from the blood circulation and thereby artificially reduce the haematocrit as measured in this study. This could also negate any haemoconcentration associated with acute transfer to increased salinity. Analysis of haemoglobin concentration and actual cell counts via light microscopy would have been highly beneficial in interpretation of the haematocrit data.

For *S. canicula* acclimated to 120% SW a significant decrease in plasma osmolality was recorded after 4 hours of the acute transfer to 100% SW (Figure 2.3.2.3). Unlike animals acclimating from 80% SW, during the acute transfer these animals did render plasma osmolality to a level equivalent to that of the control animals. This was the result of decreases in plasma urea and Cl^- (and Na^+) concentration (Figures 2.3.2.4 and 5). A major source of these decreases in osmolyte concentration may be a rapid increase in blood volume during the early stages of transfer. Such an increase in blood volume would account for the rapid decrease in blood haematocrit over the first 4 hours of the acute transfer (Figure 2.3.2.6). However, the decrease in urea occurs after 12 hours, whereas that in Cl^- occurs after 6 hours. The fact that plasma urea and Cl^- concentrations decrease at markedly different rates is illustrative of the different rates of efflux of these osmolytes. Plasma levels of urea and Cl^- are not significantly different to the control animals after 2 and 6 hours respectively. Clearly then the output of the kidney and rectal gland must be mediated throughout periods of acute transfer to hyposaline conditions so as to lower plasma concentrations of these osmolytes to levels equivalent to those of animals long term acclimated to 100% SW. Indeed, urine flow rate is significantly increased in *S. canicula* after acclimation to hyposaline conditions (Wells *et al.*, 2002). Further investigation into the activity of the rectal gland during these acute transfer periods would be of great importance in understanding the osmoregulatory response of elasmobranchs to salinity change. Rectal gland activity is discussed in detail below (Chapters 4, 5, and 6)

It is therefore evident that a multitude of osmoregulatory actions occur in *S. canicula* in response to acute salinity transfer. There is a change in blood haematocrit associated with acute salinity transfer which is suggestive of changes in blood volume. Plasma

concentrations of Cl^- and Na^+ are altered relatively quickly in response to acute transfer. This is most probably achieved through alterations in the fluxes of these ions with the environment through modifications in the activity of the rectal gland and the gut, and possibly the gills (Sections 5.1 and 4). Slightly more delayed than this change in Cl^- and Na^+ is a change in plasma urea concentration. Urea levels are likely decreased through an increase in urine flow rate, but the mechanisms for increasing plasma urea concentration do not show any effect in the first 12 hours of transfer. It is likely that the elevated plasma urea levels seen in long term acclimation to increased salinity are achieved through a decrease in urine flow rate and/or an increase in the activity of the OUC and urea synthesis. The differences between urea levels in captive and wild *C. leucas* give further support to the concept that dietary intake may be crucial for elevating plasma urea levels (Armour *et al.*, 1993a).

The magnitude of these changes in haematic parameters and the rates at which they occur during salinity transfer may well vary between different species of elasmobranch. The degree of these differences and the duration which they persist may be representative of the different capacity for euryhalinity. For *S.* canicula alterations in the plasma concentrations of Cl^- and Na^+ occur early on during salinity transfer and persist into long term acclimation. This is likely to be a reflection of the availability of these ions in the marine environment. Importantly, changes in the concentrations of these ions do not appear to be dependent on dietary intake. Changes in urea concentration occur on a larger time scale and may be highly dependent on dietary protein intake. The changes in plasma osmolyte concentrations, and more importantly those in blood haematocrit could be descriptive of changes in the blood volume of elasmobranchs during salinity transfer. The importance of changes in blood volume as a stimulus for osmoregulatory

organs has been highlighted above. Therefore practical assessment of elasmobranch blood volume would not only give a more complete picture of the changes in haematic parameters during salinity transfer, but also give further insight into the stimulation and control of elasmobranch osmoregulation. Surgical procedures and acute salinity transfers were possible in *S. canicula* but not in *C. leucas*. For these reasons blood volume was investigated only in *S. canicula*.

Chapter 3: Blood volume

3.1 Introduction

Assessment of blood volume is of key importance in fully understanding osmoregulation in elasmobranchs, as has been previously discussed (Section 2.4). Changes in blood volume are associated with changes in blood haematocrit, as variation in plasma volume alters the relative concentration of erythrocytes. Within the literature there are numerous examples of haematocrit studies on elasmobranchs (Table 3.1.1), some of which show changes with acclimation to salinity change and some of which do not. Changes in haematocrit are important because they are suggestive of, but not evidence for, changes in blood volume associated with chronic salinity transfer. Similarly, a more constant blood haematocrit during chronic acclimation to salinity change suggests a lesser affect on blood volume. A smaller degree of haemodilution may therefore be encountered by fully euryhaline species, such as *C. leucas*, as compared to partially euryhaline species, such as *S. canicula*. Given the probable central role played by changes in blood volume in the cascade of osmoregulatory processes during acute salinity transfer, smaller changes in blood volume are descriptive of tighter regulation of osmotic and diffusional fluxes. This is intuitive, greater ability to maintain haematic parameters at different salinities are naturally associated with more euryhaline elasmobranchs. Whereas, salinity transfers of high magnitude conducted on *S. canicula* tend to result in increased mortality over a period of days, suggesting that partially euryhaline elasmobranchs fail to regulate haematic parameters completely under such conditions.

Altered blood haematocrit		Stable blood haematocrit	
Species	**Reference**	**Species**	**Reference**
S. canicula	(Table 2.3.1.1)	*S. canicula*	(Hazon and Henderson 1984)
R. erinacea	(Goldstein and Forster 1971)	*C. leucas*	(Table 2.3.1.2)
		C. leucas	(Thorson et al. 1973)
		H. portusjacksoni	(Cooper and Morris 1998)
		T. testacea	(Cooper and Morris 1998)

Table 3.1.1 – State of blood haematocrit of different species of elasmobranchs in response to chronic transfer (> 72 hours) to altered environmental salinity.

Interestingly, there are conflicting records for the haematocrit of *S. canicula* at different salinities. Hazon and Henderson (1984) recorded no significant differences in the haematocrit of animals after 14 day acclimation to a range of salinities (50 – 140% SW). However, results from this study have demonstrated significant differences after 14 day acclimations between 80, 100, and 120% SW in the same species (Table 2.3.1.1). This is probably due to the larger sample size utilised in this study.

Although numerous attempts have been made to investigate blood volume in a variety of species (Hazon, *pers. comm.*), few studies have quantitatively assessed the blood volume of elasmobranchs (Table 3.1.2). Practical assessment of blood volume typically involves the introduction of a marker substance into the subject. This then mixes within the vascular space and becomes diluted. Sampling of the blood and subsequent measurement of the marker then permits a dilution factor to be calculated. However, the nature of the chosen marker is of vital importance, particularly in elasmobranchs.

Species	Blood volume (ml 100 g^{-1} BM)	Reference
Raja binoculata	8.0	(Thorson 1958)
Raja rhina	7.2	(Thorson 1958)
N. brevirostris	7.0	(Thorson 1958)
Carcharhinus nicaraguensis	6.8	(Thorson 1958)
G. cirratum	6.8	(Thorson 1958)
S. acanthias	6.8	(Thorson 1958)
	6.6	(Opdyke et al. 1975)
Hydrolagus colliei	5.2	(Thorson 1958)
S. canicula	6.8 / 4.1 *	(Tort et al. 1991)

Table 3.1.2 – Blood volume of elasmobranchs obtained using dye dilution techniques. Volumes are expressed as ml per 100 gram of body mass. (*) denotes corrected value for binding affinity of Evans blue.

All of the studies on elasmobranch blood volume to date have used a dye dilution method utilising T-1824 (Evans blue) and back-extrapolation of the concentration/time curve. This has lead to a possible overestimation of blood volume due to the binding affinity of the dye. Tort and co-workers (1991) described the possible sources of this overestimation. Once injected into the blood Evans blue binds to the protein fraction, mostly to albumin proteins (Freedman and Johnson 1969). It is well accepted that the albumin concentration in elasmobranch blood is low (Irisawa and Irisawa 1952), although Evans blue does bind to elasmobranch globulins to a greater extent than others (Tort et al. 1991). However, overall binding of Evans blue is lower than in other vertebrates with high blood serum albumin levels, with around 40% of injected die failing to bind to the protein fraction (Tort et al. 1991). Values for blood volume obtained via this method may therefore be unreliable, as protein binding of Evans blue may differ from species to species. For example, a protein fraction with some albumin properties has been recorded in some species of carcharhinids but not in others (Yanagisawa and Hashimoto 1984).

Other dye dilution experiments have utilised fluorescent-labelled hydroxyethyl starch (HES), typically with fluorescein isothiocyanate (Thomas et al. 2000; Massey et al. 2004). In general values obtained using these markers are lower than those from albumin dyes. Such studies have been limited to human clinical trials and the behaviour of the marker in elasmobranch systems is unknown.

There are inherent dangers with introducing substances into the vascular space without a complete understanding of their natural occurrence. Marker dilution assessment of blood volume depends on a uniform distribution of that marker and a predictable and

quantifiable mixing within the vascular space. Any breakdown of the marker or movement from the vascular space must be accounted for. Therefore utilising protein bound dyes and labelled starch in elasmobranch species are not robust methods of assessing blood volume, unless species and salinity specific concentrations of those substances have been recorded.

Other, non-invasive techniques have also been developed in human clinical trials, such as impedance cardiography (Von Rueden and Turner 1999), and the oesophageal Doppler (Gan 2000). Whilst these hold possibility for future assessment in non-mammalian vertebrates, current technology and costs restrict their application. Further investigation and advances in such technologies do hold great promise for assessment of blood volume in species which react adversely to anaesthesia or are difficult to confine, such as *C. leucas*.

Other than dye dilution, the other common methods of assessing blood volume in fish involve the use of radioactive markers. This can be through isotopically labelled microspheres (Kent and Olsen 1982), ^{125}Iodine (I) bovine serum albumin (BSA) (Gingerich and Pityer 1989), or ^{51}Chromium (Cr) labelled erythrocytes (Conte et al. 1963; Duff et al. 1987; Gingerich et al. 1987; Gingerich and Pityer 1989; Gingerich et al. 1990). Microspheres by their nature become trapped in the fine capillaries of tissues and as such are useful for assessing blood flow to specific organs. However, their use as a means of assessing total body blood volume is limited by this feature. Use of ^{125}I-labelled BSA in elasmobranchs raises questions similar to those outlined for Evans blue, concerning the behaviour of a substance which may naturally occur in variable quantities in the blood. The level of natural occurrence for the carrier medium greatly

influences the dynamics of the label and its degree of mixing within the vascular space. Gingerich and Pityer (1989) illustrated that assessment of blood volume in the teleost *Salmo gairdneri* via both ^{125}I-labelled BSA and ^{51}Cr-labelled erythrocytes yielded different results. Whole body blood volume was significantly lower when calculated from ^{51}Cr-labelled erythrocytes than when ^{125}I-labelled BSA or both markers were used. They concluded that it was not clear whether this disparity was due to the distribution of erythrocyte poor blood into the secondary circulation, or the result of extravascular exchange of plasma proteins. Protein permeability is high in teleost capillary membranes, and plasma protein retention in the blood has been shown to correlate directly with blood hydrostatic pressure (Hargens et al. 1974). There is therefore large scope for error when assessing blood volume using albumin bound labels, particularly in elasmobranchs which may have highly variable amounts of albumin proteins in the blood serum.

For the reasons detailed above blood assessment in this study was conducted via the use of ^{51}Cr-labelled erythrocytes. Not only do erythrocytes occur naturally in elasmobranch blood, but their concentration can be easily quantified by haematocrit. From a practical viewpoint ^{51}Cr is an ideal marker to use as it has a high energy γ emission which facilitates accurate and rapid measurements. Furthermore, the use of ^{51}Cr-labelled erythrocytes and their relative stability in the vascular space as compared to albumin bound dyes, presents the possibility of prolonged assessment over acute salinity transfer. Once fully mixed in the vascular space any changes in marker concentration would be due to either the break down of erythrocytes or the radioactive decay of the marker, both of which remain reasonably constant over the time period. Therefore upon acute salinity transfer any variation recorded in the experimental groups which is not

recorded in the control group can be assumed to represent a tangible change in the dilution of the marker resulting from a change in blood volume. Assessment of blood volume during both chronic and acute transfer was of key importance to the study as it allowed a quantification of the concentration and dilution of body fluids associated with acclimation to salinity change. As such this would give great insight into the osmotic stress and response of *S. canicula* during both chronic and acute salinity transfer. This is the fundamental factor in elasmobranch osmoregulation at different salinities.

3.2 Materials and methods

Animals used were identical to those outlined above (Sections 2.2, 2.2.1, and 2.2.2).

3.2.1 Chemicals and equipment

Chemicals and equipment used were identical to those outlined above (Section 2.2.3).

3.2.2 Surgical procedures

The coeliac and mesenteric arteries of *S. canicula* were cannulated in an identical manner as outlined above (Section 2.2.4). The size of incision was smaller (approximately 4 cm) due to the fact that no procedures were carried out on the rectal gland during this study.

3.2.3 Analysis and collection

Blood volume was assessed via modification of the ^{51}Cr-labelled erythrocyte method detailed by Gingerich and co-workers (1987). 2 ml of blood was drawn from the caudal vein of the designated donor animal and centrifuged for 5 minutes at 100 *g* and 10 °C. The plasma portion was removed and the erythrocytes were washed 3 times in volumes of 4 °C Ringer solution equivalent to that of the removed plasma, centrifuging under the same conditions. The erythrocytes were then resuspended in 4 °C Ringer solution to give a final volume of 2 ml. Then ^{51}Cr (Sodium chromate (360 – 600 mCi mg^{-1} Cr), Amersham plc, Little Chalfont, Buckinghamshire) was added to give an activity of 1.0 x 10^8 counts per minute (CPM) ml^{-1}. The erythrocytes were then left overnight in a refrigerator at 10 °C.

The erythrocytes were then centrifuged and washed as described above 4 times in 4 °C Ringer, each time retaining a 200 µl sample of the supernatant to measure three 50 µl replicates in a γ-counter (Minaxi auto-gamma 5000 series, Packard Instrument Company, Downers Grove, Il, USA) to check for haemolysis. Finally the cells were resuspended in 4 °C Ringer to give a final haematocrit of 17% for 100% SW, 13% for 80% SW, and 22% for 120% SW. Triplicate 50 µl samples were then measured in the γ-counter to accurately assess the activity of the final erythrocyte suspension before loading into a 1 ml syringe approximately 1 ml Kg^{-1} body mass for each animal. The mass of the syringes were recorded before and after discharge to accurately calculate the volumes delivered. The specific gravity of blood from donor animals acclimated to each salinity was measured to gain salinity specific mass to volume conversion factors (Table 3.3.1.1).

400 μl of blood was drawn from the mesenteric arterial cannula for osmolyte analysis prior to injection of the labelled erythrocytes. The cannula was then flushed with 320 μl of Ringer solution and 200 IU ml^{-1} heparin and the stopper pin replaced. 200 μl of blood was removed via the coeliac arterial cannula after 0.5, 1, 2, 3, and 24 hours for basal levels and after 0, 2, 4, 6, 8, and 10 hours for acute transfer; the 24 hour basal and 0 hour transfer samples being the same sample. An equivalent volume of the appropriate salinity Ringer solution was then injected via the same cannula to replace the lost volume. In order to assess blood volume triplicate 50 μl samples of whole blood were measured for radioactivity in the γ-counter. Blood volume was then calculated via the method detailed below.

3.2.4 Calculation of blood volume

For assessment of basal blood volume in *S. canicula* the mean number of CPM from the triplicate samples was plotted against time after injection of the ^{51}Cr-labelled erythrocytes, and a linear regression was performed (Curve Expert 1.3, Daniel Hyams, Hixson, TN, USA) (Figure 3.2.4.1). This regression line could be described by the following equation:

$$y = mx + c$$

Where m represents the slope of the line, c the point of intercept with the y axis, y the mean CPM, and x the time after injection. The value of c therefore represents the theoretical CPM at time 0 assuming instantaneous mixing of the labelled erythrocytes. From this value and the known activity injected into the animal a dilution factor, and therefore blood volume, could be calculated:

$$Vol = [\,[\,a / c\,] / Mass\,] * 100 \quad \text{(ml 100 g}^{-1}\text{ body mass)}$$

Where *Vol* represents blood volume, *a* represents the activity injected, and *Mass* being body mass.

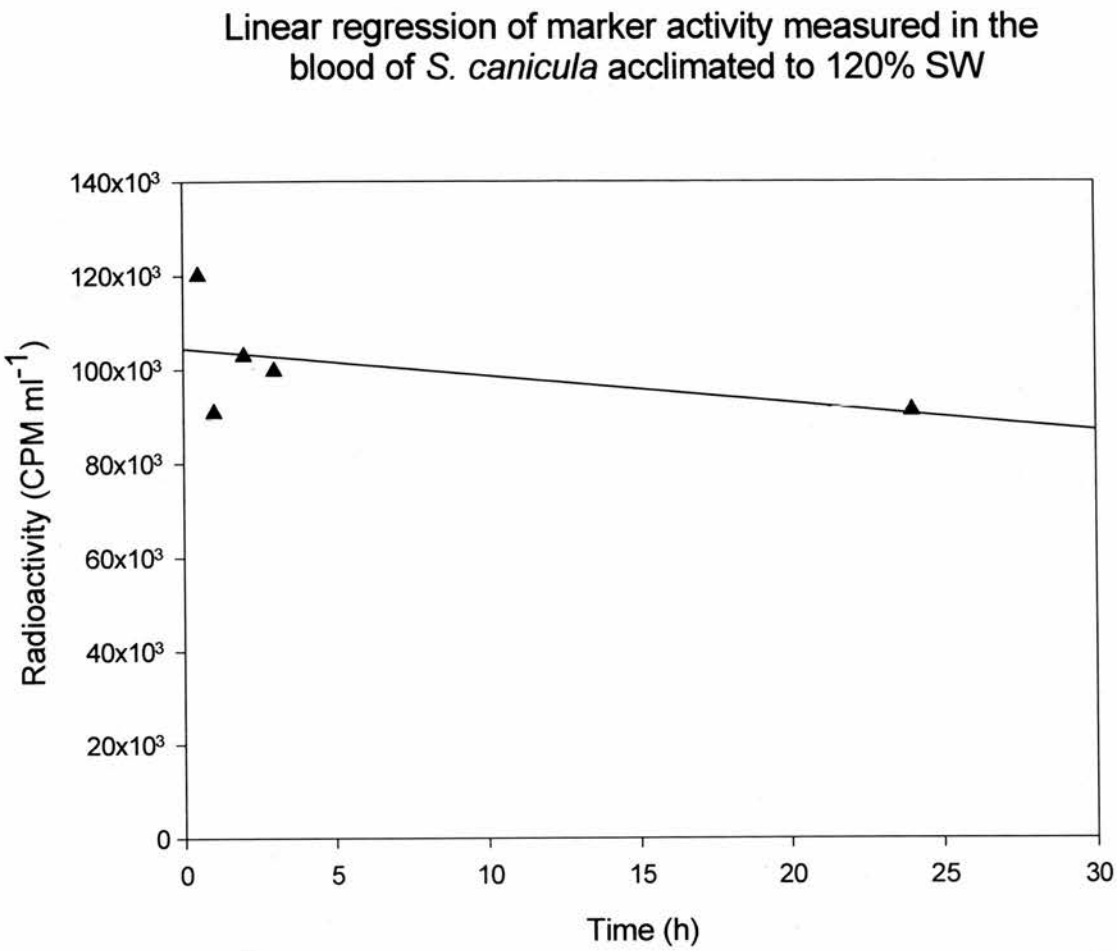

Figure 3.2.4.1 – Typical regression line drawn to calculate the theoretical marker concentration at time zero assuming instantaneous mixing and the slope value associated with marker decay during blood volume assessment in *S. canicula*.

Given that major variations in activity are removed once the labelled cells have thoroughly mixed with the systemic blood, and that the rate of decay is constant, the calculated value of the linear slope (m) can also be applied to later time points to extrapolate back to time 0. Therefore once acute transfer had begun and values for activity in the blood were calculated for each serially taken blood sample, values for the resultant changes in blood volume could be calculated individually for each time point by modifying the equation of the linear regression:

$$c = y - mx$$

This gave new values of the intercept for each time period and hence a different value for activity in the blood at time 0 assuming instantaneous mixing of the labelled erythrocytes. Given that the amount of activity injected into the animal remained constant, the same equation used to calculate blood volume from basal levels remained applicable.

3.2.5 Statistical analysis

All data are presented as means ± SEM. For basal blood volumes statistical analysis was performed via one-way ANOVA and a Tukey post hoc test (InStat) (significance was denoted as * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.005$). Data gathered during the acute transfer studies was analysed in two ways: differences between the two experimental groups and the control group were analysed via one-way ANOVA and a Tukey post hoc test (significance was denoted as * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.005$); differences between values during transfer and at time 0 within each group were analysed via a one-tailed unpaired students t-test with a Welch correction factor (significance was denoted as † $P < 0.05$, †† $P < 0.01$, and ††† $P < 0.005$) (InStat). Once differences had occurred they persisted throughout the transfer, although for clarity differences are only noted at the first and last instances.

3.3 Results

The results for blood volume in *S. canicula* are presented in two sections: a comparison of basal levels in animals acclimated to 80, 100, and 120% SW; and a comparison during acute transfer from all salinities to 100% SW.

3.3.1 Basal levels

Blood specific gravity and volume of *S. canicula* acclimated to 80, 100, and 120% SW are presented below (Table 3.3.1.1). No significant differences were seen in the specific gravity of blood taken from animals acclimated to the three salinities. Animals acclimated to 80% SW had a significantly larger blood volume than those from 100% SW. Animals acclimated to 120% SW had a highly significantly smaller blood volume than those from 100% SW.

Salinity (SW)	Blood specific gravity ($g\ ml^{-1}$)	Blood Volume ($ml\ 100g^{-1}$)
80%	0.99 ± 0.00	6.3 ± 0.2 *
100%	1.02 ± 0.01	5.6 ± 0.2
120%	1.02 ± 0.01	4.6 ± 0.2 **

Table 3.3.1.1 – Blood specific gravity and volume of *S. canicula* after >14 day acclimations to 80, 100, and 120% SW. All values are presented as means ± SEM (n = 9, 9, and 9 respectively for blood specific gravity; n = 7, 7, and 7 respectively for blood volume). Statistical analysis was performed via one-way ANOVA and a Tukey post hoc test. Significant differences from values for 100% SW were denoted as * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.005$).

3.3.2 Acute transfer levels

The data gathered on the blood volume of *S.* canicula acclimated to the three salinities during acute transfer to 100% SW are presented below (Figure 3.3.2.1). The blood volume of animals from 80% SW was significantly different from that at time 0 after 6 hours of acute transfer to 100% SW, and remained so thereafter. The blood volume of animals from 120% SW was significantly different from that at time 0 after 2 hours of acute transfer to 100% SW, and remained so thereafter. The blood volume of the control group did not change significantly during the transfer period. Blood volume in animals from 80% SW started significantly higher than, and after 8 hours was significantly lower than that of the control animals. After 2 hours of the transfer the blood volume of animals from 120% SW was no longer significantly different from that of the control group, and this remained so for the rest of the transfer period. Interestingly, the blood volume of all three groups increased after 2 hours of the transfer, although not always significantly so. At least in part, this can be attributed to the high slope values calculated for the linear regression and the method of calculation used.

The slope values for the linear regressions performed on individual animals are presented below (Table 3.3.2.1).

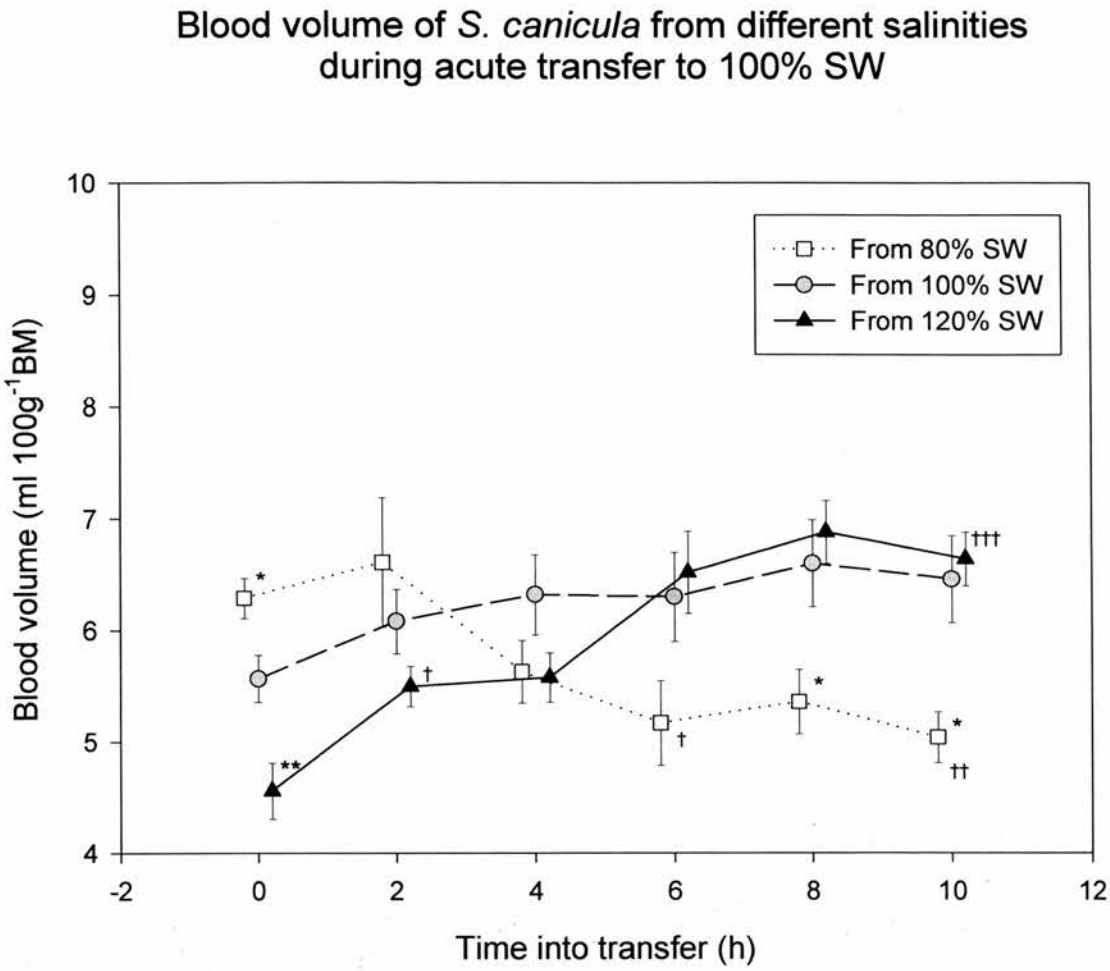

Figure 3.3.2.1 – Blood volume of *S. canicula* acclimated to 80, 100, and 120% SW during acute transfer to 100% SW. Values are presented as means ± SEM (n = 6, 7, and 8 respectively). Statistically significant differences from the control transfer were assessed via one-way ANOVA and a Tukey post hoc test (significance was denoted as * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.005$)); statistically significant differences from values at time 0 were assessed via a one tailed unpaired students t-tests with Welch correction factor (significance was denoted as † ($P < 0.05$), †† ($P < 0.01$), and ††† ($P < 0.005$)). Once differences had occurred they persisted throughout the transfer, although for clarity differences are only noted at the first and last instances.

Sample	Salinity (% SW)		
	80	100	120
1	-16.2	-11.0	-87.6
2	-68.2	-8.8	-113.9
3	-113.1	-22.7	-11.0
4	-190.2	-191.1	-53.7
5	-121.4	-139.2	-34.6
6	-7.6	-103.9	-67.5
7	-6.1	-6.3	-71.3
8	-3.1	–	-27.3
Mean ± SEM	-65.8 ± 24.7	-69.0 ± 28.5	-58.4 ± 11.9

Table 3.3.2.1 – Slope values for linear regression of blood radioactivity during basal periods.

3.4 Discussion

The specific gravities of blood from *S. canicula* acclimated to 80, 100, and 120% SW were not significantly different. This was not expected given the differences in haematocrit previously recorded for similarly acclimated animals (Table 2.3.1.1). These two sets of results suggest that the difference in mass between the erythrocyte and plasma portions of elasmobranch blood is not sufficient to result in any significant change in blood specific gravity following acclimation to altered salinity. However, this data is still important as it provided salinity specific mass to volume conversion factors for the blood volume studies on *S. canicula*.

The blood volume of 100% SW *S. canicula* was calculated as 5.6 ± 0.2 ml $100g^{-1}$ body mass (Table 3.3.1.1). This is consistent with those reported for other elasmobranchs (Table 3.1.2). It also supports the suggestion that the Evans blue method can lead to an overestimation of blood volume in elasmobranchs, although the allowance made for this may be too great. This value is slightly below the standard value (6.8 ml 100 g^{-1} body mass), and slightly above the corrected value (4.1 ml 100 g^{-1} body mass) reported for the species using Evans blue (Tort et al. 1991). The reasons for these differences are not clear. It is possible that they reflect the extravascular exchange of plasma proteins, as suggested by Gingerich and Pityer (1989). However, given the highly variable portion of albumin proteins in the blood of elasmobranchs and the lack of understanding of the *in vivo* behaviour of markers bound to such proteins (Section 3.1), the value reported here from a marker associated with naturally occurring, species specific erythrocytes can be considered the more accurate measurement.

It has also been shown that acclimation of *S. canicula* to 80% SW resulted in an increase in blood volume and an associated decrease in blood haematocrit. Conversely, acclimation to 120% SW resulted in a decrease in blood volume and an associated increase in blood haematocrit. This is the first ever measurement of the effects of salinity acclimation on elasmobranch blood volume. Given the central role which is believed to be played by changes in blood volume in elasmobranch osmoregulation (Shuttleworth 1983; Solomon et al. 1984; Solomon et al. 1985; Shuttleworth and Thompson 1986; Silva et al. 1996; Cooper and Morris 1998; Olson 1999; Silva et al. 1999; Anderson et al. 2002a; Anderson et al. 2002b) these findings are of great importance.

These results provide quantifiable evidence that the partially euryhaline species *S. canicula* experiences appreciable haemodilution and concentration during acclimation to salinity change. This is consistent with the changes in haematocrit which have been previously reported (Table 2.3.1.1). However, such changes in blood haematocrit have been shown to be species specific (Table 3.1.1), and possibly a reflection of life history. Therefore caution must be used when extrapolating the results of salinity transfer on the blood volume of *S. canicula* to elasmobranchs in general. It is possible that species with a greater degree of euryhalinity would show a reduction or even a lack of changes in blood volume upon chronic acclimation to different salinities. Clearly more species of elasmobranch need to be assessed for the affects of salinity on blood volume.

The blood volume of *S. canicula* was also assessed during acute salinity transfers from 80, 100, and 120% to 100% SW (Figure 3.3.2.1). The fact that there were no significant changes in the blood volume of animals undergoing the control transfer from 100% to

100% SW was as expected given the constant environmental conditions (Figures 2.3.2.1 and 2). This is also suggestive that the marker used to assess blood volume in this study is stable *in vivo* since there was no significant decrease in recorded activity in the blood of the control animals once complete mixing had occurred.

Animals acclimating from 80% to 100% SW started with a significantly increased blood volume and had significantly reduced this after 6 hours of the transfer. Furthermore, there appears to be overcompensation in regulatory volume decrease in these animals as blood volume was significantly lower than that of animals long term acclimated to 100% SW after 8 and 10 hours. Upon initiation of transfer these animals are in a hyposmotic state and will therefore osmotically lose water across the semi-permeable surfaces. Furthermore, these animals also have a significantly increased basal urine flow rate resulting from chronic acclimation to reduced salinity (Wells et al. 2002). These animals will continue to lose water, even after urine flow rates have returned to levels equivalent to those of chronically acclimated 100% SW animals, whilst plasma osmolality remains hyposmotic to the environment. This overcompensatory loss of blood volume then provides the stimulus for a drinking response. Indeed, the time period of this overcompensatory decrease in blood volume coincides with that in which a drinking response is typically recorded in similarly transferred *S. canicula* (Anderson et al. 2002b). The drinking response is of vital importance in increasing plasma Na^+ and Cl^- concentrations, and therefore overall plasma osmolality.

This overcompensatory decrease in blood volume in *S. canicula* undergoing acute transfer from 80% to 100% SW is not consistent with an unaltered blood haematocrit in similarly transferred animals (Figure 2.3.2.6). Such decreases in blood volume should

result in an increased blood haematocrit if the number of erythrocytes in the blood remains constant. The lack of any significant variation in the haematocrit of these animals can be explained in a number of ways. Haematocrit in this study was measured as the volume taken up by erythrocytes within a blood sample (Section 2.2.5). Given the increase in blood plasma osmolality in these animals (Figure 2.3.2.3) there may be a decrease in erythrocyte cell volume during acute transfer from 80% to 100% SW. It is possible therefore that any increase in the concentration of erythrocytes resulting from decreased blood volume is offset by a decrease in erythrocyte cell volume, and hence their proportional volume within a blood sample. In this way these two opposing factors may negate each other and thereby result in no net change in haematocrit, as measured in this study. However, these two factors also oppose each other in animals undergoing acute transfer from 120% to 100% SW, although their affects on proportional erythrocyte volume in the blood are reversed as compared to animals acutely transferring from 80% to 100% SW. The fact that there is a significant change in the haematocrit of animals undergoing acute salinity transfer from 120% to 100% SW, but not in those from 80% to 100% SW, suggests that changes in erythrocyte cell volume are not the cause for this discrepancy.

The lack of any significant changes in the haematocrit of *S. canicula* undergoing acute transfer from 80% to 100% SW is more likely due to the nature of surgical procedures performed on each group of animals and the ongoing refinement in surgical technique throughout the study. Animals used for the measurement of blood haematocrit underwent a longer period of surgery and the cannulation of 4 separate vessels: the coeliac and mesenteric arteries (Section 2.2.4), as well as the rectal gland vein and duct (Section 4.2.2). Animals used for the measurement of blood volume underwent a shorter

period of surgery, had a smaller size of initial incision, and had only the coeliac and mesenteric arteries cannulated (Section 3.2.2). Furthermore, animals used to measure haematic parameters following acute transfer from 80% to 100% SW were the first experimental group for the entire study (January 2002). Whereas animals used to measure blood volume following the same acute transfer were the penultimate experimental group for the entire study (December 2004). There was a high level of refinement in surgical techniques over this time period and the proportion of animals with visible blood clotting upon *post mortem* analysis was reduced. The formation of blood clots will necessarily reduce the measured haematocrit by removing erythrocytes from the circulating blood volume. This would account for the discrepancy between the haematocrit (Figure 2.3.2.6) and blood volume (Figure 3.3.2.1) of *S. canicula* undergoing acute transfer from 80% to 100% SW.

Animals acclimating from 120% to 100% SW started with significantly decreased blood volume and had significantly increased this after the first 2 hours of transfer. The nature of this increase in blood volume was such that after 2 hours the blood volume of these animals was not significantly different to that of animals long term acclimated to 100% SW. These results show that regulatory increases in blood volume occur very rapidly upon transfer to reduced salinity, and support the concept of this playing a stimulatory role for subsequent osmoregulatory responses. This increase in blood volume is likely a reflection of the increased gradient for the osmotic influx of water across the semi-permeable surfaces.

There were some problems with assessing the blood volume of *S. canicula* in this manner. Repeated sampling of animals and the removal of blood could have effects on

blood volume. This was minimised by replacing the blood lost during sampling with an equivalent volume of Ringer solution. It can be seen that this does affect the blood haematocrit of *S. canicula* due to the associated loss of erythrocytes (Figure 2.3.2.6). This removal of plasma erythrocytes may have artificially increased the concentration of ^{51}Cr-labelled erythrocytes in the vascular space, and hence influenced blood volume calculations. However, the quantity of erythrocytes in a 200 µl blood sample is minimal when compared to that in a blood volume of 5.6 ml 100 g^{-1}. Furthermore, no significant differences were seen in the blood volume of animals undergoing the control transfer from 100% to 100% SW.

