The endocrine control of renal function in elasmobranch fish.

By

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Declaration

I, Alan Wells hereby certify that this thesis, which is approximately 33,000 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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I was admitted as a research student in September 1999 and as a candidate for the degree of Ph.D in September 2000; the higher study for which this is a record was carried out in the University of St Andrews between 1999 and 2002.

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Abstract

1. Urine flow rate was measured *in vivo*, as fish were acclimated to reduced salinity (70% SW). Urine flow increased in a step-wise manner, whilst plasma osmolality, urea, chloride and sodium concentrations were all significantly reduced. Associated with this reduction in plasma osmolality was a significant increase in the renal clearance of ions.

2. An *in situ* perfused renal preparation has been developed and established as a viable tool for the investigation of renal function in the dogfish in fish acclimated to both SW and 85% SW. The preparation recovered from a high dose of arginine vasotocin and remained viable for a sufficient period of time to investigate the renal effects of osmoregulatory peptides.

3. On perfusion through the renal trunk preparation, arginine vasotocin and angiotensin II caused a significant glomerular antidiuresis and decreases in the tubular transport maxima for glucose and perfusate flow in trunks from both SW and 85% SW-acclimated fish. However, C-type natriuretic peptide, had the opposite effect, causing a glomerular diuresis and an increase in the tubular transport maxima for glucose trunk preparations from fish acclimated to both salinities. Tubular parameters remaincd unchanged by all three hormones under test.

4. Preliminary evidence for the existence of an intra-renal renin-angiotensin system has been presented. The angiotensin converting enzyme inhibitor captopril caused a significant glomerular diuresis and an increase in transport maxima for glucose in SW-acclimated preparations.

5. The filtering population of glomeruli was examined directly using the ferrocyanide technique. Perfusion of arginine vasotocin or angiotensin II caused a significant reduction in the proportion of filtering glomeruli. This reduction was greater than that previously predicted by the measurement of transport maxima for glucose. Large discrepancies between TmG and direct measurements of the filtering population of glomeruli have cast further doubt on the effectiveness of TmG as a measure of the functional tubular mass. These results have serious implications for the use of TmG as a measure of functional tubular mass in elasmobranch fish. Perfusion of C-type natriuretic peptide had no significant effect on the proportion of filtering glomeruli in SW-acclimated preparations and only a slight increase in preparations acclimated to 85% SW. These results strongly suggest alterations in single-nephron glomerular filtration rate.

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Abbreviations

1αOH-B	1α-hydroxycorticosterone
ACE	angiotensin converting enzyme
Ang I	angiotensin I
Ang II	angiotensin II
Ang III	angiotensin III
Ang IV	angiotensin IV
ANP	atrial natriuretic peptide
AVP	arginine vasopressin
AVT	arginine vasotocin
BK	bradykinin
BNP	brain or B-type natriuretic peptide
CD	collecting duct
CFTR	cystic fibrosis transmembrane conductance regulator
C_{H20}	free water clearance
C_{H_2O}/GFR	relative free water clearance
Cl^-/HCO_3^-	chloride/bicarbonate
CNP	C-type natriuretic peptide
C_{osm}	osmolar clearance
C_{osm}/GFR	relative osmolar clearance
CT	collecting tubule
CV	central vessel
DG	diacylglycerol
ECF	extra-cellular fluid
EDT	early distal tubule
ER	rate of excretion
FE	fractional excretion
GC	guanylate cyclase
GFR	glomerular filtration rate
GL	glomerulus
IP_3	inositol triphosphate
IS	intermediate segment
JG	juxtaglomerular
LDH	lactate dehydrogenase
LDT	late distal tubule
MRC	mitochondria-rich cell
MS222	3-aminobenzoic acid ethyl ester
Na^+/NH_4^+	sodium/ammonium
Na^+K^+ATPase	sodium/potassium ATPase
Na-K-2Cl	sodium-potassium chloride
NP	natriuretic peptide
NPR	natriuretic peptide receptor
NPY	neuropeptide Y
NS	neck segment
PAH	*p*-aminohippurate
P_{GC}	glomerular capillary hydraulic pressure
PI	1st proximal tubule
PII	2nd proximal tubule

RAS	renin angiotensin system
Scy II	scyliorhinin II
SNGFR	single nephron glomerular filtration rate
T_m	tubular transport maximum
TMAO	trimethylamine oxide
TmG	tubular transport maxima for glucose
U/P_{in}	urine/perfusate inulin concentration ratio
U/P_{osm}	U/P osmolality ratio
U-I	urotensin I
U-II	urotensin II
$\dot{V}$	urine flow rate
VIP	vasoactive intestinal peptide
VNP	ventricular natriuretic peptide

1

General Introduction

1.1. Introduction

The present study encompasses an investigation into the hormonal control of renal function in the European lesser-spotted dogfish, *Scyliorhinus canicula*. This chapter will introduce some general concepts of renal function and endocrinology, followed by a discussion of the osmoregulatory process in elasmobranch fish.

1.2. General concepts of renal function

Osmoregulation in vertebrates involves the maintenance of a stable internal environment, by the regulation of extracellular and intracellular ion concentration and water content, in the face of a variable external environment. The kidney plays a central role in successful osmoregulation, and is ubiquitous, but differs in anatomical structure, throughout the vertebrate phyla.

The kidneys of all vertebrates, with the exception of aglomerular teleost fish are constructed from the same basic units. In general, vertebrate nephrons consist of a glomerulus followed by a neck segment, a proximal tubule, an intermediate segment, a distal tubule, and finally, a collecting tubule and duct system (Dantzler, 1985). However, variations among the vertebrate classes occur in the sequence and presence of nephron segments and in the relationship of the nephrons to their blood supply

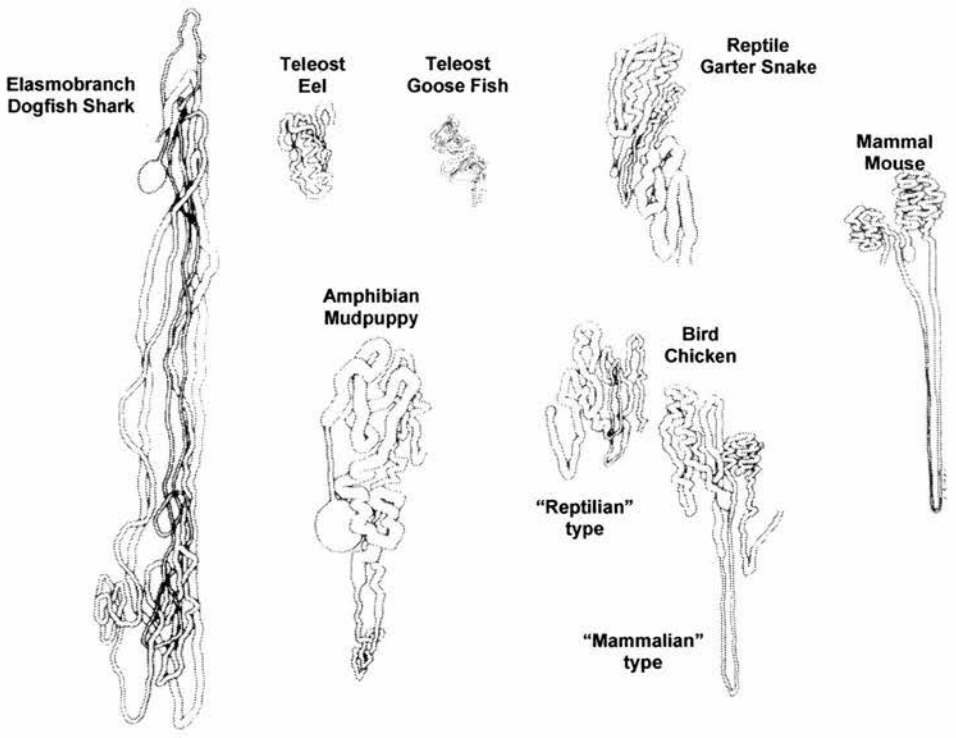

Fig. 1.1. Representations of fish (elasmobranch, *Squalus acanthias*; glomerular teleost, *Anguilla rostrata*; aglomerular teleost, *Lophius piscatorus*), amphibian (*Necturus maculosus*), reptilian (*Thamnophis sirtalis*), avian (*Gallus domesticus*), and mammalian (*Mus flavicollis*) nephrons drawn to a single scale. Adapted from Dantzler (1985).

(Dantzler, 1985). Figure 1.1. shows representations of nephrons from a variety of species, and several important variations exist. First, the nephrons of a number of fish species lack glomeruli, and urine is formed solely by secretion (See Page 12). In the mammalian nephron, the intermediate segment is arranged to form a loop of Henle, the countercurrent multiplier that allows the subsequent production of a hyperosmotic urine by the osmotic abstraction of water in the collecting duct (Lote, 1987). Nephrons of most non-mammalian vertebrates lack loops of Henle and are arranged perpendicular to the collecting ducts (see teleost, amphibian and reptilian examples in Fig. 1.1.). This arrangement would not be expected to permit them to produce urine hyperosmotic to plasma. However, the avian kidney contains two distinct nephron types; nephrons

resembling those found in the kidneys of most other non-mammalian vertebrates (reptilian-type) and nephrons resembling those found in the kidneys of mammals (mammalian-type) (Braun and Dantzler, 1972) (Fig. 1.1.). The majority of avian nephrons are reptilian-type nephrons and are located superficially in the kidney. They drain at right angles into collecting ducts and are not arranged in a manner that would be expected to permit them to produce a hyperosmotic urine (Braun, 1993). However, some nephrons are of the mammalian-type and have loops of Henle which are bound to the vasa recta and collecting ducts in parallel by a connective-tissue sheath. This arrangement would be expected to permit the avian kidney to produce a urine hyperosmotic to the plasma (Dantzler, 1985). The arrangement of the elasmobranch nephron will be discussed in much greater detail in Section 1.5.

Urine formation begins with the filtration of plasma through the glomerular capillaries. The final urine is quite different from the glomerular filtrate because, as the filtered fluid flows through the various portions of the tubule, its composition is altered. This change occurs by two general processes: tubular reabsorption and tubular secretion (Vander, 1985) (Fig. 1.2.).

The net pressure for filtration is determined by the opposition of hydrostatic pressure, the driving force for filtration, against the colloid oncotic pressure and Bowman's space hydrostatic pressure, which act against filtration. Oncotic pressure is the osmotic pressure generated by large molecules, especially proteins, in a solution (Koeppen and Stanton, 1992). As blood passes through the glomerular capillaries, capillary hydraulic pressure decreases slightly due to the resistance to flow offered by the capillaries, and oncotic pressure increases due to an increase in the protein concentration of the

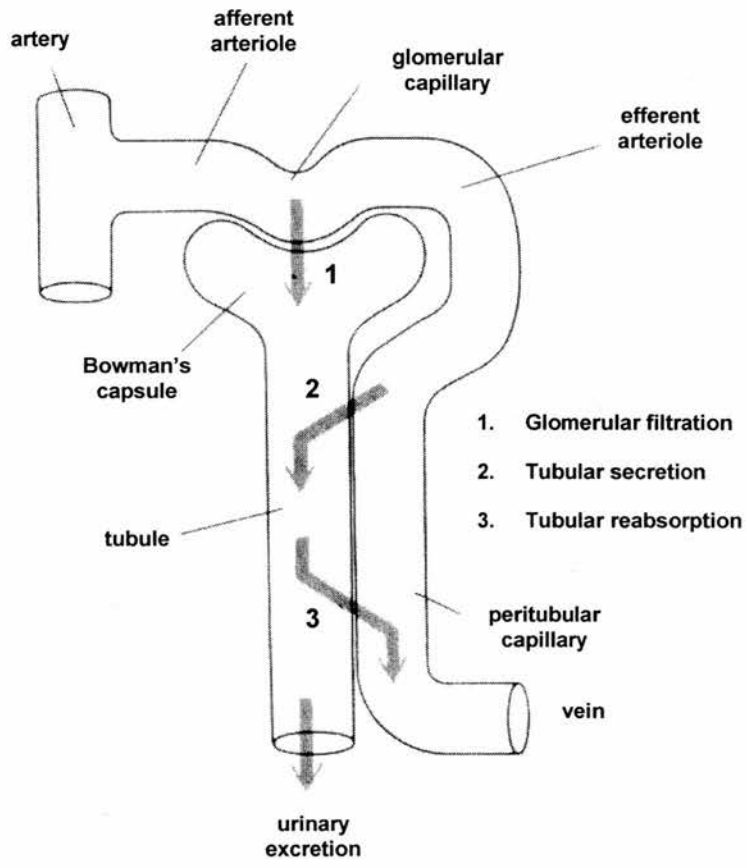

Fig. 1.2. The three basic components of renal function. Adapted from Vander (1985).

unfiltered plasma because filtration removes water and ions but not protein. At some point along the glomerular capillary, net filtration pressure may become zero, a phenomenon known as filtration pressure equilibrium (Vander, 1985). This process is illustrated diagrammatically in Fig 1.3. and is an extremely important concept in low pressure, non-mammalian systems. In the kidney of non-mammalian vertebrates the net filtration pressure may be insufficient to promote filtration, resulting in glomeruli that are arterially perfused, but not filtering. The significance of this will be discussed further in Chapter 6 and Chapter 7.

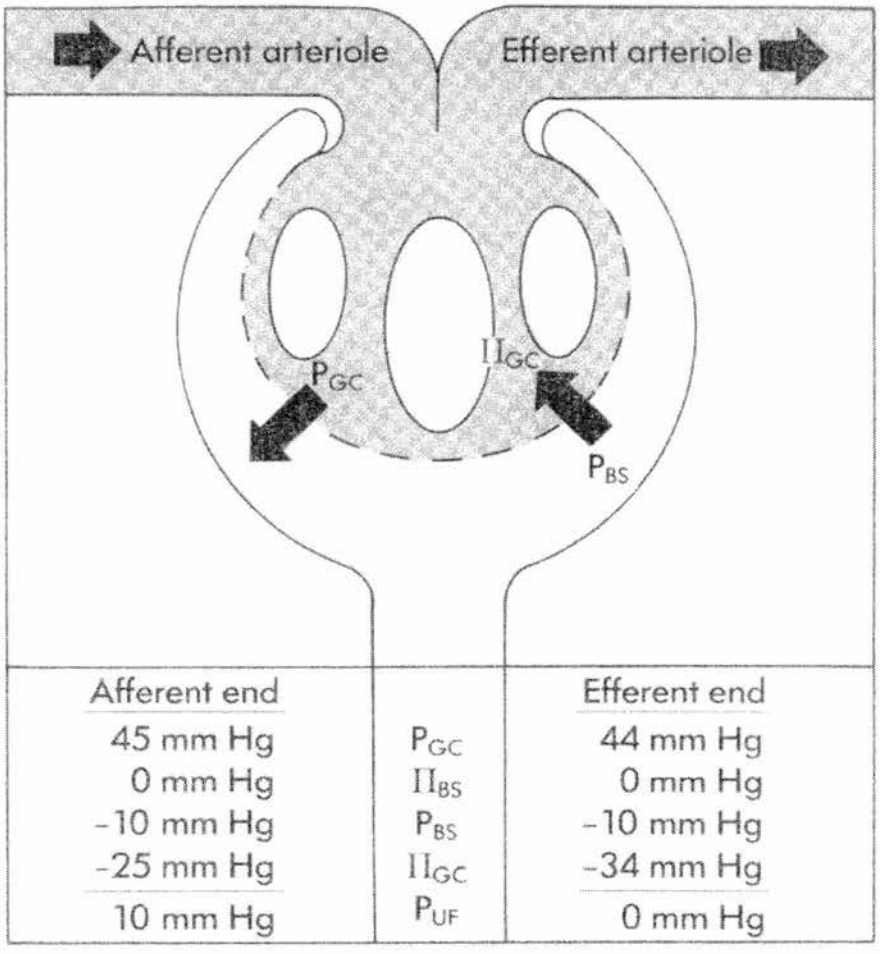

Fig. 1.3. Schematic representation of an idealised glomerular capillary and the Starling forces across the filtration barrier. P_{UF}, net ultrafiltration pressure; P_{GC}, glomerular capillary hydrostatic pressure; P_{BS}, Bowman's space hydrostatic pressure; II_{GC}, glomerular capillary oncotic pressure; II_{BS} Bowman's space oncotic pressure (adapted from Koeppen & Stanton, 1992).

The glomerular capillaries are freely permeable to water and solutes of small molecular dimensions. They are relatively impermeable to large molecules or colloids, such as the plasma proteins. The glomerular filtrate is therefore essentially protein free and contains most solutes in virtually the same concentration as in the plasma (Lote, 1987). The glomerular capillary wall is extremely specialised and consists of three discrete regions (Brenner *et al.*, 1987): the fenestrated capillary endothelium, an acellular basement

membrane area, and an overlying layer of specialised epithelial cells, or podocytes, which display a complex pattern of interdigitating foot processes (Fig. 1.4.). A thick

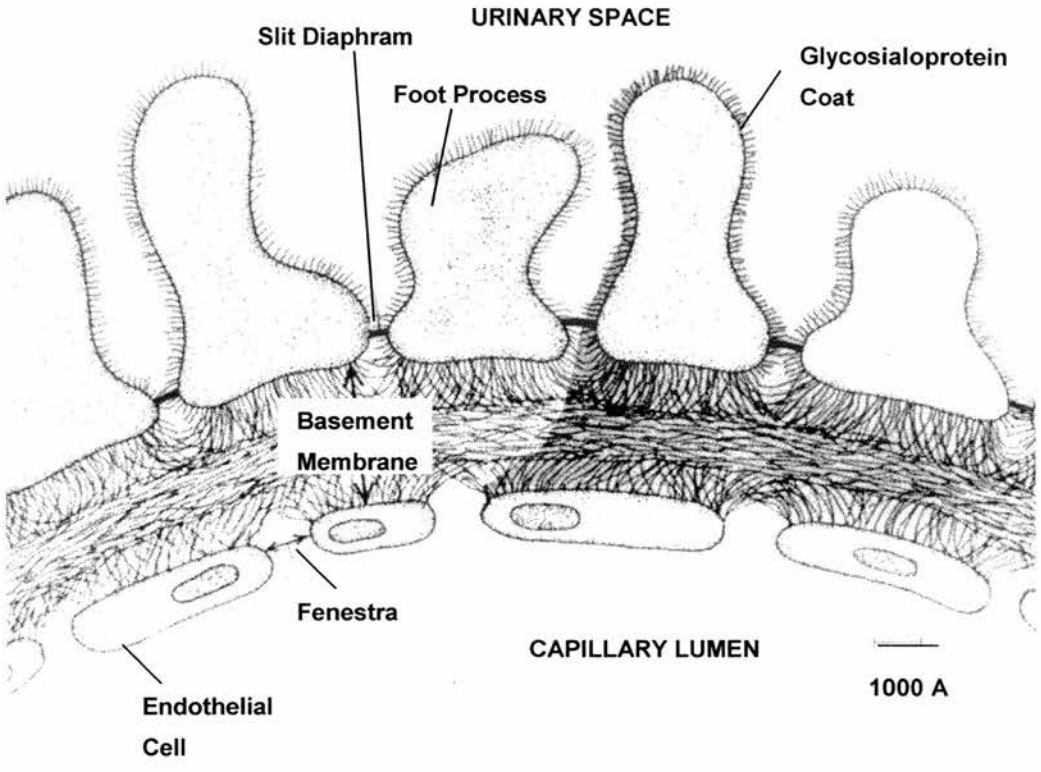

Fig. 1.4. Glomerular capillary wall in cross-section (Adapted from Brenner *et al.* 1987).

layer of extracellular material (glycosialoproteins) coats these foot processes, which partially occlude the slit diaphrams. These glycosialoproteins are negatively charged and therefore molecules are also held back on the basis of charge, with cationic molecules being filtered more readily than anionic molecules (Koeppen and Stanton, 1992). Water filtered across the glomerular barrier is thought to pass via an extracellular route, firstly through the endothelial fenestrae of the glomerular capillaries, secondly through the hydrated gel of the basement membrane and finally through the slit diaphragms of the capillary epithelial layer (Vander, 1985) (Fig. 1.4.).