This suggests that this factor did not influence the results. The erythrocytes lost during sampling could have been replaced by resuspended cells taken from the donor animals. However, the injection of Ringer solution after the removal of blood samples not only replaced the lost volume but also cleared the cannula of blood and prevented the formation of blood clots. Replacing the lost erythrocytes with cells resuspended in Ringer solution would have increased the risk of clot formation and therefore jeopardised the experiment.

There were also some problems with the calculation of blood volume during the acute transfer periods. The linear regression was calculated from blood samples taken after 0.5, 1, 2, 3, and 24 hours of injecting the labelled erythrocytes. CPM values for samples taken in the first 3 hours proved to be highly variable, due to mixing of the marker in the blood system of a predominantly sedentary animal. This early variation translated into a wide variation in the calculated slope values for the linear regression lines (Table 3.3.2.1). Due to the nature of the calculation for blood volume this variation becomes

more prevalent as time (and therefore distance from time 0) increases. Therefore whilst calculations for basal blood volume are accurate, exact values expressed for the period of acute transfer may be overestimated. This problem could be removed by replacing intensive sampling in the first three hours with single samples taken further apart during the basal study period. This would lead to a more accurate calculation of the linear regression and greater confidence in later time points. However, whilst exact values during acute transfer may have been overestimated the trends in the results are accurate, as are the values calculated for basal blood volumes in chronically acclimated animals.

Assessment of blood volume in *S. canicula* has therefore given great insight into the changes in volaemic parameters associated with both chronic and acute transfer to changes in environmental salinity. This insight is of fundamental importance in understanding the osmoregulatory responses of elasmobranchs during variations in salinity. Only through quantification of the haemodilution and concentration experienced by elasmobranchs during salinity transfer *in vivo* can the influence of volaemic change on osmoregulatory mechanism *in vitro* be validated. In particular these results have provided further evidence to support the concept of increases in blood volume occurring rapidly during acute transfer and therefore coinciding with expected periods of increased rectal gland secretion. The volaemic affects on rectal gland secretion *in vitro* have been well documented (Solomon et al. 1984; Solomon et al. 1985; Olson 1999). Furthermore, it has already been shown that variations in plasma Cl^- (and Na^+) levels also occur early during acute salinity transfer (Figure 2.3.2.4). Given these two factors it is clear than an assessment of *in vivo* rectal gland activity would give a greater level of understanding in elasmobranch euryhalinity.

Chapter 4: *In vivo* rectal gland secretion

4.1 Introduction

The rectal gland is the only organ in elasmobranchs which is capable of producing a solution with levels of Na^+ and Cl^- which are more concentrated than those of the blood plasma. Therefore it is the only means of net Na^+ and Cl^- excretion in marine elasmobranchs. As such the gland must play a pivotal role in ionic regulation, particularly during salinity transfers. The cost of NaCl secretion by the rectal gland has been estimated at just 0.5% of the standard metabolic rate (Morgan *et al.*, 1997) although this makes no account for the intermittent nature of gland activity. The metabolic cost during periods of active secretion is likely to be considerably greater. Given the changes in plasma Cl^- concentration which occur during acute transfer in *S. canicula* (Figure 2.3.2.4), the importance of the glands function cannot be overlooked.

The gland itself is a blind-ending, usually bullet-shaped tube in the dorsal mesentery, which is suspended above the valvular intestine. It is attached to the intestine postvalvularly. Rectal glands vary in size and shape depending on the species of elasmobranch, and its life history. It has been reported that glands are smaller in euryhaline, and particularly in freshwater animals, than in marine species (Oguri, 1976). Recent work has shown that in shorter FW systems where animals are more likely to be exposed to salinity gradients, there is no significant difference in rectal gland size between FW and SW individuals (Pillans and Franklin, 2004). If animals in a large and stable FW system do have proportionally smaller rectal glands, this is presumably due to the lower influxes and variations of Na^+ and Cl^- in a more dilute environment.

The rectal gland is comprised of a complex mixture of connective, nerve, and smooth muscle tissue, and at least three types of epithelia: secretory tubule, central duct and

endothelium (Valentich *et al.*, 1996). The adult rectal gland is comprised of three concentric tissue layers arranged around the lumen of the central canal (Figure 4.1.1). The outer capsule is covered by a visceral peritoneum and is permeated with blood vessels, smooth muscle, connective tissue, and a network of nerves (Bulger, 1963).

The middle secretory parenchyma consists of radially orientated tubules and an extra tubular matrix of connective tissue interspersed with capillaries and nerve fibres (Bulger, 1963). Occasionally a single tubule may transverse the entire region, but more commonly a single tubule will diverge into three to five branches as it radiates from the central canal.

This results in tubules being tightly packed in the peripheral portion of the parenchyma (Bulger, 1963). The tubules are lined with a single type of columnar cell and have a narrow lumen in this region of the secretory tissue (Eveloff *et al.*, 1979). In these dense areas there is a highly ordered radial arrangement, although some tubules may turn parallel to the lumen of the central canal (Bulger, 1963). The extra tubular matrix is compact with capillaries closely associated with, and running parallel to the tubules.

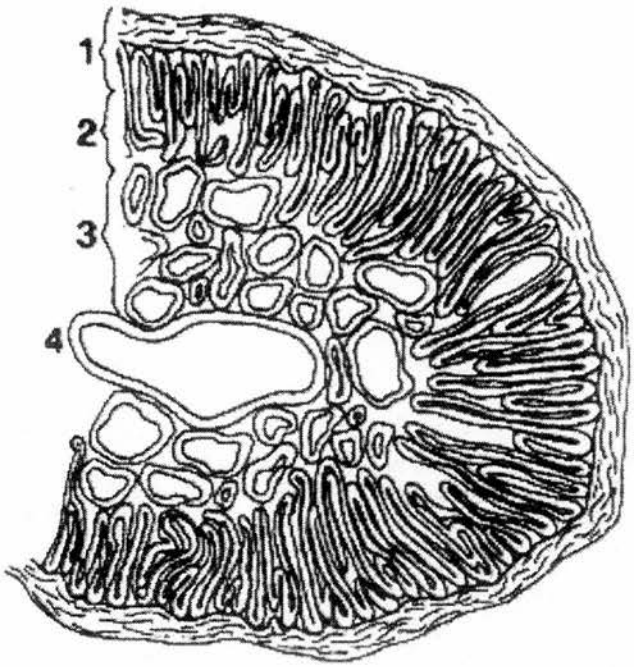

Figure 4.1.1 - Diagram of the rectal gland from *S. canicula*. Cross section showing 4 concentric zones: (1) capsular and subcapsular zone, (2) outer layer of radial tubules, (3) inner layer of branching tubules, and (4) the central canal (Masini *et al.*, 1993).

In the central region of the parenchyma, tubules are more randomly orientated and have larger tubular lumens. In this area the capillaries are often replaced by venous sinuses, and the matrix is less compact (Bulger, 1963). Nerve fibres with VIP (Section 1.11.1) immunoreactivity are closely associated with the tubular cells in *S. acanthias* (Stoff *et al.*, 1988). These nerve fibres are well ordered in the peripheral parenchyma, and ramify extensively in the venous sinusoids of the inner parenchyma (Bulger, 1963). This suggests a greater degree of neural influence, and hence an increased potential for the subsequent modification of rectal gland secretory output in this region. In the caudal end of the gland, where it is embedded in the postvalvular intestine, the secretory parenchyma is reduced and ductal epithelium predominates (Bulger, 1963).

The secretory tubules of the rectal gland generally consist of a single type, and a single layer of columnar epithelium (Eveloff *et al.*, 1979). Two varieties of cells have been categorised: 'light' and 'dark' cells, based on the density of the cytoplasmic matrix (Bulger, 1963). It remains unclear whether these are different types of cell, or if they represent different states of activity. The secretory cells have two distinctive features: numerous mitochondria and extensive basolateral membrane infoldings (Ernst *et al.*, 1981).

There are generally between one and five parallel strands of tight (occluding) junctions which separate adjacent cells. These junctions are relatively shallow but have a very high length density. The values vary according to species and life history but a typical value is seen in *S. acanthias* of 86 ± 5.7 m cm^{-2} (Forrest *et al.*, 1982). The network of junctions provide an extensive, selective paracellular diffusional pathway which is important in Na^+ secretion, as well as restricting the diffusion of other ions into the

lumen of the tubule (Section 1.5) (Forrest *et al.*, 1982). The length density of the junctions in *S. acanthias* is greater in the inner secretory parenchyma (102 ± 4.7 m cm^{-2}) than in the outer region (80 ± 6.7 m cm^{-2}), which was thought to indicate differences in regional secretory activity (Forrest *et al.*, 1982). The anatomy of the junctions remains unchanged in 68% SW acclimated *S. acanthias* as well as during maximal stimulation of perfused glands (Forrest *et al.*, 1982). Secretion rate is therefore independent of junction morphology.

The vasculature of the rectal gland has been studied using a variety of methods: vinyl acetate (Bulger, 1963), latex infusion (Hayslett *et al.*, 1974), and using scanning electron microscopy and methyl methocrylate corrosion (Kent and Olsen, 1982). The gland is supplied by the posterior-mesenteric or rectal gland artery which branches from the dorsal aorta. The artery enters the anterior (distal) third of the gland and splits into the anterior and posterior rami which travel the length of the dorsal aspect of the gland (Kent and Olsen, 1982). The exterior of the gland is encompassed by a network of paired circumferential arteries which branch off from the rami every 3 mm. These arteries in turn give rise to smaller branches, thus forming an arteriolar plexus in the outer capsule (Kent and Olsen, 1982). The large posterior ramus continues into the postvalvular intestine (Figure 4.1.2).

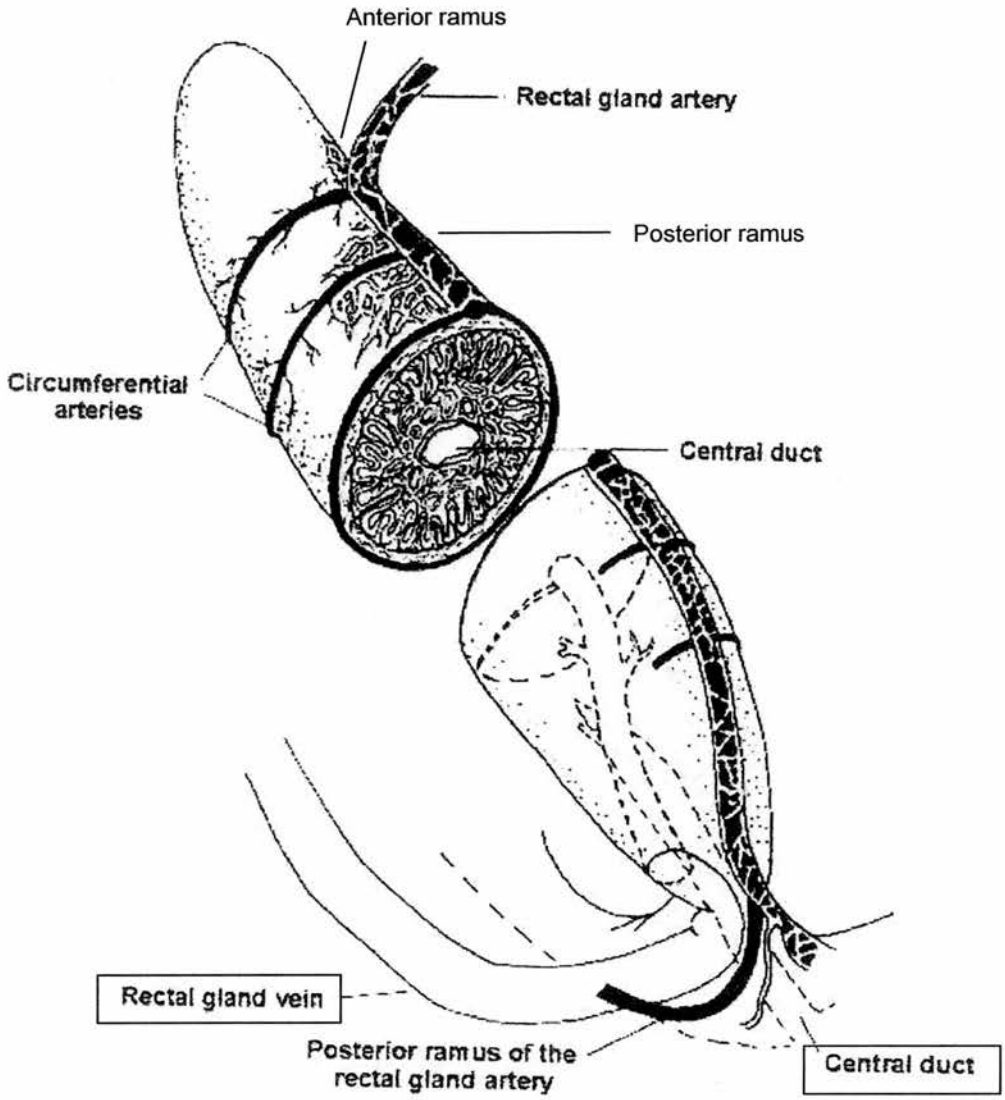

Figure 4.1.2 - Schematic diagram of the vascularisation of the rectal gland of *S. acanthias*. Vessels with boxed labels were cannulated during surgery (modified from Hazon *et al.*, 1997b).

The capsular arterioles perfuse two distinct circulations: either to the capillaries in the secretory parenchyma, or directly to the capsular venules through arteriovenous anastomoses (AVA's), thereby greatly reducing blood supply to the secretory tubules (Kent and Olsen, 1982) (Figure 4.1.3). Constrictions have been noticed in the AVA's which supports the idea that blood flow to and around the rectal gland is tightly regulated (Kent and Olsen, 1982).

The capsular venules are commonly paired on either side of the corresponding arteries and arterioles. Numerous small vessels arise from these venules and form a vascular mesh over the arterial vasculature (Kent and Olsen, 1982). These venules give rise to larger veins forming a dense venous plexus in the capsule beneath the circumferential arteries. This is drained by a series of larger veins which travel back along the structure of the rectal gland artery to posterior cardial veins, or with the posterior arterial ramus into the postvalvular intestine (Kent and Olsen, 1982).

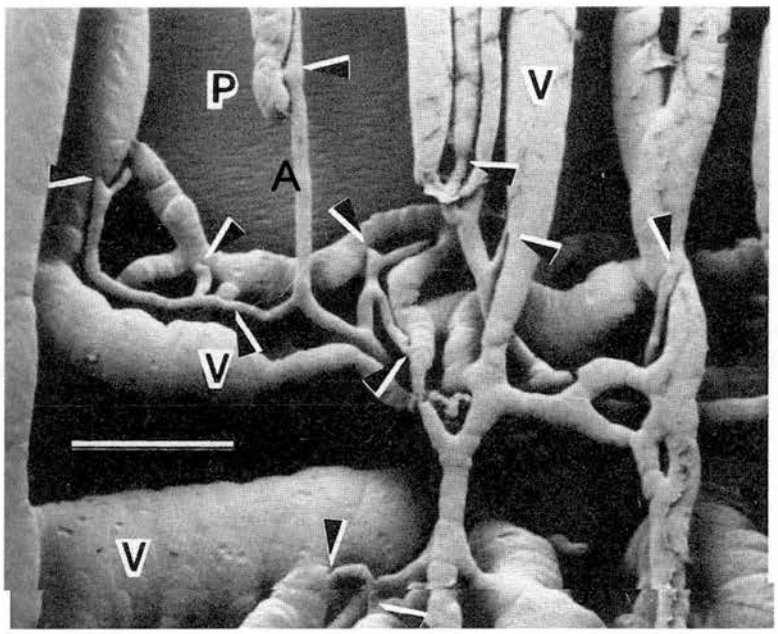

Figure 4.1.3 – Arteriovenous anastomoses in the rectal gland of *S. acanthias*. The AVA's (►) are situated between capsular arterioles (A) and venules (V), which encompass the posterior ramus (P) of the rectal gland artery. Scale bar of 700 μm (Kent and Olsen, 1982).

The fine vasculature of the secretory parenchyma consists almost exclusively of capillaries or post capillary venules. These originate in the capsular arteriolar plexus and are orientated radially through the extra tubular matrix. Anastomotic branches interconnect adjacent capillaries and these are more prevalent in the inner secretory parenchyma where the vasculature is more sinusoidal (Kent and Olsen, 1982). The secretory capillaries have a fenestrated endothelium and lie in close proximity to the basal membranes of the secretory epithelial cells (Ernst *et al.*, 1981). It has been reported that blood flow in this region is parallel with secretory flow and there is therefore no counter current multiplication of electrolytes in the rectal gland (Kent and Olsen, 1982). However, Newbound and O'Shea (2001) recently reported that flow in secretory tubules is in the opposite direction to that of the capillaries in the rectal gland of *H. portusjacksoni*, a partially euryhaline species which ventures from SW into the estuarine environment. This could therefore represent a morphological difference between euryhaline and stenohaline species permitting counter current multiplication in the secretory tubules. However, this could also be a unique feature of *H. portusjacksoni*; clearly other species with varying degrees of euryhalinity must be studied.

The innermost sinusoids coalesce into one of several main veins which boarder the rectal gland central duct. These eventually ramify into a single vein which exits the posterior of the gland in the tissue of the excretory duct (Kent and Olsen, 1982).

In summary, blood which flows along the rectal gland artery can flow in three possible routes: it can flow directly into the postvalvular intestine via the posterior ramus and effectively bypass the gland altogether. Secondly, the blood can enter the capsular sinusoids via the AVA's, resulting in partial blood flow to the secretory tissues of the

gland. Thirdly, the blood can perfuse the capillaries of the secretory parenchyma and flow out through the central vein, resulting in maximal blood supply to the secretory tissues. With such large scope for variation in blood flow to the gland it is possible that variation in blood flow is at least partly responsible for changes in rectal gland secretion rate.

Blood flow in the rectal gland has been illustrated as capable of sizeable fluctuations: out of 33 free-swimming fish, 21 showed less then 1% of total blood volume entering the gland, and 12 had between 2% and 7% (Kent and Olsen, 1982). This suggests a pattern of intermittent blood flow and highlights the role of the gland in osmotic homeostasis. It is concurrent with the intermittent nature of rectal gland activity (Burger, 1967), suggesting minimal blood flow to the gland during periods of inactivity. This theory was proven experimentally using microsphere studies of blood flow and relating them to *in vivo* rates of rectal gland secretion (Kent and Olsen, 1982).

It has been theorised that rectal gland secretion rate correlates to the concentration of Cl^- ions in the arteriovenous blood and/or changes in the degree of perfusion of blood through the rectal gland (Burger, 1962). This was questioned by the findings of laboratory work on perfused rectal glands of *S. canicula* which showed no change in blood flow even after a twenty-fold increase in Na^+ secretion (Solomon *et al.*, 1984; Shuttleworth and Thompson, 1986). Little work has been carried out regarding the possible role of vascular perfusion on rectal gland secretion rate; the majority has been carried out at the level of ion transport (Section 1.5). Recent work has shown that blood flow to the secretory epithelia of the rectal gland is greater in fish acclimating to reduced salinities than those seen in fish acclimating to increased salinities or long term

acclimated to SW (Anderson *et al.*, 2002a). It has also been shown that intravascular volume expansion is a potent, and possibly the primary stimulus for rectal gland secretion (Erlij and Rubio, 1986). This is suggestive of increased blood supply during periods of secretory activity however a direct relationship remains to be established.

Rectal gland cell volume expansion has been illustrated as a major stimulus of rectal gland secretion (Solomon *et al.*, 1985). Cell volume in the epithelial cells of the secretory tubules is regulated by the structural organisation of actin within the cell (Henson *et al.*, 1997). Transient loss of cytoskeletal (F-actin) organisation at the basolateral cell face, induced by hypotonicity, brings about the selective efflux of organic osmolytes. This produces a regulatory volume decrease in the rectal gland cells of *S. acanthias* (Ziyadeh and Kleinzeller, 1991). The cytoskeleton may also be important in mediating the response of the rectal gland to CNP (Sections 1.11 and 6.1). Silva and Epstein (2002) showed that CNP stimulation of the rectal gland in *S. acanthias* was highly dependent on the action of the actin cytoskeleton and myosin light chains. Disruption of the actin cytoskeleton or inhibition of myosin light chain kinase strongly inhibited CNP stimulated Cl^- secretion, although stimulation with VIP (via the cAMP cascade) was virtually unaffected by similar cytoskeletal effects (Silva and Epstein, 2002). Not only do these studies show the effect of volume expansion on rectal gland secretion rate, but also the complex action of hormonal and neural control factors. These are discussed in detail elsewhere (Sections 1.5, 1.8 - 12, and 6.4).

The secretion rate of the rectal gland is often unaffected by blockage of nerves, although stimulation of isolated perfused glands with veratrine was prevented by the nerve channel blockers tetrodotoxin and procaine, and this was not seen in preparations of

dispersed cells (Stoff *et al.*, 1988). The fact that the rectal gland is often unaffected by nerve blockage suggests that stimuli affecting the rectal gland are typically carried in the blood, such as hormonal cues or ion concentrations. A hormonal signal is consistent with the constant lag time between external stimuli and increases in rectal gland secretion rates which have been seen in a variety of experiments (Erlij and Rubio, 1986; Anderson *et al.*, 1995a; Anderson *et al.*, 2002a). However, given the extensive ramification of nerve fibres in the inner secretory parenchyma of the rectal gland of *S. acanthias* (Kent and Olsen, 1982), the recorded release of VIP from nerves within the rectal gland of *S. acanthias* (Silva *et al.*, 1987; Chipkin *et al.*, 1988), and the stimulatory effect of VIP on the rectal gland of *S. acanthias* (Stoff *et al.*, 1977a; Silva *et al.*, 1987), neural influences may be more prevalent in some species.

This may also represent a difference in the nature of hormonal and neural influences: hormonal signals have an associated lag time and may be involved with chronic changes in rectal gland secretion, whereas neural signals are faster acting and may be more important in acute responses. Endocrine factors which affect the rectal gland have been described elsewhere along with a model for stimulation (Sections 1.5, 1.8 - 1.12, and 6.4).

The rate of cellular secretion is controlled by regulating the permeability of the chloride-selective channel in the apical membrane of the secretory tubule cells (Riordan *et al.*, 1994). This is achieved through alterations in the intracellular concentrations of cAMP which is stimulated by hormones such as VIP and Scyliorhinin II (Sections 1.11 and 6.4) (Forrest, 1996). As has been previously stated, the activity of the Na^+K^+-$2Cl^-$ cotransporter is mediated by intracellular Cl^- concentration (Section 1.5). Elevating

intracellular Cl^- concentration or preventing it from decreasing (i.e. not permitting Cl^- secretion into the lumen) blocks the activation of the cotransporter in response to secretory stimuli. Cellular Cl^- therefore regulates its own rate of entry via the Na^+K^+-$2Cl^-$ cotransporter (Lytle and Forbush III, 1996).

One final influence on rectal gland secretions has been proposed. Elasmobranch rectal glands are surrounded by a band of smooth muscle fibres just below the capsule (Bulger, 1963; Evans and Piermarini, 2001) and, although not localised to this band, rectal glands are responsive to smooth muscle signalling agents (Evans and Piermarini, 2001). Therefore there is scope for smooth muscle contractions having an effect on the activity of the rectal gland, although no direct studies have been conducted.

Given the fundamental role of the rectal gland in osmoregulation there is a disparate amount known on secretion rates *in vivo*. It is reasonable to assume that the gland is of paramount importance for regulating Na^+ and Cl^- levels during salinity transfer. However, since the early work of J. W. Burger this area has been devoid of new insight. Rectal gland activity in *S. acanthias* has been described as variable but persistent on a day-to-day basis (Burger, 1967). The average secretion rate from SW acclimated animals was 0.47 ml Kg^{-1} h^{-1} (Burger, 1962). Acclimation to dilute SW (approximately 80%) caused an increase in rectal gland secretory rate which was measured at 1.4 ml Kg^{-1} h^{-1} on the eighth day of transfer (Burger, 1965). In order to ascertain the role of the rectal gland and how its action relates to blood volume and osmolyte concentrations during changes in salinity, secretion rates *in vivo* must be investigated. This will give further insight into the factors affecting euryhalinity in elasmobranch fish.

4.2 Materials and methods

Experiments on rectal gland secretion rate in *S. canicula* were run concurrently with those for haematic parameters (Chapter 2).

4.2.1 Chemicals and equipment

Chemicals and equipment used were identical to those outlined above (Section 2.2.3).

4.2.2 Surgical procedures

In addition to the procedures outlined for haematic parameters (Section 2.2.4) the rectal gland duct and vein were cannulated as follows. A Mersilk tie was passed between the valvular intestine and the rectal gland vein using a needle, taking care not to rupture the intestine. An incision was made in the vein anterior (downstream) of the tie. Portex polythene tubing of 0.61 mm outer diameter with an obliquely cut tip was passed into the vein until the tip was adjacent to the gland. The tie was then tightened to hold the cannula in position. The cannula was then cut at an oblique angle leaving approximately 25 mm protruding from vein. A second incision was made in the rectal gland vein slightly anterior to the first. The other end of the cannula was then passed into this and held by another tie. This procedure created a bridge in the rectal gland vein which ensured that blood flow from the gland was unaffected by cannulation of the duct, as well as providing a means of purchase during duct cannulation.

A tie was passed through the connective tissue between the rectal gland and the postvalvular intestine. An incision was then made in the rectal gland duct, which passes through this tissue, anterior to the tie. A 60 cm length of Portex polythene tubing of 0.61 mm outer diameter was passed into the duct until the obliquely cut tip just entered the

gland. The Mersilk tie was then tightened around both cannulae, fixing the duct cannula against that in the vein. In this manner the rectal gland duct was cannulated without disrupting blood flow from the rectal gland vein (Figure 4.1.2). Animals were then sutured as previously described (Section 2.2.4) and left in the salinity transfer tanks (Figure 2.2.1.1) for 24 hours after surgery.

4.2.3 Analysis and collection

Fingertips were removed from powder free, nitrile gloves and inverted. These were used as collection balloons for the rectal gland duct cannula, being affixed with Mersilk ties. Collection periods lasted for 2 hours during both basal and transfer experiments, with new balloons used for each time period. Basal collections were taken after 2, 4, 6, 8, and 24 hours; transfer collections were taken after 2, 4, 6, 8, 10, and 12 hours. After each time period the balloons had the ties removed and were placed vertically in a freezer overnight. Rectal gland fluid (RGF) was assessed gravimetrically assuming a specific gravity of 1 g ml^{-1}. Chloride concentration was measured as previously outlined (Section 2.2.5) using a 4-fold dilution in Milli Q water. After the terminal transfer collections rectal glands were removed, dried on tissue paper, and the wet weights were recorded.

Activity of the rectal gland in *S. canicula* was examined through three parameters: volume of RGF, Cl^- concentration in the RGF, and from these two the corresponding rate of Cl^- clearance from the gland. All rates have been normalised for values per gram rectal gland wet mass per hour.

4.2.4 Statistical analysis

All averages are presented as means ± SEM. Statistical analysis of basal parameters was performed via one-way ANOVA and a Tukey post hoc test. Significant differences from values for 100% SW animals were denoted as * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.005$).

Data gathered during the acute transfer studies were analysed in two ways: differences between the two experimental groups and the control group were analysed via one-way ANOVA and a Tukey post hoc test (significance was denoted as * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.005$); differences between values during transfer and at time 0 within each group were analysed via a one-tailed unpaired student's t-test with a Welch correction factor (significance was denoted as † $P < 0.05$, †† $P < 0.01$, and ††† $P < 0.005$) (InStat). Unlike the analysis for haematic parameters, any significance is always shown.

Where data has been manipulated to exclude periods of inactivity non-parametric analysis was used. Differences between the two experimental groups and the control group were analysed via a Kruskal-Wallis test and Dunn's multiple comparisons post test (significance was denoted as * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.005$); for acute transfers differences between values during transfer and at time 0 within each group were analysed via a one-tailed Mann-Whitney test (significance was denoted as † $P < 0.05$, †† $P < 0.01$, and ††† $P < 0.005$) (InStat).

4.3 Results

The results for *in vivo* rectal gland parameters are presented in two sections: a comparison of basal levels in *S. canicula* acclimated to different salinities; and a comparison of levels from *S. canicula* acclimated to the three salinities during acute transfer to 100% SW.

4.3.1 Basal secretion rates

Rectal glands from *S. canicula* which had been long term acclimated to hypo- and hypersaline conditions were not significantly different in size to control animals from SW (Table 4.3.1.1). RGF volume from 100% SW *S. canicula* was highly variable between time periods and between animals. Similar trends were observed in long termed acclimated animals from both 80% and 120% SW

The concentration of Cl^- in the RGF was significantly different between the three salinities. Cl^- concentration in the RGF is altered proportionately with environmental salinity. Despite this difference in Cl^- concentration no differences were seen in either basal RGF volume or Cl^- clearance between chronically acclimated *S. canicula* from the three salinities. Similarly, no differences were seen when analysis was conducted on periods of active secretion.

Salinity	Proportional RG mass (mg 100 g^{-1})	Basal data			Active secretion data	
		RGF volume (ml g^{-1} h^{-1})	Cl^- concentration (mmol l^{-1})	Cl^- clearance (mmol g^{-1} h^{-1})	RGF volume (ml g^{-1} h^{-1})	Cl^- clearance (mmol g^{-1} h^{-1})
80% SW	1.83 ± 0.19	0.62 ± 0.23	375 ± 7 ***	0.23 ± 0.08	0.66 ± 0.22	0.24 ± 0.08
100% SW	1.75 ± 0.10	0.34 ± 0.12	480 ± 14	0.16 ± 0.05	0.36 ± 0.11	0.17 ± 0.05
120% SW	2.01 ± 0.22	0.45 ± 0.14	589 ± 8 ***	0.27 ± 0.08	0.56 ± 0.15	0.34 ± 0.09

Table 4.3.1.1 – Rectal gland parameters from *S. canicula* acclimated to different salinities. Rectal gland mass values have been normalised and are expressed as mg per 100 g body mass. All values are presented as means ± SEM (n = 8, 7, and 8 respectively). Statistical analysis was performed via one-way ANOVA and a Tukey post hoc test for Cl^- concentration, two-tailed Mann-Whitney tests for basal RGF volume and Cl^- clearance rates, and a Kruskal-Wallis test and Dunn's multiple comparisons post test for periods of active secretion. Significant differences from values for 100% SW were denoted as * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.005$).

4.3.2 Acute transfer secretion rates

All figures for acute transfer have had the values for animals from 80 and 120% SW offset on the time axis for clarity, although the measurements are all taken at equivalent time periods. Rectal gland secretion volume remained highly variable during acute transfer to 100% SW from 80, 100, and 120% SW environments (Figure 4.3.2.1). RGF volume was significantly reduced in the control animals after 2, 6, and 8 hours of the transfer as compared to basal levels. RGF volume remained unchanged in animals acclimating to 100% SW from 80% SW. RGF volume was significantly increased in animals acclimating from 120% SW after 8 hours of the transfer. Counter intuitively, secretion rates in the early stages of transfer were significantly higher in animals acclimating to 100% SW from 80% SW as compared to the control 100% SW animals. Both experimental groups showed significant increases in RGF output between 6 – 8 hours into the transfer.

Animals acclimating to 100% SW from 80% SW significantly increased Cl^- concentration in the RGF after 2 hours of the transfer, and it remained so thereafter (Figure 4.3.2.2). *S. canicula* acclimating to 100% SW from 120% SW showed significantly decreased Cl^- concentration in the RGF after 4 hours, and it remained decreased throughout the rest of the transfer. RGF Cl^- concentration in the control animals remained unchanged throughout the transfer. Cl^- concentration in the RGF increased in animals acclimating from 80% SW and was not significantly different to that of the control 100% SW group after 6 hours. Conversely, Cl^- concentration in the RGF decreased in animals acclimating from 120% SW and was not significantly different to that of the control 100% SW group after 6 hours.

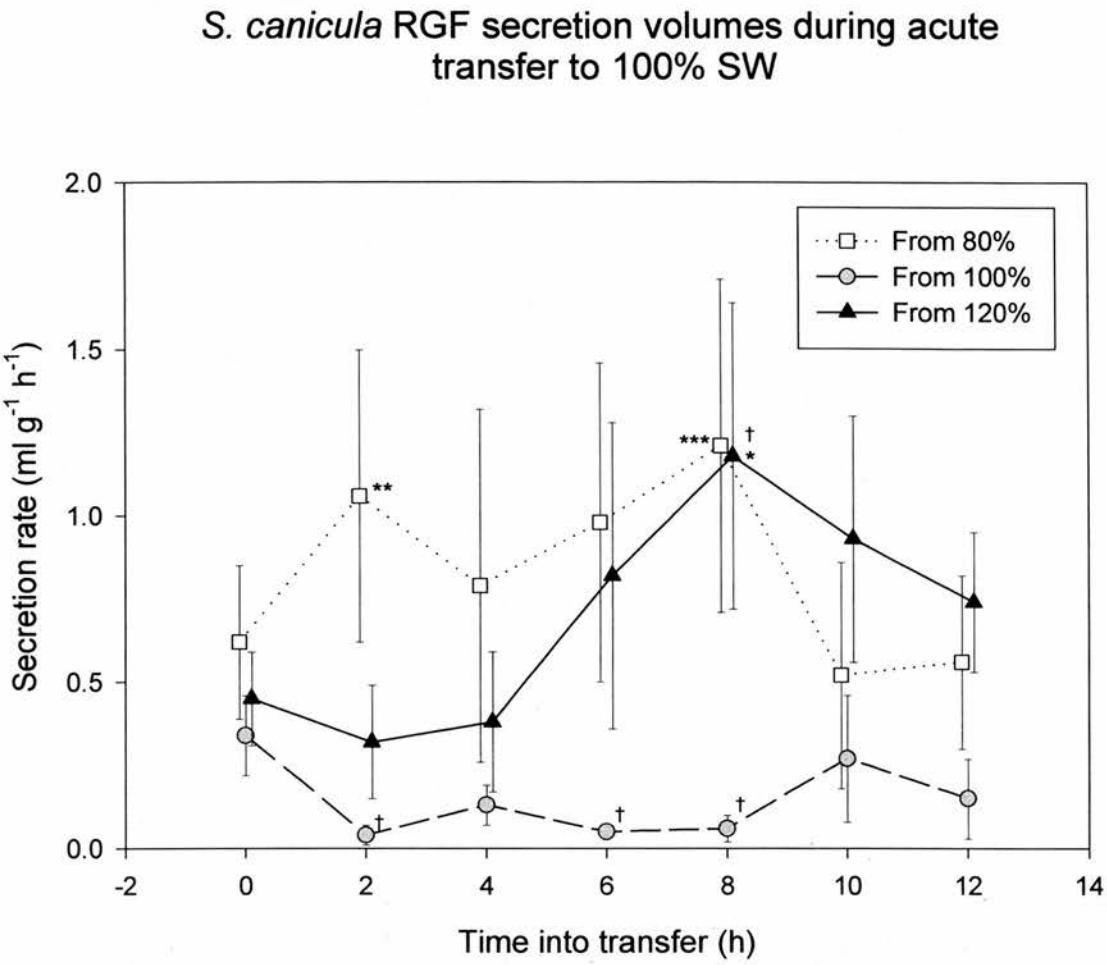

Figure 4.3.2.1 – RGF secretion rates during acute transfer to 100% SW. Values are means ± SEM (n = 8, 6, and 6 respectively). Statistically significant differences from the control transfer were assessed via one-way ANOVA and a Tukey post hoc test (significance was denoted as * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.005$)); statistically significant differences from basal values at time 0 were assessed via one tailed unpaired students t-tests with Welch correction factor (significance was denoted as † ($P < 0.05$), †† ($P < 0.01$), and ††† ($P < 0.005$)).

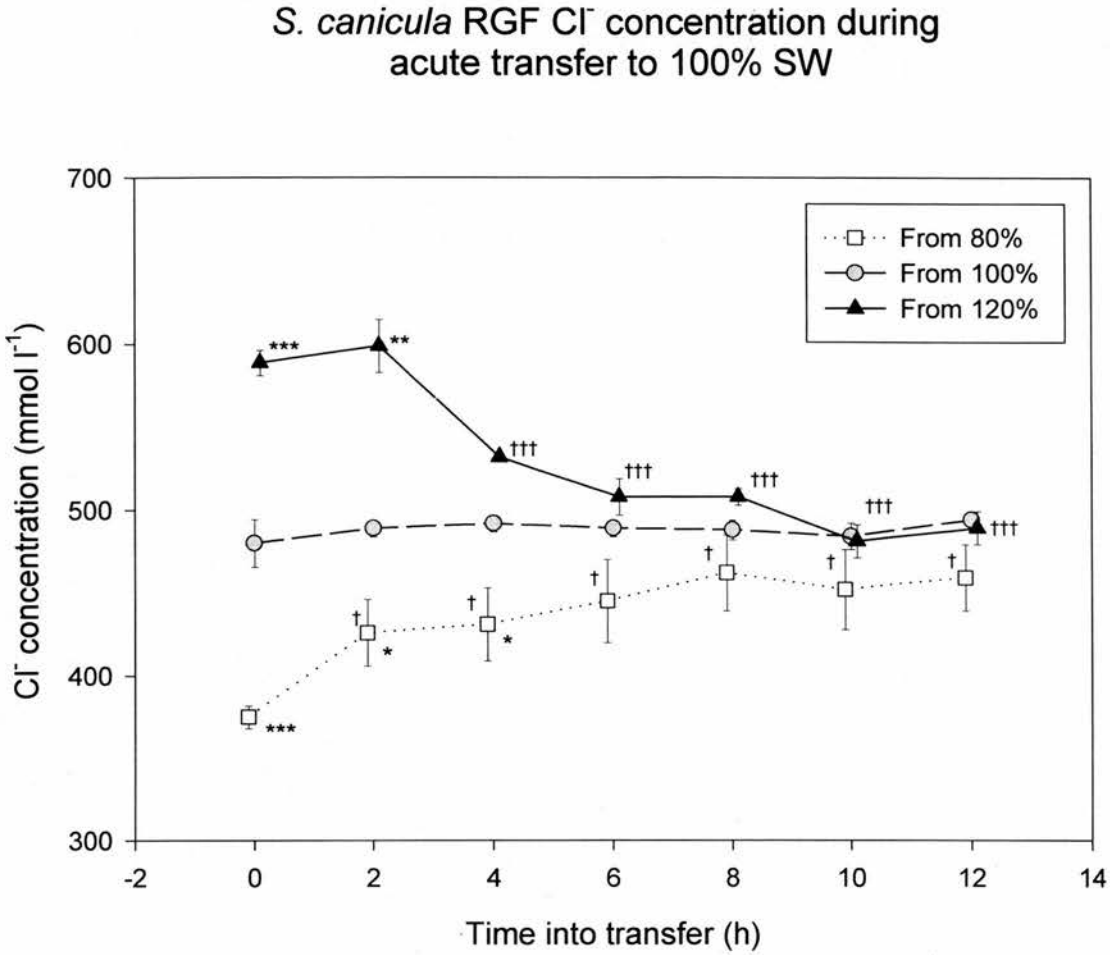

Figure 4.3.2.2 – Cl^- concentration in RGF during acute transfer. Values are means ± SEM (n = 8, 6, and 6 respectively). Statistically significant differences from the control transfer were assessed via one-way ANOVA and a Tukey post hoc test (significance was denoted as * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.005$)); statistically significant differences from basal values at time 0 were assessed via one tailed unpaired students t-tests with Welch correction factor (significance was denoted as † ($P < 0.05$), †† ($P < 0.01$), and ††† ($P < 0.005$)).

Cl^- clearance rates from the rectal gland of *S. canicula* were not significantly different from basal levels during acclimation to 100% SW from 80 and 120% SW environments (Figure 4.3.2.3). Cl^- clearance rate from the rectal gland of control animals was significantly reduced during transfer when compared to basal levels. Counter intuitively, Cl^- clearance was significantly higher in animals acclimating to 100% SW from 80% SW, but only after 2 and 8 hours when compared to the control 100% SW animals. Cl^- clearance was also significantly higher in animals acclimating to 100% SW from 120% SW after 8 hours when compared to the control 100% SW animals.

Accurate assessment of rectal gland Cl^- clearance *in vivo* is hindered greatly by the intermittent nature of gland activity. Therefore Cl^- clearance was also assessed discarding data collected during periods of inactivity (Figure 4.3.2.4). On average this resulted in assessing 5 of the possible 8 animals from 80% SW, 3 of the 6 from 100% SW, and 4 of the 6 from 120% SW. This should provide a better representation of Cl^- clearance during periods of secretion. During periods of activity Cl^- clearance from the glands of animals acclimating to 100% SW from 80% SW did not change significantly from basal levels. Clearance from the rectal glands of animals acclimating to 100% SW from 120% SW was significantly increased from basal levels after 8 and 12 hours of acute transfer. Cl^- clearance during periods of activity in the control 100% SW group was significantly reduced from basal levels after 2, 6, and 8 hours of the transfer. After 8 hours of acute transfer animals acclimating to 100% SW from 120% SW had a significantly higher rate of Cl^- clearance from the rectal gland than the control animals.

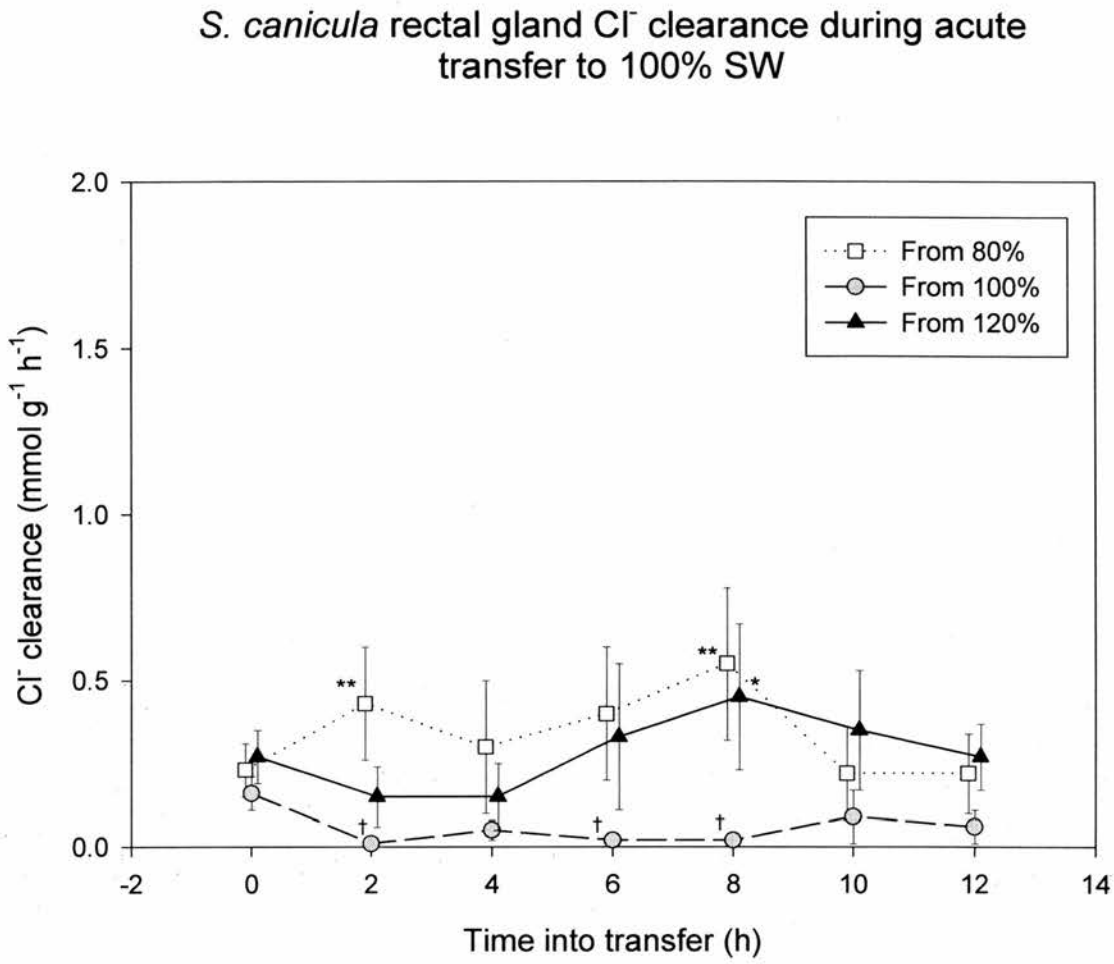

Figure 4.3.2.3 – Cl^- clearance rates from the rectal glands of *S. canicula* during acute transfer to SW. Values are means ± SEM (n = 8, 6, and 6 respectively). Statistically significant differences from the control transfer were assessed via one-way ANOVA and a Tukey post hoc test (significance was denoted as * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.005$)); statistically significant differences from basal values at time 0 were assessed via one tailed unpaired students t-tests with Welch correction factor (significance was denoted as † ($P < 0.05$), †† ($P < 0.01$), and ††† ($P < 0.005$)).

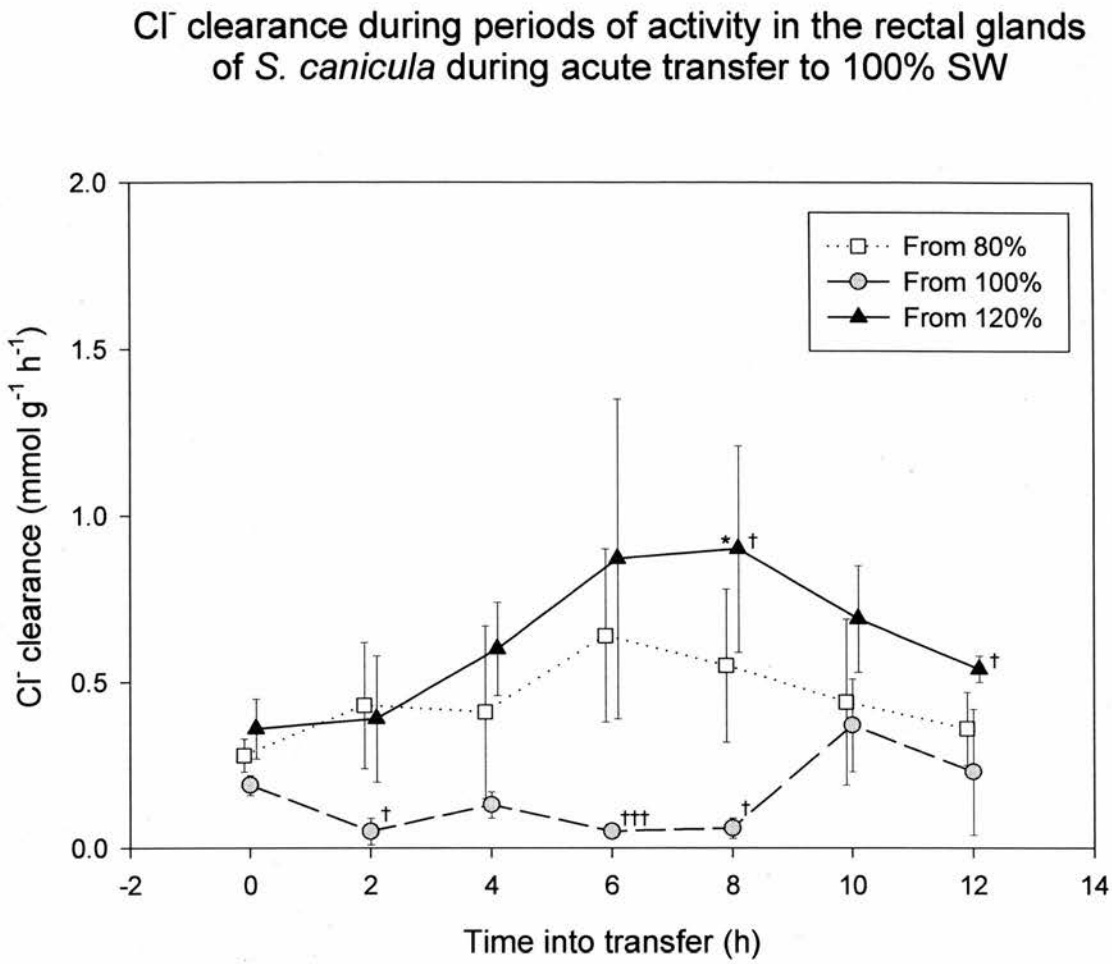

Figure 4.3.2.4 – Cl^- clearance rates during periods of activity from the rectal glands of *S. canicula* during acute transfer to SW. Values are means ± SEM (n = 8, 6, and 6 respectively). Statistically significant differences from the control transfer were assessed via a Kruskal-Wallis test with Dunn's multiple comparisons post test (significance was denoted as * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.005$)); statistically significant differences from basal values at time 0 were assessed via a one tailed Mann-Whitney test (significance was denoted as † ($P < 0.05$), †† ($P < 0.01$), and ††† ($P < 0.005$)).

4.4 Discussion

Chronic acclimation to altered environmental salinity had no effect on the relative size of the rectal gland in *S. canicula* (Table 4.3.1.1). Previous work has found that rectal glands from species inhabiting reduced salinities can be proportionally smaller than those of marine species, depending on the nature of the FW system (Oguri, 1976; Pillans and Franklin, 2004). This suggests that whilst there may be interspecies, and possible interpopulation variation in rectal gland size due to salinity, there is no intraspecific modification in this marine species. It is possible that the 3 week acclimation to salinity change used in this study is not sufficient to elicit any change in the proportional rectal gland mass of *S. canicula*. However, this is a marine species which experiences minor changes in salinity in the wild and it is therefore probable that the species would not show any change in proportional rectal gland mass even during a longer period of acclimation. The affects of long term salinity acclimation on the structure of the rectal gland are discussed below (Section 5.4).

The results presented above show that *in vivo* rectal gland activity in *S. canicula* is extremely intermittent, as has been previously reported for *S. acanthias* (Burger, 1967). This is true in all three of the salinities studied. Maximal rectal gland secretion throughout the study peaked at around 2% of total blood Cl^- levels (Tables 2.3.1.1, 3.3.1.1, 4.3.1.1, and 5.3.1.1 and Figure 4.3.2.4). These findings further support the concept of the rectal gland being of fundamental importance in maintaining ionic homeostasis. The concentration of Cl^- in the RGF is significantly increased at a higher salinity, and significantly reduced at a salinity below SW (Table 4.3.1.1). This is consistent with the concept of the rectal gland secreting a fluid around isosmotic with blood plasma and consisting largely of Cl^- and Na^+.

However, despite this increase in the concentration of Cl^- in the RGF, the rate of Cl^- clearance from the rectal gland is not different at elevated or reduced environmental salinities. This is largely due to the fact that RGF secretory volume is not consistently altered by acclimation to different salinities (Table 4.3.1.1). These findings *in vivo* are contrary to those from studies on the isolated perfused rectal glands of *S. canicula* which showed that Cl^- clearance was affected by salinity change: Cl^- clearance was decreased at elevated salinities, and increased at reduced salinities (Anderson *et al.*, 2002a). This discrepancy suggests that there are methods of mediating rectal gland secretion, exogenous to the gland, which are important during salinity change. Given that the isolated glands were perfused with Ringer solution of the appropriate salinity it is unlikely that this method of control stems from osmolyte concentrations in the perfusate/blood. It is more likely that the control of rectal gland Cl^- clearance is achieved through hormonal signals carried in the blood, as outlined elsewhere (Sections 1.5, 1.8 – 12, and 6.4). This discrepancy also highlights the importance of *in vivo* studies: for whilst the mechanics of the gland may suggest one mode of action under certain environmental conditions, actual rectal gland function may be quite different.

The fact that there were no significant differences in *S. canicula* chronically acclimated to 80%, 100%, and 120% SW for either RGF secretion or Cl^- clearance rates, and given that the rectal gland is the main source of secretion for Na^+ and Cl^-, suggest that there are proportionally similar amounts of these ions entering the animals at each of the salinities. That is to say, *S. canicula* is capable of complete osmotic acclimation to both 80 and 120% SW and experiences no changes in the relative influxes of Na^+ and Cl^- once fully acclimated. *S. canicula* is able to sustain altered levels of plasma osmolytes (such as Cl^- and Na^+) as a means of maintaining an iso/hyperosmotic state. This further

supports the findings discussed above concerning basal haematic parameters in animals which are long term acclimated to 80, 100, and 120% SW conditions (Section 2.4).

During acute transfer to 100% SW *S. canicula* modifies the concentration of Cl^- in the RGF. The concentration of Cl^- in the RGF was no longer significantly different to that of the control group in both of the experimental groups after 6 hours (Figure 4.3.2.2). This coincides with the point at which blood plasma osmolality was no longer significantly different between animals transferring from 120% SW and the control animals (Figure 2.3.2.3). However, the plasma osmolality of animals transferring from 80% SW continued to be significantly below that of the control animals throughout the transfer (Figure 2.3.2.3), and yet Cl^- concentration in the RGF of the same animals was altered to levels similar to those in the control group. This does not support the idea of the RGF being altered to be isosmotic to blood plasma.

Whilst rectal gland secretory volume was unchanged in animals acclimating from 80% SW, it was significantly increased after 8 hours in animals acclimating from 120% SW (Figure 4.3.2.1). However, this did not translate into a significant increase in Cl^- clearance, due to the large amount of variation stemming from intermittent gland activity (Figure 4.3.2.3). The significant decreases in RGF volume and Cl^- clearance seen in the control group during acute transfer are illustrative of the intermittent nature of rectal gland function.

Rectal gland secretory volume was significantly higher in the two experimental groups after 8 hours of transfer than in the control group. This did result in an associated higher rate of Cl^- clearance. Animals undergoing acute transfer from 80% SW have the

requirement to increase plasma Cl^- concentrations and so an increased frequency of secretion, and therefore an increase in Cl^- clearance, from the rectal gland is counter intuitive. Given the specific evolution of the rectal gland for the secretion of Na^+ and Cl^- it is likely that the influences over its secretory activity are highly refined. The immediate requirement to increase plasma osmolality during acute transfer to 100% SW from 80% SW is met by a series of discrete drinking events in order to increase intake of readily available Na^+ and Cl^- (Anderson *et al.*, 2002b; Hazon, *pers. comm.*). Once the initial disparity between environmental and plasma osmolalities has been reduced, internal concentrations of Na^+ and Cl^- are regulated through the action of the rectal gland. This could explain the increased frequency of rectal gland activity in these animals.

Animals undergoing acute transfer from 120% SW also increase Cl^- clearance from the rectal gland. This was expected as a means to reduce plasma Cl^- concentrations as part of the process to lower overall plasma osmolality. Results of *in vivo* rectal gland secretion from the acute transfer studies further support the findings that rapid modifications to blood plasma osmolality are largely achieved through changes in plasma Na^+ and Cl^- concentrations (Section 2.3.2). The rectal gland is of vital importance to this as both a means of regulating the influx of Na^+ and Cl^- from a drinking response, and as a means of reducing plasma concentrations of these ions to reduce overall plasma osmolality.

In order to gain further insight into the effects of salinity transfer on rectal gland secretion the results were reanalysed removing the periods of inactivity and only assessing the gland during periods of secretion (Figure 4.3.2.4). The results of the

control group showed that rectal gland Cl^- clearance is modified during constant environmental conditions. That is to say that rectal gland secretion is not purely modified by changing periods of activity and inactivity, but also by changing Cl^- clearance rates during the periods of activity. This is most likely a reflection of variations in plasma osmolality over time and the role of the gland in plasma osmotic homeostasis. Periods of inactivity were fewer in animals acclimating to salinity change than they were in the control group (Section 4.3.2), and Cl^- clearance rates during periods of activity are more variable in these groups as a result of that. Whilst there were no significant changes in Cl^- clearance in animals acclimating to 100% SW from 80% SW, animals acclimating to 100% SW from 120% SW did significantly increase rectal gland Cl^- clearance during periods of activity (Figure 4.3.2.4).

This difference between overall Cl^- clearance and that during periods of activity in the rectal glands of these animals illustrates the response of the gland throughout acute transfer to reduced salinities. The rectal gland is not necessarily active for greater periods of time during these salinity transfers, but Cl^- clearance is increased during the periods of activity. This increase acts as a means of reducing plasma Cl^- (and Na^+) concentration so as to reduced overall plasma osmolality to levels which are iso/hyperosmotic to the environment.

These findings illustrate the importance of mediating the secretion of Na^+ and Cl^- in elasmobranch osmoregulation, particularly during acclimation to changes in salinity. They also illustrate importance of *in vivo* studies in describing the overall osmoregulatory response of the rectal gland during salinity transfer. However, the wide variety of factors which influence rectal gland activity *in vivo* (which have previously

been discussed (Sections 1.5 and 1.8 – 12)) make specific analysis of individual aspects of glandular function highly complex. For this reason it becomes evident that the use of *in vitro* techniques is of great importance; allowing isolation of the rectal gland from these control factors, therefore permitting a more precise analysis of the individual processes involved in regulating active secretion. In this manner the structural changes within the rectal gland following chronic acclimation to salinity transfer were assessed.

Chapter 5: Structural changes in the rectal gland

See Pillans, R. P., Good, J. P., Anderson, W. G., Hazon, N. and Franklin, C. E. (2005). "Freshwater to seawater acclimation of juvenile bull sharks (*Carcharhinus leucas*): plasma osmolytes and Na^+/K^+-ATPase activity in gill, rectal gland, kidney and intestine." *J Comp Physiol B* **175**: 37 – 44 (Appendix 2) for details on *C. leucas*.

5.1 Introduction

Elasmobranchs modify their blood plasma osmolality, through altering the concentrations of key osmolytes, in response to changes in salinity. It has been demonstrated that Cl^- and Na^+ are two of these key osmolytes (Chapter 2), and that the action of the rectal gland is of paramount importance to altering the plasma concentrations of these ions (Chapter 4). It is therefore important to investigate potential changes in the tissues of the rectal gland which permit these changes in Cl^- concentration and secretory volume of the RGF.

As previously stated, marine elasmobranchs may face a large salt load during feeding events from the ingested food and also the imbibed SW (Section 1.4). MacKenzie (1996) investigated the effects of this salt load on the structure of the rectal gland through a comparison of histological sections from starved and recently fed (12 hours after feeding) *S. canicula*. Feeding, and the associated salt load, resulted in a 40% increase in the diameter of the central collecting duct, a 47% increase in the diameter of the central vein, and a 47% increase in the visible number of blood vessels in the capsular layer. These results suggest that there are changes in rectal gland structure associated with increased blood flow to, and blood and secretory flow from, the rectal gland during periods of high salt loading. This was further supported by more recent work conducted on blood flow to the rectal gland in *S. canicula* acclimated to different salinities. Blood perfusion of the secretory epithelia was significantly increased in the rectal glands of animals acclimated to 70% SW, as compared to that in animals from 100 and 120% SW environments. Blood perfusion in the 120% SW group did not significantly differ from that in the 100% SW group (Anderson et al. 2002a). Isolated perfused rectal glands from animals similarly acclimated to reduced salinity also show

an increase in Cl^- clearancc rates (Anderson et al. 2002a). Furthermore, this study has demonstrated previously that rectal gland Cl^- clearance *in vivo* is increased during periods of activity in animals acclimating to reduced salinity (Figure 4.3.2.4). These findings support the idea of increased blood flow to the secretory epithelia of the rectal gland during conditions which induce increases in rectal gland output. It is therefore important to ascertain whether or not acclimation to salinity change, and the associated fluxes in Cl^- and Na^+, invokes similar changes in rectal gland structure as produced in response to dietary salt loading. Histological analysis of the rectal glands from *C. leucas* captured in Lake Nicaragua and the FW system of the Rio San Juan demonstrated a thicker connective tissue, an enlarged central duct, an irregular capillary network, and a decrease in the number of glandular tubules when compared to SW captured animals (Oguri 1964; Gerzeli et al. 1976). This river system is very different to the Brisbane River which has tidal effects and a salinity gradient throughout. The population of *C. leucas* in Lake Nicaragua are therefore likely to spend a greater amount of time in a fully FW environment. Analysis of rectal gland structure in *C. leucas* from the Brisbane River acclimated to both FW and SW will therefore give further insight into the effects of salinity transfer on the structure of the rectal gland in this fully euryhaline species.

Studies by MacKenzie (1996) demonstrated changes in structural aspects of the rectal gland associated with periods of acute salt loading. In this study structural analysis was performed on the rectal glands of both a fully and a partially euryhaline elasmobranch species acclimated to different environmental salinities. This enabled investigation into the specific effects of salinity acclimation and variations in secretory output on rectal gland structure. If any effects were recorded a comparison of a fully and a partially euryhaline species could be analysed.

Changes in rectal gland structure may therefore be descriptive of changes in secretory output. Variations in secretory output are most likely to be associated with changes in Na^+, K^+-ATPase activity and/or abundance. Although the rate of cellular secretion is controlled by regulating the permeability of the chloride selective channel in the apical membrane of the secretory cells (Riordan et al. 1994), Na^+, K^+-ATPase is of vital importance to cellular secretion. This is due to the fact that the action of Na^+, K^+-ATPase is the only active process in the movement of Na^+ and Cl^- across cell membranes (Figure 1.5.1). Rosenberg (1948) states that only a transport against the combined effects of electrochemical potential and concentration gradients should be considered active. As such Na^+, K^+-ATPase can be considered as the most important protein in NaCl secretion as all subsequent processes are driven by its action.

Na^+, K^+-ATPase is an enzyme which is composed of two heterologous subunits: a catalytic α-subunit and a glycosylated β-subunit. The β-subunit spans the cell membrane once whereas the α-subunit spans up to 10 times, with both subunits exposing the *N*-terminal into the cytoplasm. Most of the mass of the α-subunit is on the cytoplasmic surface of the membrane whilst most of the mass of the β-subunit is on the extracellular surface and contains several glycosylation sites (Geering 1988). The subunits are encoded for by two independent mRNA sequences, with their synthesis being strictly coordinated (Geering et al. 1985).

The Na^+, K^+-ATPase purified from elasmobranch rectal glands has been shown to be heterogeneous, in contrast to similar methods utilised on the enzyme isolated from mammalian kidneys. Examples of this heterogeneity include biphasic spontaneous phosphorylation (Cornelius 1995a; Cornelius 1995b), inactivation by *N*-ethylmaleimide

(Esmann 1982; Esmann and Nørby 1985), and the existence of high and low binding affinities for ouabain (a Na^+, K^+-ATPase specific inhibitor) associated with the α-subunit (Silva et al. 1983; Hansen 1999). In all cases monophasic reactions are seen with Na^+, K^+-ATPase purified from mammalian kidney, but not with enzyme isolated from mammalian brain tissue which is composed of different isoforms of the α-subunit (Hansen 1976; Hansen 1986; Hansen et al. 1991). Contrastingly, the heterogeneity in ouabain binding affinity seen in the rectal gland of *S. acanthias* is not due to different isoforms of the α-subunit (Hansen 1999). The cause for this heterogeneity in Na^+, K^+-ATPase in the elasmobranch rectal gland remains unclear.

In work related to this present study analysis of rectal glands from *C. leucas* chronically acclimated to both FW and SW showed no significant differences in either mRNA levels or enzyme abundance for Na^+, K^+-ATPase (Meischke, *pers. comm.*). Furthermore, the amount of enzyme present in the tissues of the rectal gland proved so high as to be a hinderence to quantitative analysis. No differences were recorded for either mRNA levels or enzyme abundance in the intestinal tissue of the same animals. Differences were recorded in the branchial tissue regarding the localisation of the Na^+, K^+-ATPase α1 subunit with high levels on the gill filament and lamellae of FW *C. leucas* but only on the filaments of SW acclimated animals (Meischke, *pers. comm.*); patterns identical to those recorded for *D. sabina* (Piermarini and Evans 2000). Differences were also seen in Na^+, K^+-ATPase enzyme abundance in the kidney of *C. leucas* where FW acclimated animals consistently showed greater levels of immuno-fluorescence than SW acclimated animals (Meischke, *pers. comm.*).

Na^+, K^+-ATPase is responsible for maintaining the basic monovalent cation homeostasis in all vertebrate cells, and in epithelia is basolaterally located (Riordan et al. 1994). In the secretory epithelia of the rectal gland Na^+, K^+-ATPase uses the energy derived from ATP to actively pump K^+ into and Na^+ out of the cell against both electric potential and concentration gradients (Figure 1.5.1). The action of Na^+, K^+-ATPase is essential to rectal gland function as its specific inhibition by ouabain stops secretion completely (Silva et al. 1977). Secretion can be stimulated by cyclic adenosine monophosphate (cAMP) (Stoff et al. 1977b), and there is indirect evidence that this is accompanied by enhanced activity of Na^+, K^+-ATPase. For example, stimulation by cAMP is associated with an increase in ouabain-sensitive oxygen consumption by the whole gland (Silva et al. 1979), as well as increasing the rate and amount of ouabain binding (Silva et al. 1983). Furthermore, in cultured cells from the rectal gland of *S. acanthias* intracellular Na^+ concentration has been shown to decrease in response to cAMP (Lear et al. 1992). Intracellular concentrations of cAMP are also of key importance in regulating the permeability of the chloride-selective channel (Forrest 1996), the major factor controlling the rate of cellular secretion.

Due to the action of Na^+, K^+-ATPase in pumping cations against electrochemical and concentration gradients it is also of key importance in other elasmobranch osmoregulatory organs. It is of paramount importance in acid base extrusion at the surface of the gills (Figure 1.3.2) (Evans et al. 2005), and there is growing evidence for the gills of FW elasmobranchs being involved in active ion uptake (Hirose et al. 2003). In the kidney, tubular cells in the EDT have been shown to have similar characteristics to cells which actively transport Na^+ (Lacy and Reale 1991b; Lacy and Reale 1991a). Intestinal Na^+, K^+-ATPase has been shown to be of importance in teleosts during

salinity transfer (Jampol and Epstein 1970; Finstad et al. 1989), and in elasmobranchs due to the effects of dietary sodium intake illustrated in *S. canicula* (MacKenzie 1996; MacKenzie et al. 2002). It is therefore intuitive that it plays a similar role in elasmobranchs.

The response of Na^+, K^+-ATPase to salinity transfer has been investigated in other studies. Piermarini and Evans (2000) investigated changes in the activity and abundance of Na^+, K^+-ATPase in the gills and rectal gland of *D. sabina* associated with acclimation to different salinities. Activity and abundance of branchial Na^+, K^+-ATPase decreased when FW animals were acclimated to SW, levels in SW animals were lower still. Na^+, K^+-ATPase rich cells were found on both the filament and lamellae of the branchial epithelium in FW stingrays, but only on the filament of SW animals. This reflects the decrease in requirement for active ion uptake at the gills in increased salinity. Conversely, the activity and abundance of rectal gland Na^+, K^+-ATPase increased when FW animals were acclimated to SW, to levels equivalent to those found in SW animals. This reflects the increase in requirement for NaCl secretion by the rectal gland at increased salinities.

Quantitative histochemical studies on FW *C. leucas* from Lake Nicaragua have found Na^+, K^+-ATPase activity in the rectal gland to be below accurately measurable levels (Gerzeli et al. 1976). This data conflicts with that gathered on the same species from the Brisbane River where Na^+, K^+-ATPase activity was prohibitively high for quantitative analysis in FW individuals (Meischke, *pers. comm.*). This could be the result of an extended period of the life cycle in a wholly FW system such as Lake Nicaragua, or indicate that *C. leucas* is capable of greatly reducing rectal gland activity in FW as part

of its euryhaline strategy. However, it is also possible that these results reflect incomplete inhibition of Na^+, K^+-ATPase by lower concentrations of ouabain used by Gerzeli and co-workers (1976). Clearly the activity of Na^+, K^+-ATPase is of fundamental importance to the activity of osmoregulatory organs, and elasmobranchs seem able to modify either the abundance and/or activity of the enzyme in response to salinity change.

In this study the Na^+, K^+-ATPase maximal activity in key osmoregulatory organs were assessed in both a fully and partially euryhaline species. This allowed a quantitative analysis of the total amount of enzyme activity possible by each of the organs. Furthermore, comparative analysis between salinity acclimations showed any modifications in maximal Na^+, K^+-ATPase activity within the principle osmoregulatory organs. This also permitted a comparison of the degree of modification seen in a partially and a fully euryhaline elasmobranch species.

5.2 Materials and methods

Tissue modifications in *S. canicula* and *C. leucas* in response to salinity changes were assessed by two methods. Rectal gland structure was assessed by histological staining and analysis. Na^+, K^+-ATPase activity was assessed in the gills, the gut, the rectal gland, and the kidney via a maximal activity assay. The protocols for these are outlined below (Sections 5.2.2 and 3).

5.2.1 Chemical and equipment

Unless otherwise stated all chemicals used were obtained from Sigma and were identical to those described previously (Section 2.2.3). Histological sections were cut at 6μm on a microtome (rotary microtome, Leica UK, Milton Keynes, UK).

5.2.2 Histological staining and analysis

Transverse sections (approximately 2 mm) were taken from the middle of the rectal gland and tissue samples were prepared following the protocol outlined by MacKenzie (1996) using Masson's trichrome staining method (Masson 1929) (Appendix 1).

Slides were analysed following similar methods to those outlined previously (Anderson et al. 2002a). Images of each slide were captured using an M3Z binocular microscope (Wild, Heerbrugg, Switzerland) attached to a CV-255C video camera (MVD, Tokyo, Japan) and a computer running Analysis 2.11 (Norfolk Analytical, Hilgay, Norfolk). Prior to image analysis, images of all slides were isolated from the background using a 6 pixel brush tool and pure white colour (this was chosen as it did not occur naturally in the tissues of the rectal gland), with the central duct and vein being similarly isolated using a 4 pixel brush. Images were then analysed on a visual range excluding pure white with data gathered for area and mean diameter on the whole gland section, the central duct, and the central vein (Figure 5.2.2.1)

A total of 30 images were selected at random and analysed for each rectal gland, with mean values being calculated for each of the parameters. This was taken to represent the mean values of each parameter for each gland. From these values it was then possible to calculate the percentage of the cross-sectional area relating to the central duct and vein.

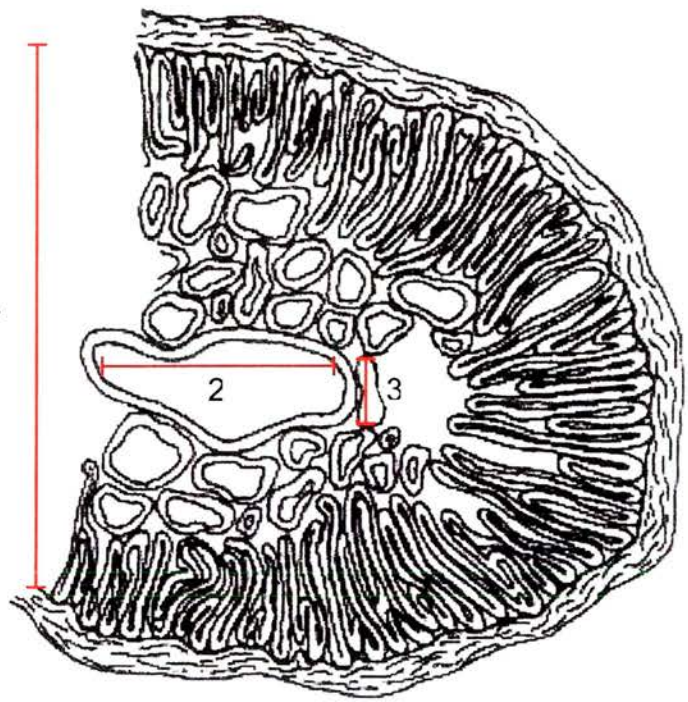

Figure 5.2.2.1 – Measurements taken for structural analysis. T-bars encompass the areas measured for the cross section of the whole gland (1), the central duct (2), and the central vein (3) (Masini et al. 1993).

5.2.3 Maximal Na^+, K^+-ATPase activity

Maximal enzyme activity was measured following the methods of MacKenzie and co-workers (2002) (Appendix 1). Tissue samples were taken in the following ways: Gill tissue was taken by scraping the anterior and posterior surfaces of the third right side gill hemibranch with a scalpel blade, removing approximately 200 mg of tissue. Intestinal mucosa was taken by removing and opening the valvular intestine and scraping the first 1 cm of the anterior section with a scalpel blade. The rectal gland was sampled using the posterior half for *C. leucas*, and the whole gland minus sections taken for respirometry studies (Chapter 6) for *S. canicula*. Approximately 200 mg of kidney tissue was taken by cross-section from the nephrogenic region. After homogenisation, tissues were filtered through 4 layers of sterile gauze and stored at -80 °C prior to assay, with a maximum of 7 days allowed prior to measurement of Na^+, K^+-ATPase activity.