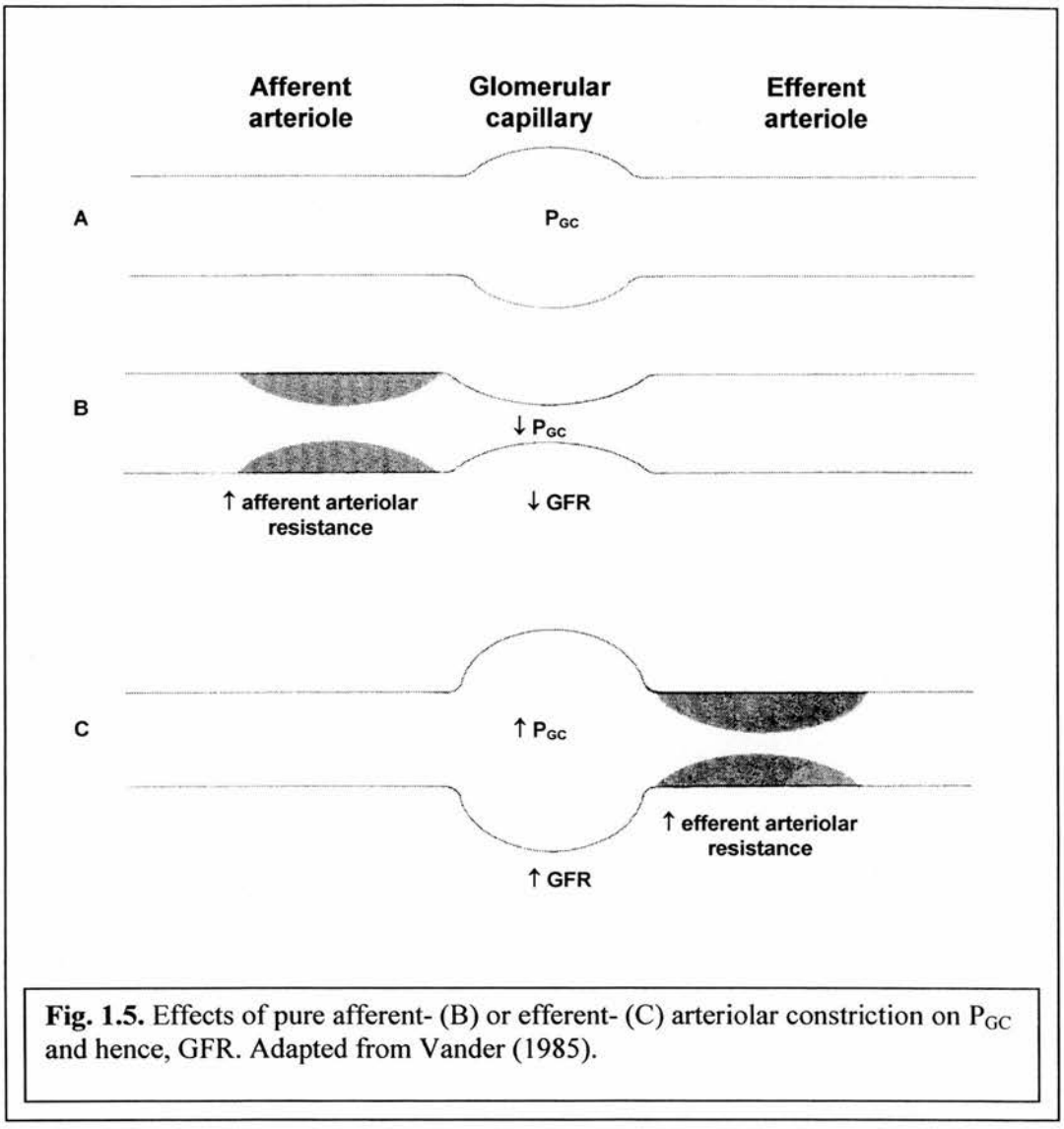

Fig. 1.5. Effects of pure afferent- (B) or efferent- (C) arteriolar constriction on P_{GC} and hence, GFR. Adapted from Vander (1985).

The rate of filtration, which results in the glomerular filtrate, is termed the glomerular filtration rate (GFR). The GFR depends not only on the net filtration pressure described above, but also on the hydraulic permeability of the glomerular membranes and the surface area available for filtration (Vander, 1985). The GFR is not fixed, but may show marked fluctuations in differing patho-physiological states, for example during acute or chronic renal failure. If all other factors remain constant, any change in the glomerular hydraulic permeability or surface area, in the hydraulic pressures within the glomerular capillaries or in the oncotic pressure of the glomerular-capillary plasma will alter GFR. For example, Figure 1.5. demonstrates how vascular constriction at the afferent or

efferent arteriole may affect glomerular-capillary hydraulic pressure (P_{GC}) and therefore GFR. An increase in afferent arteriolar constriction will tend to lower P_{GC} by causing a pressure drop between the renal arteries and glomerular capillaries. This would lead to a reduction in GFR. Conversely an increase in afferent arteriolar dilation will tend to raise P_{GC}. However, an increase in efferent arteriolar constriction would result in an increase in P_{GC} and therefore an increase in GFR. This mechanism has also been proposed to explain the stability of GFR, in the healthy mammalian kidney due to the phenomenon of autoregulation (Vander, 1985). Autoregulation is proposed to explain how the rate of blood flow through the kidney remains relatively constant in the face of changes in mean arterial pressure, in the physiological range. As perfusion pressure increases, the resistance to flow also increases, due to the smooth muscle of the afferent renal arterioles contracting to a greater degree (Fig. 1.5.). This pressure sensitive mechanism is termed the myogenic mechanism, and is related to the intrinsic tendency of vascular smooth muscle to contract when it is stretched and relax when it is shortened (Koeppen and Stanton, 1992). In addition, to the myogenic mechanism, a flow dependent mechanism known as tubuloglomerular feedback has also been proposed to explain autoregulation (Koeppen and Stanton, 1992). This involves a feedback loop in which the flow of tubular fluid and salt delivery rate is sensed by the macula densa of the juxtaglomerular apparatus (Fig 1.6.) (Guyton and Hall, 2001). For example, a decrease in sodium chloride at the macula densa initiates a signal from the macula densa that has two effects; firstly it decreases resistance of the afferent arterioles, thereby raising glomerular hydrostatic pressure (Fig. 1.5.). Secondly, it increases renin release from the juxtaglomerular cells of the efferent arteriole, resulting in the formation of angiotensin II (see Chapter 4) which constricts the efferent arterioles, thereby increasing glomerular hydrostatic pressure and returning GFR towards normal (Guyton and Hall, 2001) (Fig.

1.5. & 1.6.). GFR is therefore maintained constant across a wide range of mean arterial pressure in the mammalian kidney, but in low-pressure systems in lower vertebrates, changes in mean arterial pressure may have a profound effect on GFR.

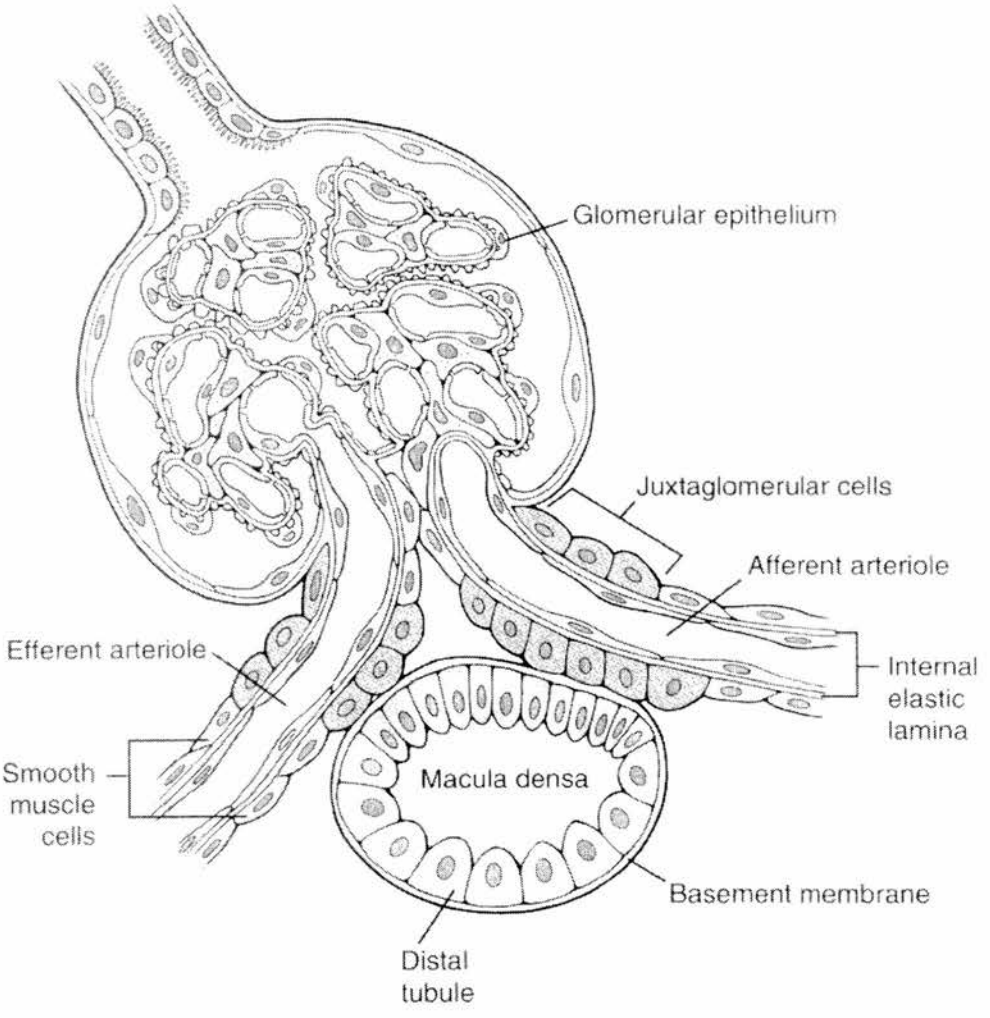

Fig 1.6. Structure of the juxtaglomerular apparatus (Guyton & Hall, 2001)

GFR can be measured by the clearance of a substance that is neither secreted nor reabsorbed by the renal tubule, and is freely filtered at the glomerulus (Levinsky and Levy, 1973). It has been suggested that any substances proposed as indices of GFR should meet the following criteria (Levinsky and Levy, 1973):

1. Be physiologically inert and non-toxic
2. Not be protein bound and be completely filterable at the glomerulus
3. Not be reabsorbed or secreted
4. Not be subject to destruction, synthesis or storage within the kidney
5. Not be excreted by the aglomerular fish
6. Have clearance constant over a wide range of plasma concentrations

A great number of materials have been proposed as indices of GFR including inulin, polyfructosan, urea, creatinine, vitamin B_{12} and various radioisotopic markers (Levinsky and Levy, 1973). For convenience, many test substances are commonly employed that fulfil the above criteria only partially. However, extensive evidence indicates that the clearance of inulin is an accurate index of GFR in most species (Levinsky and Levy, 1973). Slight tubular reabsorption has been reported in the urinary bladder and tubules of some teleost fish however, (Beyenbach and Kirschner, 1976) so care must be taken in the interpretation of results, particularly in lower vertebrates.

In non-mammalian vertebrates, changes in whole-kidney GFR appear to result primarily from changes in the number of filtering glomeruli (Dantzler, 1985) (See Chapter 6). The concept that changes in whole-kidney GFR result from changes in the number of glomeruli filtering is supported by studies showing that the tubular transport maximum (T_m) (see below), for the renal transport of glucose or *p*-aminohippurate (PAH), varies directly with whole kidney GFR (Brown *et al.*, 1980; Dantzler, 1967; Dantzler and Schmidt-Nielsen, 1966; Forster, 1942). If the changes in whole-kidney GFR resulted from equal changes in the amount filtered by each glomerulus, with all continuing to function, the T_m for glucose or PAH transport would not be expected to change, because

the mass of tissue transporting these substances would not have changed (Forster, 1942). However, in lower vertebrates, changes in the individual filtering rate of each nephron (single–nephron GFR or SNGFR) also occur (Braun and Dantzler, 1972; Brown and Green, 1987; Brown *et al.*, 1978; Brown *et al.*, 1980). Whole-kidney GFR therefore involves a balance between the number of filtering nephrons and the SNGFR.

As previously stated, two general processes; tubular reabsorption and tubular secretion alter the final composition of urine. Tubular reabsorption (Fig. 1.2.) can occur by one of a number of mechanisms. The simplest mechanism of tubular reabsorption is simple diffusion, whereby a substance moves down a concentration or electrochemical gradient, with no specific interaction between the substance and the proteins of the membrane (Vander, 1985). In addition, a substance may be reabsorbed by simple facilitated diffusion whereby the substance moves down its electrochemical gradient, but is dependent upon interaction of the substance with specific membrane proteins which facilitate its movement. This is an important mechanism for accelerating the movement of non-lipid-soluble molecules and the membrane proteins involved are termed either channels or carriers (Vander, 1985). Thirdly, active transport of a substance may occur, whereby the substance is transported against a concentration or electrochemical gradient. In this case the energy for the process may come either directly from ATP, in which case membrane bound ATPase is a component of the actual carrier mechanism, or indirectly when two substances interact simultaneously with the same membrane proteins and both are translocated across the membrane (Lote, 1987). This process is termed co-transport, and the direct source of energy is the energy liberated by the simultaneous movement of another substance down its concentration gradient (Lote, 1987).

Many of the active reabsorptive systems in the renal tubule can transport only limited amounts of material per unit time, primarily because the membrane proteins responsible for the transport become saturated. For example, glucose is not normally excreted in the urine because tubular reabsorption is complete. However, it is possible to produce urinary excretion of glucose simply by administering large quantities of glucose into the plasma. The concentration of plasma glucose at which glucose starts to appear in the urine is termed the tubular transport maxima for glucose (TmG) (Vander, 1985). From this point on, any further increase in plasma glucose is accompanied by a proportionate increase in excreted glucose because the T_m has now been reached. The TmG can be used to indirectly assess the functional tubular mass (See above).

Tubular secretory processes (Fig 1.2.), which transport substances across the tubular epithelium into the lumen, constitute a second mechanism by which secretion of a substance that is not filtered or only partly filtered may be excreted (Vander, 1985). For example, in the case of aglomerular fish, tubular secretion is the only means by which substances may be excreted in the urine, as there are no glomeruli to allow filtration to take place. The overall secretory process begins with the simple diffusion of a substance out of the peritubular capillaries into the interstitial fluid. Movement into the tubular lumen can then occur in a similar manner for that of tubular reabsorption, except in the opposite direction. A further point to note is that substances rarely show purely unidirectional flux across the tubule, and therefore tubular movement is usually referred to as 'net' reabsorption or secretion (Brenner *et al.*, 1987).

1.3. General concepts of endocrinology

Hormones are chemicals that are released from the endocrine glands, into the blood, which carry them to special sites that are physio-chemically programmed to react and respond to them. Endocrine glands are tissues that release their secretions directly into the blood passing through them. Vertebrate hormones belong to two principal classes of chemical compounds; peptide hormones and steroid hormones. Peptide hormones are made up of amino acids and range in complexity from tripeptides, to others like growth hormone that contain about 190 such units. Steroid hormones, such as those produced in the adrenal cortex and gonads are made from cholesterol. They consist of a series of carbon rings, the basic unit being the cycloperhydrophenanthrene (Bentley, 1982). The basic steroid structures are shown in Figure 1.7. These structures give rise to the 4 major classes of steroid hormones: C_{18}-steroids or oestrogens; C_{19}-steroids or androgens; C_{21}-

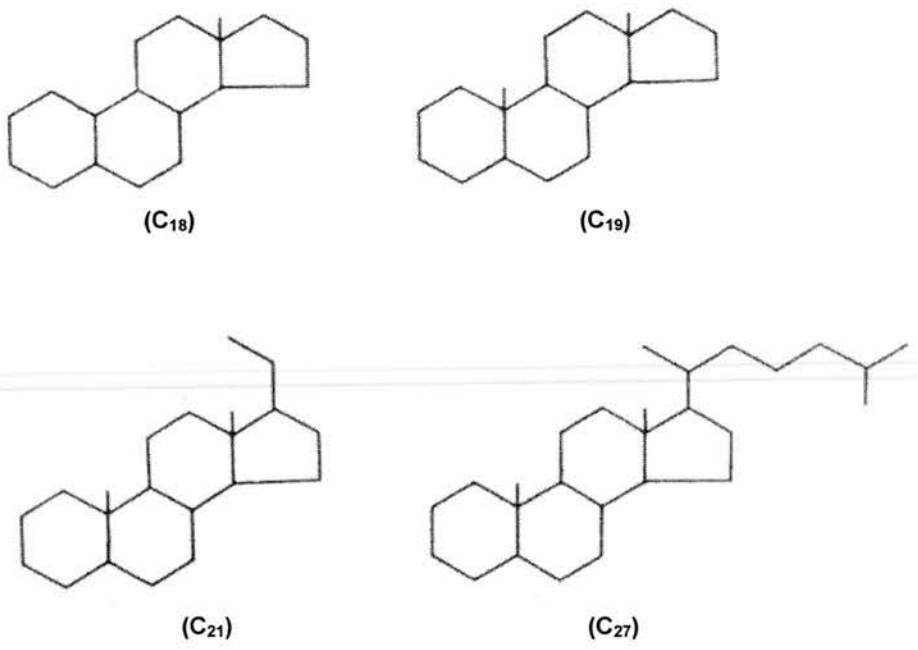

Fig 1.7. Basic steroid structures (Adapted from Kime, 1987)

steroids or corticosteroids; and C_{27}-steroids or vitamin D metabolites (Kime, 1987).