Prior to measurement of maximal Na^+, K^+-ATPase activity, samples were assessed for protein concentration. For *S. canicula* this was achieved following the method outlined by Bradford (1976) (Appendix 1). For *C. leucas* protein was determined using a Micro Lowry protein kit, Peterson's modification (TP0300, Sigma) based on the method outlined by Lowry and co-workers (1951).

From preliminary results tissue homogenates were diluted in the following ratios to give values which were included in the range of standards: gill (1:5), gut (1:15), rectal gland (1:5), kidney (1:15). Upon calculation of protein concentration the homogenates were diluted to give final concentrations of between 0.2 and 0.4 mg ml^{-1} before measurement of enzyme activity.

Maximal Na^+, K^+-ATPase activity was defined as the ouabain-sensitive component of the hydrolysis of ATP in the presence of Na^+, K^+, and Mg^{2+} (MacKenzie 1996; MacKenzie et al. 2002) (Appendix 1). Ouabain sensitive phosphate release was determined as the difference between values in the presence and absence of 2 mM ouabain. This concentration was chosen based on the complete inhibition of Na^+, K^+-ATPase activity (MacKenzie 1996).

From the results of the protein assay and the Na^+, K^+-ATPase assay, maximal activity of the enzyme could be calculated:

$$\text{Activity } (\mu\text{mol Pi mg}^{-1}\text{ Protein h}^{-1}) = \frac{\text{Ouabain sensitive phosphate release } (\text{mmol ml}^{-1}\text{ h}^{-1})}{\text{Concentration of protein in assay } (\text{mg ml}^{-1})}$$

5.2.4 Statistical analysis

All data are presented as means ± the standard error of the mean (SEM). For the data gathered on *S. canicula* statistical analysis was performed via one-way ANOVA and a Tukey post hoc test (InStat). Data gathered on *C. leucas* was analysed using a two-tailed unpaired student's t-test with Welch correction factor (InStat). Significance was denoted as * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.005$).

5.3 Results

The results for structural changes are presented in two sections: rectal gland structure and maximal Na^+, K^+-ATPase activity. Due to the differences in equipment at the two laboratories, different parameters of body and rectal gland size were taken for *S. canicula* and *C. leucas*.

5.3.1 Rectal gland structure

The body mass of *S. canicula* was not significantly different between groups of animals acclimated to 80, 100, and 120% SW (Table 5.3.1.1). Rectal glands from *S. canicula* acclimated to 80% SW were highly significantly heavier than those acclimated to 100% SW. This corresponded to a significantly larger proportion of body mass being attributable to these rectal glands, and a significantly larger cross-sectional area, as compared to glands from 100% SW acclimated animals. Rectal glands from animals acclimated to 120% SW had a highly significantly smaller proportion of body mass attributable to them than glands from 100% SW acclimated animals. There was no significant difference in cross-sectional area between glands from animals acclimated to 120 and 100% SW. There were no significant differences in mean area of either the central duct or vein of *S. canicula* acclimated to the three salinities.

C. leucas acclimated to SW had significantly smaller body masses than those acclimated to FW (Table 5.3.1.2). This was not due to any significant difference in total body length of the animals. This corresponded to a highly significantly smaller proportional body mass of the SW acclimated animals. The rectal gland of *C. leucas* was not significantly different in any measured parameters between FW and SW acclimated animals.

Salinity	Body mass (g)	RG mass (mg)	Proportional rectal gland mass (% body mass)	Cross sectional area (mm^2)	Duct area (μm^2)	Vein area (μm^2)
80% SW	559.0 ± 38.0	131 ± 12 **	0.022 ± 0.001 *	56.99 ± 7.88 *	88.2 ± 2.1	16.1 ± 4.5
100% SW	442.0 ± 46.0	64 ± 9	0.019 ± 0.001	33.78 ± 3.94	47.8 ± 6.2	9.9 ± 1.3
120% SW	406.5 ± 45.0	56 ± 9	0.013 ± 0.000 **	34.96 ± 3.59	115.7 ± 24.4	10.5 ± 1.7

Table 5.3.1.1 – Morphological parameters of *S. canicula* acclimated to 80, 100, and 120% SW. All values are presented as means ± SEM (n = 8, 16, and 8 for mass; and 6, 5, and 6 for area respectively). Statistical analysis was performed via one-way ANOVA and a Tukey post hoc test. Significant differences from values for 100% SW were denoted as * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.005$).

Salinity	Body mass (Kg)	Total length (cm)	Proportional body mass (g cm^{-1})	Cross sectional area (mm^2)	Duct area (μm^2)	Vein area (μm^2)
FW	3.6 ± 0.2	84.6 ± 2.0	42.1 ± 1.5	50.18 ± 4.59	243.5 ± 48.9	20.2 ± 6.0
SW	2.9 ± 0.2 *	80.1 ± 1.7	36.0 ± 1.4 **	54.64 ± 4.64	260.6 ± 52.8	31.8 ± 8.5

Table 5.3.1.2 – Morphological parameters of *C. leucas* acclimated to FW and SW. All values are presented as means ± SEM (n = 12 and 10 for whole body; and 4 and 5 for rectal gland respectively). Statistical analysis was performed via a two-tailed unpaired student's t-test and Welch correction factor. Significant differences from values for FW were denoted as * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.005$).

Due to the observed effects of the intracellular and blood volumes on rectal gland size in *S. canicula* at the different salinities, and those anticipated for *C. leucas* in FW and SW, rectal gland structure was further analysed normalising for cross-sectional area and investigating the relative sizes of the central duct and vein (Figures 5.3.1.1 and 2). In this manner the sizeable interindividual variations in rectal gland size were removed permitting a more accurate analysis of rectal gland structure. In *S. canicula* the proportional area of the central duct as compared to total gland cross sectional area was highly significantly larger in animals acclimated to 120% SW than those acclimated to 100% SW, there was no difference in the proportional size of the central vein. No significantly differences were seen in the proportion of either feature between animals acclimated to 80 and 100% SW. In the rectal glands of *C. leucas* there were no significant differences in the proportion of either feature between FW and SW acclimated animals.

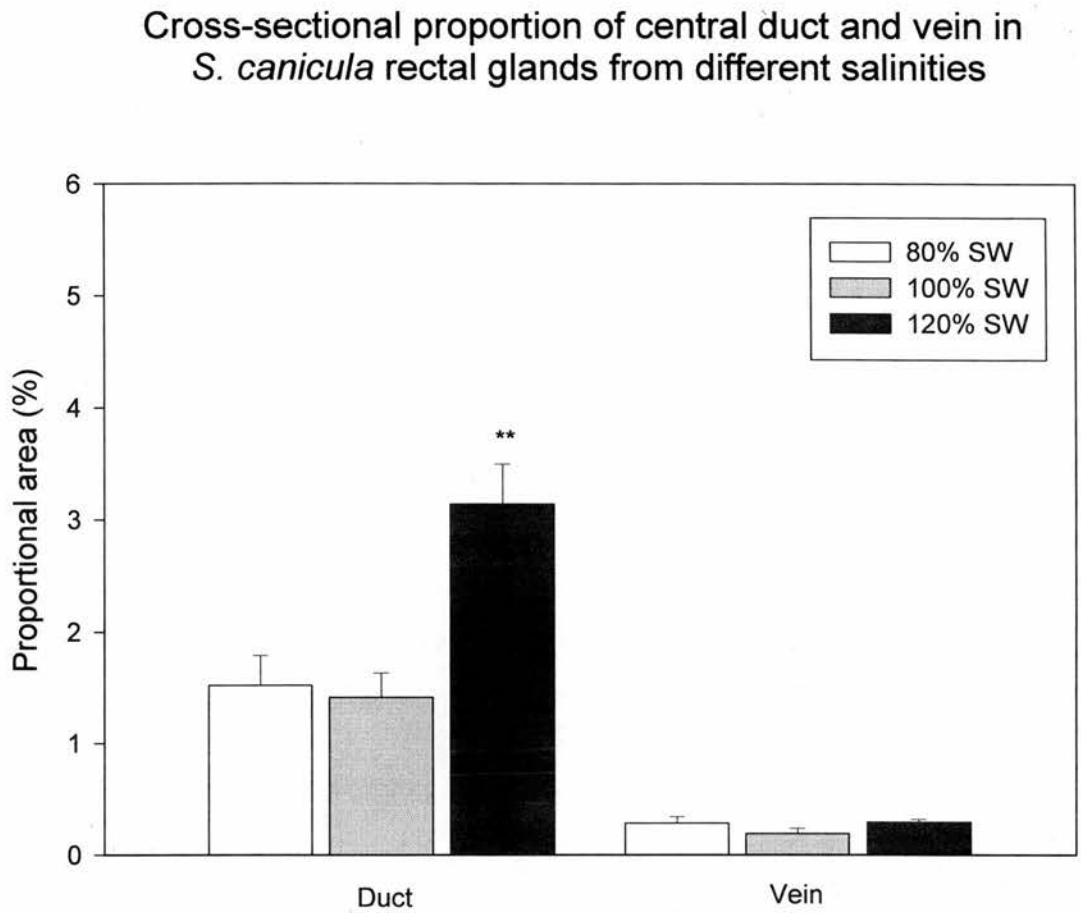

Figure 5.3.1.1 – Proportional area of the central duct and vein from rectal glands of *S. canicula* acclimated to 80, 100, and 120% SW. All values are presented as means ± SEM (n = 6, 5, and 6 respectively). Statistical analysis was performed via one-way ANOVA and a Tukey post hoc test. Significant differences from values for 100% SW were denoted as * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.005$).

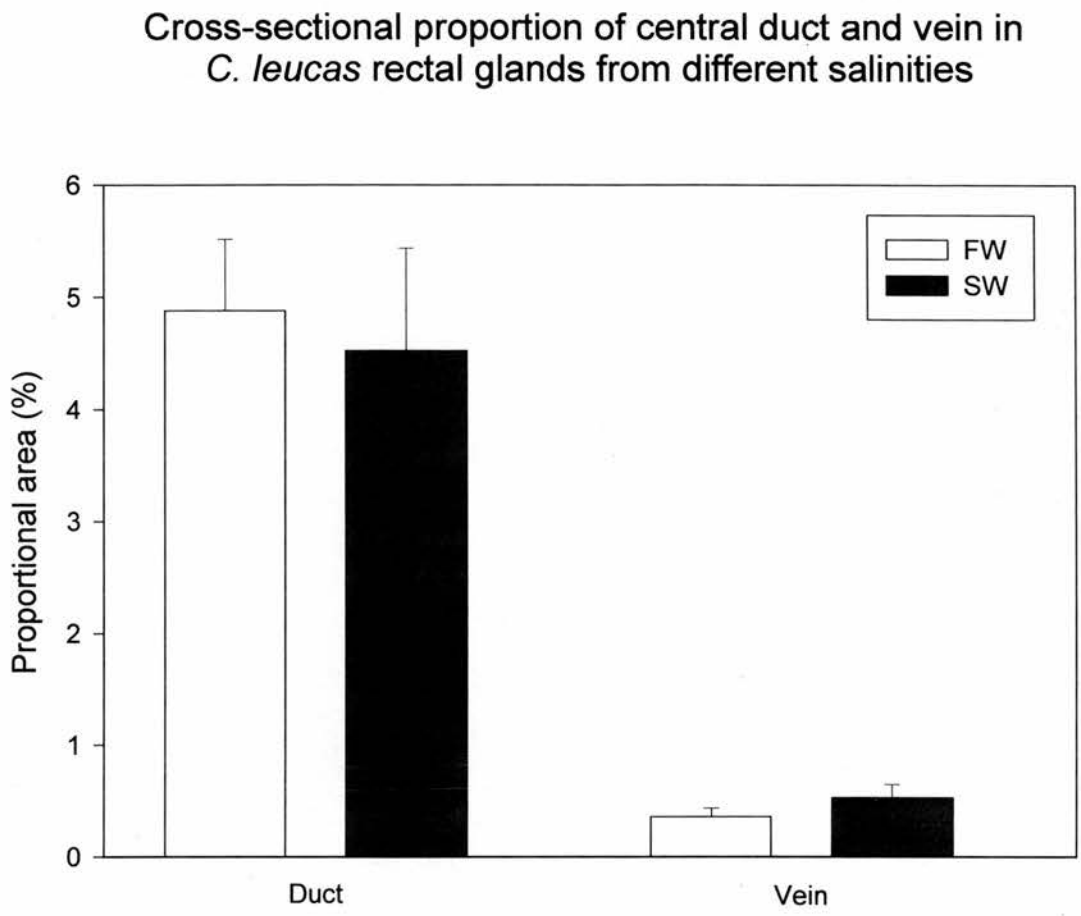

Figure 5.3.1.2 – Proportional area of the central duct and vein from rectal glands of *C. leucas* acclimated to FW and SW. All values are presented as means ± SEM (n = 4 and 5 respectively). Statistical analysis was performed via a two-tailed unpaired student's t-test and Welch correction factor. Significant differences from values for FW were denoted as * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.005$). No significant differences were recorded.

5.3.2 Maximal Na^+, K^+-ATPase activity

Maximal enzyme activity in the osmoregulatory tissues of *S. canicula* was lowest in the gills and in the intestine, and higher in the kidney and rectal gland respectively (Figure 5.3.2.1). Variation in maximal activity was low in the gill tissues from all salinities, and greater in the other three tissues. There were no significant differences in tissue maximal Na^+, K^+-ATPase activity between the different environmental salinities.

In *C. leucas* the gills and the intestine showed the lowest maximal activities of Na^+, K^+-ATPase for both FW and SW acclimated animals, with no significant differences between the two salinities (Figure 5.3.2.2). Maximal activity in FW acclimated animals was highest in the kidney, whereas the highest values for SW animals were in the rectal gland. Maximal enzyme activity was extremely significantly increased in the rectal glands of SW acclimated animals as compared to that of FW *C. leucas*. Conversely, maximal activity of Na^+, K^+-ATPase was highly significantly decreased in the kidneys of SW acclimated animals as compared to that of FW *C. leucas*.

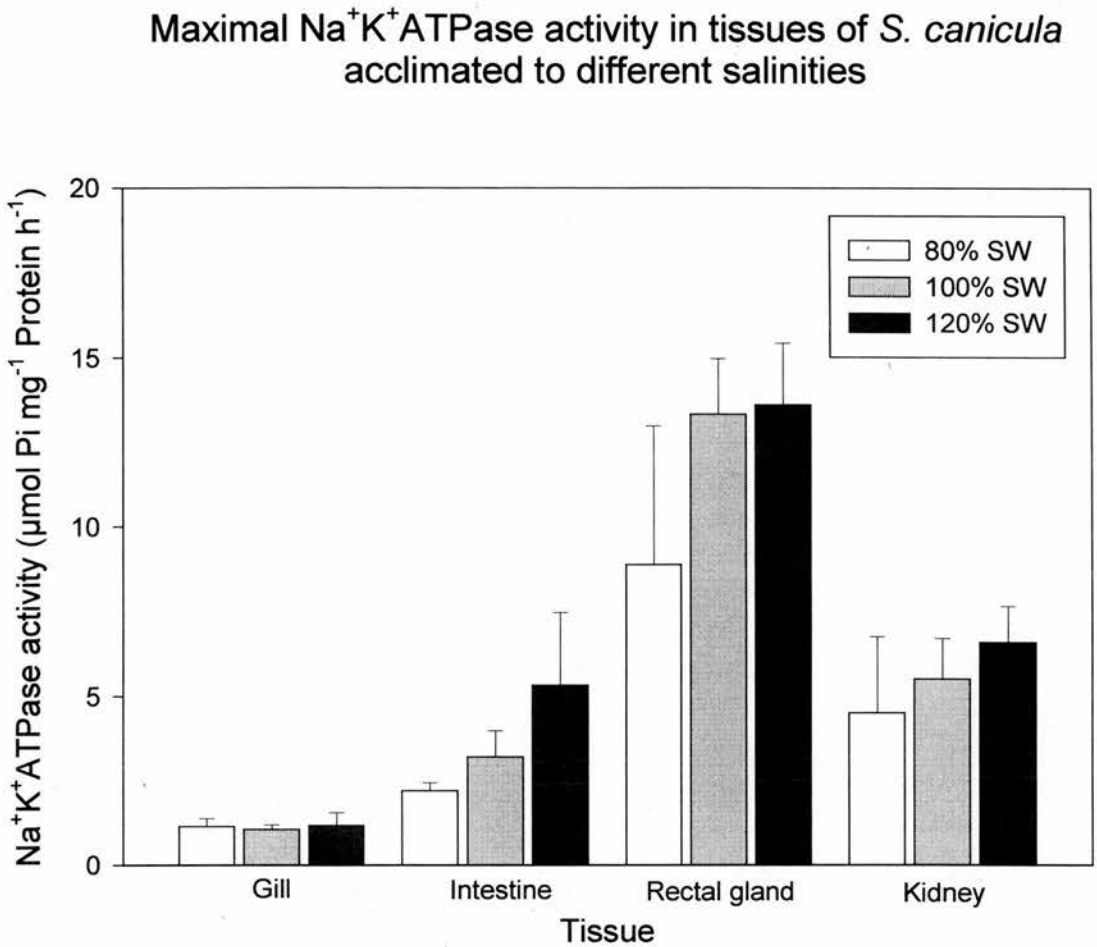

Figure 5.3.2.1 – Maximal Na^+, K^+-ATPase activity in the osmoregulatory tissues of *S. canicula* acclimated to 80, 100, and 120% SW. All values are presented as means ± SEM (n = 7, 5, and 7 for gill; 5, 8, and 5 for intestine; 4, 8, and 5 for rectal gland; and 4, 10, and 5 for kidney respectively). Statistical analysis was performed via one-way ANOVA and a Tukey post hoc test. Significant differences from values for 100% SW were denoted as * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.005$). No significant differences were recorded.

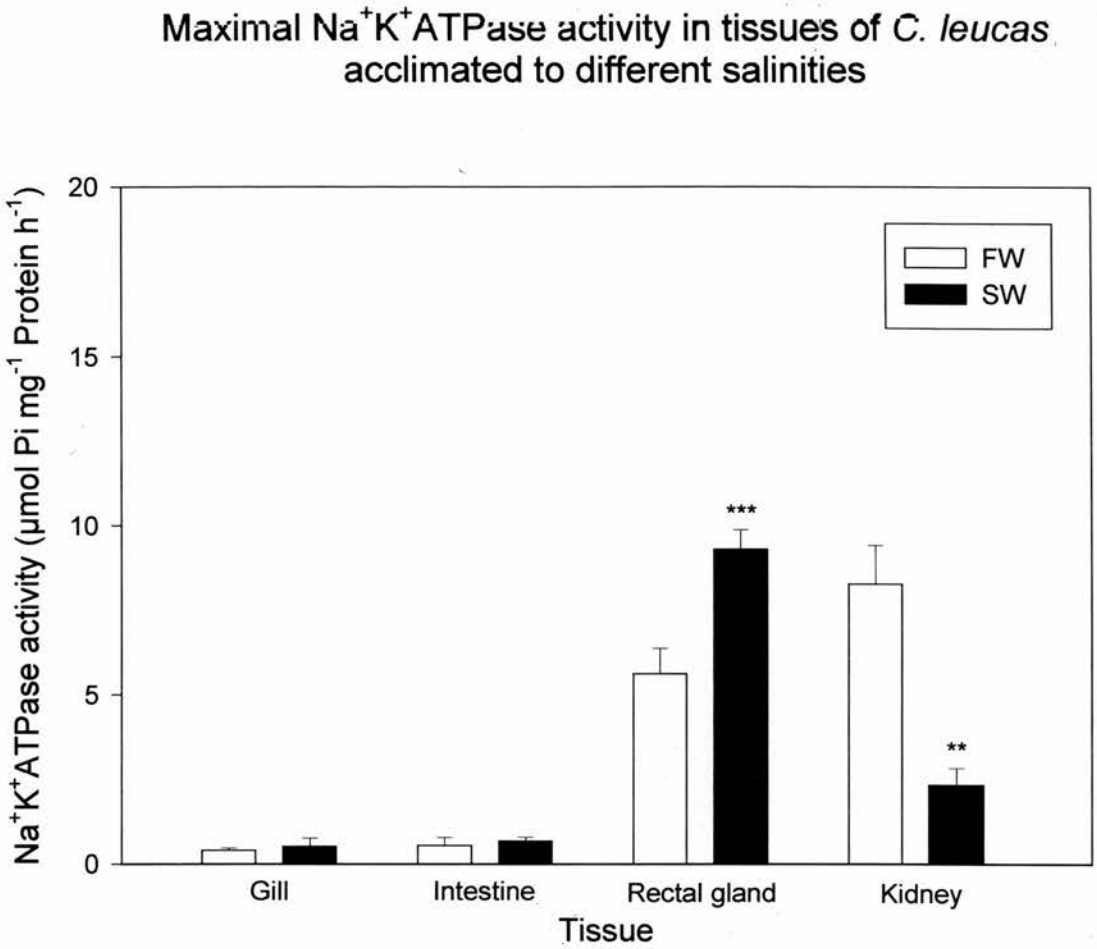

Figure 5.3.2.2 – Maximal Na^+, K^+-ATPase activity in the osmoregulatory tissues of *C. leucas* acclimated to FW and SW. All values are presented as means ± SEM (n = 6 and 4 for gill; 5 and 4 for intestine; 9 and 8 for rectal gland; and 5 and 6 for kidney respectively). Statistical analysis was performed via a two-tailed unpaired student's t-test and Welch correction factor. Significant differences from values for FW were denoted as * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.005$).

5.4 Discussion

The body mass of elasmobranchs is altered as a result of acclimation to changes in salinity (Table 5.3.1.2). The proportional body mass of *C. leucas* is significantly lower in SW than in FW acclimated animals. This is in part due to the haemoconcentration associated with acclimation to increased salinity which has been previously described (Chapter 3) in which a reduction of intra- and extracellular volumes results in a decrease of total body mass. This reduction in proportional body mass in SW acclimated *C. leucas* may also due to the metabolic breakdown of muscle tissue as a substrate for urea synthesis as animals undergoing acute transfer from FW to SW must increase urea concentration in the blood plasma. This could lead to a greater degree of muscle catabolism than is seen in captive FW *C. leucas*. The effect of captive salinity transfer on urea levels in *C. leucas* is described in depth below (Section 7.1.3).

Total length data should have been gathered for *S. canicula* in order to analyse any variation in proportional change in body mass associated with salinity acclimation in this species. However, there is no significant difference in the haematocrit of *C. leucas* acclimated to FW and SW (Table 2.3.1.2) and chronic acclimation to salinity transfer does affect body mass in this species; *S. canicula* does show changes in haematocrit associated with salinity change (Table 2.3.1.1) and therefore experiences a greater degree of concentration/dilution of body fluids associated with salinity change, as compared to *C. leucas*. Furthermore, given that rectal gland mass (Table. 5.3.1.1) and blood volume (Table 3.3.1.1) are both increased through acclimation to reduced salinity, it seems reasonable to assume that proportional body mass would also increase. Earlier studies have indeed demonstrated salinity acclimation having similar effects on body mass in this species (Hazon 1982). No significant differences were seen in the body

mass of *S. canicula* acclimated to the different salinities because the animals were of a wide range of age and size; unlike *C. leucas* which were all juveniles and showed very little variation in total length.

Interestingly, as elasmobranchs become proportionally heavier with acclimation to reduced salinities, the relative mass of the rectal gland increases disproportionately to whole body mass (Table 5.3.1.1). This could be a reflection of the highly vascular nature of the rectal gland, and given that blood flow to the secretory epithelia is increased in animals acclimated to reduced salinity (Anderson et al. 2002a), this most likely accounts for some of the increase in the proportional mass. Some of this increase in mass may also be due to intracellular volume expansion, but given that any such increase would likely occur in most cells of the body this would not account for the disproportionate increase in rectal gland mass relative to body mass. Increases in intracellular volume and blood volume and flow to the gland would explain the increase in cross-sectional area seen at reduced salinities in *S. canicula*. However, it is also possible that these represent natural variation in rectal gland size within *S. canicula*. It is impossible to know the exact effects of salinity transfer on rectal gland cross sectional area as measurements can only be taken *post mortem*, thereby preventing any before and after measurements. The fact that no such variation is seen in the cross-sectional area of rectal glands from *C. leucas* chronically acclimated to FW and SW suggests that the degree of dilution and concentration experienced by this species, in both intracellular and blood volumes, is less than that in *S. canicula*. This is further supported by the fact that the blood haematocrit of *S. canicula* is affected by acclimation to salinity change (Table 2.3.1.1), whilst that of *C. leucas* is unaffected by acclimation to a larger change in salinity (Table 2.3.1.2).

The analysis of rectal gland histology also shows variations between salinities and species. Although there was much variation in the cross-sectional area of the central duct in the rectal gland of *S. canicula*, there were no significant differences between the three salinities. It must be borne in mind that changes in the area of the central duct may not represent actual structural changes; they may reflect the secretory state of the glands at the time of excision. For example, a gland that was actively secreting upon excision may have a larger central duct due to the volume of RGF being passed through it and the elasticity of the duct. Although rectal glands of both species had similar cross-sectional areas, the glands from *C. leucas* had significantly larger central ducts than those of *S. canicula*. Furthermore, there was no significant difference in the proportional area of the central duct in *C. leucas* rectal glands from FW and SW (Figure 5.3.1.2). The fact that there was no difference in duct area between a rectal gland with no requirement to secrete Na^+ and Cl^- (FW acclimated *C. leucas*) and one with a requirement for intermittent active secretion (SW acclimated *C. leucas*), suggests that differences in duct area are due to changes in structure and not due to changes in secretion rate and duct elasticity. However, this cannot be taken as the case for all species of elasmobranchs; the different orientation of blood flow recorded in the rectal glands of *S. acanthias* (Kent and Olsen 1982) and *H. portusjacksoni* (Newbound and O'Shea 2001) are illustrative of how rectal gland structure can vary between species.

In rectal glands of *S. canicula* acclimated to 120% SW the central duct encompassed significantly more of the cross-sectional area when compared to glands from 80 and 100% SW (Figure 5.3.1.1). No changes were seen in the proportional area of the central vein in either species upon acclimation to changes in salinity. This demonstrates that the

partially euryhaline *S. canicula* may modify the structure of the rectal gland in response to elevated salinity, but no modification is made in the rectal gland of the fully euryhaline *C. leucas* in response to a greater increase in salinity.

MacKenzie (1996) found that the maximum diameter of both the central duct and vein were increased in *S. canicula* 12 hours after feeding when compared to starved animals. This indicates that rectal gland structure changes in response to an increased salt load, presumably to accommodate the resulting increase in rectal gland secretory output, and that these changes in structure persist 12 hours after the feeding event. The results of this study also suggest that the rectal gland structure of *S. canicula* changes in response to a requirement to increase Na^+ and Cl^- clearance. There is a significant increase in the proportional size of the central duct, presumably as a result of increased RGF secretion, and this change in structure persists for at least 14 days after salinity transfer. The increase in blood flow to the secretory epithelia of the rectal gland 4 days after transfer to reduced salinity illustrated previously (Anderson et al. 2002a), does not result in a persistent increase in the proportional size of the central vein 14 days after salinity transfer.

The lack of modification in rectal gland structure seen in *C. leucas* can be explained by a number of reasons. It is possible that any changes in structure associated with an increased requirement for the clearance of Na^+ and Cl^- do not persist after 7 days. Certainly a more rapid ability to respond to changes in salinity would seem intuitive for a fully euryhaline species which may move from FW to SW in a matter of hours. Also, because the central duct is proportionally larger in the fully euryhaline *C. leucas* as compared to the partially euryhaline *S. canicula*, increases in the relative size of the duct

in response to acclimation to higher salinities may not be necessary to increase Na^+ and Cl^- clearance in this species. Lacking any need to change structure in order to increase rectal gland secretion rate would also decrease the time taken to acclimate to changes in salinity in a fully euryhaline species; more so than an increased rate of structural change. The fact that the proportional area of the central duct in FW *C. leucas* (which have little requirement for rectal gland secretion to maintain osmotic homeostasis) is over double that of SW *S. canicula* (which do require rectal gland secretion (Chapter 4)), and that removing the requirement for structural change would give the largest decrease in rectal gland response time to salinity change, are compelling reasons to conclude that *C. leucas* does not change rectal gland structure in response to salinity transfer, unlike *S. canicula*. This may be a crucial difference with regard to the degree of euryhalinity in the two species.

The changes seen in the rectal gland structure of *S. canicula* are not coupled with any significant change in the maximal activity of Na^+, K^+-ATPase in rectal gland homogenates (Figure 5.3.2.1). It is important to state that this study assessed maximal enzyme activity and not the level of actual activity *in vivo*. Therefore any differences in maximal activity reflect an increased capacity for the movement of Na^+ and K^+, and not necessarily an increase in the rate of movement of these ions *in vivo*. Changes in maximal activity can therefore be explained by two reasons: either the abundance of Na^+, K^+-ATPase in the tissues remains constant and there is a change in the amount of pumps able to be recruited in the assay, or there has been a change in the level of genetic expression for the pumps and this has translated to an actual change in the abundance of the Na^+, K^+-ATPase protein. Given that values for the mRNA expression and abundance of Na^+, K^+-ATPase were found to be high in both FW and SW

acclimated *C. leucas* with levels of fluorescence hindering accurate quantification (Meischke *pers. comm.)*, it is most likely that the differences in Na^+, K^+-ATPase maximal activity represent variation in the recruitment of available pumps. This is discussed further below.

For *S. canicula* acclimated to all salinities maximal enzyme activity was highest in the rectal gland (Figure 5.3.2.1). This is illustrative of the role the enzyme plays in the active movement of Na^+ and Cl^-, as well as the importance of the rectal gland as a means of secreting these ions. No significant differences were seen in the maximal activity of Na^+, K^+-ATPase in any of the osmoregulatory tissues of *S. canicula* acclimated to the three salinities. This is probably a reflection of the salinity changes which are naturally experienced by the species. *S. canicula* faces moderate salinity changes in the wild and the results of this study suggest that the osmotic responses to those changes are achieved without modifying either the abundance or recruitment of Na^+, K^+-ATPase. It is also possible that modifications in *S. canicula* may not persist after 14 days at altered salinity and therefore no differences in enzyme maximal activity were recorded. Alternatively the changes in salinity used in this study may not have been sufficient to elicit any modification Na^+, K^+-ATPase recruitment or abundance. Certainly the salinity transfers employed would not have resulted in a persistent reversal of the fluxes of Na^+ and Cl^- between the animals and the environment, as was the case for *C. leucas*. Given that *S. canicula* cannot survive in full FW, and mortality rates are high with salinity transfers below 60% SW, clearly the species is unable to modify the action of the osmoregulatory organs as completely as fully euryhaline elasmobranchs. This would support the idea that *S. canicula* is unable to modify Na^+, K^+-ATPase abundance or recruitment due to the natural salinity range of the species, rather than the

salinity changes not being great enough to elicit changes in activity. Further studies on the species should investigate a wider range of environmental salinities over a time course of acclimation periods in order to accurately assess the limitations of modifying Na^+, K^+-ATPase maximal activity in *S. canicula*.

Given that *S.* canicula does not alter the abundance and/or recruitment of Na^+, K^+-ATPase, controlling the activity of active ion transport in the osmoregulatory tissues must therefore be achieved by some other means. In the case of the rectal gland this can be through greater periods of activity/inactivity and changes in the vascular perfusion of the secretory epithelia (Anderson et al. 2002a) which result in modifications in Cl^- concentration and clearance via the RGF (Section 4.3).

Modifications in the maximal activity of Na^+, K^+-ATPase were seen in the osmoregulatory tissues of *C. leucas* acclimated to FW and SW (Figure 5.3.2.2). Although no significant differences were seen in rectal gland structure, there was a significant increase in enzyme maximal activity in the rectal gland of animals acclimated to SW. This increase in rectal gland Na^+, K^+-ATPase maximal activity reflects the requirement for active secretion of excess Na^+ and Cl^- in the SW environment. This increased requirement is, in part, met by an increase in either the recruitment or expression of Na^+, K^+-ATPase in the tissues of the rectal gland.

Acclimation from FW to SW results in a reversal of the concentration gradients for Na^+ and Cl^- between the environment and the internal fluids of *C. leucas*. FW animals face a continual loss of these ions across the semi-permeable surfaces and there is therefore no requirement for their active secretion from the rectal gland. Such high levels of maximal

activity in a possibly inactive gland may provide an important degree of plasticity in the osmoregulatory mechanisms of a fully euryhaline elasmobranch. If the enzymes responsible for active rectal gland secretion are functional in FW this would dramatically decrease the time taken for glandular secretion to commence/increase in response to salinity transfer. It is therefore likely that regulation of secretion by the rectal glands of FW *C. leucas* is achieved through mechanisms such as reduced blood flow (Shuttleworth and Thompson 1986; Anderson et al. 2002a) and controlling the permeability of the apical chloride channel (Riordan et al. 1994), as a means of reducing the loss of Na^+ and Cl^- in FW.

Conversely in SW, there is a continual influx of Na^+ and Cl^- across the semi-permeable surfaces and the rectal gland actively secretes in order to maintain plasma concentration levels of these ions. This active secretion is facilitated in part by a significant increase in the recruitment and/or abundance of Na^+, K^+-ATPase in the secretory epithelia of the rectal gland. These findings are consistent with those for the fully euryhaline *D. sabina* which showed elevated activity and expression of Na^+, K^+-ATPase in the rectal glands of SW acclimated and SW captured animals as compared to those from FW (Piermarini and Evans 2000). The levels of mRNA expression and enzyme abundance for Na^+, K^+-ATPase in the rectal glands of *C. leucas* utilised for this study were investigated in a related study (Meischke, *pers. comm.*). When taken in conjunction with the results presented here for maximal Na^+, K^+-ATPase activity in the rectal glands of *C. leucas* acclimated to FW and SW they suggest that alterations in rectal gland activity are primarily achieved through post-translational mechanisms. Furthermore such mechanisms permit FW acclimated *C. leucas* to maintain high levels of Na^+, K^+-ATPase in the rectal gland without the energetic costs associated with their activity.

When compared to the results of *D. sabina* this may well represent separate evolution of different methods which permit full euryhalinity in elasmobranch fish.

There was no significant difference in the maximal activity of Na^+, K^+-ATPase in the gills of FW and SW acclimated *C. leucas*. This is surprising given that recent investigations into the localisation and activity of transport enzymes have shown significant increases in branchial tissue of FW, as opposed to SW acclimated *D. sabina* (Piermarini and Evans 2000; Piermarini and Evans 2001; Piermarini et al. 2002). The implications of this are discussed below (Sections 7.1.1 and 2).

Maximal activity of Na^+, K^+-ATPase in the intestine of *C. leucas* was unaffected by acclimation to SW (Figure 5.3.2.2), suggesting no modification in the intestinal tissues as a result of salinity transfer. This is surprising given that elasmobranchs increase drinking rate as a means of elevating plasma osmolality during acclimation to increased salinity (Anderson et al. 2002b), particularly given the magnitude of change from FW to full SW. No other studies have investigated intestinal Na^+, K^+-ATPase activity in elasmobranchs during salinity change, although activity is generally higher in SW acclimated teleosts (Jampol and Epstein 1970; Nielsen et al. 1999). The implications for this finding in fully euryhaline elasmobranchs are discussed below (Section 7.1.3).

There were significant differences in maximal Na^+, K^+-ATPase activity in the kidney tissue of *C. leucas* acclimated to FW and SW (Figure 5.3.2.2). Maximal activity of Na^+, K^+-ATPase was significantly reduced in *C. leucas* after 7 day acclimation to SW, reflecting the different roles of the kidney in FW and SW environments. The ability to reduce urinary loss of osmolytes is an important factor in the maintenance of ion

balance in FW elasmobranchs, particularly given the magnitude of the gradient for the influx of water. Exposure of other fully euryhaline elasmobranchs to decreased salinities has increased urine flow rates and decreased urine osmolality, resulting in an increase in absolute free-water clearance (Payan et al. 1973; Janech et al. 1998; Janech and Piermarini 2002).

The decreased maximal activity of Na^+, K^+-ATPase seen in the kidneys of SW acclimated *C. leucas* suggests that the enzyme has a more important role in active ion reabsorption in FW, than in ion secretion in SW when the rectal gland is active. Furthermore, plasma Na^+ and Cl^- concentrations in FW *C. leucas* are in part dependent on active renal reabsorption rates, maintained by high Na^+, K^+-ATPase maximal activity. This high rate of maximal activity is also likely to increase Na^+ linked urea reabsorption (Section 1.6).