Target tissues recognise appropriate hormones from the blood perfusing them by means of specialised high affinity proteins called receptors. For most peptide hormones, these receptors are located on the external surface of the plasma membrane, therefore these hormones, which are hydrophilic, do not need to pass through the lipid bilayer of the cell membrane. Lipophilic hormones, such as thyroid and steroid hormones diffuse freely through the plasma membrane and interact with internal receptors.

It is generally accepted that peptide hormones bind to receptors on the cell membrane, causing a conformational change in membrane G proteins. These activate enzymes inside the cell, resulting in the synthesis of second messengers, such as cyclic adenosine monophosphate, (cAMP), intracellular calcium ions, diacylglycerol (DG) or inositol triphosphate (IP_3), which activate the cellular response (Slaunwhite, 1988) (Fig. 1.8.).

Steroid hormones and thyroid hormones are poorly soluble in water but readily soluble in lipids. They are therefore transported in the blood, bound to plasma proteins. Steroid hormones pass easily through the plasma membrane into the cell where they bind to specific receptor proteins, although the precise location of these receptors remains controversial. In the case of thyroid hormones and sex hormones the receptor is in the nucleus, while in the case of the glucocorticoids, the receptor is in the cytoplasm. After the hormone binds to the receptor, the complex is translocated to the nucleus where it controls transcription of specific genes (Greenstein, 1994) (Fig. 1.8.). Both steroid and peptide hormones interact with their target cells through a primary interaction with

receptor proteins which recognise the hormones selectively, thus conferring specificity of response.

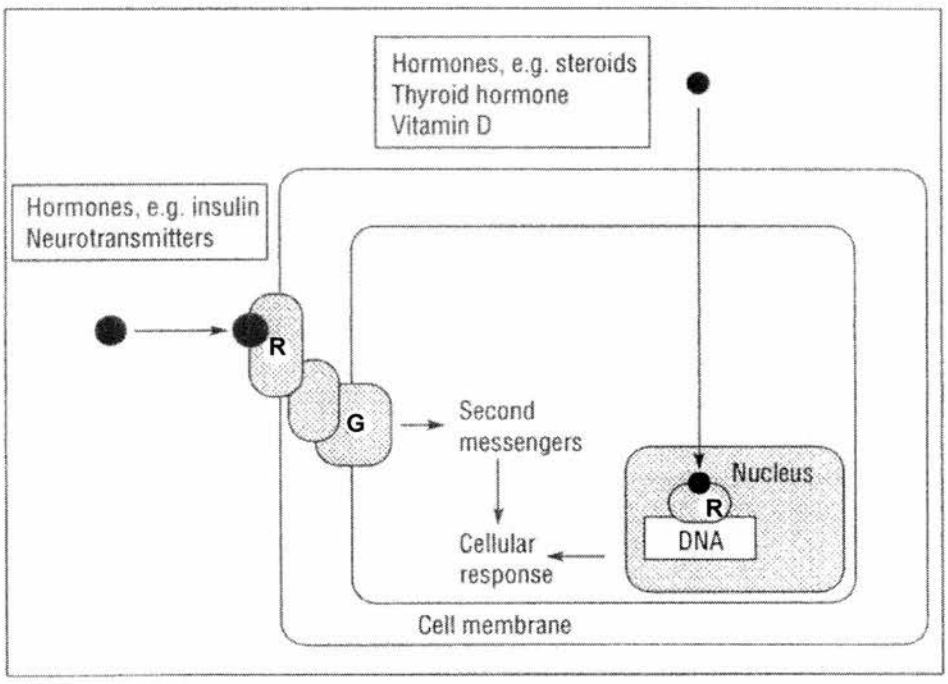

Fig. 1.8. Mechanisms of hormone action; *R*, receptor; *G*, G-protein (Adapted from Greenstein, 1994).

1.4. Elasmobranch Osmoregulation

The class Chondrichthyes, or cartilaginous fishes, includes two subclasses: the Holocephali or ratfishes and the Elasmobranchii, including sharks and rays. Their common features include a cartilaginous skeleton and an osmoregulatory strategy based mainly on urea rather than purely on salt as in bony vertebrates. This introduction will focus only on the osmoregulatory processes of elasmobranch fish.

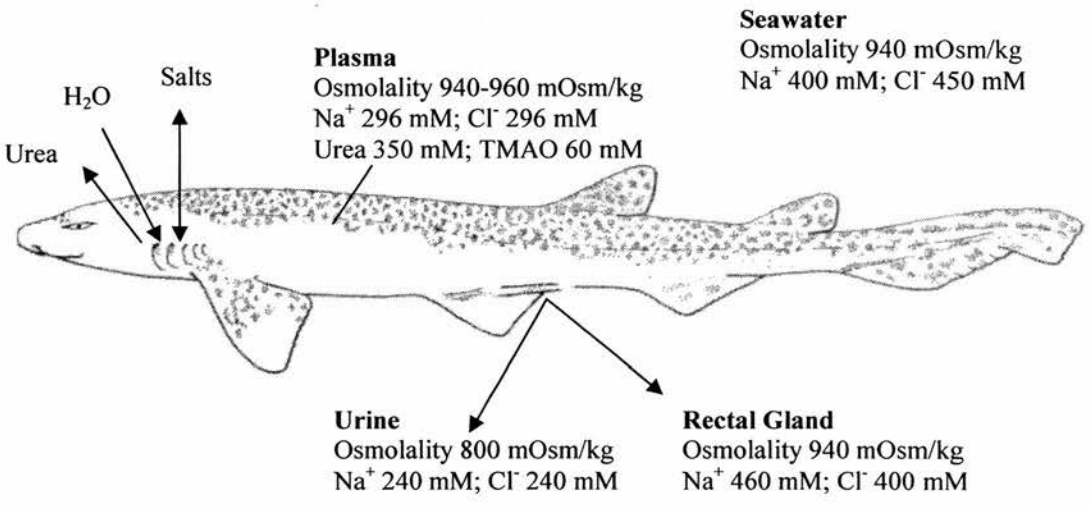

Fig. 1.9. Summary of osmolyte regulation in a typical elasmobranch

Fig. 1.9. illustrates the key aspects of the elasmobranch osmoregulatory strategy. The roles of the major organs involved in osmoregulation will be discussed in detail later in this chapter. The major difference from most marine vertebrates is that marine elasmobranchs maintain their plasma slightly hyperosmotic to the surrounding medium (Smith, 1936) resulting in a slight influx of water across the gills. Elasmobranch fish face a continuous influx of sodium chloride, particularly across the gills, and this excess salt is removed by a specialised salt-secreting gland, the rectal gland (See section 1.6.). The major function of the elasmobranch kidney is the retention of urea (See below and

Section 1.5.), and whilst the gills are relatively impermeable to urea, they are still quantitatively the most important site of urea loss (Section 1.7.).

Elasmobranchs maintain their plasma hyperosmotic to the surrounding medium, in part because sodium and chloride concentrations are higher than in other vertebrates, but mainly by the retention of high concentrations of nitrogenous compounds which quantitatively constitute approximately 40-50% of the total plasma osmolality. The most significant non-protein nitrogenous compounds in elasmobranch extracellular and intracellular fluids are urea, trimethylamine oxide, and amino acids. Urea, which is formed in the hepatic ornithine urea cycle (Anderson and Casey, 1984), is the major nitrogen end product and major osmolyte in all marine and euryhaline elasmobranchs. It constitutes approximately 35% of the total osmolality of both extracellular and intracellular fluids and its concentration is regulated in response to external salinity. High concentrations of urea are maintained in elasmobranchs by both the relative impermeability of the gill membranes to urea (Section 1.7.) and the reabsorption of urea by the kidneys (Section 1.5.). Trimethylamine oxide (TMAO) is the second most important nitrogenous end product, contributing 5-20% of the osmolality of plasma and muscle. It also plays an important role in counteracting the toxicity of urea to proteins. Like urea, TMAO is reabsorbed by the kidneys of elasmobranchs, with up to 95% of the filtered load being reabsorbed (Cohen *et al.*, 1958). Amino acids play an important role in intracellular osmoregulation and cell volume regulation (See Page 19).

Urea is an effective protein-denaturing agent and therefore its use as an osmolyte appears paradoxical. Physiological concentrations of urea are known to have adverse effects on the structure and function of many proteins and enzymes (Yancey *et al.*,

1982; Yancey and Somero, 1978; Yancey and Somero, 1980). However, some elasmobranch proteins, such as haemoglobin, have adapted to the adverse effects of urea (Martin *et al.*, 1979), and some elasmobranch proteins even require the presence of urea in order to function optimally, such as (M4) lactate dehydrogenases (LDH) (Yancey and Somero, 1978). Many elasmobranch enzymes do not function optimally in the presence of urea and these enzymes rely on the additional presence of methylamine compounds to counteract the adverse effects of urea (Yancey and Somero, 1980). TMAO is the major methylamine compound, making up approximately 90% of all methylamines in elasmobranchs (Vyncke, 1970), but betaine and sarcosine are also important. The inhibition of a number of enzymes from tissues of elasmobranchs by urea is largely or fully offset by TMAO, betaine and sarcosine (Yancey and Somero, 1980). It is interesting to note that the stabilising effects of these methylamine osmolytes occur to the same degree with proteins of non-elasmobranchs, which are not normally subjected to high urea concentrations. This suggests that this effect is probably derived from fundamental physiochemical interactions between solutes and proteins, rather than from special adaptations in particular enzymes (Yancey and Somero, 1980). This was confirmed in recent thermodynamic studies (Zou *et al.*, 2002). The authors showed that low concentrations of TMAO stabilise the quaternary structure of proteins to offset the effects of urea. The stabilising effect of methylamines is maximal at an approximate 2:1 ratio between urea concentration and the summed concentration of methylamine osmolytes. This 2:1 ratio is consistently found in marine elasmobranchs, even in specimens acclimated to half strength seawater (Yancey and Somero, 1980). The 2:1 relationship between urea and methylamines has been confirmed in a number of tissues using proton nuclear magnetic resonance spectrometry to quantify TMAO (Bedford *et al.*, 1998).

Amino acids also play an important role in intracellular osmoregulation and cell volume regulation in elasmobranchs. In vertebrates, most cells are isosmotic with their extracellular fluid and therefore changes in extracellular fluid osmolality present an osmotic stress to the cells. A corresponding change in intracellular fluid osmolality is therefore necessary for maintenance of cell volume and composition and for the functional integrity of the cell (Perlman and Goldstein, 1988). Urea and TMAO are both freely diffusable across plasma membranes (Fenstermacher *et al.*, 1972) and these molecules therefore remain in equilibrium between intracellular and extracellular fluid. In contrast, free amino acids constitute 1% of the total osmolality in extracellular fluid and 19% in intracellular fluid (Perlman and Goldstein, 1988). Acclimation to dilute seawater led to a marked decrease in cellular concentration of free amino acids, allowing muscle cell volume to remain constant during extracellular fluid expansion and dilution (Forster and Goldstein, 1976; Goldstein, 1981). Cells therefore maintain themselves isosmotic with the extracellular fluid mainly through the regulation of free amino acids. Any fall in cellular free amino acid concentration tends to be due to reductions in the high levels of a few amino acids, rather than a general decrease in free amino acid concentration (Goldstein, 1981). The amino acids which are important for intracellular osmoregulation are β-alanine and sarcosine in muscle, β-alanine and taurine in erythrocytes and taurine and glutamate in brain (Boyd *et al.*, 1977). The reason that these specific amino acids are accumulated and regulated intracellularly as osmolytes is assumed to be because they are relatively metabolically inert (Perlman and Goldstein, 1988). They are not found in protein, so changes would not disrupt protein biosynthesis, nor do they contribute to the major metabolic pathways of the cell.

Therefore their unique metabolic properties may have led to their selective uses in intracellular osmoregulation (Perlman and Goldstein, 1988).

In elasmobranchs, plasma sodium and chloride concentrations are generally higher than those found in marine teleosts, but are still considerably lower than that of seawater. The fish face a continuous influx of sodium and chloride, particularly across the gills. Salt loading is likely to be greatest during feeding when the ionic content of the food, together with seawater imbibed during feeding adds to the basal salt influx (Hazon *et al.*, 1997b). Depending on species, the diet of elasmobranchs can vary greatly, from carnivorous, for example the Bullshark, *Carcharinus leucas*, to entirely planktonic in the case of the Basking Shark, *Cetorhinus maximus*. In this respect, the diet may have a profound effect, especially if the diet consists primarily of invertebrates, which have a high salt content, as is the case in the species under examination in the present study, *Scyliorhinus canicula* (Wheeler, 1969).

Elasmobranchs also regulate their plasma osmolality in relation to changes in environmental salinity, but the plasma osmolality is still maintained at a level slightly hyperosmotic to the environment. On transfer to 50% seawater the little skate, *Rana erinacea*, showed a decrease in plasma urea concentration (Goldstein and Forster, 1971). This reduction was attributed to increased renal clearance and decreased biosynthesis of urea. Similarly, a decrease in plasma urea in the lip shark, *Hemiscyllium plagiosum*, was attributed to a reduction in biosynthesis (Wong and Chan, 1977). Plasma urea, sodium and chloride levels were shown to decrease in *S. canicula*, following gradual transferral to reduced salinity (Hazon and Henderson, 1984). The plasma clearance of urea was increased and derived urea production rates declined.

Additionally, an increase in plasma urea, attributed to depressed plasma clearance rates, was observed following transferral of *S. canicula* to 140% seawater (Hazon and Henderson, 1984). These studies suggest that regulation of plasma urea is a critical factor in the overall homeostasis of elasmobranch plasma. In a later study, dogfish acclimated to 130% seawater and fed on a low protein diet had no apparent ability to increase plasma urea concentration (Armour *et al.*, 1993a). Instead, these fish adopted an alternative mode of osmoregulation that involved retaining increased plasma sodium and chloride concentrations. The urea synthetic ability of these fish was compromised and osmoregulatory ability was impaired. This study emphasised that sodium chloride and urea are independently regulated in elasmobranch fish. In order to osmoregulate in the face of either changes in environmental salinity or dietary salt load, elasmobranchs must co-ordinate the activities of kidney, rectal gland, gill and gut and these tissues will now be considered in detail.

1.5. Kidney

Elasmobranchs kidneys are elongate, paired structures lying along the dorsal wall of the body cavity (Figure 1.10.) (Chester-Jones, 1957). The elasmobranch kidney is unusual in that the interrenal gland and chromaffin bodies are distinct from the renal tissue (Fig.

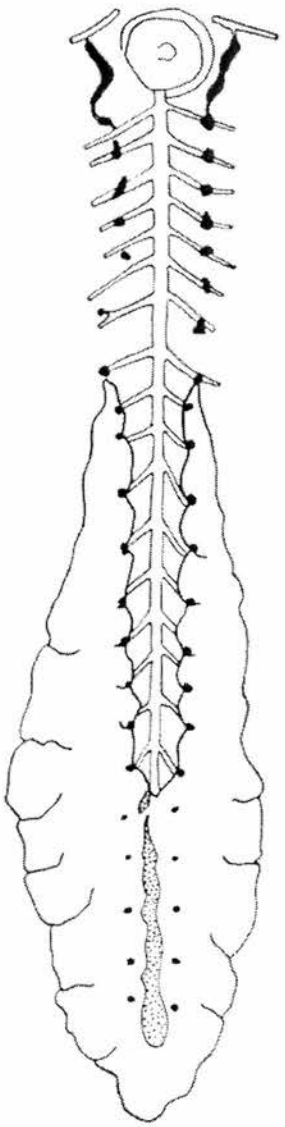

Fig. 1.10. Ventral view of the aorta, kidneys, chromaffin bodies, and interrenal of *Scyllium catulus*. Chromaffin tissue, black; cortical tissue stippled. (From Chester Jones, 1957, after Vincent, 1897).

1.10.). Blood supply to the kidneys of elasmobranchs originates from two sources: arterial and a renal portal system. Portal blood mixes freely with blood from the glomerular vasa efferentia before leaving the kidney via the renal veins. There is also evidence of a glomerular bypass shunt wherein blood may pass from the afferent to the post glomerular circulation thereby avoiding filtration (Brown and Green, 1992).

The functional element of elasmobranch renal tissue, the nephron, is a very long and complex tubular structure. The renal tissue of elasmobranchs is separated into two distinct zones; a sinus zone, where tubules are loosely packed and segregated from each other by large blood sinuses, and a zone of lateral bundles, where tubule segments are packed tightly into discrete bundles (Lacy and Reale, 1995). The transition between the two zones is marked by large renal corpuscles. Each individual nephron takes its course in both zones, forming two hairpin loops in the bundle zone and two extended convolutions in the sinus zone (Hentschel *et al.*, 1998) (Fig. 1.11.). In the sinus zone, proximal and distal tubules belonging to different nephrons extend in a common bed of sinusoidal capillaries of the venous renal portal system (Hentschel, 1988). In contrast, the portions of each nephron that penetrate into the bundle zone are separated from each other, as each bundle is enclosed in a sheath of squamous epithelial cells (Hentschel, 1987). Ultrastructural analysis of the nephron in little skate, *R. erinacea* (Lacy and Reale, 1991a; Lacy and Reale, 1991b) and *S. canicula* (Hentschel *et al.*, 1993; Hentschel *et al.*, 1998) provides strong evidence for a countercurrent system involving highly specialised and diverse epithelial transport. The nephron begins at the urinary pole of the renal corpuscle, with a neck segment followed by proximal, intermediate, and distal segments and finally a collecting duct (Fig. 1.11.). Each of these segments is further subdivided into subdivisions based on subtle morphological differences in the

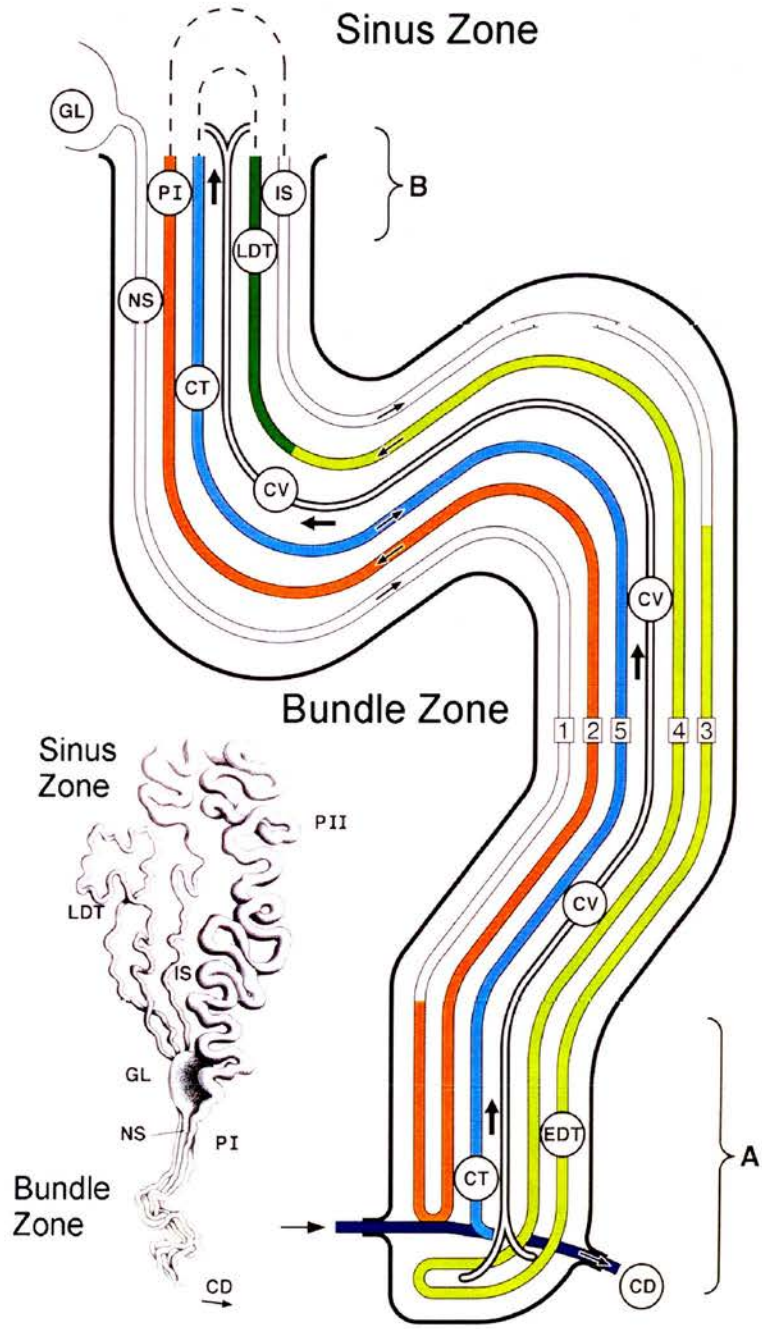

Fig. 1.11. Schematic drawing of the arrangement of nephron segments and portion of the collecting tubule-collecting duct system inside the bundle (adapted from Hentschel *et al.*, 1998). Five tubular profiles and a central vessel are enclosed in the bundle sheath (*thick line*). The course of the nephron in the mesial zone is indicated by broken lines (see inset for presentation of nephron segments in the mesial tissue zone). Thick arrows show the direction of supposed flow in the central vessel (*CV*), thin arrows indicate the direction of flow inside the renal tubule segments. Colour code: *red*-proximal tubule segment (*PIa*); *light green*-early distal tubule (*EDT*); *dark green*-late distal tubule (*LDT*); *light blue*-collecting tubule (*CT*); *dark blue*-collecting duct (*CD*). (*GL*-glomerulus; *IS*-intermediate segment; NS-neck segment; *PII*-proximal tubule segment). Inset shows a young nephron from *S. stellaris*. The bundle sheath and the central vessel are not shown.

epithelial membrane. 16 morphologically distinguishing features were assigned to the nephron tubule including, presence of flagella, tight or gap junctions, brush borders, mitochondrial density, basolateral invagination and tubular dimension and rigidity (Lacy and Reale, 1991a; Lacy and Reale, 1991b).

Elasmobranchs produce a urine that is hypo-osmotic to blood plasma. The heterogeneity of tubular epithelial cells may be an adaptation for the retention of urea. Approximately 95% of filtered urea is reabsorbed in the elasmobranch kidney (Boylan, 1967). It has long been speculated that a carrier-mediated process is involved in elasmobranch renal urea reabsorption. The fact that urea is reabsorbed against a sizeable concentration gradient has led to the proposal that the reabsorption mechanism is active, possibly coupled to the movement of sodium (Hays *et al.*, 1977; Schmidt-Neilsen and Rabinowitz, 1964). Urea reabsorption has been shown to be extremely specific, with only 35% of the urea analogue, thiourea, being reabsorbed in the elasmobranch nephron (Boylan, 1967). This led to the suggestion of an active urea reabsorption mechanism and micropuncture studies have implicated the second proximal segment (PII) (Fig. 1.11.) as a possible site of sodium-linked urea reabsorption (Stolte *et al.*, 1977). It has been shown that the kidney of *S. acanthias* contains at least one protein capable of urea transport and there is preliminary evidence for a similar urea transport protein in *R. erinacea* (Smith and Wright, 1999). The question however remains as to whether facilitative transporter proteins are solely responsible for urea reabsorption in the elasmobranch kidney. A passive (thermodynamically dissipative) model of urea reabsorption has been proposed (Friedman and Herbert, 1990) which relies on the presence of a countercurrent multiplication system and differential permeabilities of tubular segments to urea, water and sodium, as occurs in mammalian species. The renal

countercurrent bundles and the microvasculature of the elasmobranch nephron were recently examined in detail in *Scyliorhinus canicula* and *Raja erinacea* (Hentschel *et al.*, 1998). This study identified a single lymph capillary-like vessel which originates from a blind-ended rami at the tip of the bundle and runs in close contact with the collecting tubule along the entire bundle, before merging with the venous sinusoidal capillaries of the peritubular blood circulation (Fig. 1.11.). It was suggested that this central vessel provides a channel for the convective flow of sodium chloride rich-fluid to the portal system, and may therefore be involved in countercurrent exchange of urea, from the collecting tubule urine to the fluid in the central vessel (Hentschel *et al.*, 1998). This could account for the low concentration of urea in the fluid of the collecting duct as observed by micropuncture (Stolte *et al.*, 1977). It is clear that detailed analysis of urea permeabilities, and sites of transport along the elasmobranch nephron are required to determine whether urea reabsorption is active, passive or both.

The kidney also plays a role in the regulation of sodium and chloride plasma concentration. Ultrastructural studies have demonstrated that tubular cells in the early distal tubule (EDT) (Fig. 1.11.) have characteristics very similar to cells that are known to actively transport sodium (Lacy and Reale, 1991a). It has been shown that the loop 3 in the EDT exhibits similar characteristics of active sodium and chloride absorption associated with amphibian and mammalian renal diluting segments (Friedman and Herbert, 1990).

Renal micropuncture techniques have implicated loop 2 in the second proximal region (PII) as a site of sodium-linked urea reabsorption (Stolte *et al.*, 1977). In addition, isolated perfused PII tubules from *S. acanthias* have also been shown to actively secrete

sodium and chloride ions (Beyenbach and Fromter, 1985) and this appears to drive net fluid secretion (Sawyer *et al.*, 1985). However, the ultrastructural studies used as evidence for active tubular reabsorption in Loop 2 (Endo, 1984; Lacy *et al.*, 1975) could just as easily be applied in support of active tubular secretion (Henderson *et al.*, 1988) as no directional element to this tubular movement was established. Histochemical studies have only isolated Na^+K^+ATPase activity in the early and late distal tubules and the collecting duct (Endo, 1984; Hebert and Friedman, 1990). It is therefore clear that further study is required to determine whether sodium is reabsorbed or secreted across the tubular epithelium in the PII segment.

1.5.1. Hormonal control of renal function

Early studies, using primarily pharmacological doses of catecholamines, presented conflicting results on elasmobranch GFR. Recently a clear glomerular diuresis caused by adrenaline has been demonstrated in *S. canicula* (Brown and Green, 1987). Elasmobranchs possess a range of novel peptides, some of which may be involved in kidney function. The renal effects of arginine vasotocin (AVT), angiotensin II (Ang II) and C-type natriuretic peptide (CNP) will be discussed in Chapters 3, 4 and 5 respectively. GFR may also be modulated by other endogenous peptide hormones including vasoactive intestinal peptide (VIP) and prolactin (Yokota and Benyajati, 1988) which both appeared to significantly increase GFR.

1.6. Rectal Gland

Elasmobranchs are equipped with a highly specialised salt secreting organ known as the rectal gland. This gland is situated at the caudal end of the peritoneal cavity and it secretes a fluid isosmotic to blood plasma. This fluid however, is almost entirely composed of sodium chloride and therefore the rectal gland functions to remove these ions from the plasma (Burger and Hess, 1960). The complex vasculature of the rectal gland is illustrated in Fig. 1.12. The rectal gland receives a blood supply from the rectal gland artery, which arises from the dorsal aorta. It is drained via a central venous sinus,

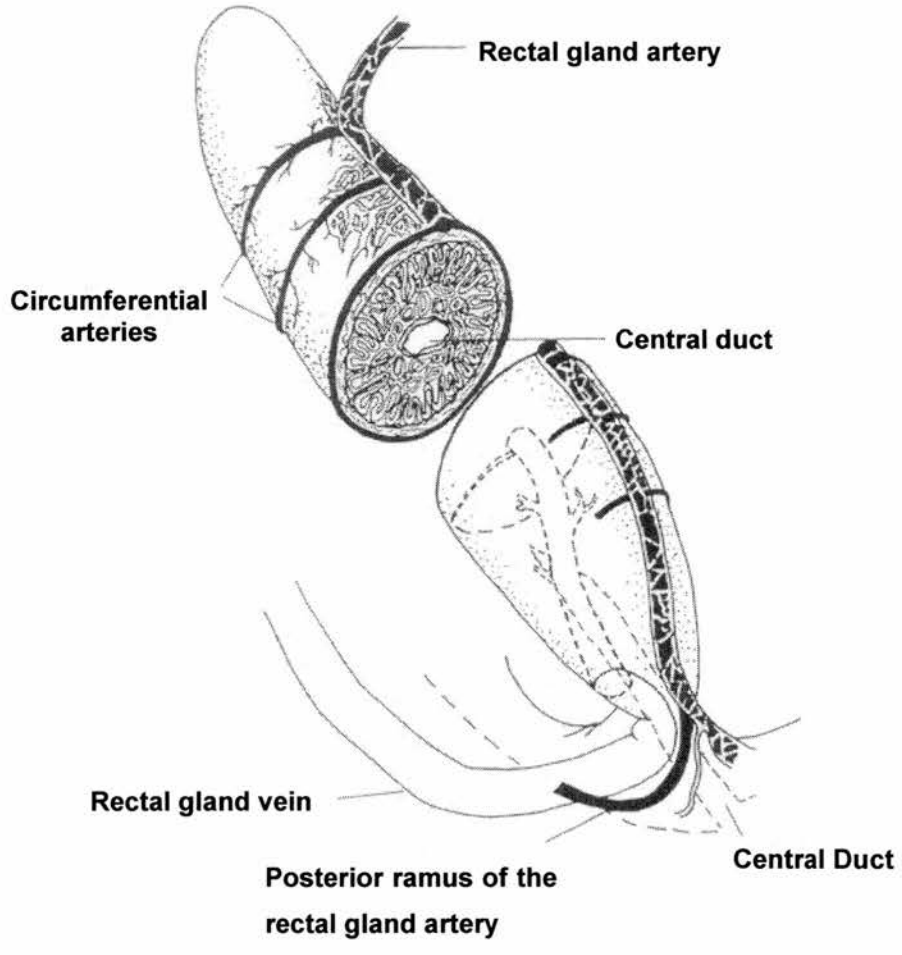

Fig. 1.12. Schematic diagram of the vascularization of the rectal gland (Hazon *et al.* 1997b)

which follows the intestine anteriorly upon exiting the gland. The central duct drains the rectal gland fluid and opens into the lower intestine anterior to the rectum (Kent and Olsen, 1982).

The structure of the rectal gland in sharks has been subdivided into 4 regions: an outer capsule, covered by peritoneum, with the principal arterial and venous systems; a narrow sub-capsular layer consisting of smaller arterial and venous vessels, closely connected through arterio-venous anastomoses; a layer of secretory tubules; and a central region of branching tubules arranged around the central canal (Bonting, 1966; Kent and Olsen, 1982; Masini *et al.*, 1994).

The net secretion of sodium chloride by rectal gland secretory epithelia is brought about through the combined action of four transport pathways: the basolateral Na^+K^+ATPase pump; the basolateral Na-K-2Cl co-transporter; potassium channels in the basolateral membrane; and chloride channels in the apical membrane.

1. Na^+K^+ATPase has been localised on the cytoplasmic side of the basolateral membrane of the secretory cells (Goertemiller and Ellis, 1976). This location was confirmed by autoradiographic techniques in which [^{3}H]-ouabain binding was high in the basolateral membrane with the highest density of enzyme sites close to the mitochondria (Eveloff *et al.*, 1979). Ouabain binding characteristics in the perfused rectal gland of *S. acanthias* were significantly altered after stimulation of the gland with cyclic adenosine monophosphate (cAMP) and theophylline (Silva *et al.*, 1983). It was suggested that two classes of binding site, with different affinities for ouabain, were present in the rectal gland. Stimulation of the gland appeared to

increase the affinity of the high affinity binding sites, suggesting a change in the configuration of Na^+K^+ATPase or its distribution within the cell membrane (Silva *et al.*, 1983). This apparent heterogeneity in binding cannot be explained by isoform diversity, and to date no genuine explanation for this enzymatic diversity is available (Hansen, 1999). Na^+K^+ATPase actively transports K^+ into the cell in exchange for Na^+. Na^+ is transported out of the cell into the extracellular space on the blood side of the epithelium, providing a steep electrochemical gradient for the passive re-entry of Na^+ into the tubular cell. This provides the energy to simultaneously drive the coupled, uphill translocation of K^+ and two Cl^- via the sodium-potassium chloride co-transport system (Olson, 1999).

2. The presence of a sodium-potassium chloride (Na-K-2Cl) co-transport system has been demonstrated on the basolateral membrane but appears to be excluded from the apical side of the cell (Lytle *et al.*, 1992). Transepithelial chloride secretion involves first accumulation of chloride inside the cell against its electrochemical potential by the action of Na-K-2Cl, followed by passive movement of chloride into the lumen by apical chloride channels (Hannafin *et al.*, 1983). The presence of the Na-K-2Cl co-transporter was further demonstrated using a series of inhibitors (Greger and Schlatter, 1984a; Greger and Schlatter, 1984b). These studies demonstrated that the Na-K-2Cl co-transporter is the only quantitatively important site of sodium entry into the rectal gland tubule cell.
3. Potassium channels, located on the basolateral membrane of the rectal gland epithelia (Greger and Schlatter, 1984b) provide a reservoir for continuous recirculation of potassium through the cell via the Na^+K^+ATPase pump and the Na-K-2Cl co-transporter (Greger *et al.*, 1987a).

4. Chloride channels on the apical membrane permit the passive loss of chloride ions into the duct lumen. Two chloride channels have been identified using patch-clamping techniques. These are, a large conductance channel considered only to be open when the rectal gland was stimulated (Greger *et al.*, 1987b), and a small conductance channel, which may be active both in non-stimulated and stimulated cells and may account for low chloride conductance in the unstimulated rectal gland (Goglein *et al.*, 1987). A third chloride channel with close homology to human cystic fibrosis transmembrane conductance regulator (CFTR) was also identified and located in the rectal gland of *S. acanthias* (Marshall *et al.*, 1991). This channel was activated by the stimulation of isolated tubules with cAMP (Hanrahan *et al.*, 1993). The currently accepted pathway for sodium chloride secretion is summarised in Fig. 1.13.

Thus, the movement of chloride ions is not directly coupled to an energy consuming process. Conversely, sodium is actively transported out of the secretory cell into the intracellular space but secretion of sodium from the intracellular space into the tubular fluid is a passive, paracellular process, driven by the negative potential in the lumen, established through chloride secretion (Olson, 1999).

The role of blood flow through the secretory tissue of the rectal gland, and its influence on secretion rate is discussed in Chapter 4.

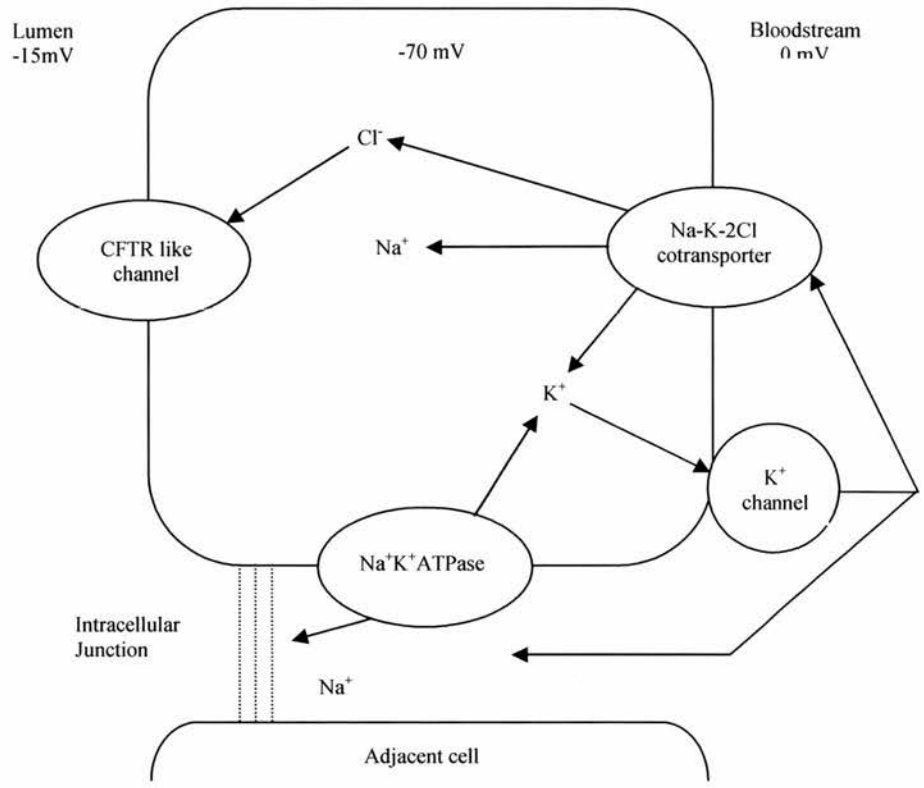

Fig 1.13. Schematic diagram showing the currently accepted pathways and membrane proteins involved in the active secretion of NaCl from the rectal gland epithelium (Hazon *et al.* 1997b).