The fact that the partially euryhaline *S. canicula* does not significantly alter the maximal activity of Na^+, K^+-ATPase in the osmoregulatory organs in response to salinity change, and that the fully euryhaline *C. leucas* does, gives great insight into the mechanisms which determine the osmoregulatory capacity of elasmobranchs at different salinities. Given the differences in rectal gland structure and maximal Na^+, K^+-ATPase activity between the two species further investigation into the rectal gland was conducted in order to gain a greater understanding of the modifications within the tissue following chronic acclimation of both partially and fully euryhaline elasmobranchs.

Chapter 6: Rectal gland respirometry

6.1 Introduction

The secretory activity of the rectal gland has already been investigated in *S. canicula* (Chapter 4), but the nature of *C. leucas* and its reaction to anaesthetic prevented similar studies to be conducted. Also, the intermittent nature of the gland proved a major limitation during *in vivo* assessment. Furthermore, *in vivo* experiments are not ideal for studying individual endocrine control factors as it is impossible to isolate the effects of introduced substances. Therefore an *in vitro* technique was required.

Another method of assessing rectal gland activity is through respirometry studies and investigating the O_2 consumption of the gland. Such studies have been conducted on both *S. canicula* (Shuttleworth and Thompson 1980) and *S. acanthias* (Morgan et al. 1997). Shuttleworth and Thompson (1980) conducted respirometry studies on tissue from the rectal gland, spleen, and kidney of SW *S. canicula*. They discovered that whole tissue O_2 consumption in the spleen (94 ± 25 µl O_2 g^{-1} h^{-1}) was significantly lower than that in the rectal gland (234 ± 59 µl O_2 g^{-1} h^{-1}) and the kidney (248 ± 61 µl O_2 g^{-1} h^{-1}). This compares to a value of 27.9 µl O_2 g^{-1} h^{-1} for whole animal O_2 uptake measured by Butler and Taylor (1975). The rate of O_2 uptake in these tissues was higher than that of the whole animal; this is due to the fact that a large proportion of body mass is associated with tissues with low O_2 consumption, such as skeletal elements and body fluids.

The markedly higher O_2 consumption rates of the rectal gland and the kidney reflect a higher metabolic rate in these tissues which is largely due to active osmolyte transport in these osmoregulatory tissues (Sections 1.5 and 6). These tissues also have higher maximal activities of Na^+, K^+-ATPase, even compared to other osmoregulatory tissues

(Figure 5.3.2.1). Conversely the low O_2 consumption seen in tissue from the spleen indicates a lower metabolic activity, reflecting its role in the storage and release of red blood cells.

Morgan and co-workers (1997) investigated the O_2 consumption in the rectal gland and gills of *S. acanthias*. It was found that O_2 consumption in the rectal gland (14.2 ± 1.2 μmol O_2 g^{-1} h^{-1}) was significantly higher than that in the gill (9.6 ± 1.4 μmol O_2 g^{-1} h^{-1}). Again this is consistent with the maximal activity of Na^+, K^+-ATPase in these tissues in *S. canicula* (Figure 5.3.2.1). The addition of 0.5 mM ouabain and the resulting inhibition of Na^+, K^+-ATPase were associated with a 54.9 and 21.8% reduction in rectal gland and gill O_2 consumption respectively. Furthermore, the residual levels of O_2 consumption in the two tissues did not differ (Morgan et al. 1997). This suggests that different levels of Na^+, K^+-ATPase activity or abundance are the cause of higher O_2 consumption in the rectal gland of *S. acanthias*.

Shuttleworth and Thompson (1980) also investigated the effects of 10^{-4} M ouabain on basal O_2 consumption in tissue from the rectal gland, spleen, and kidney. The inhibition of Na^+, K^+-ATPase with ouabain significantly reduced O_2 consumption rates in all three tissues. The proportion of whole tissue O_2 consumption associated with Na^+, K^+-ATPase was calculated as 22.1, 20.2, and 41.1% in the rectal gland, spleen, and kidney respectively. Given that maximal activity of the enzyme is higher in the rectal gland than in the kidney (Figure 5.3.2.1) these findings suggest that a large proportion of O_2 consumption in the rectal gland is associated with other metabolic processes. The differences in ouabain-sensitive O_2 consumption during basal secretion in the rectal glands of *S. acanthias* and *S. canicula* (54.9 and 22.1% respectively) either reflect a

difference in rectal gland structure between the two species, differences in the degree of ouabain inhibition of Na^+, K^+-ATPase, or differences in the secretory activity of the glands sampled between the two species. It has been demonstrated in *S. canicula* that 10^{-4} M ouabain does not completely inhibit Na^+, K^+-ATPase, unlike concentrations above 1 mM (MacKenzie 1996). This may have lead to an underestimation of the relative O_2 consumption by Na^+, K^+-ATPase in the rectal gland of *S. canicula*.

Supporting the finding of high maximal Na^+, K^+-ATPase activity in the rectal gland is the increase in rectal gland O_2 consumption following administration of cAMP (0.05 mmol l^{-1}) and theophylline (0.25 mmol l^{-1}). A 5-fold increase in O_2 consumption was associated with these substances, and this was entirely abolished by the coupled administration of ouabain. Interestingly, the same administration of cAMP and theophylline had no effect on O_2 consumption in either the spleen or the kidney (Shuttleworth and Thompson 1980). Similar concentrations of these substances have been shown to increase secretory activity in isolated perfused rectal glands (Silva et al. 1977; Stoff et al. 1977b), as well as increase ouabain binding (Shuttleworth and Thompson 1978). These findings suggest that cAMP and theophylline specifically stimulate the activity of Na^+, K^+-ATPase in the tissues of the rectal gland. This is consistent with other studies which have suggested that cAMP activates Na^+, K^+-ATPase thereby reducing cellular concentrations of Na^+ in cultured rectal gland cells (Lear et al. 1992). Furthermore, O_2 consumption in the rectal gland is increased by the addition of theophylline alone, to levels equivalent to those associated with the coupled administration of cAMP (Shuttleworth and Thompson 1980). Theophylline inhibits the breakdown of cAMP by phosphodiesterases. This illustrates the stimulatory effect of endogenous cAMP on Na^+, K^+-ATPase in the rectal gland, as well as suggesting the

continued production of this cAMP in incubated tissue slices. These findings are contrary to those of Stoff and co-workers (1977b) who reported a synergistic effect of cAMP and theophylline on Cl^- secretion rates during coupled administration on isolated perfused rectal glands. Theophylline may therefore also have effects on other proteins involved in ion transport which do not require increased O_2 consumption during periods of active secretion.

The results presented by Shuttleworth and Thompson (1980) are not only illustrative of the intermittent nature of rectal gland activity, but also of the large scope for increased activity of Na^+, K^+-ATPase during stimulated periods of active secretion. The hormonal regulation of Na^+, K^+-ATPase has been reviewed by Gick and co-workers (1988) and they drew a distinction between factors which act in minutes (through altering ion permeability or direct activation of the enzyme), and those which act over hours (through changes in pump abundance). Due to the nature of respirometry studies on sections of isolated glands the action of fast acting factors on Na^+, K^+-ATPase activity are of greater importance.

One such group of fast acting factors are vasopressins (such as AVT in elasmobranchs (Section 1.8.2)) which regulate urine flow rate, GFR, and tubular transport maxima for glucose in the kidney (Amer and Brown 1995; Wells et al. 2002). Similar antidiuretic hormones have been shown to increase Na^+ entry into cells via the number of functional Na^+ channels, and hence stimulate the action of Na^+, K^+-ATPase in a variety of vertebrates (Mendoza et al. 1980; Li et al. 1982; Reznik et al. 1985).

Another group of fast acting factors are catecholamines (Section 1.12) which have been shown to induce cAMP-mediated stimulation of Na^+, K^+-ATPase activity in skeletal muscle which were independent of changes in Na^+, K^+-ATPase abundance (Clausen and Hansen 1977). Studies on vertebrate cerebral cell cultures have shown that catecholamine stimulation of Na^+, K^+-ATPase activity can also be independent of cAMP levels (Wu and Phillips 1980). Clearly more research is required into the effects of catecholamines on Na^+, K^+-ATPase, particularly in elasmobranchs.

Thyroid hormones, such as thyroxine and triiodothyronine, have also been shown to stimulate O_2 consumption and active Na^+ and K^+ transport in number of vertebrate tissues (Gick et al. 1988).

CNP is the only natriuretic peptide in elasmobranchs (Sections 1.11.1 and 4.1) and it has been shown to bind with high affinity to two different receptors in the plasma membranes of rectal gland cells: a clearance receptor and a guanylate cyclase-linked GC-B type receptor (Gunning et al. 1993). CNP is a potent stimulant of the enzyme guanylate cyclase increasing its intracellular activity in the rectal gland of *S. acanthias* (Gunning et al. 1993). Guanylate cyclase converts guanosine triphosphate (GTP) into cyclic guanosine monophosphate (cGMP). However, perfusion of isolated rectal glands with cGMP does not stimulate chloride secretion (Silva et al. 1987). Silva and co-workers (1996) noted that exogenous cGMP had an inconsistent effect of stimulating short-circuit current in cultured rectal gland cells. Later work showed that CNP significantly increased short-circuit current in cultured rectal gland cells (Silva et al. 1999). It would appear therefore that intracellular cGMP is important in mediating the effects of CNP, although the precise action of this is unclear.

The mode of stimulation for rectal gland secretion by CNP is highly complex, although a generalised model can be drawn (Figure 6.1.1). In *S. acanthias* CNP acts as a stimulus for the release of VIP from rectal gland nerves, although CNP also has well documented direct effects on both isolated tubules (Solomon et al. 1993; Solomon et al. 1995a; Solomon et al. 1995b), and cultured rectal gland cells (Karnaky et al. 1992; Karnaky et al. 1993; Silva et al. 1999). In the same species, CNP can also act through increased activity of guanylate cyclase, as detailed above; although the actions of other natriuretic peptides have also been isolated from any increase in intracellular cGMP (Budzik et al. 1987; Barrett and Isales 1988; Lear et al. 1990). This raises the possibility of CNP acting on rectal gland cells via a pathway independent of cGMP.

One pathway via which this may occur is the inositol phosphate pathway, as work by Ecay and Valentich (1990) demonstrated that VIP increased inositol phosphate formation. Investigation conducted by Silva and co-workers (1999) suggested that at least part of the stimulatory action of CNP may occur via a similar route. Specific inhibition of protein kinase C (PKC) completely removed the cGMP-independent stimulatory effect of CNP in isolated perfused rectal glands of *S. acanthias*. However, pharmacological activation of PKc did not stimulate Cl^- secretion in similar preparations (Silva et al. 1999). It is possible therefore that the synergistic action of cGMP and PKC are required for increasing Cl^- secretion in response to CNP in the tissues of the rectal gland.

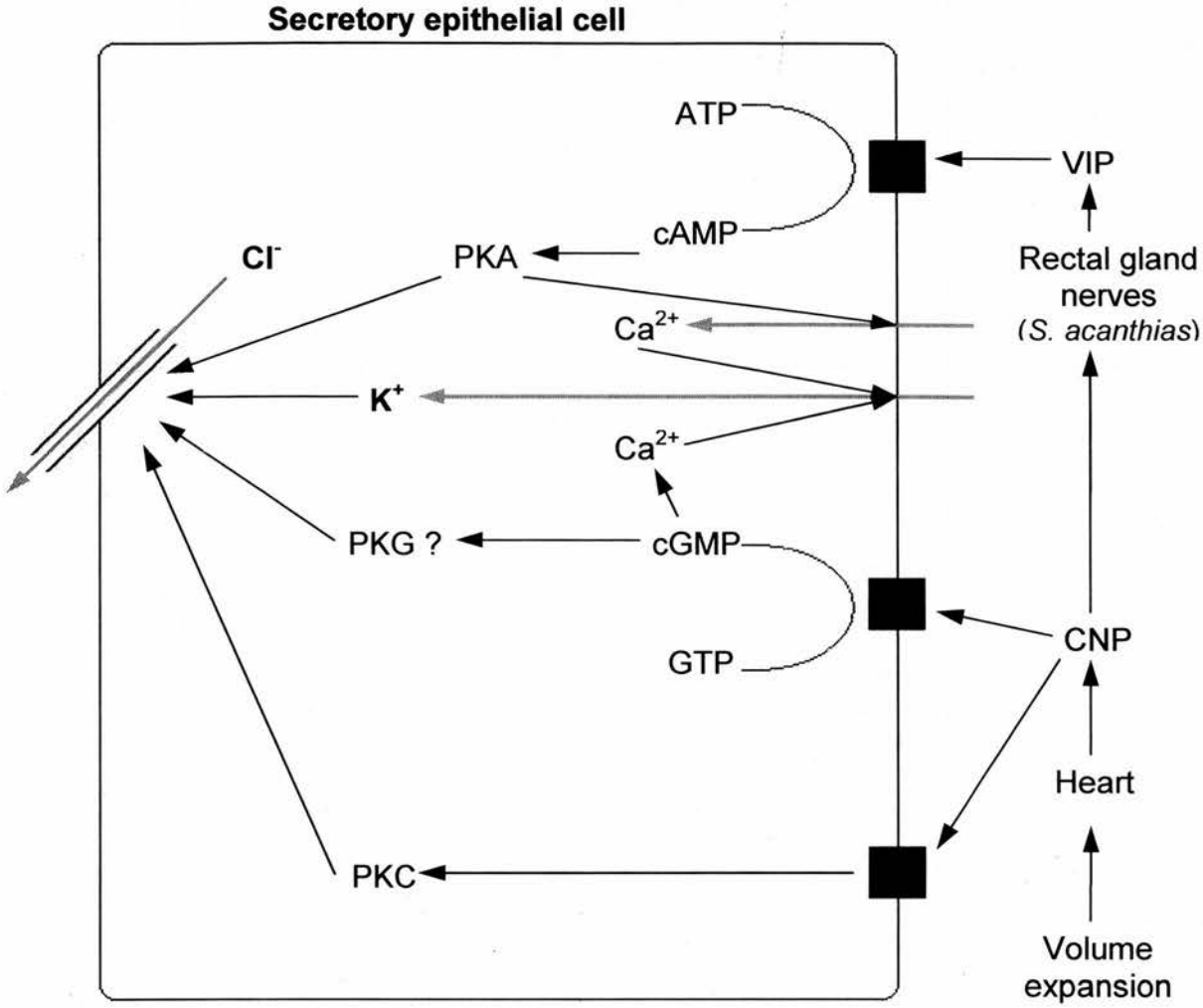

Figure 6.1.1 – Generalised model of stimulation by CNP on rectal gland secretory cells. Volume expansion causes the release of CNP from the heart. CNP binds to a guanylate cyclase B-type receptor stimulating the production of cGMP. This causes a rise in the release of intracellular stores of Ca^{2+} which increases basolateral K^+ conductance, which in turn stimulates Cl^- secretion. cGMP also acts via an undetermined route to cause an increase in Cl^- secretion, possibly via protein kinase G (PKG). CNP also has a parallel stimulatory action on protein kinase C (PKC). In the case of *S. acanthias* CNP also stimulates release of VIP which increases cAMP levels which stimulates protein kinase A (PKA). This causes a separate increase in Ca^{2+} influx as well as having direct effects on Cl^- secretion (Warth et al. 1998; Silva et al. 1999).

CNP may also affect Cl^- secretion rate through alterations in intracellular Ca^{2+} as outlined above (Warth et al. 1998) (Figure 6.1.1). It has been demonstrated that Ca^{2+} can stimulate NaCl secretion from isolated rectal gland tubules of *S. acanthias*. Intracellular Ca^{2+} concentration can be elevated by carbachol stimulated store release. This release of Ca^{2+} from intracellular stores is independent of cAMP and acts to increase basolateral K^+ conductance, thereby stimulating Cl^- secretion. Intracellular Ca^{2+} concentration can also be elevated in response to cAMP, by increasing the rate of transmembrane influx via protein kinase A (Warth et al. 1998). Intracellular Ca^{2+} may therefore also be important in mediating the response of the rectal gland to CNP. This also suggests that Ca^{2+} may be important in two distinct methods of stimulating rectal gland secretion (Figure 6.1.1).

Furthermore, Ca^{2+} has been found to directly reduce rectal gland secretion. This can occur via two distinct modes of action: constriction of the rectal gland artery and a reduction in blood perfusion, and also through a reduction in intracellular Ca^{2+} concentration by reduced influx (Fellner and Parker 2002). Ca^{2+} could play a major role in controlling the activity of the rectal gland through a combination of modifying blood flow and tubular secretion.

Despite the lack of a definitive mode of action, CNP has well documented effects of the osmoregulatory tissues of elasmobranchs (Sections 1.11.1, 4.1). There is also wide scope for the modulation of these effects. For these reasons CNP was the chosen stimulant for this study which investigated the effects of salinity acclimation on the respiratory parameters of rectal glands from the partially euryhaline *S. canicula* and the fully euryhaline *C. leucas*.

In this study the O_2 consumption by rectal glands was measured in a partially (*S. canicula*) and a fully (*C. leucas*) euryhaline species of elasmobranch in order to assess any differences associated with acclimation to salinity change. Furthermore, following from the study of maximal Na^+, K^+-ATPase activity in the rectal glands of both species (Chapter 5), the O_2 consumption associated with this enzyme was also measured. The effect of CNP on both parameters was also measured in order to gain further understanding of the role of this hormone in the endocrine control of rectal gland function in different elasmobranch species.

6.2 Materials and methods

6.2.1 Chemicals and equipment

Unless otherwise stated all chemicals used were obtained from Sigma. Recipes for Ringer solutions used on both species were identical to those detailed previously (Section 2.2.3).

Respirometry experiments on *S. canicula* were conducted at 11 °C using 1302 O_2 electrodes and a 928 6-channel measurement system (Strathkelvin Instruments Ltd., Glasgow). The data was analysed using 928 O_2 system version 2.2 (Strathkelvin Instruments Ltd.). Respirometry chambers were kept at a constant temperature via a model LTD6 refridgerated bath (Grant Instruments Ltd., Cambridge). All solutions were also kept at a constant temperature via this method and bubbled with air for 10 minutes before use.

Respirometry experiments on *C. leucas* were conducted at 23 °C using a 781 O_2 electrode and metre (Strathkelvin Instruments Ltd.), and a microrespirometer sampling at 4 Hz (Strathkelvin Instruments Ltd.). The analogue signal was sent to a PowerLab 4/20 (AD Instruments Pty. Ltd., Castle Hill, NSW, Australia) running Chart 5.0 software (AD Instruments Pty. Ltd.). The microelectrode was inserted into a glass respiration chamber which was water-cooled to a constant temperature via a model LTD6 refridgerated bath (Grant Instruments Ltd.). All solutions were also kept at a constant temperature via this method and bubbled with air for 10 minutes before use.

6.2.2 Tissue sampling

Sampling protocols for both species were as follows: 4 transverse sections were cut from the middle portion of the rectal gland, approximately 1 mm in thickness. For studies on *S. canicula* all slices were placed in Ringer solution of the appropriate salinity and kept in the water bath. For experiments on *C. leucas* where slices were analysed individually, all slices were placed in Ringer and stored at 4 °C in a fridge until 15 minutes before use, at which time they were placed into Ringer solution in the water bath to acclimate.

After respirometry experiments all tissue slices were blotted dry on tissue paper and had the wet mass recorded.

6.2.3 Data collection

Upon setup all electrodes were calibrated for daily atmospheric pressure: for *S. canicula* data was obtained from Leuchars weather station (BBC 2003); for *C. leucas* data was obtained from the University of Queensland weather station (Geography 2003). Use of a multi-channel system allowed all 4 slices from the rectal glands of *S. canicula* to be studied at the same time; for *C. leucas* slices were analysed individually. For simplicity the protocol used for one chamber of the study on *S. canicula* is described below, with changes in protocol for *C. leucas* being noted.

660 µl of Ringer solution of the appropriate salinity was added to the respirometry chamber (200 µl for *C. leucas*), and the O_2 consumption of the electrode was measured for 15 minutes in order to compensate for this during tissue studies. The respirometry chamber was then thoroughly rinsed with Milli Q before being refilled with Ringer and the tissue introduced. O_2 consumption was again measured for a 15 minutes period. The tissue was then removed and placed in Ringer whilst the respirometry chamber was rinsed again. The chamber was then filled with one of the following solutions: 10^{-8} M CNP in Ringer solution, 10^{-10} M CNP, 10^{-12} M CNP, or just Ringer solution. The CNP used was homologous for *S. canicula* and was kindly donated by Prof. Y. Takei. Subsequent analysis of CNP in *C. leucas* demonstrated an identical amino acid sequence (Takei, *pers. comm.*). The tissue was then replaced in the chamber and O_2 consumption was again measured for 15 minutes. The tissue was then removed, the chamber rinsed, and then refilled with the same solution as before plus 2mM ouabain. The tissue was replaced in the chamber and O_2 consumption was measured for a final 15 minutes.

The concentration of CNP used in any particular electrode was rotated to avoid bias in the results (with *C. leucas* the order in which the CNP concentrations were used was rotated as a single electrode was used). The concentrations of CNP used were chosen based on previous work investigating affects on the rectal gland (Anderson et al. 2002a). The concentration of ouabain was chosen based on previous work on Na^+, K^+-ATPase in the rectal gland (Pillans et al. 2005) (Chapter 5).

O_2 consumption was calculated via linear regression on the final 10 minutes of each 15 minute period of observation as consumption was most consistent during this period (Figure 6.2.3.1). Rates were then normalised per gram of rectal gland wet mass.

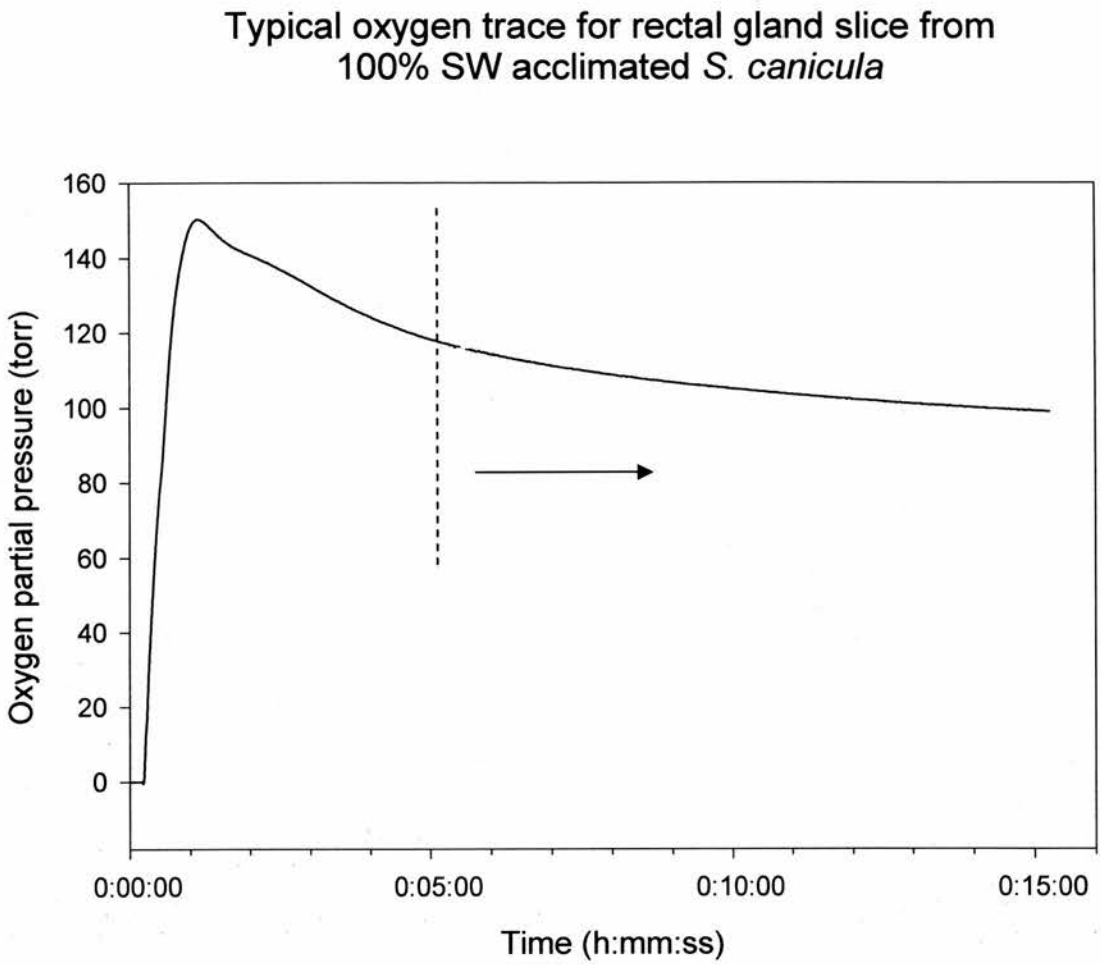

Figure 6.2.3.1 – Typical trace for O_2 partial pressure in respirometry chamber showing the section utilised for analysis (right of dotted line).

6.2.4 Statistical analysis

All data are presented as means ± the standard error of the mean (SEM). For the tissue controls analysis was performed via repeated measures ANOVA and a Tukey post hoc test. For analysis between salinities, data gathered on *S. canicula* was analysed via one-way ANOVA and a Tukey post hoc test, data gathered on *C. leucas* was analysed using a two-tailed unpaired student's t-test with Welch correction factor (InStat). Significance was denoted as * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.005$). For analysis on the effects of CNP data on both species were compared with basal levels using a one-tailed paired student's t-test (InStat). Significance was denoted as † ($P < 0.05$), †† ($P < 0.01$), and ††† ($P < 0.005$). For the section specific results and proportional increases, data gathered on *S. canicula* was analysed via one-way ANOVA and a Tukey post hoc test. Data gathered on *C. leucas* was analysed using a two-tailed unpaired student's t-test with Welch correction factor (InStat). Significance was denoted as * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.005$).

6.3 Results

For ease of presentation the results for *S. canicula* and *C. leucas* are described separately.

6.3.1 *S. canicula*

O_2 consumption by the rectal gland of 100% SW acclimated animals remained constant for the first 2 hours after excision (Figure 6.3.1.1). This time represents double that taken to measure consumption experimentally.

Whole tissue O_2 consumption was not significantly different between rectal glands from animals acclimated to the three environmental salinities (Figure 6.3.1.2). O_2 consumption was unaffected by all three concentrations of CNP in rectal glands from *S. canicula* acclimated to all three salinities.

O_2 consumption by Na^+, K^+-ATPase was defined as the ouabain sensitive portion of whole tissue O_2 consumption. Na^+, K^+-ATPase O_2 consumption was not significantly different between rectal glands from animals acclimated to the three environmental salinities (Figure 6.3.1.3). O_2 consumption by Na^+, K^+-ATPase was unaffected by all three concentrations of CNP in the rectal glands from *S. canicula* acclimated to all three salinities.

Oxygen consumption of 100% SW acclimated *S. canicula* rectal glands

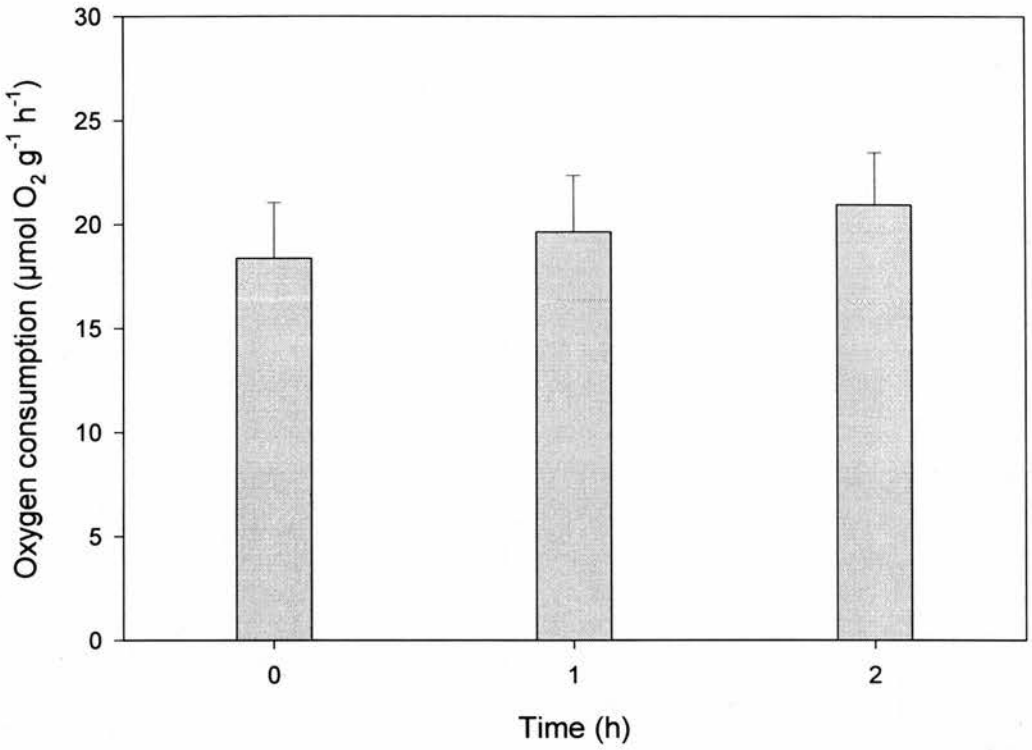

Figure 6.3.1.1 – Whole tissue O_2 consumption in *S. canicula* rectal gland slices from 100% SW acclimated animals. Values are presented as means ± SEM (n = 10). Statistical analysis was performed via repeated measures ANOVA and a Tukey post hoc test (significance was denoted as * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.005$)). No significant differences were recorded.

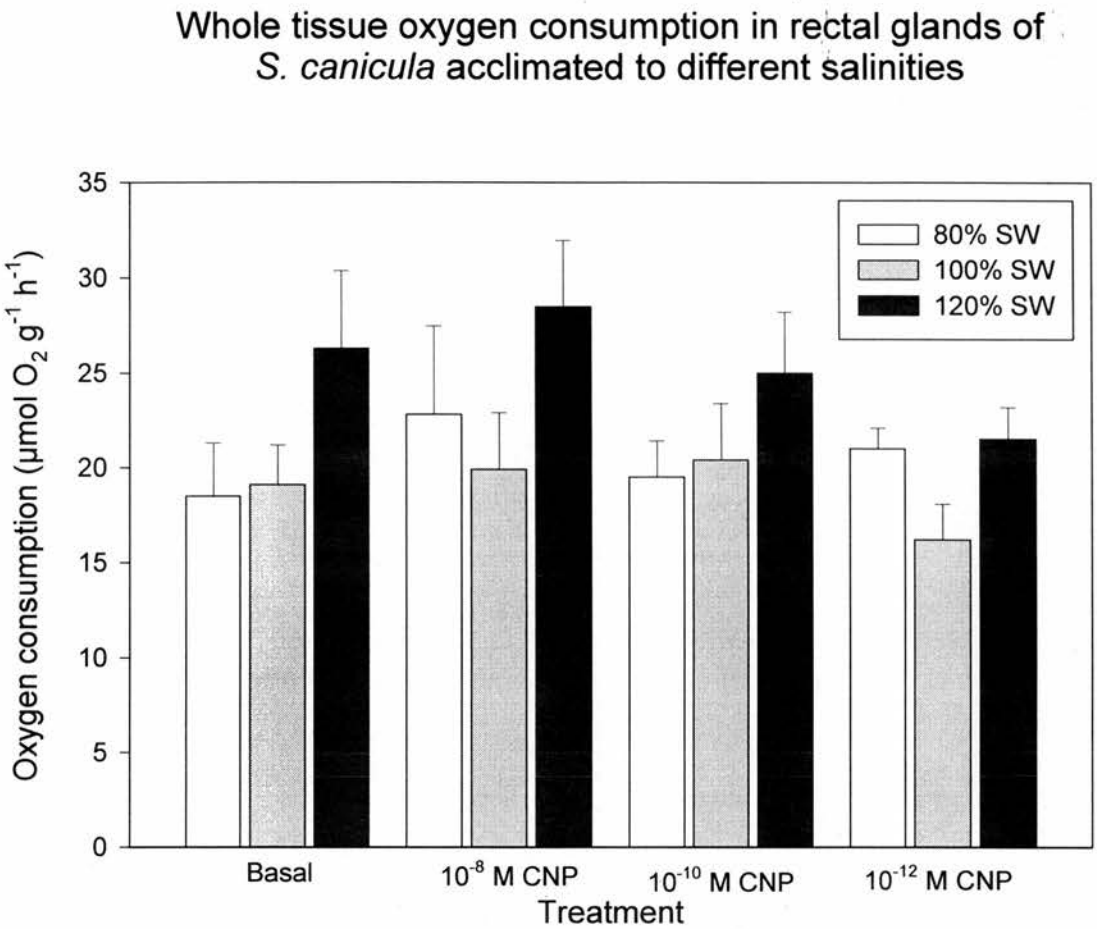

Figure 6.3.1.2 – O_2 consumption in rectal glands from *S. canicula* acclimated to 80, 100, and 120% SW. Values are presented as means ± SEM (n = 8, 14, and 8 respectively). Statistically significant differences between salinities were assessed via one-way ANOVA and a Tukey post hoc test (significance was denoted as * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.005$)); within groups, statistically significant differences from basal values were assessed via one tailed paired students t-tests with Welch correction factor (significance was denoted as † ($P < 0.05$), †† ($P < 0.01$), and ††† ($P < 0.005$)). No significant differences were recorded.

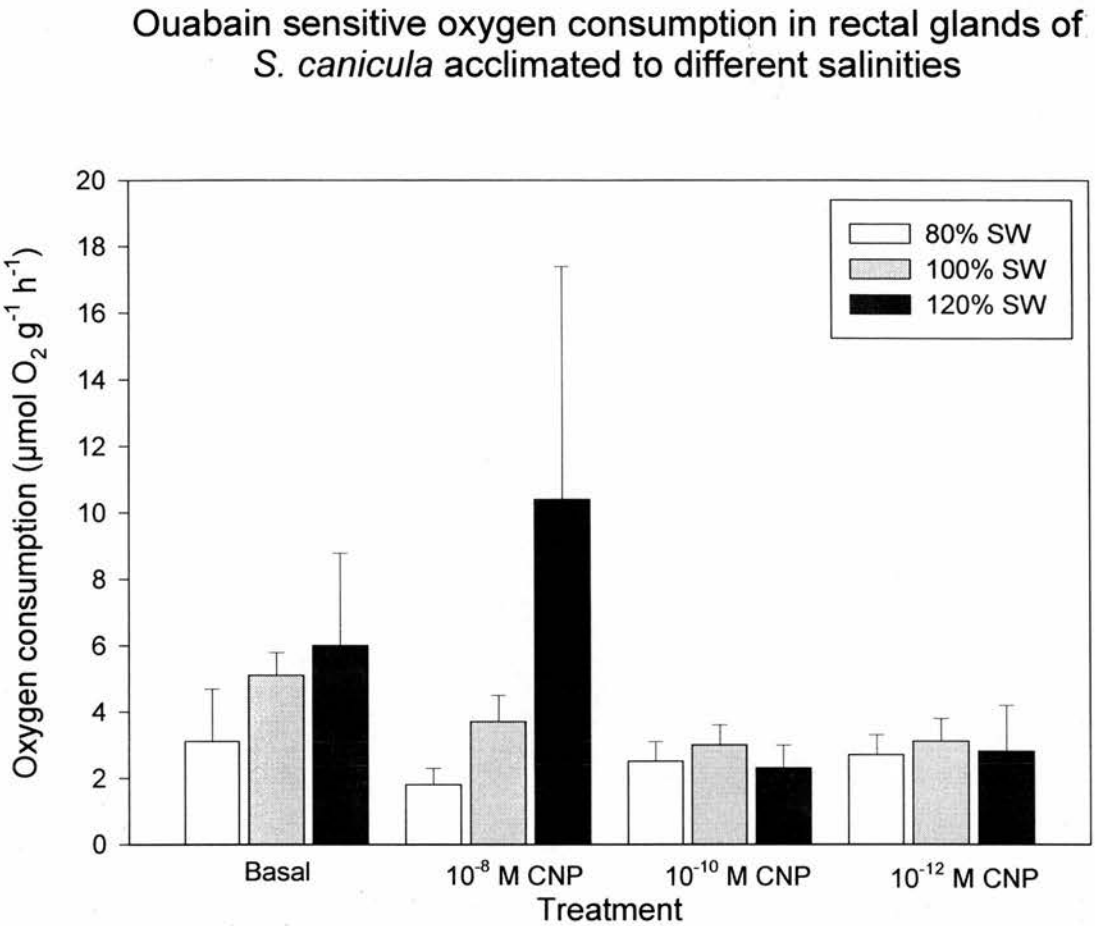

Figure 6.3.1.3 – O_2 consumption of Na^+, K^+-ATPase in the rectal glands of *S. canicula* acclimated to 80, 100, and 120% SW. Values are presented as means ± SEM (n = 7, 7, and 5 respectively). Statistically significant differences between salinities were assessed via one-way ANOVA and a Tukey post hoc test (significance was denoted as * (P < 0.05), ** (P < 0.01), and *** (P < 0.005)); within groups, statistically significant differences from basal values were assessed via one tailed paired students t-tests with Welch correction factor (significance was denoted as † (P < 0.05), †† (P < 0.01), and ††† (P < 0.005)). No significant differences were recorded.