1.6.1. Hormonal control of rectal gland function

The intermittent activity of the rectal gland (Burger and Hess, 1960; Holt and Idler, 1975) suggests that it is regulated by stimulatory and inhibitory endocrine and/or neuroendocrine control mechanisms. Vasoactive intestinal peptide (VIP)-like and bombesin-like immunoreactive nerve fibres have been observed following the radially arranged secretory tubules and also around the central excretory duct of the rectal gland of *S. acanthias* (Holmgren and Nilsson, 1983). Immunoreactivity towards VIP was also found in the secretory cells of the rectal gland of *S. canicula* (Masini *et al.*, 1994). VIP stimulated secretion of chloride in the rectal gland of *S. acanthias*, by stimulating the

accumulation of the second messenger, cAMP. Although cAMP has been implicated in the stimulation of rectal gland secretion in general, the mode of action remains unclear (Lear *et al.*, 1992; Shuttleworth and Thompson, 1980; Silva *et al.*, 1977; Stoff *et al.*, 1977). There appears to be a major difference in the nature of gut peptides involved in rectal gland secretion in *S. acanthias* and *S. canicula*. Whilst VIP clearly stimulated chloride secretion in tubules of *S. acanthias*, this has not been reported in *S. canicula* or other elasmobranch species (Shuttleworth and Thorndyke, 1984; Thorndyke and Shuttleworth, 1985). A homologous gut peptide was isolated from *S. canicula* that showed potent stimulatory activity in the rectal glands of *S. canicula*, *R. erinacea*, and *S. acanthias* (Shuttleworth and Thorndyke, 1984). This fraction was tentatively named 'rectin' but was not characterised. A peptide was subsequently isolated and characterised from *S. canicula*, and was found to be the tachykinin, Scyliorhinin II (Scy II) (Anderson *et al.*, 1995a). It was proposed that rectin was in fact Scy II and that Scy II plays a central role in the control of rectal gland secretion.

The role of Ang II and natriuretic factors in rectal gland function will be discussed in Chapters 4 and 5 respectively. *In vitro* perfusion studies have identified two neuropeptides involved in the inhibition of rectal gland secretion. Somatostatin was found to inhibit chloride secretion in *S. acanthias* after stimulation by cAMP and forskolin (Silva *et al.*, 1985). Somatostatin inhibited VIP-stimulated chloride secretion, but did not alter the specific binding of VIP to rectal gland cells. Rather, VIP-stimulated accumulation of cAMP was inhibited, but the precise site of action remains unclear (Silva *et al.*, 1985). A further study on the action of somatostatin, showed that bombesin, a neurotransmitter present in the nerves within the rectal gland, inhibits the release of chloride stimulated by either VIP or cAMP. This inhibitory effect parallels its

effect on the release of somatostatin, and it was concluded that the effect of bombesin was caused by the release of somatostatin (Silva *et al.*, 1990). The second neuropeptide involved in the inhibition of rectal gland secretion is Neuropeptide Y (NPY). In perfused rectal glands NPY inhibited secretion stimulated by VIP, forskolin or cAMP and theophylline (Silva *et al.*, 1993).

The elasmobranch corticosteroid, 1α-hydroxycorticosterone (1α-OH-B) has also been implicated in the control of rectal gland secretion. Following interrenalectomy, a decrease in volume, osmolality, and sodium and chloride concentrations of rectal gland fluid was observed (Holt and Idler, 1975).

1.7. Gills

The anatomy of elasmobranch gills has been studied by several authors (Metcalfe and Butler, 1986; Olson and Kent, 1980; Wright, 1973). There are usually five pairs of gills with each gill consisting of a gill arch bearing two parallel alternating series of gill filaments on its lateral borders. The two series of filaments are joined by an interbranchial septum, composed of muscular and connective tissue. Dorsal and ventral

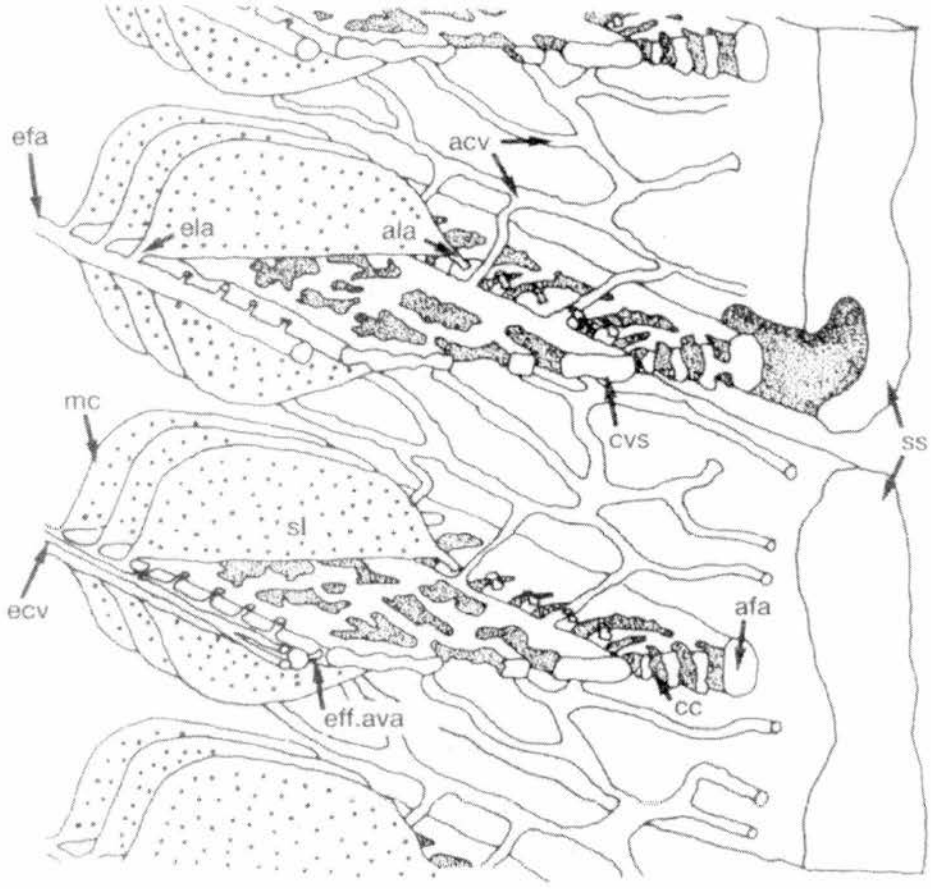

Fig 1.14. A semi-diagramatic representation of the gill vascular anatomy of the dogfish, presented as a solid cast of the blood vessels (adapted from Metcalfe & Butler, 1986). The figure illustrates portions of two consecutive gill filaments from one hemibranch.
(afa), afferent filament artery; (cc), corpus cavernosum; (ala), afferent lamellar arteriole; (sl), secondary lamella; (ela), efferent lamellar arteriole; (efa), efferent filament artery; (eff.ava), efferent arterio-venous anastomosis; (cvs) central venous sinus; (acv) afferent companion vessel; (ecv) efferent companion vessel; (mc), marginal channel; (ss) septal sinus.

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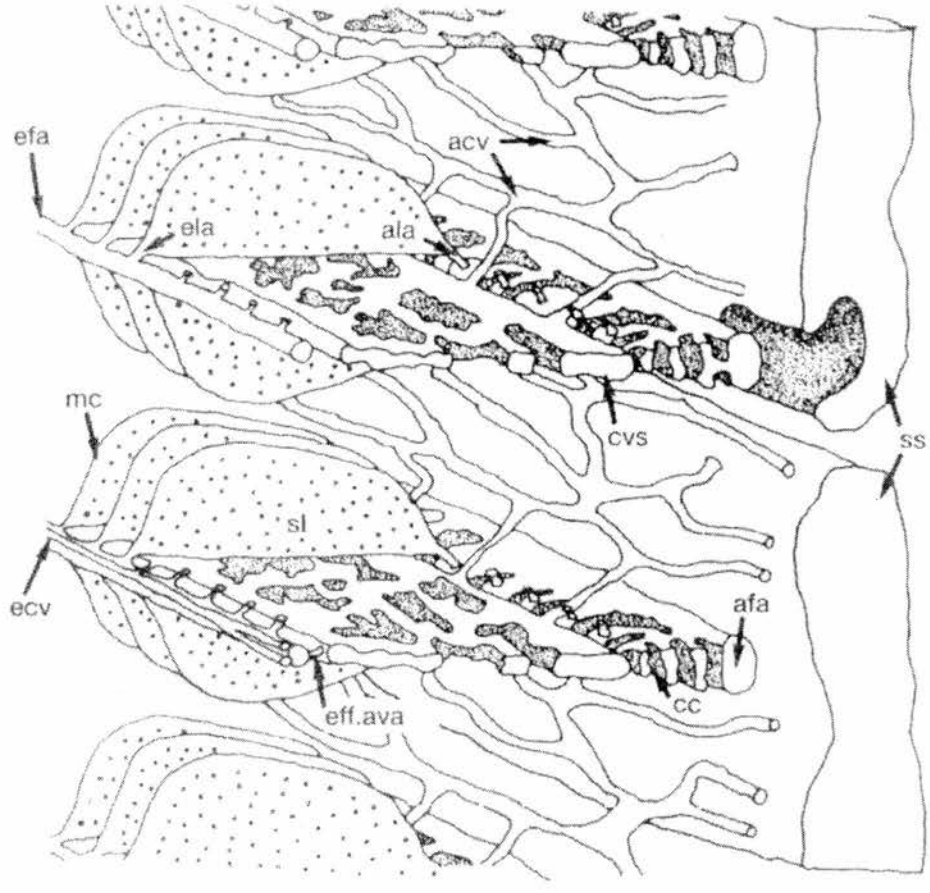

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surfaces of each gill filament have a row of secondary lamella and these are the principal sites of gas exchange (Figure 1.15.). Arterioarterial vasculature, which supplies the secondary epithelium, is controlled by sphincters located on efferent primary artery and also on both afferent and efferent arteries (Laurent and Dunel, 1980). The gill vasculature anatomy is represented in Figure 1.14.

Chloride cells, or mitochondria-rich cells (MRCs) (Figure 1.15.), have been identified in elasmobranch gills (Doyle and Gorecki, 1961) and have been described as pear shaped cells with the narrower end in contact with the external medium (Wright, 1973). These cells are characterised by smooth endoplasmic reticulum, many mitochondria, and basolateral plasma membrane infoldings (Wright, 1973). More recently, comparative studies of *R. clavata* and *S. canicula* have revealed two types of chloride cells (Laurent and Dunel, 1980). Some chloride cells are deeply buried and connect to the external milieu by a narrow opening; in the second type the apical membrane of the chloride cell protrudes outward. Both these cell types lack a tubular system as found in teleosts, and this is replaced by numerous basolateral infoldings (Laurent and Dunel, 1980). Numerous mitochondria are peripherally disposed in close relationships with the infoldings of the basolateral cell membrane. Despite the presence of chloride cells, elasmobranch branchial Na^+K^+ATPase activity has been reported as negligible in comparison with that of marine teleosts (Jampol and Epstein, 1970). This is perhaps unsurprising given the role of the specialised salt secreting rectal gland (Section 1.6). In a recent study, strong Na^+K^+ATPase immunoreactivity was associated with the basolateral membrane of MRCs (Wilson *et al.*, 2002), suggesting a possible role for the dogfish gill in salt secretion. However, in the same study, dogfish were able to maintain ionic balance after rectal gland removal despite MRCs being unresponsive in terms of

changes in number, fine structure and Na^+K^+ATPase activity. This indicates that the renal diuresis and natriuresis observed by Burger (1965), perhaps in concert with a decrease in branchial permeability, were sufficient to compensate for rectal gland

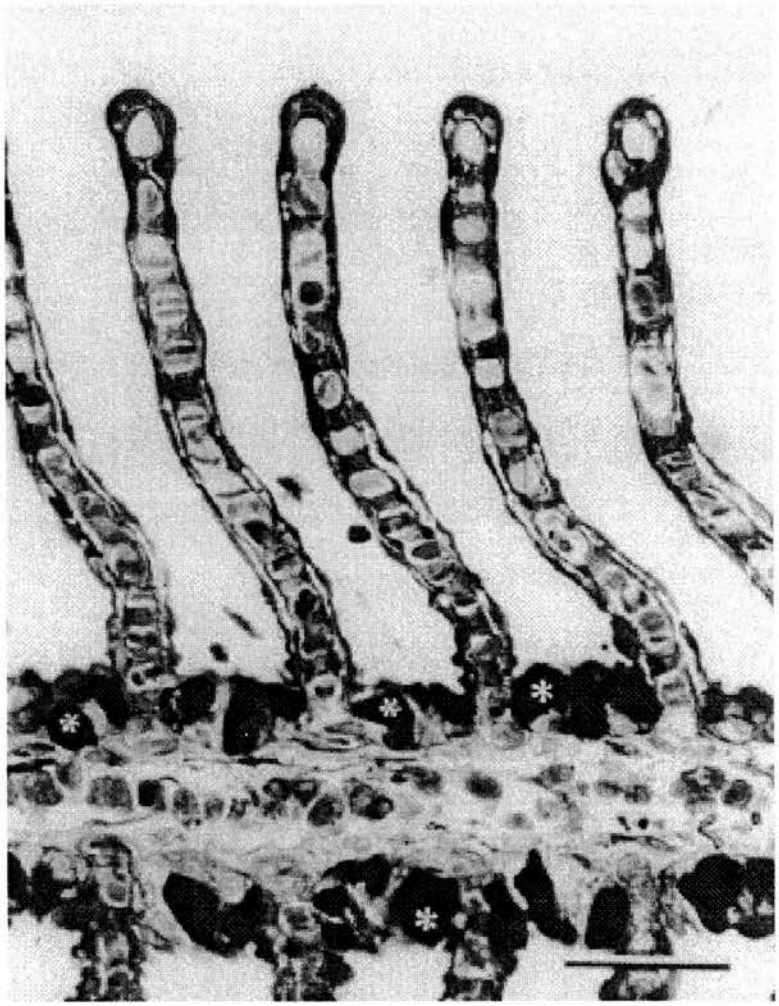

Fig 1.15. Light micrograph of dogfish gill (Wilson *et al.* 2002). Some of the large, darkly staining MRCs in the interlamellar spaces are labeled with asterisks (*). Scale bar 50 μm.

removal. The specific role of MRCs, or chloride cells, in the elasmobranch gill has therefore yet to be clarified (Laurent and Dunel, 1980; Wilson *et al.*, 2002).

The urea gradient across the dogfish gill is reported as one of the largest chemical gradients to exist across a biological membrane (Boylan, 1967). The precise underlying mechanism for this low permeability coefficient has been poorly studied. It is currently considered that some sort of physical barrier minimises urea loss across gill epithelia. Modifications of the phospholipid bilayer membrane have been reported to be responsible, in part, for decreasing the gill permeability to urea in *S. acanthias* (Fines *et al.*, 2001). In this study, high levels of cholesterol in the basolateral membrane were reported to contribute to this effect. In addition, there is evidence supporting the existence of urea transporters in elasmobranch gill. Recent urea analogue and inhibitor studies have suggested the presence of an inwardly directed active urea transporter (Fines *et al.*, 2001; Part *et al.*, 1998; Wood *et al.*, 1995). This system actively transports urea out of the gill epithelial cells back into the blood against the urea concentration gradient. A homologue to the elasmobranch urea transporter in *S. acanthias* has also been identified in elasmobranch gill tissue (Smith and Wright, 1999). Further investigation is required to determine the role of this homologue in gill tissue. Despite such an apparently efficient system of urea retention, urea loss is still quantitatively greatest across the gill due to the large concentration gradient between elasmobranch plasma and seawater (Shuttleworth, 1988).

The elasmobranch gill epithelium has been much less studied with respect to movement of sodium and chloride ions than teleost gill epithelia. There is however a large inward concentration gradient for sodium and chloride ions between seawater and plasma. The absence of a substantial electropotential gradient led researchers to postulate that elasmobranchs were subject to a constant diffusional uptake of sodium and chloride ions

across the gill epithelia (Bentley *et al.*, 1976; Maetz and Lahlou, 1966; Payan and Maetz, 1973).

Active sodium influx across the gill epithelium has been reported (Bentley *et al.*, 1976), and while this may seem paradoxical in the light of the constant diffusional uptake of sodium, it may be related to acid-base balance. The elasmobranch gill is considered to be the primary site for acid-base regulation in elasmobranchs (Cross *et al.*, 1969). A component of sodium influx was proposed where the influx of sodium was coupled to the efflux of H^+ as part of the exchange mechanism regulating acid-base balance (Payan and Maetz, 1973). During periods of severe hypercapnia it is necessary for elasmobranchs to actively extrude ammonia through the sodium/ammonium (Na^+/NH_4^+) exchanger (Claiborne and Evans, 1992). This results in a loss of ammonia required for the production of urea and an influx of sodium, but in cases of severe hypercapnia the capacity to restore acid-base balance quickly would outweigh the loss of nitrogen (Claiborne and Evans, 1992). This is particularly important in the light of the extremely poor buffering capacity of elasmobranch plasma (Murdaugh and Robin, 1967). Chloride efflux has been shown to be regulated through a chloride/bicarbonate (Cl^-/HCO_3^-) exchange, where chloride efflux is coupled to bicarbonate influx during periods of hypercapnia (Randall *et al.*, 1976).

1.7.1. Hormonal control of gill function

There is very little information available with regards to hormonal control of ion transport in elasmobranch gills. In *S. acanthias* and *S. canicula* the responses of branchial vasculature to both adrenaline and noradrenaline appears to be a β–adrenoreceptor-mediated vasodilation (Capra and Satchell, 1977; Davies and Rankin,

1973). The physiological effects of angiotensin II and CNP in the elasmobranch gill will be discussed in Chapters 4 and 5 respectively.

1.8. Intestine

The elasmobranch gut consists of the oesophagus, the stomach, the spiral intestine and the rectum (Figure 1.16.) (Nilsson and Holmgren, 1988). The stomach can be divided histologically into a cardiac stomach and a pyloric stomach. The cardiac stomach can be further subdivided into a proximal part possessing a striated muscle wall, and a distal part with only a smooth muscle wall. The elasmobranch gut is densely innervated and many neuropeptides implicated in the control of gut motility in mammals have been identified (Holmgren and Nilsson, 1983) (Section 1.8.1.).

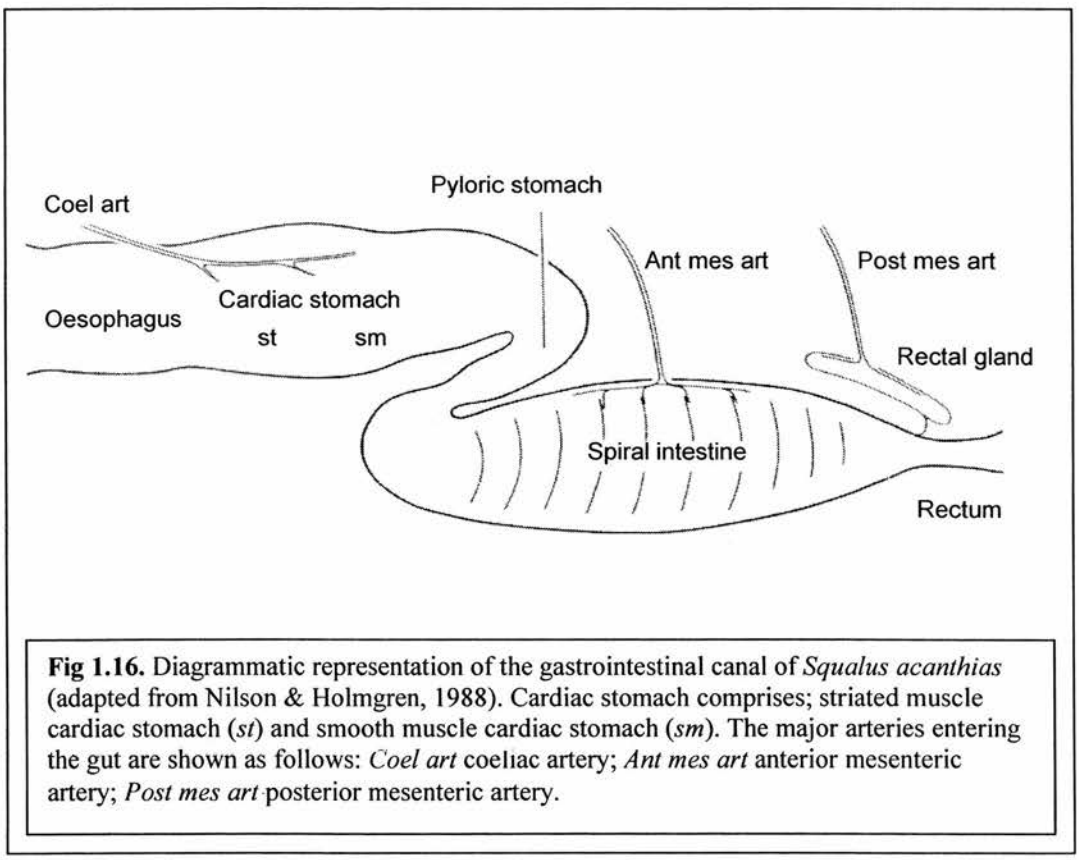

Fig 1.16. Diagrammatic representation of the gastrointestinal canal of *Squalus acanthias* (adapted from Nilson & Holmgren, 1988). Cardiac stomach comprises; striated muscle cardiac stomach (*st*) and smooth muscle cardiac stomach (*sm*). The major arteries entering the gut are shown as follows: *Coel art* coeliac artery; *Ant mes art* anterior mesenteric artery; *Post mes art* posterior mesenteric artery.

Marine elasmobranchs maintain body fluid osmolality slightly hyperosmotic to that of the surrounding medium. As a consequence, there is a lack of osmotic water loss from the animal and therefore elasmobranchs were classically thought not to drink (Smith,

1936). In the light of this early work, there has been very little work on the osmoregulatory role of the elasmobranch gut. However, drinking has been demonstrated in elasmobranchs (Hazon *et al.*, 1989) and pharmacological manipulation of the endogenous renin angiotensin system (Chapter 4) resulted in an increase in drinking. Furthermore, drinking rates of *S. canicula* have been shown to vary in response to changes in environmental salinity (Hazon *et al.*, 1997b) and in response to Ang II and CNP (Anderson *et al.*, 2001b; Anderson *et al.*, 2001c).

As previously stated, when the osmoregulatory ability of dogfish was impaired in fish transferred to 130% seawater and fed on a low protein diet, the fish adopted an alternative mode of osmoregulation that involved the retention of increased plasma sodium and chloride concentrations (Armour *et al.*, 1993a). This raises the possibility that, in some circumstances, elasmobranch fish may increase plasma sodium and chloride by drinking from the environment (Hazon *et al.*, 1999). There is very little information regarding the role of the elasmobranch gut in osmoregulation, and there is a clear requirement to examine the possible mechanisms of sodium, chloride and water absorption.

1.8.1. Hormonal control of the intestine

As mentioned in Section 1.4. the diet of elasmobranch fish may have a profound effect on the loading of sodium and chloride. Salt loading is likely to be greatest during feeding when the ionic content of the food together with seawater imbibed during feeding adds to the basal salt influx (Hazon *et al.*, 1997b). The salt load must be removed from the animal via the rectal gland (Section 1.6.). Indeed, following a single feeding event, Na^+K^+ATPase activity in rectal gland homogenates from *S. canicula*

increased over 40-fold, 9 hours after starved dogfish were fed (MacKenzie *et al.*, 2002). The elasmobranch gut is densely innervated and many neuropeptides involved in controlling gut motility in mammals have been identified (Holmgren and Nilsson, 1983). Bombesin-, gastrin/CCK-, 5-hydroxytryptamine-, neurotensin-, somatostatin-, and VIP-like immunoreactivity were all observed in the gut of *Squalus acanthias* (Holmgren and Nilsson, 1983). A marked increase in the secretory activity of the rectal gland of *S. acanthias* can be obtained by VIP, and this effect is blocked by somatostatin (Stoff *et al.*, 1979). In addition the homologous gut peptide, Scy II has also been shown to increase rectal gland secretory activity in *S. canicula* (Anderson *et al.*, 1995a). This suggests that these peptides could be released, following feeding in order to remove the associated salt load.

The roles of Ang II and CNP in the control of drinking are discussed in Chapters 4 and 5 respectively.

1.9. Other peptides involved in elasmobranch osmoregulation

The caudal portion of the spinal cord of elasmobranch fish incorporates a diffuse neuroendocrine system called the Caudal Neurosecretory System. Neurosecretory cells were first identified in the spine of a skate (Speidel, 1919). In elasmobranchs, the nerve terminals of the neurosecretory neurons are not concentrated into a compact urophysis, as occurs in teleosts, but these neurons project into diffuse neurohaemal areas on the ventral surface of the posterior spinal cord (Fridberg, 1962). Although Urotensin I (U-I) and Urotensin II (U-II) were originally regarded as being found only in lower vertebrates, the peptides have a wide phylogenetic distribution across the vertebrate lineage, including mammals. The human ortholog of U-I is Urocortin, which is synthesised in selected regions of the brain (Conlon, 2000).

Urotensin II (U-II) has been purified and characterised from extracts of the caudal portion of the spinal column in an elasmobranch fish, *S. canicula* (Conlon *et al.*, 1992). In addition, U-II has been isolated from the brain of the skate *Raja rhina* (Waugh and Conlon, 1993) and evidence for a Urotensin I (U-I) -related peptide was provided in *S. canicula* (Waugh *et al.*, 1995).

U-II has been shown to have cardiovascular actions in *S. canicula* (Hazon *et al.*, 1993). Bolus injections of synthetic dogfish U-II resulted in sustained and dose-dependent increases in arterial blood pressure and pulse pressure. The administration of phentolamine, an adrenergic blocking agent, completely abolished the pressor response, suggesting that the U-II effect was at least in part mediated by catecholamines (Hazon *et al.*, 1993). However, the prolonged nature of this pressor response led the authors to

suggest that, after an initial pressor response mediated by catecholamines, there may be a slowly developing direct effect of U-II. In the same study, U-II also produced a dose dependent contraction of isolated rings of vascular muscle from the afferent branchial artery, but the threshold dose for this contraction was relatively high, and therefore the physiological significance of this direct action of U-II is unclear.

More recently, the cardiovascular effects of dogfish U-I have been examined in *S. canicula*. The predominant cardiovascular effect of bolus intra-arterial injections of dogfish U-I was to produce a dose-dependent increase in arterial blood pressure (Platzack *et al.*, 1998). However, this pressor response was initially proceeded by a transient fall in arterial blood pressure. It was suggested that this was caused by the peptide exercising a direct relaxant effect on vascular smooth muscle that was rapidly reversed by the constrictor action of catecholamines (Platzack *et al.*, 1998).

To date research on the possible osmoregulatory role of thyroid hormones in elasmobranchs is extremely limited. Removal of the thyroid in the euryhaline stingray, *D. sabina* caused an elevation of plasma osmotic concentration and urea levels (De Vlaming *et al.*, 1975), but it was not clear whether thyroid hormones increased urea efflux or modified urea metabolism. Replacement therapy with thyroxine returned urea levels to normal in these thyroidectomized animals (De Vlaming *et al.*, 1975).

1.10. Aims

This chapter has provided an introduction to the general concepts of renal function and endocrinology, and has focussed on elasmobranch osmoregulation. Further, more detailed information will be provided in the introductions to the individual experimental chapters. It would appear that, in order to osmoregulate in response to changes in external salinity or dietary salt loading, the elasmobranch requires the combined actions of the kidney, rectal gland, gills and intestine. The hormonal influences on the elasmobranch kidney have been poorly studied and data on the effects of hormones *in vivo* can be difficult to interpret because of the potential compensatory actions of a range of factors that may increase or decrease blood pressure and thereby change GFR and renal function. The aim of this study was to focus on the kidney of *S. canicula* in terms of the hormonal control osmoregulation. The specific aims were:

1. To assess renal function *in vivo* in terms of acclimation to dilute SW in order to provide baseline data for comparison with work to be performed *in vitro*.
2. To use the information gained from the *in vivo* work to develop and verify an *in situ* perfused renal trunk preparation in *S. canicula*, to allow the assessment of the renal effects of a range of osmoregulatory peptides.
3. To use the *in situ* perfused trunk preparation to examine the renal effects of AVT, Ang II and CNP on urine flow rate, GFR, TmG and tubular function.
4. To investigate the possible presence of an intra-renal renin angiotensin system in *S. canicula*.
5. To examine changes in the filtering population of glomeruli directly, using the ferrocyanide method, following perfusion with AVT, Ang II and CNP.

2

In vivo renal function and technique development

2.1. Introduction

The majority of elasmobranch species tend to be regarded as stenohaline marine fish. However, many species of sharks, rays and skates enter and live in brackish and freshwater as part of their natural life cycle (Goldstein *et al.*, 1968; Smith, 1936; Thorson, 1970; Thorson *et al.*, 1973). Furthermore, a range of species have previously been shown to successfully acclimate to dilute SW under laboratory conditions (Goldstein and Forster, 1971; Hazon and Henderson, 1984; Sulikowski and Maginniss, 2001; Tierney *et al.*, 1998; Wong and Chan, 1977). One of these species, the European lesser-spotted dogfish, *Scyliorhinus canicula*, is widespread around the coast of the UK and is a widely used experimental species. Integral to the process of acclimation to dilute SW is the necessity to vary urine output in order to control extracellular fluid volume (Brown and Green, 1987). Female *S. canicula* are well suited for renal function studies, particularly in relation to changes in external salinity, as they are available in large numbers, adapt well to captive conditions and have readily accessible urinary papillae. In contrast, male fish cannot be used as it is impossible to collect urine without contamination from sperm, as the reproductive ducts and urinary ducts both exit into the urinary papilla.

Investigations into the hormonal control of renal function in elasmobranch fish have largely concentrated on *in vivo* techniques (Benyajati and Yokota, 1990; Brown and

Green, 1987). However, data on the effects of hormones *in vivo* can be difficult to interpret because of the potential compensatory actions of a range of factors (e.g. paracrine and endocrine) that may increase or decrease blood pressure and thereby change GFR and renal function. In addition, *S. canicula* possesses very long and convoluted urinary sinuses and urine output tends to occur during periods of spontaneous swimming activity rather than as a continuous flow. In order to avoid at least some of these complications and to determine the actions of individual peptides on kidney function, previous studies in teleost fish have utilised a perfused trunk preparation with the kidney *in situ* (Amer and Brown, 1995; Dunne and Rankin, 1992). These preparations have several advantages; they are relatively easy to establish, they allow the control of variables including, pressure, perfusion flow and peptide concentration and they eliminate the influence of any systemic factors.

The perfused kidney method was initially established in rats (Weiss *et al.*, 1959) and was later used to study kidney function in amphibia (Deeds *et al.*, 1977; Deeds *et al.*, 1978). Unlike mammals, where the kidney is excised and immersed in a bath filled with physiological saline (Lebowitz *et al.*, 1992; Weiss *et al.*, 1959), the fish kidney has to be perfused *in situ*, as the complex renal vascular architecture means it cannot be removed from the trunk with the dorsal aorta intact. Renal function of the trout has been examined using this method by several authors (Amer and Brown, 1995; Brown *et al.*, 2000; Dunne and Rankin, 1992; Pang *et al.*, 1983).

To date, a perfused kidney preparation has never been successfully developed in an elasmobranch fish. The aim of this chapter was to assess renal function *in vivo*, in dogfish acclimated to dilute seawater, and then to use this information to aid in the

development and verification of an *in situ* perfused renal trunk preparation in *S. canicula.* This preparation would allow an assessment of the effects of a range of osmoregulatory peptides on renal function to provide insights into the role of the kidney during acclimation to varying environmental salinities.

2.2. Materials and Methods

2.2.1. Animals

European lesser-spotted dogfish, *Scyliorhinus canicula* (600-1100 g) were obtained from waters around Millport on the West Coast of Scotland, from waters around Aberdeen on the East Coast of Scotland or from waters around Bangor on the north west coast of Wales. The fish were transported to the Gatty Marine Laboratory and stocks were maintained in aerated free-flowing seawater (osmolality 940 mOsm kg^{-1}; Na, 399 mM, K, 8.5 mM, Ca, 12 mM; Mg, 45 mM; Cl, 368 mM) under a natural photoperiod at ambient temperature (5-16°C). For experiments involving fish acclimated to 85% SW animals were acclimated to reduced salinity for 3 days prior to use. The experimental fish were not fed for at least 2 weeks prior to experimentation. Female fish were used in all experiments involving the collection of urine due to the anatomy of the urinary system (See Section 2.1.). All procedures were carried out in accordance with UK Home Office regulations (Animals (Scientific Procedures) Act, 1986).

2.2.2. Chemicals and Equipment

All chemicals were obtained from Sigma (Sigma Chemical Company, Poole, Dorset) unless otherwise stated. All solutions and buffers were made using deionised water (MilliQ reagent water system, Millipore (UK) Ltd., Watford, Herefordshire).

2.2.3. In vivo urine collection

Female dogfish were anaesthetised by immersion in 0.015% 3-aminobenzoic acid ethyl ester (MS222), buffered with an equal amount of sodium bicarbonate, and the urinary

papilla was cannulated (PE 50, Portex). The cannula was attached with a suture around the papilla and held in place with further sutures onto the anal fin and dorsal fin. A latex bag was attached to the cannula in order to collect urine (See Figure 2.1.). Once cannulated, fish were placed in 40 litre experimental tanks and urine was collected at 24-hour intervals. This long clearance period was designed to overcome variations in urine output caused by inconsistent urine flow from the urinary sinuses during periods of swimming activity. In order to maintain water quality during acclimation, two 40 litre tanks were placed in a rack, one approximately 60 cm above the other. Seawater was pumped from the bottom tank to the top tank via a biological canister filter (Fluval 403) and then returned to the bottom tank by overflow. Fish were placed in the top tank and any salinity adjustments were made using only the bottom tank. This recirculating system maintained water quality in the tanks throughout the experimental period and prevented the disturbance and possible stress of fish associated with daily water changes. During initial experiments cannulation of animals was attempted throughout the experimental acclimation period. However, it soon became clear that there were several problems with this experimental design. There was a tendency for the cannulae to become blocked or work free from the urinary papilla as a function of time, making the results recorded later in the experiment unreliable. Therefore in all subsequent experiments the fish were cannulated for a maximum of three days. The experimental fish were held in a 300 litre stock tank (containing recirculating, filtered SW), for 2 days and then the water composition was adjusted to 90% SW for a further 3 days. After 3 days in 90% SW the fish were then acclimated to 70% SW in 5% salinity decrements. On reaching each experimental salinity 4 fish were removed from the stock tank, and the urinary papilla was cannulated. The fish were then placed in the 40 litre experimental tanks and urine was collected for three days. Blood samples were taken

from the caudal blood sinus, using heparinised syringes, at the end of each three-day experimental period and immediately centrifuged to obtain plasma.

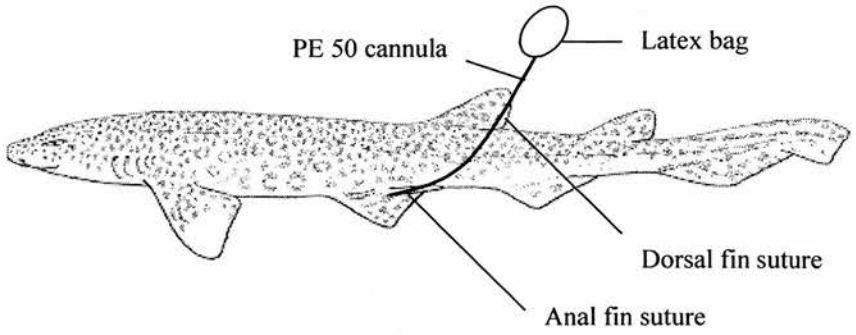

Fig 2.1. Diagrammatic representation of the experimental set-up for the *in vivo* collection of urine.