Variance within groups was high for both whole tissue O_2 consumption and that associated with Na^+, K^+-ATPase. In an effort to reduce the effects of this the slice specific effects of CNP were analysed as a percentage change between basal and CNP stimulate O_2 consumption on each slice from each rectal gland (Figure 6.3.1.4). This removed possible sources of variation such as secretory states of glands upon excision and natural variation between individuals. CNP significantly increased whole tissue O_2 consumption in the rectal glands of *S. canicula* from all three salinities above basal levels. This was the case for all concentrations of CNP studied although no dose dependent effect was seen.

The proportion of whole tissue O_2 consumption which was ouabain sensitive was calculated, thereby giving a percentage of O_2 consumption associated with Na^+, K^+-ATPase which was specific to each slice (Figure 6.3.1.5). This was not significantly different between rectal glands from animals acclimated to the three environmental salinities. The proportion of O_2 consumed by Na^+, K^+-ATPase was unaffected by all three concentrations of CNP in the rectal glands from *S. canicula* acclimated to all three salinities.

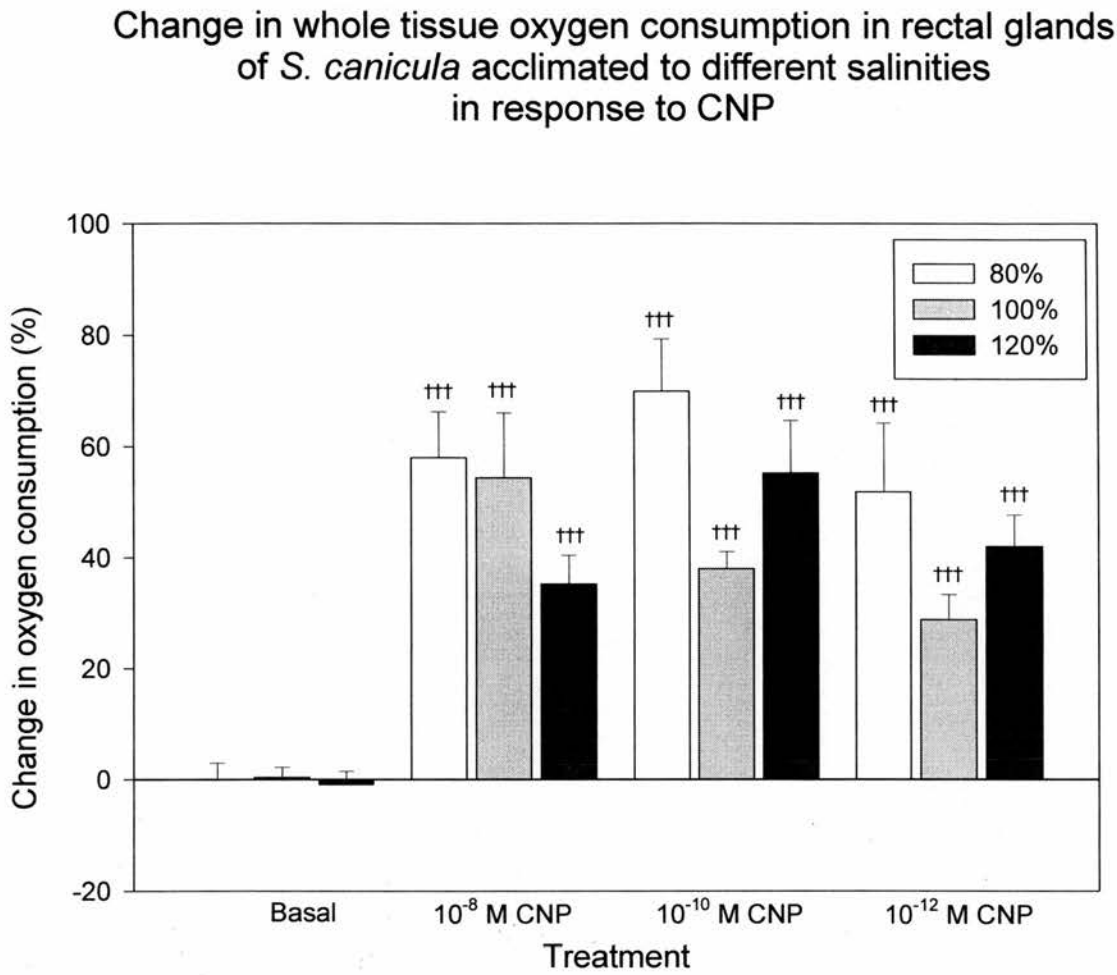

Figure 6.3.1.4 – The effect of CNP on O_2 consumption in individual sections of rectal glands from *S. canicula* acclimated to 80, 100, and 120% SW. Values are presented as means ± SEM (n = 7, 7, and 5 respectively). Statistically significant differences from the control transfer were assessed via one-way ANOVA and a Tukey post hoc test (significance was denoted as * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.005$)); within groups, statistically significant differences from basal values were assessed via one tailed unpaired students t-tests with Welch correction factor (significance was denoted as † ($P < 0.05$), †† ($P < 0.01$), and ††† ($P < 0.005$)).

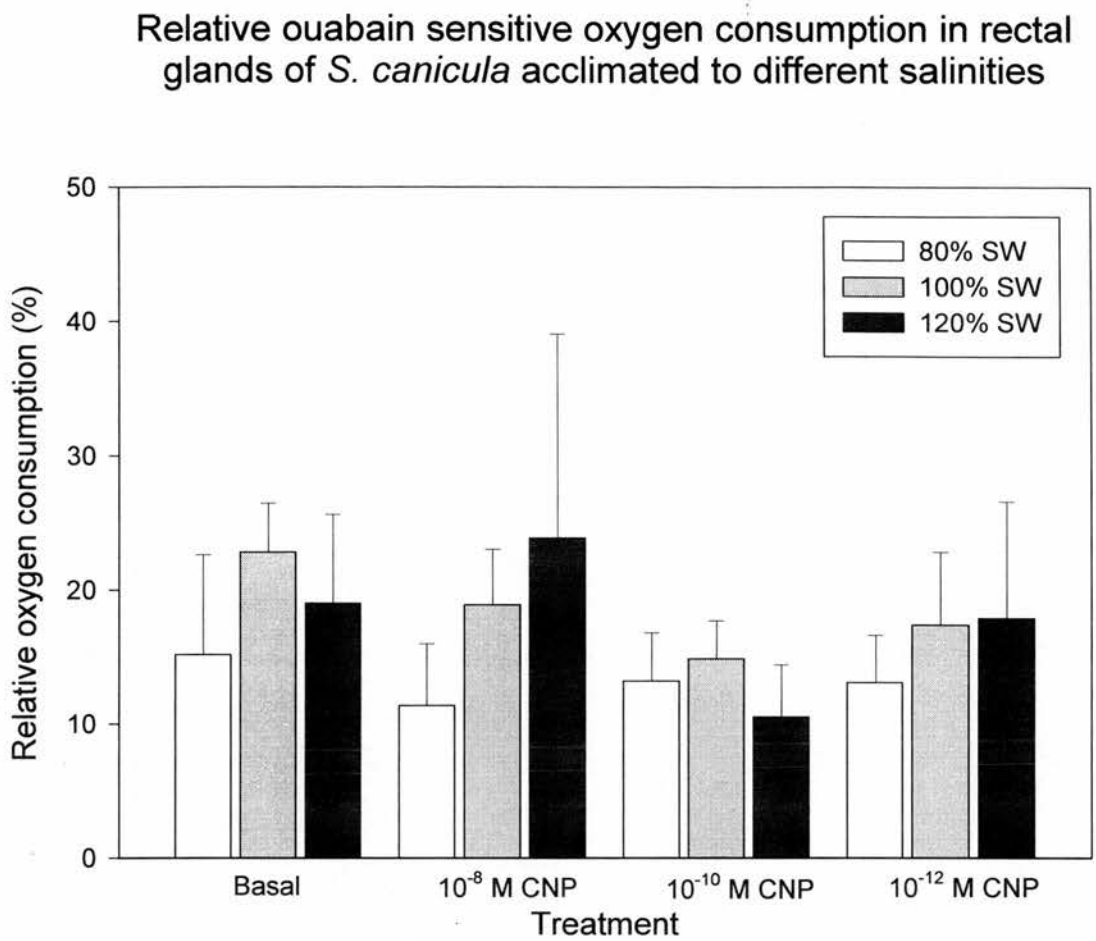

Figure 6.3.1.5 – Relative O_2 consumption of Na^+, K^+-ATPase in the rectal glands of *S. canicula* acclimated to 80, 100, and 120% SW. Values are presented as means ± SEM (n = 7, 7, and 5 respectively). Statistically significant differences from the control transfer were assessed via one-way ANOVA and a Tukey post hoc test (significance was denoted as * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.005$)); within groups, statistically significant differences from basal values were assessed via one tailed paired students t-tests with Welch correction factor (significance was denoted as † ($P < 0.05$), †† ($P < 0.01$), and ††† ($P < 0.005$)). No significant differences were recorded.

6.3.2 *C. leucas*

O_2 consumption in the rectal glands of both FW and SW acclimated animals remained constant for up to 4 hours after excision (Figure 6.3.2.1). This represents a greater amount of time than that taken to perform all experiments on excised tissue.

Consumption of O_2 in glands from FW acclimated animals was significantly higher than that of SW (Figure 6.3.2.2), and remained so for up to 4 hours (Figure 6.3.2.1). The three concentrations of CNP had no significant effects on the O_2 consumed by rectal glands of FW and SW acclimated animals. However, the variation in consumption within each salinity group was increased sufficiently to remove any significant differences between FW and SW glands.

The O_2 consumption associated with Na^+, K^+-ATPase was not significantly different between FW and SW acclimated animals (Figure 6.3.2.3). The O_2 consumed by Na^+, K^+-ATPase was unaffected by all three concentrations of CNP in both FW and SW acclimated *C. leucas*. Variance within salinity groups was again increased with the addition of CNP to the respirometry chamber.

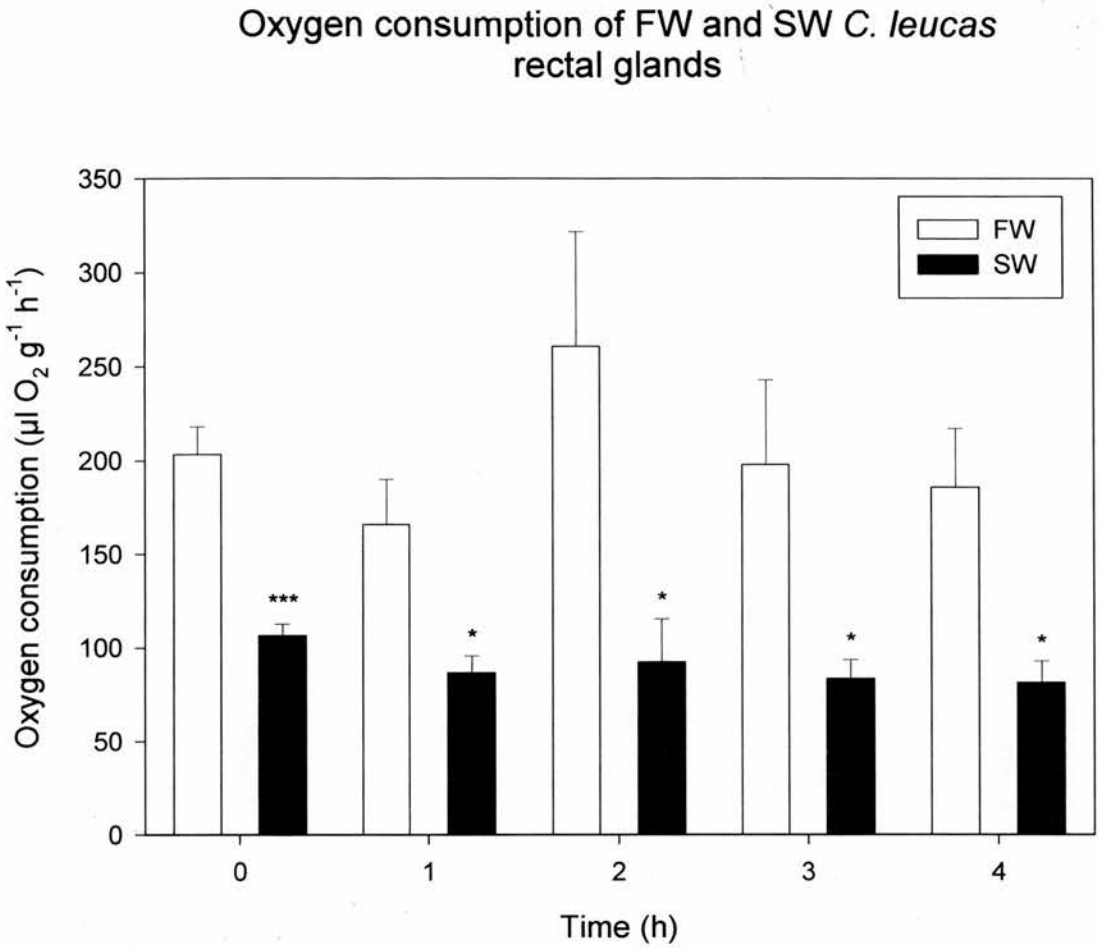

Figure 6.3.2.1 - Whole tissue O_2 consumption in *C. leucas* rectal gland slices from FW and SW acclimated animals. Values are presented as means ± SEM (n = 6). Statistical analysis within groups was performed via repeated measures ANOVA and a Tukey post hoc test (significance was denoted as † ($P < 0.05$), †† ($P < 0.01$), and ††† ($P < 0.005$)). No significant differences were recorded. Statistical analysis between FW and SW values was performed via a two-tailed unpaired student's t-test with Welch correction factor (significance was denoted as * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.005$)).

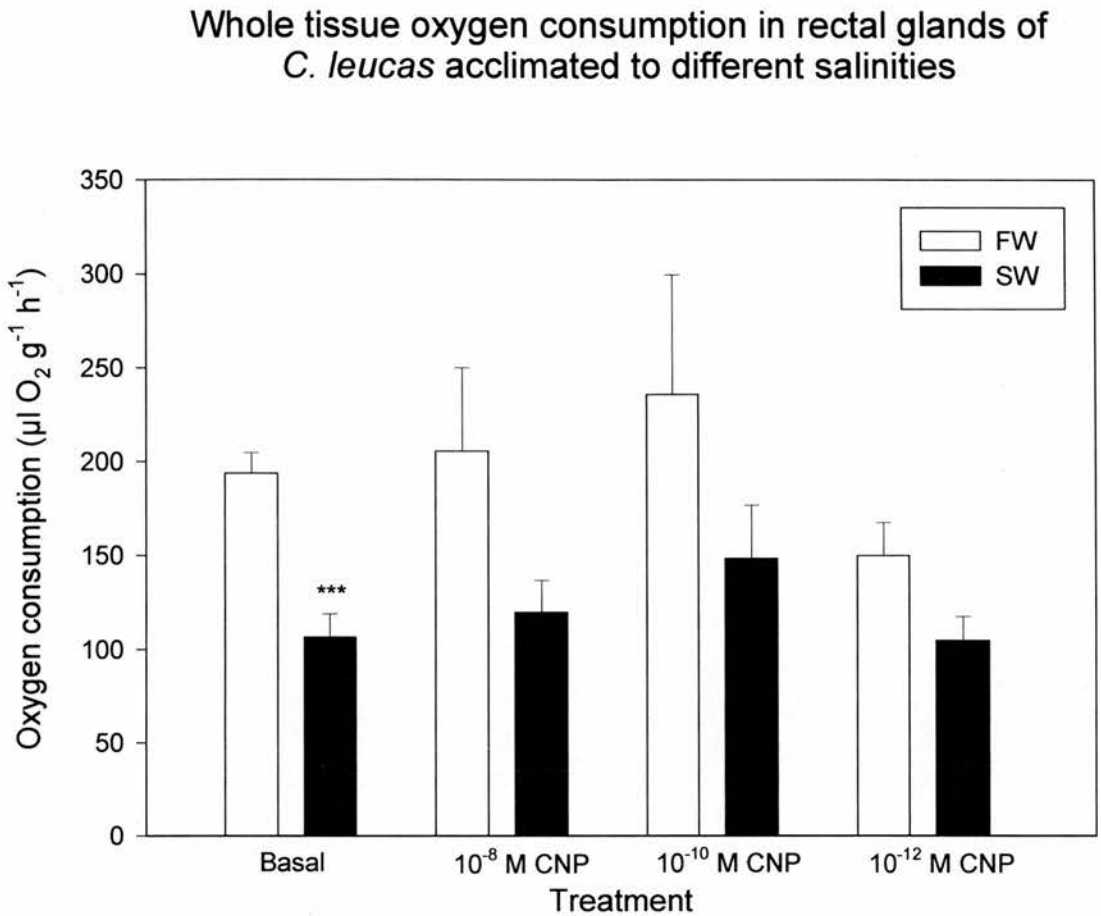

Figure 6.3.2.2 – O_2 consumption in the rectal glands of *C. leucas* acclimated to FW and SW. Values are presented as means ± SEM (n = 6). Statistically significant differences between salinities were assessed via two-tailed unpaired student's t-tests with Welch correction factor (significance was denoted as * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.005$)); within groups, statistically significant differences from basal values were assessed via one tailed paired students t-tests with Welch correction factor (significance was denoted as † ($P < 0.05$), †† ($P < 0.01$), and ††† ($P < 0.005$)).

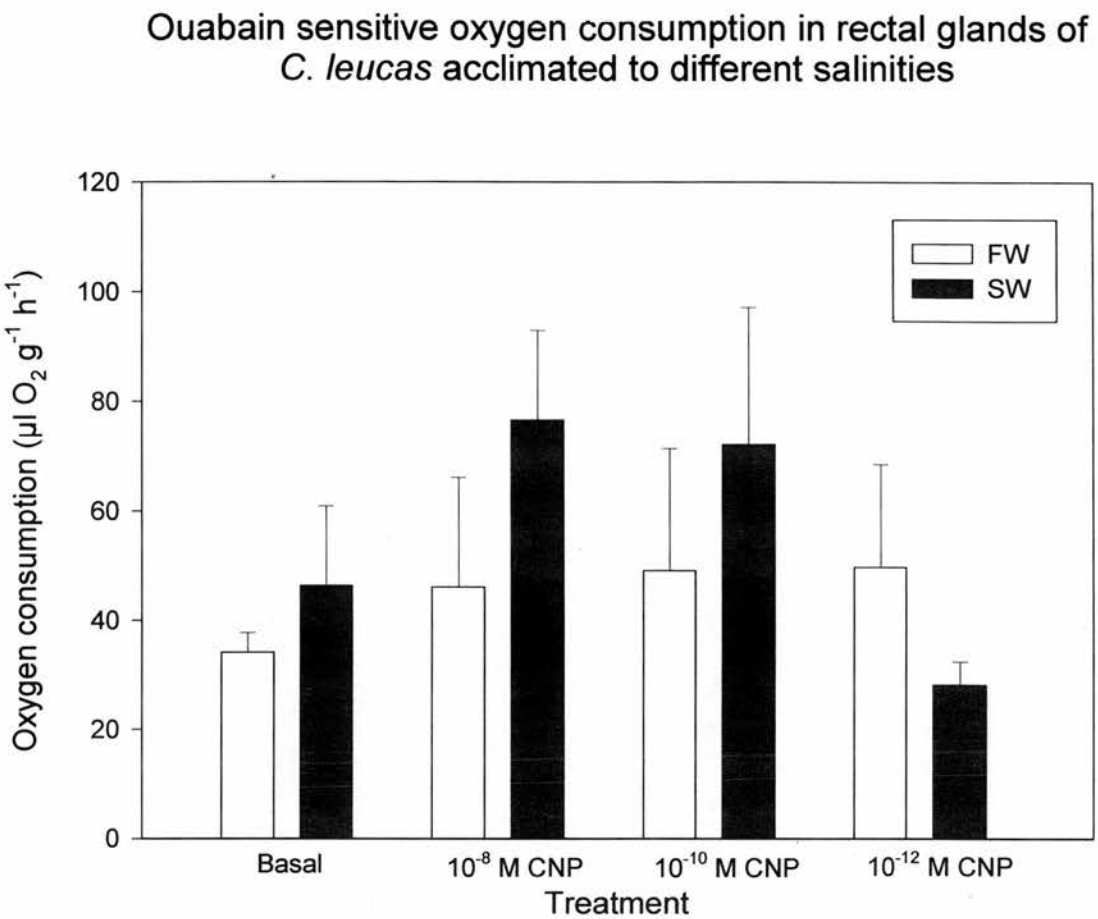

Figure 6.3.2.3 – O_2 consumption of Na^+, K^+-ATPase in the rectal glands of *C. leucas* acclimated to FW and SW. Values are presented as means ± SEM (n = 6). Statistically significant differences between salinities were assessed via two-tailed unpaired student's t-tests with Welch correction factor (significance was denoted as * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.005$)); within groups, statistically significant differences from basal values were assessed via one tailed paired students t-tests with Welch correction factor (significance was denoted as † ($P < 0.05$), †† ($P < 0.01$), and ††† ($P < 0.005$)). No significant differences were recorded.

So as to minimise the effects of variance between individual glands, the data was again analysed for specific effects of CNP on each slice in each gland (Figure 6.3.2.4). CNP caused significant increases in whole tissue O_2 consumption in the rectal glands of both FW and SW acclimated *C. leucas*. This was seen after administration of all concentrations of CNP, although no dose dependent response was recorded.

The O_2 consumed by Na^+, K^+-ATPase was again analysed as a proportion of that consumed by the whole gland (Figure 6.3.2.5). The relative O_2 consumption of Na^+, K^+-ATPase in the rectal glands of FW acclimated *C. leucas* was not significantly different to that in SW acclimated animals. In FW acclimated animals the relative O_2 consumption of Na^+, K^+-ATPase was unaffected by the three concentrations of CNP. However, significant increases were seen in the relative O_2 consumption of Na^+, K^+-ATPase in the rectal glands of SW acclimated *C. leucas* in response to CNP. Administration of 10^{-8} M CNP resulted in a near 3-fold increase in the percentage of O_2 being consumed by Na^+, K^+-ATPase; whilst 10^{-10} M CNP produced a near doubling of the same parameter. These concentrations of CNP also increased the relative O_2 consumption of Na^+, K^+-ATPase in the rectal glands of SW acclimated *C. leucas* to significantly higher levels than those of SW acclimated glands under the same conditions. It can therefore be stated that CNP has a significant effect on the proportional O_2 consumption in the rectal glands of SW acclimated *C. leucas*, but has no effect on FW animals.

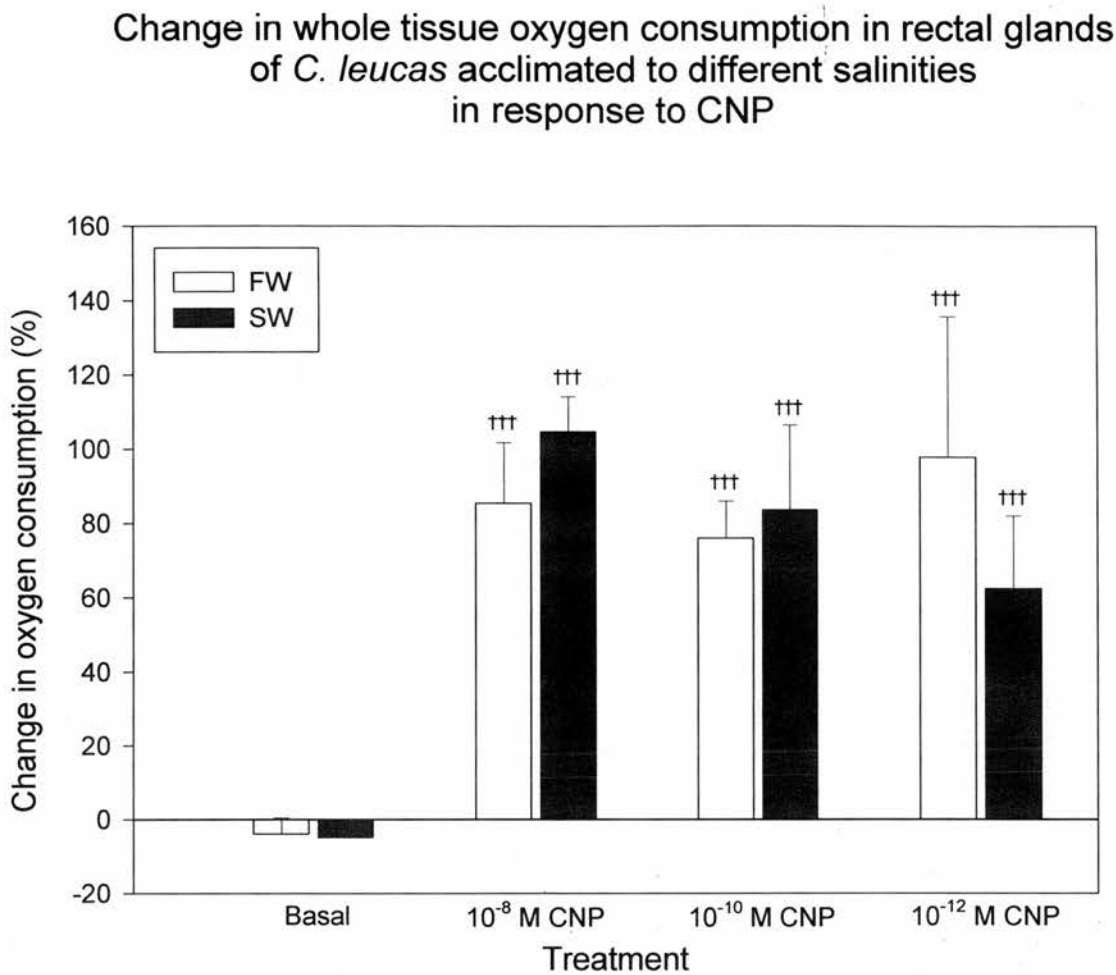

Figure 6.3.2.4 - The effect of CNP on O_2 consumption in individual sections of rectal glands from *C. leucas* acclimated to FW and SW. Values are presented as means ± SEM (n = 6). Statistically significant differences between salinities were assessed via two-tailed unpaired student's t-tests with Welch correction factor (significance was denoted as * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.005$)); within groups, statistically significant differences from basal values were assessed via one tailed unpaired students t-tests with Welch correction factor (significance was denoted as † ($P < 0.05$), †† ($P < 0.01$), and ††† ($P < 0.005$)).

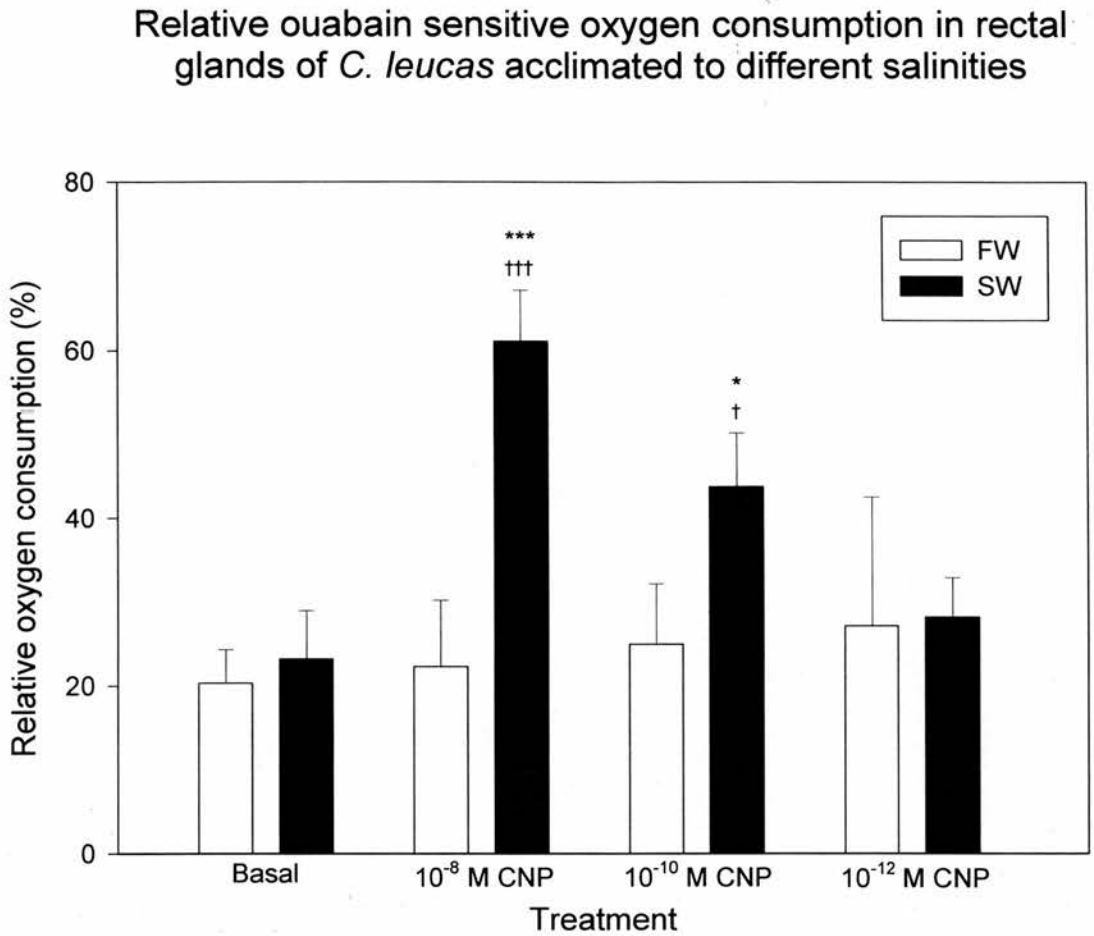

Figure 6.3.2.5 – Relative O_2 consumption of Na^+, K^+-ATPase in the rectal glands of *C. leucas* acclimated to FW and SW. Values are presented as means ± SEM (n = 6). Statistically significant differences between salinities were assessed via two-tailed unpaired student's t-tests with Welch correction factor (significance was denoted as * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.005$)); within groups, statistically significant differences from basal values were assessed via one tailed paired students t-tests with Welch correction factor (significance was denoted as † ($P < 0.05$), †† ($P < 0.01$), and ††† ($P < 0.005$)).

6.4 Discussion

The recorded values for O_2 consumption in the rectal glands of *S. canicula* and *C. leucas* were comparable to those published previously for *S. canicula* and *S. acanthias* (Shuttleworth and Thompson 1980; Morgan et al. 1997). Ouabain sensitive O_2 consumption was significantly lower than that of the whole tissue for both species in all salinities. This is also consistent with previous respirometry experiments on elasmobranch rectal glands (Shuttleworth and Thompson 1980; Morgan et al. 1997). The lower concentration of ouabain used by Shuttleworth and Thompson (1980) as compared to the present study did not lead to any large scale changes in the estimation of the ouabain sensitive proportion of O_2 consumption by the rectal gland of *S. canicula*.

Whole tissue O_2 consumption in the rectal gland of *S. canicula* was unchanged by acclimation to 80 and 120% SW (Figure 6.3.1.2). This is consistent with previous results which have demonstrated that both RGF volume and Cl^- clearance rate from the rectal gland are also unchanged by similar salinity acclimations (Table 4.3.1). This is again suggestive that there is no increase in rectal gland activity *in vivo* associated with long term acclimation to salinity transfer in *S. canicula*. It is possible that the magnitude of these salinity changes are not sufficient to elicit any long term changes in rectal gland O_2 consumption. However, it must again be stated that salinity transfers of larger magnitude increase animal mortality and this is highly suggestive that changes in rectal gland O_2 consumption are unlikely to be recorded with such transfers.

The ouabain-sensitive portion of rectal gland O_2 consumption in *S. canicula* was also unaffected by acclimation to hypo- or hypersaline conditions (Figures 6.3.1.3 and 5).

This is consistent with previous results which have shown that the maximal activity of Na^+, K^+-ATPase is not significantly different between the three environments (Figure 5.3.2.1).

Whole tissue O_2 consumption in the rectal glands of *C. leucas* acclimated to FW was significantly higher than that of SW acclimated animals (Figure 6.3.2.2). This seems counter intuitive due to the reversal of the concentration gradients for Na^+ and Cl^- between *C. leucas* and the environment as the animals acclimate from FW to SW. In FW the gradient is for the efflux of these ions and so there is an associated requirement for the retention of Na^+ and Cl^- in the body fluids. Conversely, in SW the gradient is for the influx of these ions and so there is an associated requirement for the active secretion of Na^+ and Cl^- from the rectal gland. The rectal glands of SW animals also have significantly higher maximal activity of Na^+, K^+-ATPase (Figure 5.3.2.2) (Pillans et al. 2005), and presumably higher rates of RGF secretion and Cl^- clearance, in order to facilitate this need for active rectal gland secretion. The fact that whole tissue O_2 consumption is higher in the rectal glands of FW acclimated animals could therefore be the result of higher activity of some other metabolic process or processes in the tissue. It could be explained by a higher overall metabolic rate in FW acclimated animals due to the requirement to maintain a larger disparity between internal osmolality and that of the surrounding environment. Indeed, the maintenance of such large concentration gradients for Na^+, Cl^-, urea, TMAO, and other plasma osmolytes must result in a substantial energy demand. This energy demand may be met by a higher basal metabolic rate in FW acclimated *C. leucas*.

Similar studies on teleost species have shown varied results. Some species show a decrease in whole animal O_2 consumption associated with acclimation to decreased salinities (Wood et al. 2002b; Sardella et al. 2004), whilst others show no change in either whole animal O_2 consumption (Morgan and Iwama 1998) or that of the gills (Stagg and Shuttleworth 1982). This is illustrative of the interspecies variation that can occur when measuring salinity induced changes in biological parameters.

However, it is more likely that higher O_2 consumption in rectal glands from FW acclimated *C. leucas* reflect the methods of regulating glandular secretion in this environment. Juvenile *C. leucas* require the plasticity in osmoregulatory organs to move freely between hypo- and hyperionic environments. This is particularly true of animals in the Brisbane River system which has a high tidal influence and a relatively short length of completely FW which is accessible to elasmobranchs, as compared to other areas of study for the species (Thorson et al. 1973; Sosa-Nishizaki et al. 1998; Taniuchi et al. 2003; Pillans and Franklin 2004).

One source of high O_2 consumption could be the constriction of rectal gland blood vessels, so as to minimise secretory output from the gland in FW. Attempts at isolated perfused rectal gland studies in FW *C. leucas* proved difficult due to constriction/blockage of the rectal gland artery and erratic pressure and activity within the gland (unpublished finding). Blood flow to the rectal gland of *S. canicula* has been shown to be modified during periods of active secretion (Anderson et al. 2002a). It is possible therefore that the higher O_2 consumption in rectal glands of FW acclimated *C. leucas* is at least in part due to constriction of blood vessels.

Another possible cause of higher rectal gland O_2 consumption in FW is the smooth muscle layer surrounding the gland. Work conducted by Evans and Piermarini (2001) demonstrated that this layer is responsive to contractile stimuli. It is also possible therefore that the increased O_2 consumption in rectal glands of FW acclimated *C. leucas* is at least in part due to contraction of this smooth muscle layer as a means of further restricting blood supply to the gland.