2.2.4. In situ perfusion of the kidney

Fish were killed with a sharp blow to the head followed by pithing to prevent subsequent movement of the trunk. The fish were immediately weighed and blood was collected in heparinised syringes by puncturing the caudal blood sinus. The fish were decapitated with a clean cut immediately behind the pectoral fins and the trunk was placed ventral side up. A polythene cannula (tapering to ~1.5 mm diameter), with vacuum-filtered Ringer's solution flowing through it, was immediately inserted into the dorsal aorta. Dogfish Ringer's solution contained (in mM) 240 NaCl, 7 KCl, 4.9 $MgCl_2$, 0.5 Na_2HPO_4, 0.5 Na_2SO_4, 360 Urea, 60 TMAO, 10 $CaCl_2$, 23 $NaHCO_3$, pH 7.6; and was continuously bubbled with 95% O_2: 5% CO_2. For the first five minutes of perfusion this Ringer's solution contained 20 IU heparin/ml. The urinary papilla was

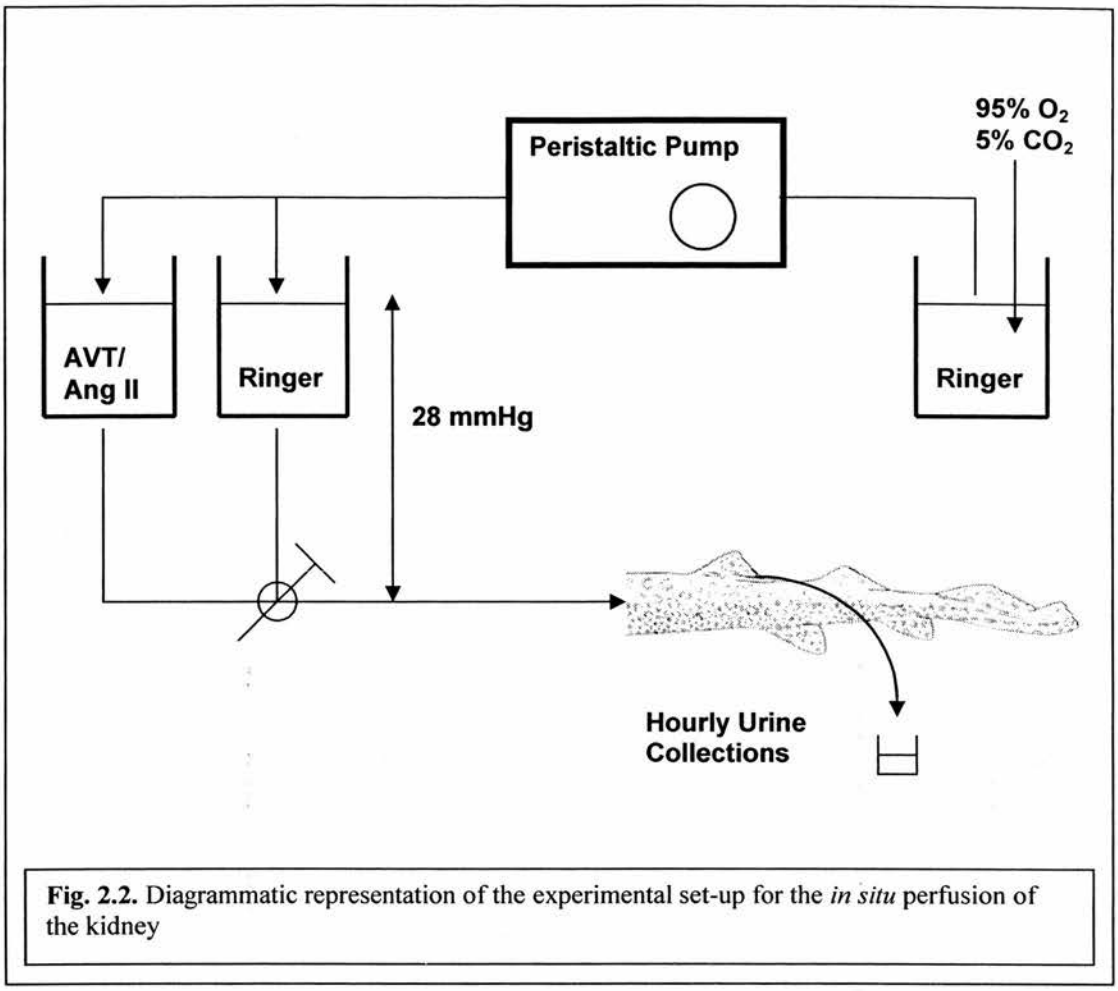

Fig. 2.2. Diagrammatic representation of the experimental set-up for the *in situ* perfusion of the kidney

cannulated with PE 50 (Portex) polythene cannula to allow the collection of timed urine samples. The trunks were then opened along the mid-ventral line to expose the dorsal wall of the body cavity, behind which the kidney is located. Blood vessels that did not lead directly to the kidney were either clamped or cauterised to prevent leakage from the trunk preparation. 85% SW-acclimated trunk preparations were set up in an identical manner, except fish were pre-acclimated to 85% SW for three days prior to use in the preparation. Ringer was diluted to match the plasma composition of fish acclimated to 85% SW.

Ringer's solution was delivered from a constant pressure head (28 mmHg), which was maintained from a reservoir via a peristaltic pump (Watson Marlow 503S, Watson

Marlow, Falmouth, UK). Tissue perfusate flow was estimated from the delivery of fluid from the pump required to maintain a pressure head of 28 mmHg. The experimental set-up is summarised in Figure 2.2.

2.2.5. Verification of the in situ perfused trunk preparation.

Kidney preparations were perfused with Ringer's solution containing inulin (0.25 g/l), to monitor inulin clearances as a measure of GFR (Section 2.2.11. & 2.2.13.). Tubular transport maxima for glucose (TmG) were determined, as a measure of functional tubular mass, by addition of glucose (5 g/l) to the perfusate (Section 2.2.12 & 2.2.13.). Urine flow was allowed to stabilise for at least one hour before a series of one-hour urine samples were collected into pre-weighed microcentrifuge tubes. Urine flow rates were determined gravimetrically, assuming a specific gravity of 1. In order to ensure that the trunk preparations remained viable for the duration of the experiment urine samples were collected for 6 hours during initial experiments. Hourly urine samples were collected and analysed in order to determine that urine flow rate, GFR, TmG and all tubular parameters remained stable.

2.2.6. Assessment of recovery from hormonal perfusion

In order to ascertain whether the *in situ* perfused trunk preparation recovered to basal levels of urine flow rate and GFR following perfusion with hormone, a further experiment was performed. The kidneys of 5 animals were perfused according to section 2.2.4. and 2.2.5. Urine flow was allowed to stabilise for at least one hour before two, one-hour urine samples were collected into pre-weighed microcentrifuge tubes. A high dose of 10^{-8} M AVT was then added to the perfusate and two further one-hour urine samples were collected. Following this hormonal perfusion, the preparation was

then perfused with Ringer's solution containing no hormone for a final two hours in order to determine whether urine flow rate and GFR recovered to pre-hormone levels.

2.2.7. Measurement of osmolality

Blood samples (1 ml) from the *in vivo* experiments were placed in microcentrifuge tubes and immediately centrifuged at 13000 rpm for 1 minute to obtain plasma (MSE Microcentaur, MSE, UK). The plasma was analysed for osmolality (Roebling Osmometer, Camlab, Cambridge, UK), within 1 hour of collection using only fresh, unfrozen plasma. 50 µl samples were used in all cases. The remainder of each sample was then frozen at –20◈C for future analysis. All other analyses were performed on freshly thawed samples that had been frozen for not longer than 2 months. Urine and perfusate osmolalities were also measured as detailed above.

2.2.8. Urea assay

Urea was analysed spectrophotometrically, on samples diluted 1 in 40 using a commercial diagnostic kit (Sigma Kit No. 640). Urea standards (2.5, 5, 7.5, 10, 12.5 mmol/l) were made up in Milli-Q water in order to construct a standard curve for the determination of unknowns (See Appendix 2). 100 µl of each standard or 10 µl of a 1 in 40 dilution of plasma, urine or Ringer's solution, was added in duplicate to 4 ml cuvettes, containing 0.25 ml urease solution. The tubes were allowed to stand at room temperature (20-24°C) for 15-20 minutes to allow urea to be hydrolysed to ammonia. Then 0.5 ml of phenol nitroprusside, 0.5 ml of alkaline hypochlorite and 2.5 ml Milli-Q water were added to each cuvette, mixing after each addition. Ammonia reacts with alkaline hypochlorite and phenol, in the presence of sodium nitroprusside, to form indophenol in a concentration dependent manner, which can be measured

spectrophotometrically. The cuvettes were left at room temperature for 20-30 minutes to allow the colour change to occur and then absorbance was read at 570 nm.

2.2.9. Measurement of chloride

Chloride concentration of plasma, urine and perfusate was measured using a Corning Chloride Analyser 925 (Corning Ltd., Essex, UK) calibrated for a 20 µl sample. Plasma samples were diluted by a factor of 2 with Milli-Q water and results were multiplied by the same factor to give the chloride concentration in mmol/l.

2.2.10. Measurement of sodium

Sodium concentration of plasma, urine and perfusate was measured in samples that were diluted by a factor of 4 with Milli-Q water (total volume 200 µl) using a Corning 480 Flame Photometer (Corning Ltd., Essex, UK). Results were then multiplied by the same factor to give the sodium concentration in mmol/l.

2.2.11. Inulin assay

The use of inulin as a marker for GFR is discussed in Section 1.2. Inulin was analysed by the spectrophotometric assay of Schriener (1950) as used by Amer & Brown (1995). The determination of inulin by resorcinol depends upon the hydrolysis of inulin to fructose. This causes a concentration dependent colour change of resorcinol. Inulin standards (0.5, 1, 2, 3, 4 mg/100 ml) were made up in MilliQ water and used to construct a standard curve (See Appendix 3). 500 µl of each standard or 20 µl of urine or Ringer's solution, diluted with 480 µl MilliQ, was added in duplicate to 5 ml pyrex test tubes (BDH). In all cases it was necessary to measure inulin in both Ringer's solution containing glucose and Ringer's solution with no glucose added, as glucose

interferes with the inulin assay. This allowed a correction factor to be used to correct urine values for this interference. 500 µl resorcinol solution (0.1% resorcinol in 95% ethanol) was then added to all tubes, followed by 1.5 ml 30% HCl. All tubes were placed in a water bath at 80°C for 25 minutes, cooled on ice and then absorbance read at 405 nm.

2.2.12. Glucose assay

Transport Maxima for Glucose (TmG) can be used as an indication of the functional tubular mass of the whole kidney (See Section 1.2.). Glucose of perfusate and urine samples was assayed by use of a glucose oxidase/peroxidase kit (Sigma Kit No 510A). Glucose standards (100, 200, 300 mmol l^{-1}) were made up in Milli-Q water, and used to construct a standard curve (See Appendix 4). 100 µl of a 4 fold dilution of urine or Ringer's solution were added in duplicate to 1.6 ml cuvettes. 1 ml of combined enzyme colour reagent (containing peroxidase, glucose oxidase and o-Diansidine) was added to each cuvette. This results in a concentration dependent colour change in oxidised o-Diansidine from which absorbance is read at 450 nm after 45 minutes at room temperature.

2.2.13. Calculations

Glomerular filtration rate. GFR was calculated from:

$$GFR = \dot{V} \times U_{in}/P_{in} \text{ (ml/kg/h).}$$

Where $\dot{V}$ is urine flow rate and U_{in} and P_{in} are urinary and perfusate inulin

concentrations, respectively.

In addition, the urine/perfusate inulin concentration ratio (U/P_{in}) provides information on the amount of water reabsorbed by the renal tubule. The concentration of inulin filtered by the glomeruli will equal that in the plasma/perfusate. Since inulin is neither reabsorbed from, nor secreted into, the tubular lumen, its concentration in tubular fluid can increase only due to water being reabsorbed from the various tubular segments (Valtin, 1983). For example, if the concentration of inulin in urine is twice as great as that in plasma, 50 % of the filtered water must have been reabsorbed. This would represent a U/P_{in} of 2. The % of the filtered volume of water reabsorbed by the renal tubule is given by:

$$1-\left(\frac{1}{U/Pin}\right) \text{x } 100 \text{ (\%)}.$$

Where U/P_{in} is the urinary/perfusate inulin concentration ratio.

Osmolar clearance (C_{osm}) and Free Water Clearance (C_{H2O}). Osmolar clearance represents the rate at which osmotically active solutes are cleared from plasma and is given by the following equation:

$$C_{osm} = [U_{osm}/P_{osm}] \text{ x } \dot{V} \text{ (ml/kg/h)}.$$

Where U_{osm} is osmolality of urine, P_{osm} is osmolality of perfusate and $\dot{V}$ is the urine flow rate. The same equation is used to calculate the clearance of ions (Chapters 3, 4 & 5).

If the urine is isotonic to plasma, then C_{osm} is equal to the urine flow rate. If the urine osmolality is lower than the plasma osmolality, C_{osm} must be less than the urine flow

rate, and the urine volume must therefore be made up of an additional volume of free water. This "free water clearance" (C_{H20}) can be calculated from:

$$C_{H20} = \dot{V} - C_{osm} \text{ (ml/kg/h)}.$$

Where $\dot{V}$ is the urine flow rate and C_{osm} is the osmolar clearance.

In addition, the relative osmolar clearance (C_{osm}/GFR) and relative free water clearance (C_{H20}/GFR), can be calculated by dividing C_{osm} or C_{H20} by the glomerular filtration rate. This allows the investigator to denote the clearance of osmolytes or free water as a percentage of the amount filtered in order to ascertain whether changes in C_{osm} or C_{H20} result from alterations in tubular water reabsorption or purely as a result of changes in GFR. The relative clearance of ions, also referred to as the fractional excretion of ions, is calculated using the same equation (See Chapters 3, 4 & 5).

Transport Maxima for Glucose. TmG is measured as the difference between filtered and excreted glucose as follows:

$$[GFR \times P_g] - [\dot{V} \times U_g] \text{ (µg/kg/h)}.$$

Where $\dot{V}$ is the urine flow rate, GFR is the glomerular filtration rate and U_g and P_g and glucose concentrations of urine and perfusate respectively.

2.2.14. Data and Statistical Analysis

All data are presented as mean ± SEM. One-Way ANOVA followed by Tukey's post hoc test was used to assess changes between *in vivo* parameters, and in the basal

perfused trunk preparation (INSTAT, computer statistical package). Paired *t*-tests were used to assess physiological changes in the *in situ* perfused trunk preparation following the addition of peptide to the perfusate (INSTAT). Comparisons were made between the final urine collection immediately prior to peptide administration and the final clearance period during peptide administration. Significance was denoted as:

$$*P < 0.05$$

$$** P < 0.01$$

$$***P < 0.005$$

2.3. Results

2.3.1. In vivo urine collection

Figure 2.3. shows urine flow rate during stepwise acclimation to 70% SW. Urine flow increased in a step-wise manner from a SW control value of 0.082 ± 0.030 ml/kg/h to a maximal value of 0.727 ± 0.094 ml/kg/h in 70% SW.

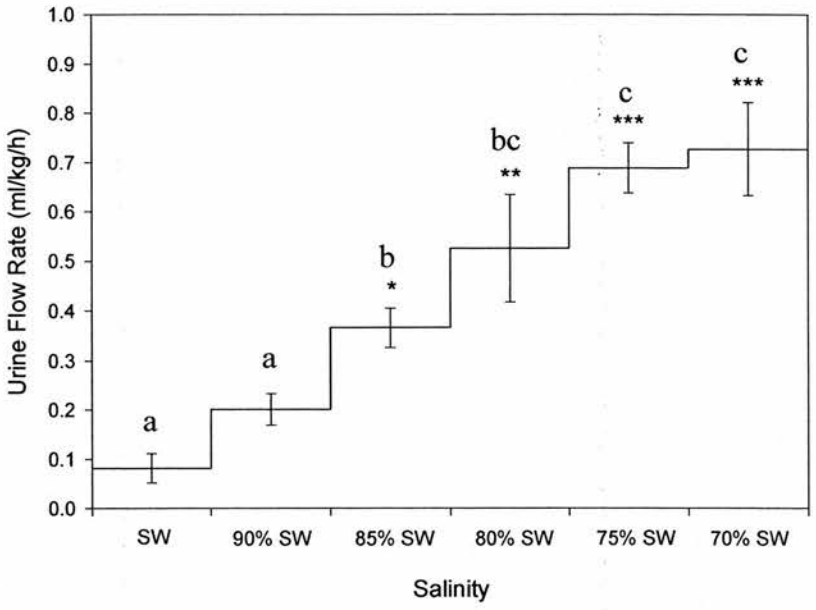

Fig 2.3. Urine flow rate of *in vivo* cannulated dogfish on stepwise acclimation to dilute seawater. Values are means ± SEM (n = 4). * ($P < 0.05$), **($P < 0.01$) and *** ($P < 0.005$) indicate statistically significant difference from urine flow rate in SW acclimated fish. Data sets between which no significant difference was observed are signified by letters.

2.3.2. Plasma and urine parameters on acclimation to reduced salinity

Plasma osmolality, urea, chloride and sodium concentrations were all significantly reduced on acclimation from SW to 70% SW (Figure 2.4. & Table 2.1.). Plasma osmolality decreased from 939 ± 1 mOsm in SW to 665 ± 2 mOsm in 70% SW, but remained slightly hyperosmotic to the surrounding medium, except in SW (Table 2.1.). Plasma urea concentration decreased from 327 ± 5 mmol l^{-1} to 226 ± 3 mmol l^{-1}, chloride concentration decreased from 266 ± 3 mmol l^{-1} to 211 ± 3 mmol l^{-1} and sodium concentration decreased from 263 ± 6 mmol l^{-1} to 219 ± 2 mmol l^{-1}.

% SW	SW Osmolality (mOsm)	Plasma Osmolality (mOsm)
100	940	939
90	844	853
85	801	809
80	747	764
75	707	719
70	656	665

Table 2.1. Osmolality of SW and plasma during environmental transfer.

Urine osmolality, urea, chloride and sodium concentrations are shown in Figure 2.5. Urine osmolality decreased from 921 ± 2 in SW to 641 ± 17 in 70% SW. There was also a significant drop in chloride and sodium concentration, but urine urea concentration remained unchanged by acclimation to 70% SW. When the concentrations of these ions are coupled with the increase in urine flow rate observed in Figure 2.3. there is a resulting increase in the clearance of urea, chloride and sodium from these animals (Figure 2.6.).