The ouabain-sensitive portion of rectal gland O_2 consumption was not significantly different between FW and SW acclimated *C. leucas* (Figures 6.3.2.3 and 5). This may appear to be inconsistent with previous studies which have shown that maximal activity of Na^+, K^+-ATPase in the rectal gland is significantly higher in SW acclimated animals (Figure 5.3.2.2) (Pillans et al. 2005). However, such studies represent maximal activities and it is possible that Na^+, K^+-ATPase activity, and hence ouabain-sensitive O_2 consumption, only differ between rectal glands of FW and SW acclimated *C. leucas* during periods of active secretion.

Natriuretic peptides such as CNP have been shown to have stimulatory effects on the secretory action of the elasmobranch rectal gland (Sections 1.11.1 and 6.1). However, whilst whole tissue O_2 consumption was significantly increased by CNP (Figure 6.3.1.4), the ouabain-sensitive portion of this in the rectal gland of *S. canicula* was unaffected by administration of three concentrations of CNP (Figures 6.3.1.5). Therefore it can be stated that any increase in O_2 consumption upon administration of CNP is not associated with a proportional increase in the activity of Na^+, K^+-ATPase. If indeed there is any increase in the activity of Na^+, K^+-ATPase, is also coupled with a proportional increase in other metabolic processes.

The effects of pharmacological agents on the tissues of the rectal gland have been shown to be highly dependent on the preparations used. Equimolar concentrations of CNP and VIP have roughly similar effects on Cl^- secretion in intact glands (Solomon et al. 1992a), but in dispersed tubules CNP produces less than half the respiratory stimulation of VIP (Solomon et al. 1993; Solomon et al. 1995a; Solomon et al. 1995b). Also, elevated intracellular concentrations of cGMP result in a slow increase in short-circuit current in isolated cells (Karnaky et al. 1991), but perfusion of isolated glands with high concentrations of cGMP showed no such increase (Silva et al. 1999). Furthermore, Stoff and co-workers (1977b) reported a synergistic effect on active Cl^- transport with administration of theophylline and cAMP in isolated perfused glands, where as treatment with cAMP caused no further increase in O_2 consumption of rectal gland sections after administration of theophylline (Shuttleworth and Thompson 1980).

In light of such discrepancies the results for ouabain-sensitive O_2 consumption in the rectal gland of *S. canicula* following administration of CNP do not seem extraordinary. It has already been suggested that the stimulatory effect of CNP via guanylate cyclase may require the coupled activation of PKC (Section 6.1). It is possible that the mode of administration of CNP in these studies effects glandular response. Introducing CNP into the surrounding medium of an isolated tissue section is not equivalent to perfusing an isolated gland. It is possible that administered CNP must pass through the circulatory system in order to have a stimulatory action on active secretion. However, this is unlikely given the documented stimulatory action of CNP on cultured rectal gland cells from *S. acanthias* (Karnaky et al. 1992; Karnaky et al. 1993). Therefore, although these results for *S. canicula* are not extraordinary, they are unexpected and unclear.

As well as being a potent stimulus for rectal gland secretion, CNP also has proven vasodilatory effects (Bjenning et al. 1992; Anderson et al. 2002a). Therefore, if the high O_2 consumption recorded in the rectal glands of FW acclimated *C. leucas* is in part due to vasoconstriction, administration of CNP could be expected to reverse this effect. However, CNP significantly increased the O_2 consumption of rectal glands from FW acclimated animals (Figure 6.3.2.4). It is possible that any decrease in O_2 consumption resulting from vasodilation was masked by a larger increase in consumption due to the stimulatory effect of CNP on the secretory tubules of the rectal gland. However, administration of CNP did not significantly alter ouabain-sensitive O_2 consumption (Figure 6.3.2.3), or its proportion in whole tissue consumption (Figure 6.3.2.5). If therefore, the high O_2 consumption of rectal glands from FW acclimated *C. leucas* is the result of vasoconstriction, either such constrictions are non-responsive to CNP, or they are coupled with an increase in O_2 consumption from another aspect of rectal gland function.

Not only did CNP affect whole tissue O_2 consumption in the rectal gland of SW acclimated *C. leucas* (Figure 6.3.2.4), it also had effects on the proportion of ouabain-sensitive O_2 consumption (Figure 6.3.2.5). This is consistent with the increased maximal activity of Na^+, K^+-ATPase in the rectal glands of these animals (Figure 5.3.2.2) (Pillans et al. 2005). It is also consistent with the increase in the abundance and activity of Na^+, K^+-ATPase in the rectal glands of SW acclimated *D. sabina* (Piermarini and Evans 2000).

The fact that administration of CNP results in an increase in the proportion of whole tissue O_2 consumption associated with Na^+, K^+-ATPase, but does not result in any

significant increase in the ouabain-sensitive O_2 consumption of SW rectal glands is intriguing. This is a reflection of the amount of variation in these parameters between individual animals acclimated to SW upon administration of CNP. Only through a proportional analysis do any trends appear. This variation could be explained by different states of activity in the glands upon excision, as well as natural variation in the species. It could be that a gland excised during a period of active secretion shows a greater response to CNP than a gland excised during a quiescent period. If this is the case it would also explain the relative lack of response in glands from FW acclimated *C. leucas* which are presumably quiescent for a much larger amount of time.

The fact that the O_2 consumption associated with Na^+, K^+-ATPase increases disproportionately to whole tissue consumption in SW acclimated *C. leucas* but not in FW animals, and that both animals have similar basal values, suggests a modification in the response of the rectal gland to CNP during acclimation to increased salinity. This could be achieved through alterations in the abundance and/or sensitivity of CNP receptors in the membranes of rectal gland secretory cells. There is no requirement for active rectal gland secretion in FW *C. leucas* and so increasing the number of receptors for this stimulatory hormone during SW acclimation seems plausible. An increased abundance of hormone receptors in response to changes in salinity has been reported in teleosts. SW acclimated *A. anguilla* have a three-fold higher Ang II receptor concentration than those acclimated to FW (Marsigliante et al. 1997). Furthermore, Katafuchi and co-workers (1994) showed that CNP-specific receptor expression was enhanced in FW *A. japonica*. Increased CNP receptor expression can therefore be induced by changes in environmental salinity in teleosts. Given that CNP is the most highly conserved of all the natriuretic peptides (Takei 1999), and is the only one

recorded in elasmobranchs (Schofield et al. 1991; Suzuki et al. 1991a; Suzuki et al. 1994), elasmobranchs may well increase receptor expression during long term acclimation to different salinities.

It is also possible that whilst the rectal gland of SW acclimated *C. leucas* respond to CNP via both cGMP and PKC pathways (thereby facilitating a synergistic action on Cl^- secretion), rectal glands of FW acclimated *C. leucas*, and those of *S. canicula* acclimated to the three environmental salinities do not respond via one of these. The fact that whole tissue O_2 consumption is still increased in this latter group suggests that these glands are responsive to CNP and that one of the pathways is probably functional. However, the lack of a disproportionate increase in Na^+, K^+-ATPase O_2 consumption suggests that either there is no increase in Cl^- secretion associated with administration of CNP in these glands, or that any increase is directly proportional to the stimulatory effects of CNP on other active metabolic processes. However, this variation in response to CNP seen in *C. leucas* represents a tangible difference in the endocrine physiology of fully euryhaline elasmobranchs when compared to a partially euryhaline species. The significance of this finding cannot be underestimated when assessing the nature of euryhalinity in elasmobranch fish.

Chapter 7: General discussion

7.1 General discussion

Marine elasmobranch fish typically maintain a blood plasma osmolality slightly hyperosmotic to the environment, in captivity it may be iso- or slightly hyposmotic. This strategy typically involves plasma concentrations of Na^+ and Cl^- below that of the surrounding environment, and the retention of urea and methylamines in the body fluids (Ballantyne et al. 1987). The result of this is an osmoregulatory strategy focussed on the retention of urea and methylamines and regulation of the influx and efflux of Na^+, Cl^-, and water.

One of the key objectives of this study has been to investigate the scope for modifications in this osmoregulatory strategy in both a partially and a fully euryhaline species. A comparison of the two was conducted by analysing the responses of partially euryhaline species to salinity change, evaluating the limitations of these responses, and highlighting the modifications in fully euryhaline species.

7.1.1 Partially euryhaline elasmobranchs

It has been shown that partially euryhaline elasmobranchs acclimate acutely to changes in environmental salinity with significant changes occurring in all major blood plasma parameters during the first 12 hours (Figures 2.3.2.3 – 6 and 3.3.2.1) , with the notable exception of elevating plasma urea concentration during acclimation to increased salinity. All of the alterations in plasma osmolyte concentration (Table 2.3.1.1), blood volume (Table 3.3.1.1), and blood haematocrit (Table 2.3.1.1) persist in chronically acclimated animals. These occur through altered concentration and osmotic gradients with the environment and the resultant fluxes across semi-permeable membranes. These fluxes are regulated by the principle osmoregulatory organs.

In order to assess the roles of the principle osmoregulatory organs during both acute and chronic acclimation to salinity change, it is necessary to have an understanding of the general response of partially euryhaline elasmobranchs to both decreases and increases in environmental salinity. The generalised response of a partially euryhaline elasmobranch (such as *S. canicula*) to a decrease in environmental salinity is described below (Figure 7.1.1.1). Immediately following the commencement of transfer to reduced salinity the animal encounters an increase in the gradient for the osmotic influx of water, resulting in an increased influx of water. This osmotic water influx leads to a haemodilution and an increase in blood volume (Figure 3.3.2.1). This increase in blood volume may be the initial stimulus for the osmoregulatory response of elasmobranchs to reduced salinity. Also associated with this haemodilution is a decrease in blood haematocrit (Figure 2.3.2.6).

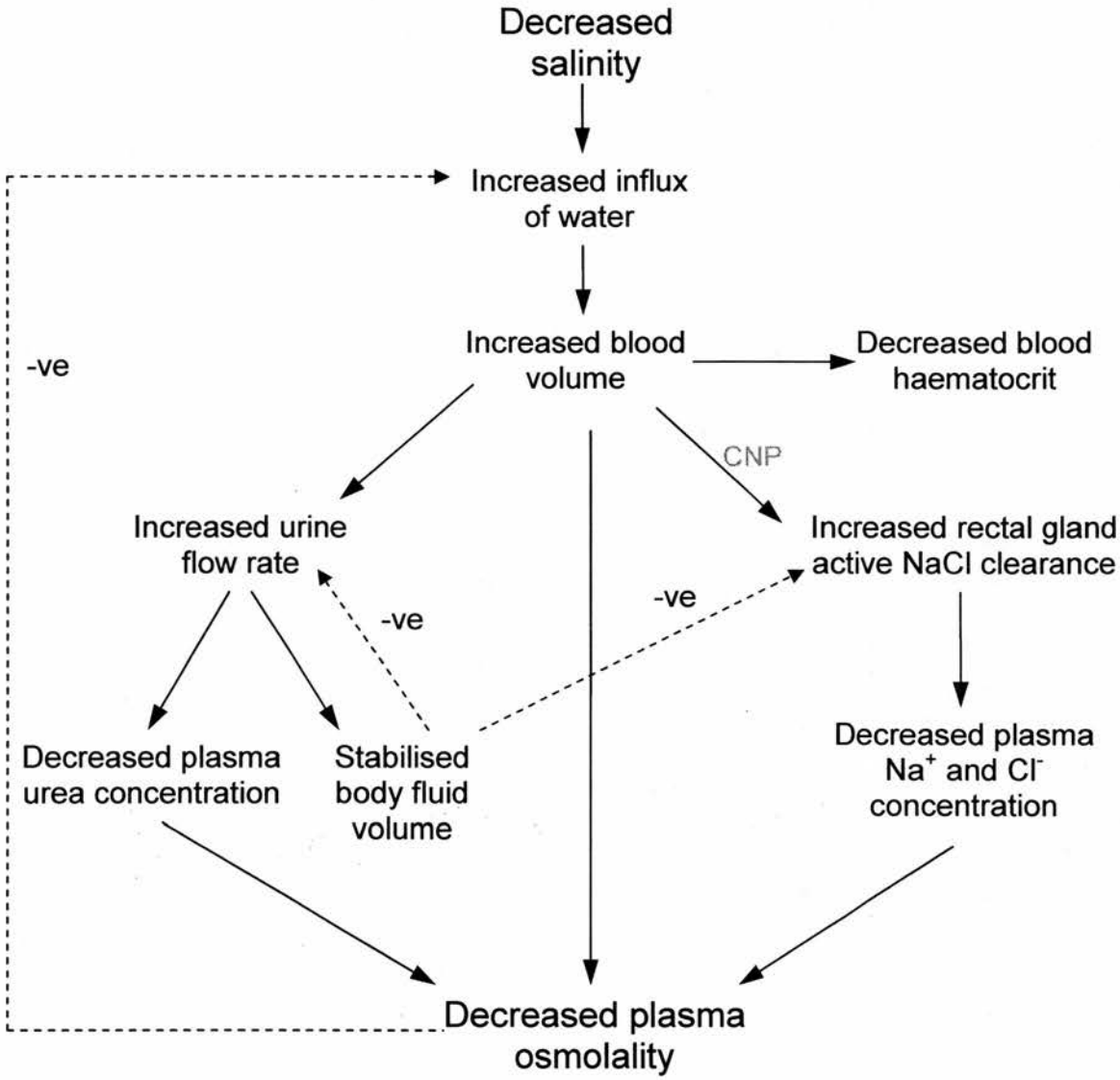

Figure 7.1.1.1 – Generalised acute response of *S. canicula* to decreased environmental salinity, showing putative negative feedback effects (-ve), see text for details.

In response to an increase in blood volume CNP is released from the heart and stimulates active secretion of NaCl from the rectal gland (Solomon et al. 1992b; Anderson et al. 2002a) (Figure 4.3.2.4). This results in the significant reduction of plasma Na^+ and Cl^- concentrations (Figure 2.3.2.4) as part of the homeostatic process to lower overall plasma osmolality (Figure 2.3.2.3) to around isosmotic to the environment.

Urine flow rates are also elevated during the early stages of salinity transfer to facilitate a decrease in plasma urea concentration and regulate the increased influx of water. Such changes in urine flow rates persist after 3 days at reduced salinity (Wells et al. 2002). In *S. canicula* this increase in urine flow rate results in a decrease in plasma urea levels (Figure 2.3.2.5) although this is not as rapid as the decrease seen in plasma Na^+ and Cl^- levels resulting from the additional action of the rectal gland on total salt excretion. Increased urine flow rate also acts to re-establish volaemic stasis in a reduced salinity environment. The actions of the rectal gland and kidney therefore regulate the osmotic consequences of transfer to reduced salinity.

The generalised response of a partially euryhaline elasmobranch (such as *S. canicula*) to an increase in environmental salinity is described below (Figure 7.1.2). Immediately following the commencement of transfer to elevated salinity the animal will encounter an increase in the gradient for the osmotic efflux of water, resulting in a decreased body fluid levels. This osmotic efflux leads to a haemoconcentration and a decrease in blood volume (Figure 3.3.2.1). A decrease in blood volume is a potent stimulus for the initiation of a drinking response (Anderson et al. 2002b) and has a negative impact on rectal gland activity. Furthermore, blood volume starts to decrease after 2 - 3 hours in *S.*

canicula acclimating to these conditions (Figure 3.3.2.1); this is within the same time frame at which a drinking response is typically recorded (Anderson et al. 2002b). This drinking response acts to increase plasma Na^+ and Cl^- levels, as well as counter act the loss of body fluids. The consequences of the drinking response may then be moderated through the action of the rectal gland and the kidney to establish an increase in plasma osmolality (Figure 2.3.2.3), eventually rendering it to a level around isosmotic to the environment. Any initial increase in plasma osmolality may be achieved primarily through imbibed Na^+ and Cl^-, with increased plasma urea concentrations occurring somewhat later after the initial salinity transfer. This delayed increase in plasma urea concentration reflects the requirement for increased hepatic urea production (Hazon and Henderson 1984).

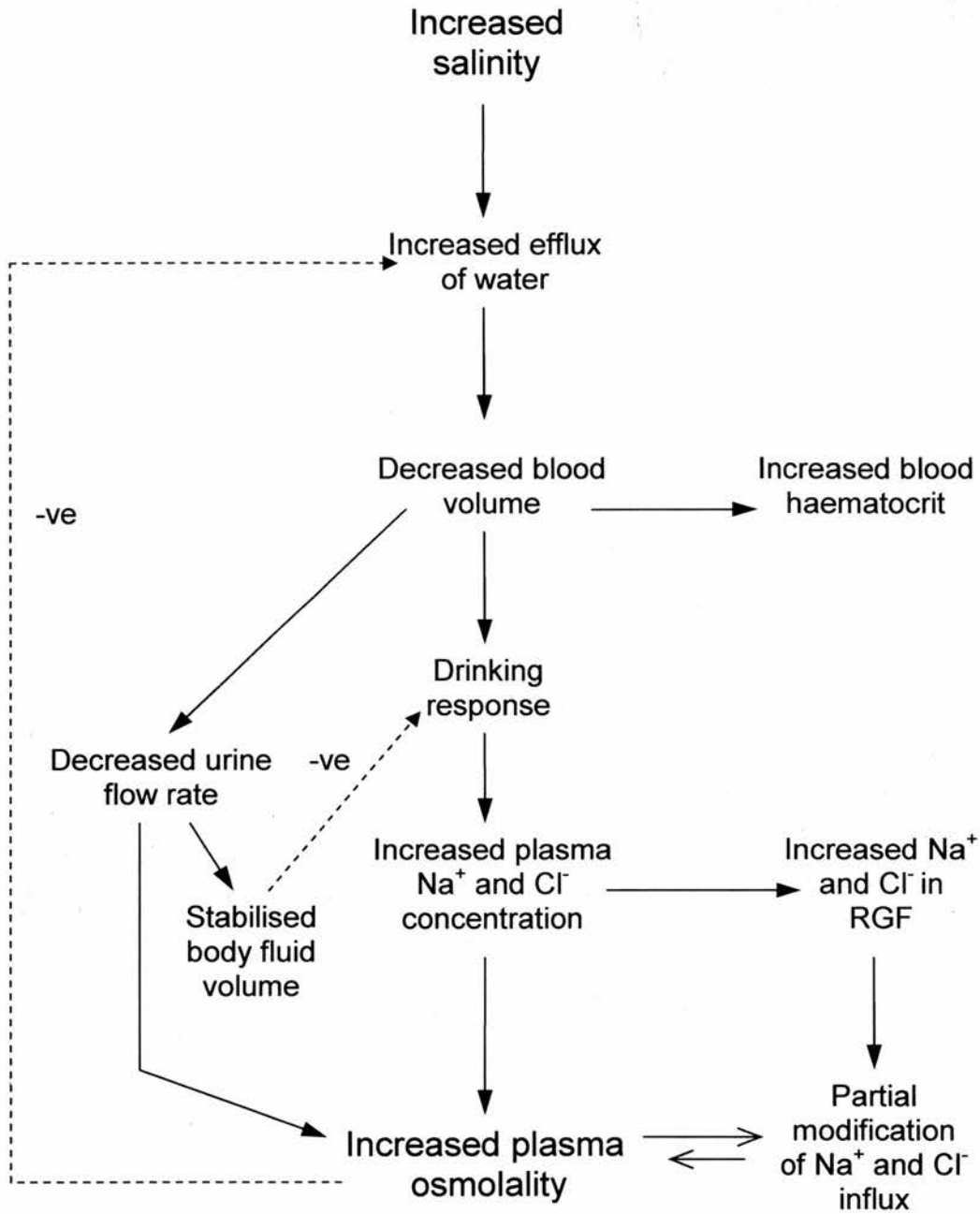

Figure 7.1.1.2 – Generalised acute response of *S. canicula* to increased environmental salinity, showing putative negative feedback effects (-ve), see text for details.

From the general response of a partially euryhaline elasmobranch to both increases and decreases in environmental salinity the central importance of changes in blood volume can readily be surmised. Not only as a means of stimulation for osmoregulatory responses, but also as a means of assessing a species capacity for chronic volaemic regulation. Salinity transfers of high magnitude conducted on *S. canicula* may result in mortality over a period of days, suggesting that partially euryhaline elasmobranchs fail to regulate haematic parameters completely to such conditions. This would be associated with larger scale changes in blood volume than those demonstrated in this study which utilised smaller changes in salinity (Table 3.3.1.1).

7.1.2 Limitations in partially euryhaline elasmobranchs

The failure of partially euryhaline elasmobranchs to adequately regulate haematic parameters in low salinity and FW must be a function of the principle osmoregulatory organs. The possibility of active ion uptake by the gills of elasmobranchs has already been discussed (Section 1.3). There was no modification in the maximal activity of Na^+, K^+-ATPase in the gills of *S. canicula* following chronic acclimation to 80 or 120% SW environments (Figure 5.3.2.1). This suggests that partially euryhaline species may not have the capacity to alter the abundance and/or recruitment of Na^+, K^+-ATPase, and any changes in active branchial ion transport occur within the capacity of the enzyme levels present in SW acclimated animals. The intake and retention of Na^+ and Cl^- and the retention of urea becomes increasingly important as environmental salinity decreases. As the availability of Na^+ and Cl^- in the environment decreases so does the gradient for their diffusional influx into elasmobranchs across the semi-permeable surfaces. Indeed, continued decreases in environmental salinity result in elasmobranchs becoming hyperionic to the environment. Under these environmental conditions elasmobranchs face a continual efflux of Na^+ and Cl^- (and urea) across the semi-permeable surfaces and active branchial uptake of these ions may form a critical part of an elasmobranch osmoregulatory strategy (Section 7.1.3). Given this reversal of Na^+ and Cl^- concentration gradients, a lack of modification in the branchial abundance and/or recruitment of Na^+, K^+-ATPase are unlikely to facilitate active ion uptake. Any increased intake of Na^+ and Cl^- must therefore be achieved by some other mechanism.

The other possible source of Na^+ and Cl^- intake is via the intestine. The importance of the intestine has already been highlighted with regard to a drinking response and increasing plasma Na^+ and Cl^- concentrations following transfer to increased salinity

(Figure 7.1.1.2). However, once salinity has decreased to the point where chronically acclimated elasmobranchs are hyperionic to the environment these animals are also faced with a requirement for Na^+ and Cl^- intake to regulate the loss of these ions across the semi-permeable surfaces. A drinking response in this scenario would not achieve a net increase in the intake of Na^+ and Cl^- as the imbibed water is hypoionic to blood plasma; it would however compound the influx of water. Animals in this environment would already face a sizable influx of water due to their hyperosmotic and hyperionic states. In the light of this any increase in drinking rate must be regulated by the action of the kidneys; this is discussed in detail below. Once the environmental salinity is reduced to that of FW and plasma Na^+ and Cl^- levels cannot be increased through drinking, dietary intake of these ions becomes of vital importance; particularly if there is no active uptake of Na^+ and Cl^- at the gills. There was also no modification in the maximal activity of Na^+, K^+-ATPase in the intestine of *S. canicula* following chronic acclimation to 80 or 120% SW environments (Figure 5.3.2.1). This is suggestive that partially euryhaline elasmobranchs are unable to modify the abundance and/or recruitment of Na^+, K^+-ATPase in response to changes in environmental salinity. It is possible that these ions are taken up via other reabsorptive mechanisms in the intestine (Section 7.1.3); however a large dietary intake and active predatory behaviour, or a selective diet high in Na^+ and Cl^- levels would be required to achieve this.

One of the primary focuses of this study has been the role of the rectal gland in elasmobranch euryhalinity. In SW, elasmobranchs are hypoionic and face a continual influx of Na^+ and Cl^- from the marine environment. At reduced salinities this gradient is reversed as the animals become hyperionic to the environment and face a continual efflux of Na^+ and Cl^-. It is therefore apparent that increasing rectal gland activity in

response to a requirement for excretion of Na^+ and Cl^-, and decreasing rectal gland activity in response to a requirement for retention of Na^+ and Cl^- are of key importance. The relatively small scale changes in environmental salinity and the associated changes in NaCl secretion rates experienced by partially euryhaline species such as *S. canicula* may be facilitated by relatively crude changes within the rectal gland. Rectal gland secretion rate in such species appears to be modified by changes in blood perfusion (Anderson et al. 2002a), secretory volume (Figure 4.3.2.1) (Anderson et al. 1995a; Anderson et al. 2002a), Cl^- concentration (Figure 4.3.2.2), and structure (Figure 5.3.1.1). Partially euryhaline elasmobranchs may not be capable of altering Na^+, K^+-ATPase abundance and/or recruitment within the rectal gland (Figure 5.3.2.1), nor modifying its response to endocrine control factors such as CNP (Figures 6.3.1.5) following acclimation to salinity change.

Regulation of rectal gland activity is of vital importance at lower salinities. The reversal of the concentration gradients for Na^+ and Cl^- associated with elasmobranchs in low salinities, and the requirement for retention of these ions, necessarily imposes a requirement to modify rectal gland secretory activity. *S. canicula* acclimating to reduced environmental salinity do not significantly alter rectal gland secretory activity *in vivo* (Section 4.3.2). This may reflect little capacity for the modification of Na^+, K^+-ATPase at the cellular level (Chapters 5 and 6). If partially euryhaline elasmobranchs do have a lower capacity for large scale decreases in rectal gland activity than fully euryhaline species, this would significantly limit the range of salinities they could inhabit.

The presence of a specialised NaCl secreting rectal gland in elasmobranchs removes the need for the production of concentrated urine from the kidney. This is crucial in

understanding euryhalinity in elasmobranchs as it permits renal function to focus on the retention of plasma osmolytes and volaemic regulation. In SW elasmobranchs the major role of the kidney is urea retention, however, Na^+ may be actively retained via Na^+-linked urea reabsorption (Section 1.6) (Stolte et al. 1977; Hentschel et al. 1998). As such the kidney of partially euryhaline species has evolved towards this with approximately 70 – 99% of filtered urea being reabsorbed (Kempton 1953; Boylan 1967). It has been shown that for *S. canicula* the relative level of urea in blood plasma decreases disproportionately at reduced salinities, more so than for fully euryhaline species such as *C. leucas* (Figure 7.1.2.1 and Table 2.3.1.3). This may well reflect an inability of partially euryhaline elasmobranchs to increase urea retention whilst simultaneously increasing urine flow rates in order to maintain volaemic stasis during acclimation to reduced salinities.

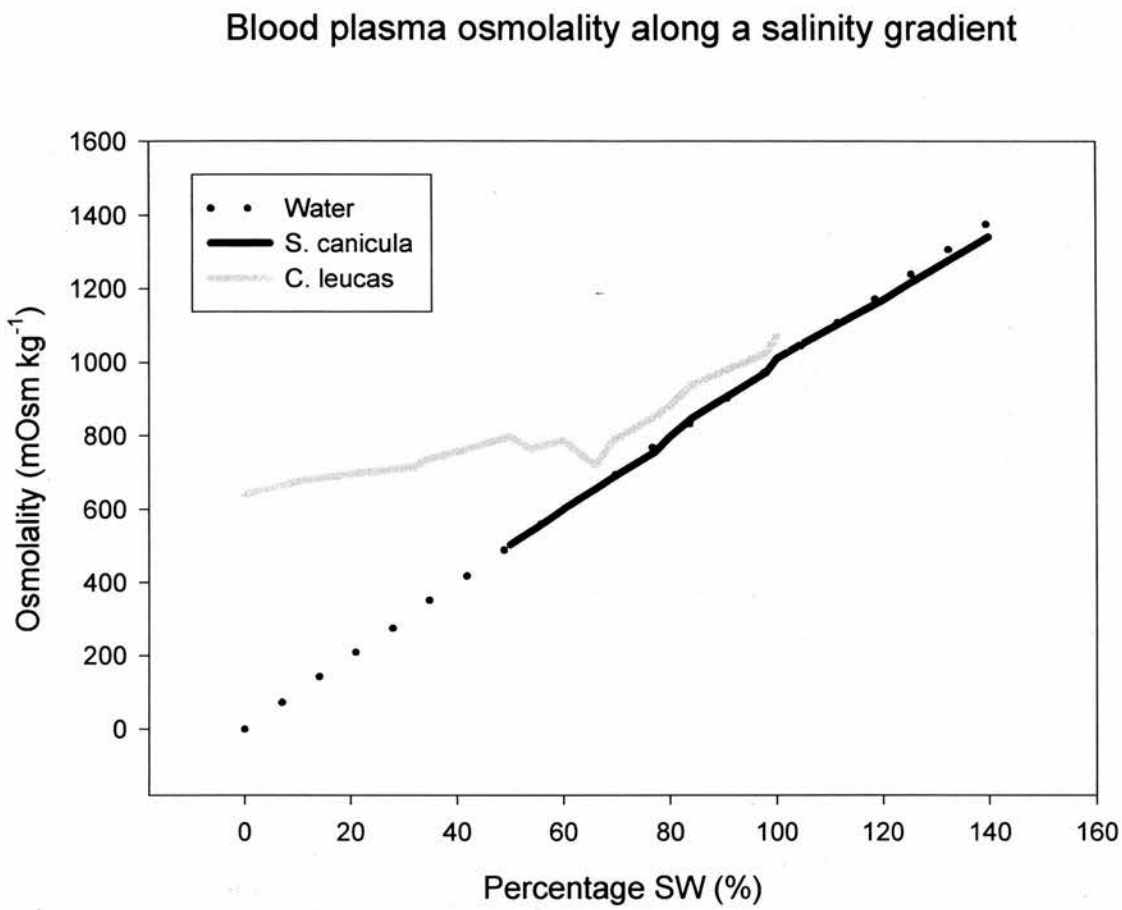

Figure 7.1.2.1 – Descriptive comparison of plasma osmolalities. Values for *C. leucas* are from wild sampled animals in the Brisbane River (Pillans and Franklin 2004). Values for *S. canicula* are cumulative results from St Andrews University (Hazon, *pers. comm.*).

As environmental salinity decreases the major requirement of renal function changes. In FW elasmobranchs there is also a need for urea retention, but the major function of the kidney is the regulation of the massive influx of water which the animals face. This must be countered by high urine flow rates from the kidney in order to maintain volaemic homeostasis. This results in a somewhat paradoxical situation in FW where the kidneys must produce urine to regulate blood volume, but increasing urine flow will necessarily increase the excretion of urea, Na^+, and Cl^-. Acclimation to FW environments therefore requires a decrease in the fractional excretion of plasma osmolytes from the kidney in order to maintain elevated plasma osmolality during periods of increased urine flow rate (Smith 1931a; Janech and Piermarini 2002). Partially euryhaline elasmobranchs may be limited by a comparatively small capacity for this, as compared to truly euryhaline species.

In this light, the evolution of the stenohaline FW potamotrygonid rays towards a strategy which does not require the retention of urea, and hence avoids the two somewhat opposing requirements on kidney function, seems highly efficient considering the large influx of water continually faced by these FW elasmobranchs. However, the inability of stenohaline FW elasmobranchs to retain elevated levels of urea in the blood plasma appears to be the key factor dictating their range of salinity tolerance (Thorson 1970; Thorson et al. 1978; Brooks et al. 1981; Wood et al. 2002a).

It is evident therefore that there are two main requirements of the elasmobranch kidney, osmolyte retention and volume regulation; although both are important in SW and FW, their relative importance changes during acclimation from one environment to the other. It has been demonstrated that there are no significant changes in the maximal activity of

Na^+, K^+-ATPase in the kidney of *S. canicula* as a result of chronic acclimation to salinity change (Figure 5.3.2.1). This suggests that partially euryhaline elasmobranchs may be unable to modify the abundance and/or recruitment of Na^+, K^+-ATPase during salinity transfer. This could be descriptive of an inability to adequately retain plasma osmolytes whilst simultaneously regulate the volaemic influx associated with transfer to FW.

7.1.3 Fully euryhaline elasmobranchs

One notable difference between different species of elasmobranchs is the presence or absence of variation in blood haematocrit (Table 3.1.1). This is important as significant changes in blood haematocrit are suggestive of, but not evidence for, changes in blood volume associated with chronic salinity transfer. A smaller degree of haemodilution may therefore be encountered by fully euryhaline as compared to partially euryhaline species during chronic transfer to reduced salinity. This is suggestive of a more complete regulation of haematic parameters during acclimation to altered environmental salinity. It is intuitive that a more rapid and complete modification in haematic parameters would be a response associated with fully euryhaline elasmobranch species. The reduction or lack of change in blood volume which may be experienced by fully euryhaline species is highly suggestive of tighter control over water fluxes with the environment. This is also suggestive of a greater scope for the modification of function in principle osmoregulatory organs.

However, there was no modification in the abundance and/or recruitment of Na^+, K^+-ATPase in the gills of *C. leucas* between FW and SW acclimations (Figure 5.3.2.2). Recent investigations into the localisation and activity of Na^+, K^+-ATPase have shown significant increases in branchial tissue of FW, as opposed to SW acclimated *D. sabina* (Piermarini and Evans 2000; Piermarini and Evans 2001; Piermarini et al. 2002). Indeed, these findings prompted Piermarini and co-workers (2002) to suggest that the location and abundance of transport enzymes in chloride cells of the gill epithelia favoured the active uptake of Na^+ and Cl^- from FW. These findings in the fully euryhaline *D. sabina* are not consistent with the data presented in this study for the fully euryhaline *C. leucas* (Figure 5.3.2.2).

Given that the protocols for acclimation from FW to SW were similar in the studies on *D. sabina* (Piermarini and Evans 2000) and *C. leucas* (Section 2.2.2.1), this can be taken to represent a physiological difference between these two fully euryhaline species. Either *C. leucas* does not alter maximal branchial activity of Na^+, K^+-ATPase in response to acclimation to SW, or changes occur after 7 days of transfer, or they occur earlier and have returned to basal levels by the 7 day sampling period. Discerning which of these scenarios is the case is not possible from the current study, but the fact that the two species display these differences may have implications for the evolution of different strategies which allow an elasmobranch to be fully euryhaline. The evolutionary distance between the Batoids and Carcharhinids is certainly adequate for the separate evolution of a fully euryhaline physiology (Figure 1.1.1).

These different strategies may be a reflection of the different ecological niches filled by *C. leucas* and *D. sabina*. The highly active predatory behaviour of *C. leucas* may result in sufficient intake of Na^+ and Cl^- through the diet and therefore changes in branchial abundance and/or recruitment of Na^+, K^+-ATPase in order to obtain these ions from the environment are not required. This is in contrast to the less active *D. sabina* which may have a far lower dietary requirement, being a non ram ventilating species, and therefore augments dietary salt uptake with branchial reabsorption of Na^+ and Cl^- from the FW environment. The importance of dietary intake for euryhaline elasmobranchs is discussed below.

Given the importance of urea retention in the FW environment (Section 7.1.2) and the fact that despite having the lowest permeability for urea the gills are the major site of efflux (Boylan 1967; Wood et al. 1995), it seems intuitive that fully euryhaline

elasmobranch species may have evolved mechanisms to further reduced branchial urea loss in FW. Branchial urea permeability has not been measured in fully euryhaline species although research on partially euryhaline species has consistently demonstrated no significant alterations in branchial urea fluxes upon acclimation to reduced salinity (Goldstein et al. 1968; Goldstein and Forster 1971; Payan et al. 1973). Evidently comparative studies on fully euryhaline species would be of great importance in this area.

As stated above, fully euryhaline elasmobranchs have evolved means to increase the intake of Na^+ and Cl^- in a FW environment. For certain species, modifications in the abundance, activity, and orientation of Na^+, K^+-ATPase upon acclimation may be of great importance for this. Partially euryhaline elasmobranchs may not have the capacity for such alterations in their branchial structure. Therefore, an increased ability for the branchial intake of Na^+ and Cl^- may be a fundamental difference between partially and euryhaline elasmobranchs, particularly when other means of ionic intake are minimal.

The importance of the intestine during elasmobranch salinity transfer has already been highlighted with regards to drinking rate (Section 7.1.1). However, there was no modification in the abundance and/or recruitment of Na^+, K^+-ATPase in the intestine of either a partially or fully euryhaline species (Figures 5.3.2.1 and 2). Elasmobranch drinking rates are considerably lower than those of teleosts (Table 1.4.1) and it is possible that the uptake of Na^+ and Cl^- from these volumes can be achieved without increasing the abundance and/or recruitment of Na^+, K^+-ATPase. However, there are no measurements for the drinking rates of fully euryhaline elasmobranchs during transfer from FW to SW. In the current study the difficulties associated with anaesthesia,

surgery, and confinement of *C. leucas* have previously been discussed (Section 2.4), and these prevented studies of drinking rates in these animals. However, the rate of drinking in FW acclimated euryhaline elasmobranchs undergoing salinity transfer is expected to be high considering the increase in plasma Cl^- concentration which is seen after 7 days in SW (around 90 mmol l^{-1} in *C. leucas*). If fully euryhaline elasmobranchs do have a greater drinking response than partially euryhaline species, there does not appear to be any modification in Na^+, K^+-ATPase associated with this.

As previously discussed (Section 7.1.2), dietary intake may also be a key source of osmolytes for some euryhaline species. Ingested food not only provides a source of Na^+ and Cl^- but also provides protein, the substrate for urea synthesis. The negative effects of reduced feeding on *C. leucas* have already been discussed (Section 1.4); similar, though less dramatic effects have also been recorded in *S. canicula* (Armour et al. 1993a). The lack of increase in abundance and/or recruitment of Na^+, K^+-ATPase may not necessarily demonstrate a lack of modification within the tissues of the intestine to the increased dietary intake of FW *C. leucas*. Inorganic ions can also be absorbed via coupled transport with other nutrients. Although elasmobranch specific research in this area is non-existent, inorganic ions such as Na^+ are known to be important in the coupled transport of nutrients such as amino acids (Smith and Lane 1971) and carbohydrates (Farmanfarmaian et al. 1972) in teleost species. Furthermore, many such transporters in the gastrointestinal tract of vertebrates are induced or repressed by changes in substrate concentrations (Diamond 1991). It is therefore possible that increased dietary intake may lead to increased uptake of Na^+ and Cl^- without the requirement for alterations in the abundance or activity of Na^+, K^+-ATPase. If Na^+-

linked absorption of other nutrients is indeed important for ion uptake from the intestine the detrimental effects of low food intake could be compounded in FW.

Another method of analysing the effects of dietary intake is to examine growth rates. It has been reported that growth has the lowest priority of energy requirements in elasmobranchs, whilst basal metabolism has the highest (Gruber 1984). Therefore, by analysing growth rates of captive elasmobranchs and comparing them to values from wild populations an assessment of feeding regimes and dietary intake becomes possible. Gruber (1984) found that *N. brevirostris* fed 12-15% of body mass per week (BM wk^{-1}) gained weight and those fed 10% BM wk^{-1} lost weight. From this it was estimated that a feeding rate of around 11.5% BM wk^{-1} was required to maintain body mass. Such estimates are highly species specific due to the variable growth rates between species (Table 7.1.3.1).

Species	Environment	Growth rate (cm yr^{-1})	Reference
E. taurus	Captive	17.5	(Gruber 1980)
	Captive	24.4	(Gilmore et al. 1983)
C. leucas	Wild	12 – 18	(Thorson and Lacy 1982)
	Wild	15 – 20	(Branstetter and Stiles 1987)
	Captive	28 - 42	(Schmid et al. 1999)

Table 7.1.3.1 – Growth estimates in different populations of elasmobranch species.

This problem is compounded further by the fact that growth rates can vary greatly during different stages of the life cycle. This is well illustrated in *C. leucas* where growth rates are around 18 and 16 cm yr^{-1} in the first two years respectively, but then subsequently reduce to 12 cm yr^{-1} (Thorson and Lacy 1982). However, *C. leucas* used in this study were taken from a juvenile population and work conducted by Schmid and co-workers (1999) showed that feeding rates of 3.5% BM wk^{-1} did not significantly decrease growth rates of captive SW animals. Although exact calculations of feeding rates were not taken in this study, the dietary regime used would have been around 15 - 25% BM wk^{-1}, far exceeding the minimum rate required for long term SW acclimated animals. Despite this, captive FW acclimated *C. leucas* had around 40 mmol l^{-1} less urea in the blood plasma than wild sampled FW animals, and captive SW acclimated animals had around 65 mmol l^{-1} less than wild sampled SW animals (Tables 1.2.2 and 2.3.1.2). The possible reasons for this disparity have been previously discussed (Section 2.4).

The metabolic requirements of a ureotelic hyperosmotic strategy in FW may well result in a significantly higher energy demand than in SW (Figures 6.3.2.1 and 2). If this is indeed the case, the fact that *C. leucas* displays higher growth rates in the first 2 years of the life cycle (Thorson and Lacy 1982; Schmid et al. 1990), and that this period is usually spent in FW (Thorson 1972; Thorson et al. 1973; Taniuchi et al. 2003), may result in a sizeable energy demand and dietary intake.

In FW, fully euryhaline elasmobranchs therefore require the active intake of osmolytes from an ion poor environment. Without the possible modification of branchial tissue for active ion uptake, this demand must be met by an increased intake via the intestine. In

ion poor FW this translates to a high requirement of dietary intake and the associated levels of active predatory behaviour. Furthermore, this increased dietary intake also requires digestive processing and absorption from the intestine. The three types of intestinal valves in elasmobranchs have already been described (Section 1.4), but no research has been conducted on the prevalence of particular types in euryhaline species. Each type of valve has a set of associated digestive parameters, such as time of passage and absorption rate. Although comparative research between the parameters of each valve type is sparse, the ring valve has the most absorptive surface area for unit length (Martin 2003b). Given that different types of intestinal valve have different consequences for digestive and absorption rates, there is scope for morphological adaptation within the gut of fully euryhaline species which require high dietary intakes as a source of Na^+ and Cl^-. Similarly to the case with branchial tissue, differences in the ability to increase ion and protein intake via the intestine may also be of crucial importance in determining the degree of euryhalinity in elasmobranch species. Clearly more research is required into this area.

Given the importance of the retention of Na^+ and Cl^- in low salinity environments, there must be modifications in the rectal gland of fully euryhaline species which restrict secretory activity during these periods. Stenohaline FW elasmobranchs have evolved a degenerate rectal gland which does not secrete Na^+ and Cl^- (Thorson et al. 1978). This removes part of the energetic cost of osmoregulation in FW, but necessarily prohibits inhabitation of hyperionic environments because of an inability to secrete excess Na^+ and Cl^-. Fully euryhaline elasmobranchs, such as *C. leucas*, have therefore presumably evolved means of minimising the energetic costs of retaining Na^+ and Cl^- in FW, whilst

maintaining the osmoregulatory plasticity to enable the excretion of these ions in hyperionic environments.

It has already been described how the evolution of the rectal gland permitted specialisation in the renal tissue through removing the requirement for concentrated urine in order to achieve net NaCl secretion (Section 7.1.1). Although this evolution has permitted refinements in kidney function which have facilitated changes in urea retention and free water clearance, it has also allowed radiation in the physiology and endocrine control of the rectal gland which have facilitated changes in Na^+ and Cl^- secretion rates in different environments.

The larger scale salinity changes tolerated by fully euryhaline elasmobranchs are a reflection of a capacity for more fundamental changes rectal gland physiology. As with the kidney, fully euryhaline elasmobranchs are able to significantly alter the abundance and/or recruitment of Na^+, K^+-ATPase in the rectal gland during chronic acclimation to salinity changes (Figure 5.3.2.2). Given the increased concentration gradients experienced by ureotelic elasmobranchs in FW and the associated increased energetic cost of osmoregulation, the ability to significantly reduce the metabolic cost associated with active transport mechanisms utilising Na^+, K^+-ATPase could be a deciding factor in meeting these energetic requirements. Whilst such reductions do occur in the rectal glands of FW euryhaline elasmobranchs, they do not affect the plasticity of the gland as enzyme maximal activity levels are significantly elevated after 7 days in full SW (Figure 5.3.2.2). This again emphasises the importance of maintaining plasticity in the rectal gland for euryhaline elasmobranchs.

As previously stated, this up regulation in enzyme activity may be achieved by changes in pump abundance or changes in pump recruitment, or a combination of both. The work conducted by Piermarini and Evans (2000) on *D. sabina* demonstrated that euryhaline species can achieve this plasticity by modifications in the abundance of Na^+, K^+-ATPase during acclimation to SW. Increased enzyme activity could also be brought about by increased recruitment of previously inactive units of Na^+, K^+-ATPase. The fact that the rectal glands of FW acclimated *C. leucas* did not increase the relative O_2 consumption of Na^+, K^+-ATPase in response to the known stimulant CNP whilst those of SW acclimated animals did (Figure 6.3.2.5) could be suggestive of a component of total Na^+, K^+-ATPase activity being inactive in FW. Indeed, the fact that Na^+, K^+-ATPase abundance was so high in FW acclimated *C. leucas* that it prevented accurate quantification via Western blotting supports the idea of post-transcriptional regulation of Na^+, K^+-ATPase activity in these animals (Meischke, *pers. comm.*). This contrast with Na^+, K^+-ATPase levels in the rectal glands of *C. leucas* and *D. sabina* upon chronic acclimation to FW and SW again suggests separate evolution of different mechanisms which permit full euryhalinity in elasmobranch fish, as does the situation in the gills of these two species described above.

The results presented in this study demonstrate the latent plasticity of the rectal gland of *C. leucas* in FW and that through modification of the glands reaction to endocrine signals during acclimation to SW secretory rate can be increased. This is suggestive that chronic acclimation to salinity change in fully euryhaline elasmobranchs may result in modifications of enzyme abundance and activity, and subtle variation in the response of secretory tubules to endocrine control factors; rather than simply modifying the relative

periods of activity and inactivity as may be the case with partially euryhaline species (Section 4.3.2).

The greater sophistication of potential modifications for rectal gland function, and the specificity of their nature seen in fully euryhaline elasmobranchs are of fundamental importance for Na^+ and Cl^- balance in both FW and SW. More importantly they permit regulation of function whilst maintaining the necessary plasticity for a fully euryhaline degree of salinity tolerance. Elasmobranchs which do not possess the capacity for these modifications in rectal gland function are necessarily more limited to a smaller range of environmental salinities.

It has already been demonstrated that the fully euryhaline species *C. leucas* is capable of modifying the abundance and/or recruitment of Na^+, K^+-ATPase in the kidney during acclimation to different salinities (Figure 5.3.2.2), the consequences of this for active ion and urea reabsorption have also been discussed (Sections 1.6, 5.4 and 7.1.1). If fully euryhaline species are able to alter the activity of Na^+, K^+-ATPase in response to salinity change, it is possible that they are able to make similar changes in the activity of facilitated urea transporters. Evidence of such transporters has been gathered in both a marine (*R. erinacea*) (Smith and Wright 1999; Morgan et al. 2003) and a euryhaline species (*D. sabina*) (Janech et al. 2003). Furthermore, levels of SkUT have been shown to decrease in the kidney of *R. erinacea* in response to decreased salinity (Morgan et al. 2003). It is possible that less euryhaline species are unable to upgrade the required transporters to facilitate active urea retention in FW. Interestingly, there was 71% sequence identity between the two urea transporters which raises the possibility of functional changes in protein structure within the kidney of different elasmobranch

species. It is possible that these permit different amounts of osmolyte reabsorption in the kidney during periods of increased urine flow rate. Clearly more comparative analysis is required to understand the differences in renal structure between partially and fully euryhaline species which permit the latter to survive in FW.

Physiological modifications in the renal tissue are undoubtedly an important factor in the salinity tolerance of elasmobranchs. The ability of the kidney to regulate the increased influx of water and its greater capacity for active urea and osmolyte reabsorption are a defining feature of fully euryhaline elasmobranchs.

7.1.4 Summary

Comparison of the plasma osmotic profiles of the partially euryhaline *S. canicula* and the fully euryhaline *C. leucas* highlights the differences in their response to salinity transfer (Figure 7.1.2.1). The major difference during acclimation to FW environments is the ability of fully euryhaline species to retain elevated levels of urea, Na^+ and Cl^- in the blood plasma. This is the result of key differences in the major osmoregulatory tissues of partially and fully euryhaline elasmobranch species. These differences are summarised below (Table 7.1.4.1).

Furthermore, the differences seen in the modifications of osmoregulatory tissues in *C. leucas* and *D. sabina* in response to chronic salinity acclimation are suggestive of alternative evolutionary adaptations which permit true euryhalinity. If this is indeed the case a comparative study of these two species could give further insight into the underlying mechanisms which define the degree of euryhalinity in elasmobranch species. Future assessment of blood volume in both of these species, and the magnitude of changes which occur during salinity transfer would also provide much needed insight into the osmoregulatory strategy of fully euryhaline elasmobranchs. Future work focussing on comparative studies of different fully euryhaline species would be of great importance in expanding understanding of the osmoregulatory modifications which facilitate euryhalinity in elasmobranch fish.

	Partially euryhaline	Fully euryhaline
Blood	Altered blood volume and haematocrit	Regulated haematocrit (and possibly blood volume)
Gills	Little modification for active Na^+ and Cl^- uptake No change in urea efflux	Possible modification for branchial reabsorption of Na^+ and Cl^- Possible decrease in urea efflux
Intestine	Little modification in Na^+, K^+-ATPase	Possible specialisation of dietary intake
Rectal gland	Modification of RGF volume, Cl^- concentration, and duct structure.	Plasticity of secretory activity Modifications in Na^+, K^+-ATPase and response to CNP
Kidney	Changed renal dynamics Little modification in Na^+, K^+-ATPase	Modifications in Na^+, K^+-ATPase Decrease in fractional osmolyte excretion at higher urine flow rates

Table 7.1.4.1 – Summary of modifications to chronic salinity transfer in partially and fully euryhaline elasmobranchs.

This study has therefore given insight into the underlying osmoregulatory mechanisms which dictate the degree of salinity change tolerated by individual elasmobranch species. In particular the variations of protein activity and endocrine control of the rectal gland between species of different levels of euryhalinity have been highlighted. This increases understanding of the energetics of elasmobranchs during both acute and chronic acclimations to salinity change. Increased understanding of these processes is of vital importance in explaining the ecophysiology of elasmobranch fish. An increased level of understanding in elasmobranch physiology is of great importance given the financial impact they have on both eco-tourism and public aquaria, and the rapidly declining populations of elasmobranchs in the wild, due to overexploitation by man.

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Appendix 1: Protocols

Blood volume

Anaesthetic

For induction use 120 ppm MS-222 with an equal amount of $NaHCO_3$ to buffer. 1ppm is 0.001g in 1L, so for a 5L induction bath use 0.6g of each.

For maintenance dose use 50 ppm, or 0.25g of each in a 5L volume.

Upon induction opercular rate will slow, the fish will lose equilibrium, and surgical level of anaesthesia is reached when there is no reaction to a firm pinch on the dorsal fin.

Surgery

Cut 60cm lengths of cannula, stretch out the ends, and then cut with a scalpel blade to give an oblique end. Ensure all cannulae are filled with heparinised ringer (200IU ml^{-1}) prior to surgery (cannula volume $\approx$ 0.16ml).

Obtain a body mass for each animal prior to surgery for calculation of irradiated cell injection later.

Make an incision in the flank of the animal, starting just posterior to the pectoral fin, around 5cm in length. Cauterise the incision to prevent internal bleeding during the experiment. Retract the stomach and part of the intestine to expose the coeliac and mesenteric arteries.

The *coeliac artery* runs parallel to the splenic vein, along the stomach wall to the spleen. Place a tie around the artery and pull tight. This fills the artery and gives purchase to put tension in the artery prior to incision.

Place another tie around the artery, *upstream* of the first, do not tighten.

Make the incision between the two ties, feed in the cannula as far as possible (try to feed through into the dorsal aorta), and pull the second tie tight to trap the cannula.

Ensure there is pressure in the cannula from the blood vessel and then secure the cannula with a third tie and another knot with the first tie. Clear the cannula of blood using heparinised ringer and place a *yellow* pin in the end.

The *mesenteric artery* lies at the top of the intestine, posterior to the junction of the coeliac artery and the dorsal aorta.

Cannulate in the same manner as described above, use *black* pin for cannula.

Suture up the animal and place in tank for 24 hours to recover, ensuring filters are running and air hose is turned on.

Preparation of blood cells (after Gingerich *et al*, 1987)
Draw 2ml of blood from donor animal and put in 0.6ml pony vial, centrifuge at 300rpm for 5 minutes in the *Mistral*. Remove the plasma portion and retain in a separate tube for osmolyte analysis.

Wash the blood cells three times in volumes of 4°C ringer solution equivalent to that of the plasma. Take care when resuspending the cells, they are delicate. Then resuspend to give a final volume of 2ml. Add ^{51}Cr to give activity of 1.0×10^8 CPM ml^{-1} (1.67MBq $\approx$ 1.0×10^8 CPM); mix for 1 minute in a vortex at 1000 rpm, then place in fridge overnight.

Wash the blood cells three times as before in volumes of 4°C ringer, each time retaining a 200µl sample of the supernatant to check in the gamma counter for haemolysis (Program 5). Then resuspend the cells in 4°C ringer to give a final haematocrit of 17% for 100%SW, 13% for 80% SW, and 22% for 120% SW.

Using the mesenteric cannula, draw a 400µl blood sample from experimental fish to give initial osmolyte and haematocrit data. Then inject 1.0ml Kg^{-1} of irradiated cell suspension and flush the cannula with 320µl of ringer + heparin (200IU ml^{-1}) solution. *Weigh the syringe* containing the cell suspension before and afterwards to get exact volume delivered.

Basal

Using the coeliac cannula withdraw 200µl of blood and replace with equivalent volume of ringer + heparin solution after 0.5, 1, 2, and 3 hours. *Gently* shake the sample to mix before allequoting three replicates of 50µl into 0.6ml pony vials, then measure activity in the gamma counter. Theoretical blood volume at time zero can be measured by extrapolating a linear regression from all four time points. Take water samples at the start and end of the basal run.

Acute transfer

Start the flow of 100% SW into the tanks. Take a water sample and a blood sample after 0,2,4,6,8, and 10 hours. Replace the lost volume as described above. Measure three 50µl replicates of the blood samples as before in the gamma counter. Plot the actual counts for the time periods against those expected assuming constant decay from the basal values. Any variation between the two must be due to changes in the dilution factor of the radiolabel.

Chemicals and solutions

^{51}Chromium

Sodium chromate (*Amersham* CJS1-1mCi)

350 – 600 mCi/mg Cr; available in 37, 74 and 185MBq amounts (37MBq ml^{-1}).

Ringer solution

NaCl	240 mM (*Sigma* S-9625)
KCl	7 mM (*Sigma* P-9333)
$MgCl_2.6H_2O$	4.9 mM (*Sigma* M-2670)
$Na_2HPO_4.2H_2O$	0.5 mM (*BDH* 301574J)
Na_2SO_4	0.5 mM (*Sigma* S-6264)
Urea	360 mM (*Aldrich* 20,888-4)
TMAO	60 mM (*Sigma* T-0514)
$CaCl_2$	10 mM (*Sigma* C-4901)
$NaHCO_3$	2.3 mM (*Sigma* S-6014)
Glucose	1g/100ml (*Sigma* G-7528)

Add $CaCl_2$ penultimately and $NaHCO_3$ last. Add the glucose on the day of experimentation.

Heparin

Monoparin (*CP Pharmaceuticals Ltd*, Wrexham)

Ampoules contain 1ml of heparin solution, 5000IU ml^{-1}. For experiments the concentration in the ringer is 200IU ml^{-1}. Therefore add 400µl to 9.6ml of ringer solution.

Anaesthetic

Ethyl 3-aminobenzoate methanesulfonate salt (*Sigma* A-5040)

$NaHCO_3$ (*Sigma* S-6014)

For induction use 0.6g of each in 5l of SW; use 0.25g of each in the same volume for maintenance.

<u>Conversion factors:</u>

1.67MBq = 1 x 10^8 CPM

1mCi = 37MBq

Monthly limit for the lab of ^{51}Cr is 200MBq

Rectal gland fixation for histology

Stage one

1) Take a thin, complete cross section of the rectal gland and immediately place in Bouins. This is left overnight.

2) This is then transferred to 75% ethanol.

Samples can be stored like this until return to St Andrews.

Stage two

3) Tissue samples are dehydrated using two changes of 96% and two of absolute ethanol, followed by two of chloroform, each for 30 minutes. This is then stored overnight.

4) Tissues are then impregnated with three changes of paraffin over a period of 4 hours and embedded in moulds in Gurrs Paramat (paraffin wax mixed with synthetic polymers). Blocks were cooled in the fridge before trimming away the excess wax.

5) Sections are cut at 6μm on a microtome (rotary microtome, Leica UK, Milton Keynes, UK) and mounted on acid cleaned slides by floating out of a water bath (46°C) of previously boiled distilled water.

6) Dry the slides in an incubator at 45°C for at least 24 hours after which they are de-waxed in two 3 minute changes of xylene. Rinse with absolute ethanol and rehydrate to distilled water through a descending series of alcohol washes (96%, 75%, and 35% ethanol).

7) Stain using Masson's trichrome method (Masson, 1929): filtered celestine blue for 10 minutes, rinsed in distilled water, then stained a further 10 minutes in Mayer's haemalum.

8) After a 5 minute wash under running water slides are stained in a yellow mordant for 3 minutes. This is washed off under running tap water for between 1-5 minutes: the time off washing is controlled by checking the slide under the microscope for clear nuclear staining.

9) Red blood cells and cytoplasm are stained by a 5 minute immersion in acid fuchsin and ponceau 2R. After a brief wash under tap water place the slides in 1% Phosphomolybdic acid to remove the red dye from the connective tissue.

10) Connective tissue is stained with 1% Aniline blue for 10 minutes and then washed in 1% acetic acid for 1-2 minutes.

11) Briefly rinse the slides with 96% ethanol, and then dehydrate with absolute ethanol. The slides are then cleared in xylene and cover slipped.

Chemicals and Reagents

Bouins solution

Piric acid

Formaldehyde (*Sigma* F-8775)

Glacial acetic acid (*Sigma* A-0808)

75% Picric acid v/v, 20% formaldehyde v/v, 5% glacial acetic acid v/v

Celestine blue

Celestine blue dye (*Sigma* C-7143)

5% Iron alum (*Sigma* F-3629)

Glycerine (*Sigma* S362158)

0.5g Celestine blue dye in 100ml 5% Iron alum, warmed to dissolve, filtered when cooled. Add 14ml glycerine.

Mayer's Haemalum

Haematoxylin (*BDH* 261103G)

Absolute ethanol

Distilled water

Aluminium potassium sulphate (Al_3KSO_4) (*BDH* 100093E)

Sodium iodate (NaI) (*Sigma* S-8379)

Chloral hydrate (*Sigma* C-8383)

Citric acid (*Sigma* C-0759)

1g Haematoxylin dissolved in 10ml absolute ethanol, added to 1 litre of distilled water containing 50g aluminium potassium sulphate and 200g sodium iodate. Allow to stand overnight and add 50g chloral hydrate and 1g citric acid. Boil for 5 minutes, cool, and filter.

Yellow mordant

Lissamine Fast Yellow dye (*Sigma* L-5382)

Orange G dye (*Sigma* 86008)

Picric acid

Distilled water

Absolute ethanol

75% ethanol

400mg of Lissamine fast yellow dye and 400mg Orange G dye added to 160ml saturated Picric acid and 40ml distilled water, mixed and stirred at room temperature. For a working solution add 30ml of this stock to 70ml 75% ethanol.

Ponceau acid fuchsin

Ponceau 2R (*Sigma* 199761)

Acid fuchsin (*Sigma* F-8129)

Distilled water

Glacial acetic acid (*Sigma* A-0808)

2g Ponceau 2R and 1g Acid fuchsin added to 200ml distilled water. Add 3ml of glacial acid and mix, store at room temperature.

Aniline blue

Aniline blue dye (*Aldrich* 415049)

Acetic acid (*Sigma* A-0808)

1% Aniline blue dye in 1% acetic acid.

Na+K+ATPase Assay

Day One

1) Make up homogenisation buffer and keep on ice.

2) Kill fish by blow to head and subsequent severing of the spinal column.

3) Remove 3rd gill arch on RHS of animal.

4) Place gill on foil over an ice-cold petri dish and scrape the cells off of the afferent surface using a scalpel blade. Weigh the scrapings.

5) Homogenise the cells in 1ml homogenisation buffer with a glass homogeniser using 30 double strokes. Use another 1ml to rinse the plunger.

6) Filter the homogenate through four layers of gauze loaded into a sterile 5ml syringe. Depress the plunger to expel as much homogenate as possible into a clean ice-cold microcentrifuge tube.

7) Repeat process for efferent gill surface, rectal gland, Intestine, and Kidney. Freeze overnight in the -70°C freezer.

Day Two - Maximum of 4 fish per run (space in centrifuge)

1) When defrosted, centrifuge the filtered homogenate at 4°C for 30 min at 16,000rpm. Make up protein standards.

2) After centrifugation, keep tubes on ice, draw off the supernatant and discard. Add 1ml homogenisation buffer to the pellet and resuspend using the action of the pipette, followed by 30 double strokes of the micropestle.

Bradford protein assay – Rational Function curve

3) Set up 9 LP4 tubes labelled 2-10

4) Remove an aliquot of 2mg/ml BSA from the freezer. This is the first standard.

5) Add 400ul Milli Q to tubes 2-10

6) Take 600ul of stock, add to tube 2 and vortex mix. Next take 600ul of standard 2 and add to tube 3 etc. This gives the following standards.

Tube No.	1	2	3	4	5	6	7	8	9	10
BSA (mg/ml)	2	1.2	0.72	0.432	0.259	0.155	0.093	0.056	0.034	0.02

7) Add 50ul standard or diluted homogenate* to 1.6ml cuvettes in duplicate.

8) Prepare a further 2 cuvettes containing 50ul Milli Q to use as blanks.

9) Add 1ml Bradford reagent and shake the cuvettes for 15 min (Luckham Shaker).

10) Read colour change at 595nm on spectrophotometer in the Fish Lab. (15342)

*The homogenate must be diluted in order that all samples contain between 0.2 and 0.4 mg/ml protein. A dilution of approximately 1 in 5 appears to be ideal for gills and rectal gland, use 1 in 10 for kidney and intestine; but this should be checked before each assay. 2ml diluted homogenate is required for the assay and this should be frozen at -20°C overnight.

Day Three - Maximum 4 fish per run of assay (space in water bath & centrifuge)

1) Set up duplicate assay tubes (LP4) on ice for three separate groups: zero time, [+ ouabain] and [- ouabain].

2) Add 50ul Milli Q to the zero time and [-ouabain] tubes, and 50ul ouabain to the [+ouabain] tubes.

3) Add 500ul ice-cold 10% TCA to the zero time tubes.

4) Add 50ul assay buffer to all tubes followed by 300ul diluted membrane homogenate.

5) Add 50ul 200mM KCL and 50ul 30mM ATP to all tubes to start the enzyme reaction.

6) Incubate tubes in a water bath at 24°C for 1 hour.

7) Make up phosphate standards using 10mM K2PO4 as the stock. Label 3 sets of LP4 tubes 1-15. Add 400ul stock to 1600ul 5% TCA to make the first standard (2mM). Then make a 6:4 serial dilution transferring 1200ul to the subsequent tube, with each tube containing 800ul 5% TCA. Pipette 200ul of each standard into the 2 remaining sets of LP4's to make a duplicate standard curve. Prepare 2 blank tubes by using 200ul 5% TCA.

No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
[P] uM	2000	1333	889	593	395	263	176	117	78	52	35	23	15	10	7

8) Place the tubes back on ice, add 500ul ice cold 10% TCA to the +ouabain and - ouabain tubes to stop the reaction.

9) Vortex mix all tubes and place on ice for 25 minutes.

10) Centrifuge the tubes at 2400rpm for 5 min at 4°C, to spin down the precipitate.

11) Dispense 200ul samples from the supernatant of each tube into LP4 tubes alongside the phosphate standards.

12) Add 750ul Milli Q to all tubes including the phosphate standards followed by 200ul molybdate reagent. Finally add 50ul DILUTED stannous chloride and vortex mix. Transfer the contents of each tube into 1.6ml cuvettes and shake for 25 min before reading at 690nm.

Quadratic Fit curve

13) The protein standard curve should be repeated alongside the phosphate curve to check the protein content of each sample.

Chemicals and Reagents

Homogenisation Buffer

N-[2- hydroxythyl]piperazine-N-[2-etyhlanesulfonic acid] (*Sigma* H-3375)
Ethylenediamine-Tetraacetic acid (*Sigma* – EDS)
Dithiothreitol (*Sigma* D-5545)
Phenylmethylsulphonyl Fluoride (*Sigma* P-7626)
Ethanol

First a 50mM Hepes and 1mM EDTA solution was made by dissolving 4.766g Hepes and 0.146g EDTA in 400ml Milli Q. This was brought up to pH 7.4 with the drop wise addition of 5M NaOH. The volume was then made up to 500ml with Milli Q and then stored in the fridge.

On the day of homogenisation 0.015g dithiothreitol was dissolved in 10ml Milli Q to make a 10mM DTT solution and 0.024g PMSF was also dissolved in 4ml absolute ethanol resulting in a 34mM solution. Prior to homogenisation 0.5ml DTT and 1.5ml PMSF solutions were added to 48ml of the 50mM HEPES/EDTA stock to make the homogenisation buffer. The components and the final buffer should be kept on ice throughout.

BSA Stock Solution

Bovine Serum Albumin (*Sigma* - A-2153)

0.08g BSA dissolved in 40ml Milli Q and then split into 1ml aliquots, stored at -20°C before being defrosted as required.

Bradford Reagent

Coomassie Brilliant Blue (Brilliant Blue G) (*Sigma* B-131)

Ethanol

Orthophosphoric acid (*Sigma* P-6560)

0.1g Coomassie Brilliant Blue was dissolved in 50ml 95% ethanol, before the addition of 100ml 85% orthophosphoric acid, and the solution made up to 1L with Milli Q. The solution was then filtered using Whatman No. 1 filter circles and stored protected from light.

Phosphate Stock

Potassium Phosphate (KH_2PO_4) (*Sigma* P-5379)

0.027g KH_2PO_4 dissolved in 20ml Milli Q.

TCA

Trichloroacetic acid (*Sigma* T-4885)

10g in 100ml Milli Q = 10%

5g in 100ml Milli Q = 5%

Ouabain

Ouabain (*Sigma* O-3125)

0.1169g ouabain dissolved in 100ml Milli Q (2mM). Caution coshh 5 – face-mask and gloves to be worn at all times.

Na+K+ATPase Assay Buffer

Histidine (*Sigma* H-8000)

NaCl (*Sigma* S-9625)

NaN_3 (*Sigma* S-8032)

$MgCl_2$ (*Sigma* M-2670)

1.162g Histidine was first dissolved in 20ml Milli Q. This requires stirring with heat. 1.75g NaCl and 0.203g $MgCl_2$ were then added, the pH checked (pH 7.2) and then made up to 25ml with Milli Q. Once cooled to room temperature 0.016g NaN_3 was added was added. It is important to allow to cool to avoid the production of noxious gas. The assay buffer was stored for 1-2 weeks protected from light at room temperature.

Potassium Chloride

Potassium Chloride (*Sigma* P-9333)

1.491g dissolved in 100ml Milli Q.

ATP

Adenosine Triphosphate (*Sigma* A-5394)

0.165g ATP dissolved in 10ml Milli Q. pH 6.8-7.0 with tris base.

Tris Base

Trizma (*Sigma* A-5394)

1.211g Trizma dissolved in 100ml Milli Q

Molybdate Reagent

Ammonium Molybdate (*Sigma* A-7302)

Sulphuric acid (*Sigma* S-1526)

2g Ammonium Molybdate was dissolved in 100ml Milli Q. In a fume hood, on ice, 22.2ml conc. sulphuric acid was carefully added drop-wise, and then made up to 200ml.

Stannous Chloride

Stannous Chloride (*Sigma* S-2752)

Hydrochloric Acid (*Sigma* H-7020)

Stock 1M $SnCL_2$ solution was made by dissolving 2g stannous chloride in 10ml conc. hydrochloric acid. This was stored at -20°C. On the day of the assay add 50ul of the main stock is added to 2.5ml Milli Q - ADD ACID TO WATER!

Analysis

- Subtract [Pi] values for +Ouabain from –Ouabain giving μM Pi produced by 200μl of supernatant.
- Multiply by 4.5 (have only sampled 200μl of total 900μl) to give value for total volume of supernatant, i.e. for 300μl of homogenate.
- Multiply by 3.333 to convert value for 300μl of homogenate to value per ml.
- Divide by concentration of protein (mg ml^{-1}) in diluted homogenate as derived from Bradford's assay.
- This gives $Na^{+}K^{+}$ATPase activity in μmol Pi mg^{-1} protein $hour^{-1}$ (divide by 1000 to give mmol Pi mg^{-1} h^{-1}).

Rectal gland respirometry

1) Ensure electrodes are turned on *at least* 1 hour before calibration, turn on water bath at 11°C.
2) Change PTFE tape on electrode holders to ensure good seal with chambers.
3) Make up ringer solution, ringer with ouabain, and also put 200ml Milli Q in a conical flask. Bubble all with air for 10 minutes.
4) Set experimental parameters on software: oxygen solubility factor at 11°C is 2.1900 $\mu mol\ l^{-1}\ torr^{-1}$; measure O_2 in torr; calculate rate in $\mu mol\ O_2\ h^{-1}$. Check air pressure (1013 mB = 760.0 torr).
5) Set up electrodes (remember to check electrolyte) and calibrate (High point for saturated O_2 water (Torr) = [atmospheric pressure (Torr) – 9.8] x 0.2096) (NB: 1013 Pascals = 760.0 Torr). When calibrating, overfill the perspex chambers and turn in the electrodes 1800°, this will leave no air bubbles in the respirometry chamber and result in a final volume of 660µl.
6) Sacrifice animal, *weigh it*, take blood and obtain plasma; remove, blot, and weigh rectal gland.
7) Cut transverse sections of rectal gland using scalpel (approximately 1 mm), discarding extreme anterior and posterior sections. Place slices in ringer and store in water bath.
8) Fill the perspex chambers with ringer as for calibration and screw in electrodes. Measure oxygen consumption in $\mu mol\ O_2\ h^{-1}$ for 15 minutes. Prepare peptide concentrations whilst this is going on.
9) Rinse out the chambers with distilled water, then refill with ringer and place tissue slice in ringer. Repeat recordings for another 15 minutes.
10) Remove slices and store in ringer whilst rinsing out chambers. Fill chambers with next solution and replace tissue slices in correct chambers. Repeat until all solutions have been tested.
11) The following solutions should be used for each of the 4 chambers:

    #1 – Just ringer/ringer + tissue/ringer + tissue/ringer + ouabain

    #2 – Just ringer/ringer + tissue/ringer + 10^{-8}/ringer, 10^{-8} + ouabain

    #3 – Just ringer/ringer + tissue/ringer + 10^{-10}/ringer, 10^{-10} + ouabain

    #4 – Just ringer/ringer + tissue/ringer + 10^{-12}/ringer, 10^{-12} + ouabain

12) *Tissue respiration remains constant for at least 1 hour* (Morgan *et al*, 1997).

13) *Tissue respiration remains constant for at least 9 hours* (Shuttleworth *et al*, 1980).

14) At the end of all trials blot and weigh rectal gland slices.

Analysis

Oxygen consumption rates are estimated using linear regressions of the final 10 minutes of each trace. Values are expressed in μmol O_2 g wet mass^{-1} h^{-1} (μl O_2 g wet mass^{-1} h^{-1} for *C. leucas*).

Chemicals and solutions

Zero oxygen solution

Sodium sulphite (*BDH* 103574F)

Milli water

On day of experiment, add 15ml of Milli Q to 0.03g of Sodium sulphite.

Dogfish ringer solution

See chart on lab wall.

1g of Glucose (*Sigma* G-7528) per 100ml of ringer

Ringer with ouabain

Ouabain (*Sigma* O-3125)

Add 0.1169g of ouabain per 100ml of ringer

Electrode electrolyte

Disodium hydrogen phosphate dihydrate ($Na_2HPO_4.2H_2O$) (*BDH* 301574J)

Potassium dihydrogen phosphate (KH_2PO_4) (*BDH* 102034B)

Potassium chloride (KCl) (*Sigma* P-9333)

Thymol ($C_{10}H_{14}O$) (*Sigma* T-0501)

Milli Q Water

Weigh out 5.31g $Na_2HPO_4.2H_2O$, 2.6g KH_2PO_4, and 1.04g KCl. Make the volume up to 100ml with Milli Q. Add a small crystal of Thymol (to inhibit bacterial growth), shake vigorously and leave for 12 hours to allow Thymol to enter solution. Filter into an airtight container.

Appendix 2: Publication arising from study