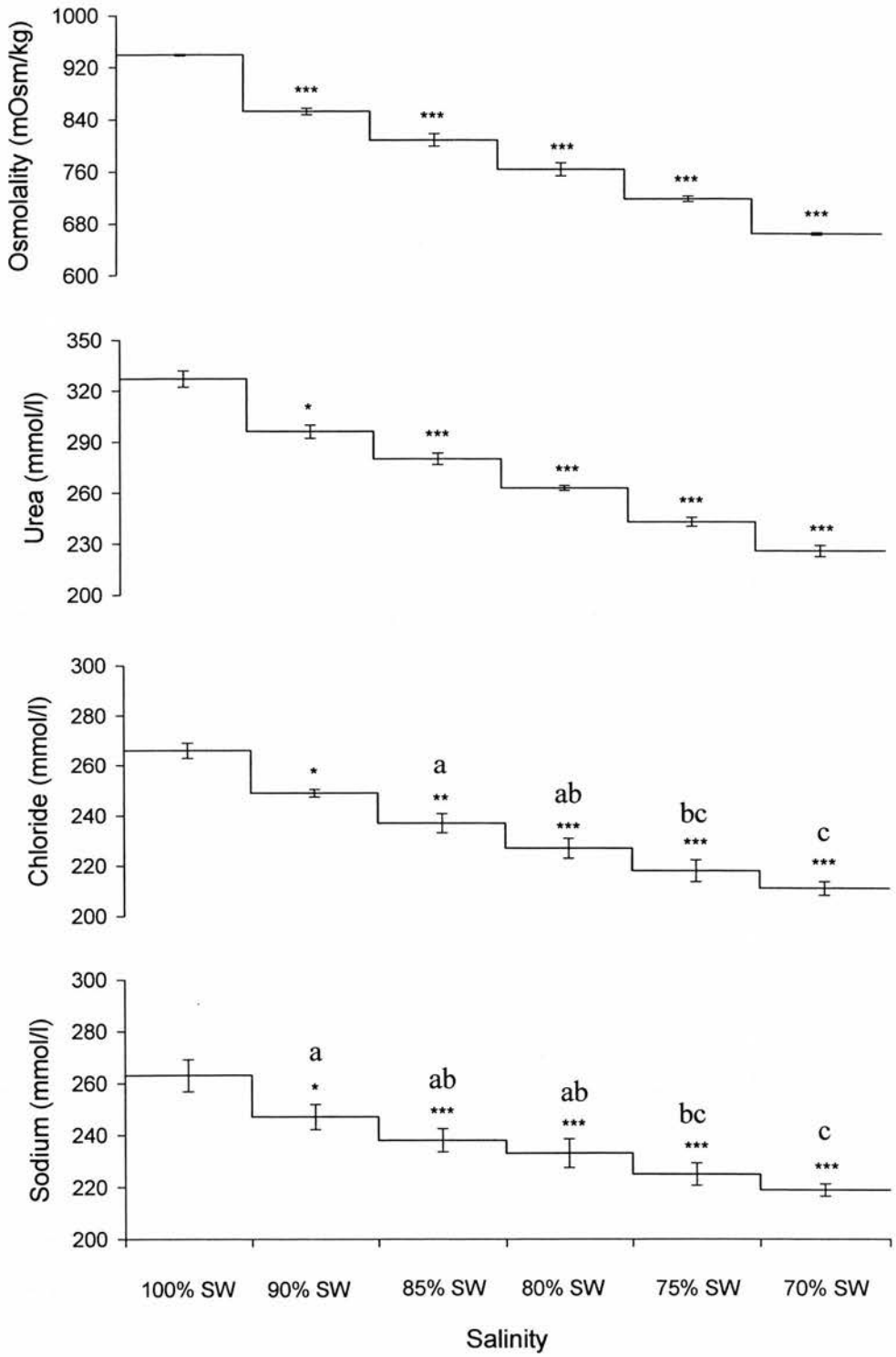

Fig 2.4. Plasma osmolality, urea, chloride and sodium in fish acclimated stepwise from SW to 70% SW. Values are means ± SEM (n=4). * ($P < 0.05$), **($P < 0.01$) and *** ($P < 0.005$) indicate statistically significant difference from urine flow rate in SW acclimated fish. Data sets between which no significant difference was observed are signified by letters.

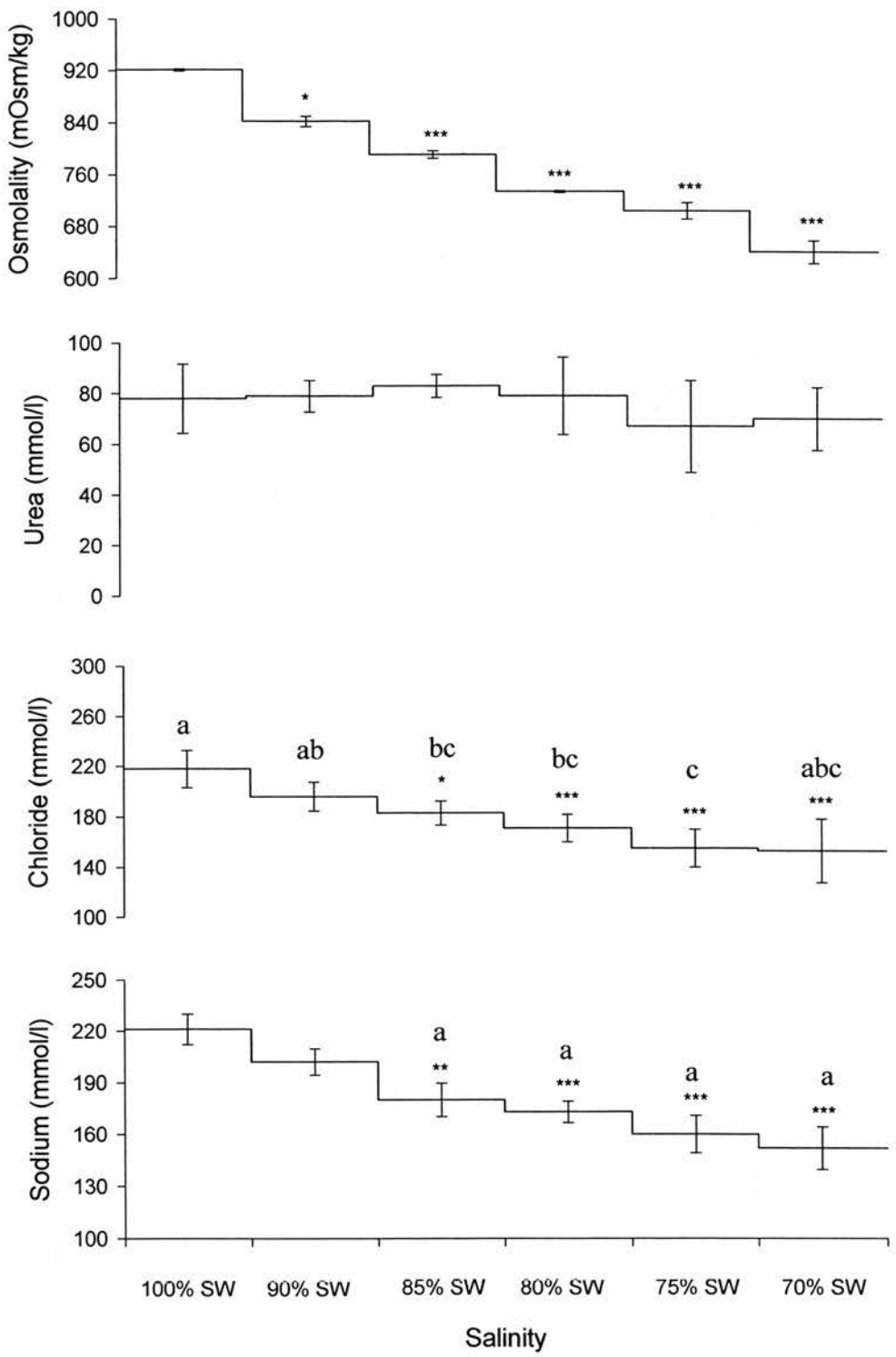

Fig 2.5. Urine osmolality, urea, chloride and sodium in fish acclimated stepwise from SW to 70% SW. Values are means ± SEM (n=4). * ($P < 0.05$), **($P < 0.01$) and *** ($P < 0.005$) indicate statistically significant difference from urine flow rate in SW acclimated fish. Data sets between which no significant difference was observed are signified by letters.

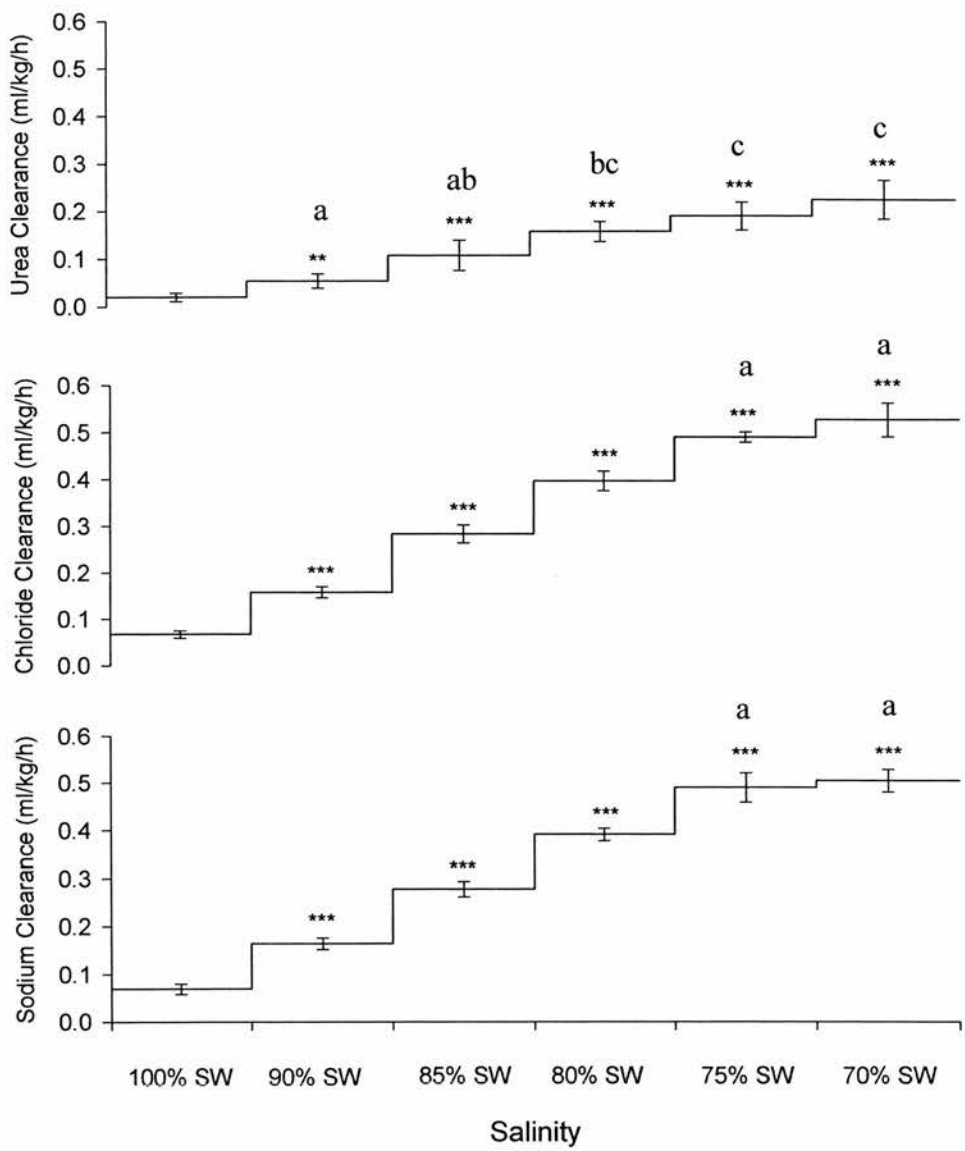

Fig 2.6. Renal urea, chloride and sodium clearance in fish acclimated stepwise from SW to 70% SW. Values are means ± SEM (n=4). **($P < 0.01$) and *** ($P < 0.005$) indicate statistically significant difference from renal clearance in SW acclimated fish. Data sets between which no significant difference was observed are signified by letters.

2.3.3. In situ perfusion of the kidney

Initial experiments, performed to ensure that the trunk preparations remained viable for the duration of the experiment are shown in Figure 2.7. The stability of urine flow rate,

GFR and TmG in control preparations that did not receive any peptide is clearly demonstrated in fish acclimated to both SW and 85% SW. It is also clear that the urine flow rates for *in situ* preparations of 85% SW acclimated fish were significantly greater than 100% SW acclimated fish ($P < 0.05$). Therefore the diuresis observed *in vivo* upon acclimation of fish to 85% SW (Fig. 2.3.) was maintained in the *in situ* preparations.

Time (h)	U/P_{in}	U/P_{osm}	C_{osm}/GFR, %	C_{H20}/GFR, %
1	1.5 ± 0.1	0.99 ± 0.01	67 ± 3	0.3 ± 0.1
2	1.5 ± 0.1	0.99 ± 0.02	69 ± 4	0.4 ± 0.1
3	1.5 ± 0.1	0.99 ± 0.01	68 ± 4	0.3 ± 0.1
4	1.6 ± 0.2	0.99 ± 0.04	69 ± 3	0.3 ± 0.2
5	1.6 ± 0.1	0.99 ± 0.01	67 ± 3	0.3 ± 0.1
6	1.5 ± 0.1	0.99 ± 0.02	66 ± 3	0.5 ± 0.2

Table 2.2. Tubular function in the SW-acclimated *in situ* perfused trunk preparation perfused for 6 hours. Values are mean ± SEM. U/P_{in}, urine/perfusate inulin concentration ratio; U/P_{osm}, urine/perfusate osmolality ratio; C_{osm}/GFR, relative clearance of osmolytes; C_{H2O}/GFR, relative free water clearance.

2.3.4. Tubular parameters

Tubular function is summarised in Table 2.2. U/P_{in} U/P_{osm}, C_{osm}/GFR and C_{H20}/GFR all remain stable for the duration of the experimental period. U/P_{in} indicates that a mean 35% of the filtered volume was reabsorbed by the renal tubule. Urine was slightly hypotonic relative to plasma with a mean U/P osmolality ratio of 0.99. Hence there was a small relative free water clearance of less than 1%. The relative osmolar clearance demonstrates that an average of 68% of filtered osmolytes were excreted.

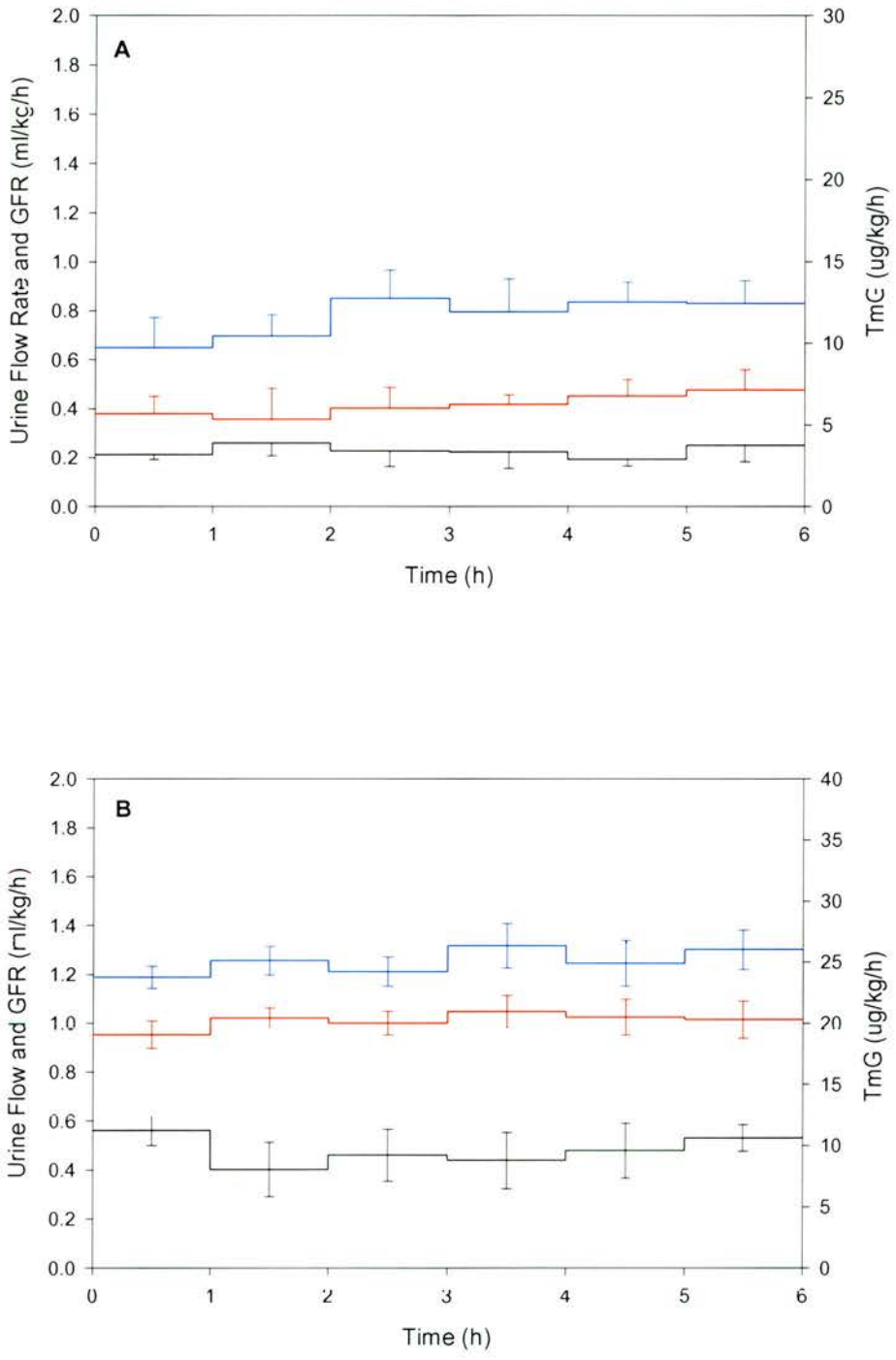

Fig 2.7. Urine flow rates, GFR and TmG of *in situ* perfused dogfish kidneys perfused for 6 hours in fish acclimated to SW (A) and 85% SW (B). In both graphs, the blue line represents TmG, the red line represents GFR and the black line represents urine flow rate. Values are means ± SEM (n = 6).

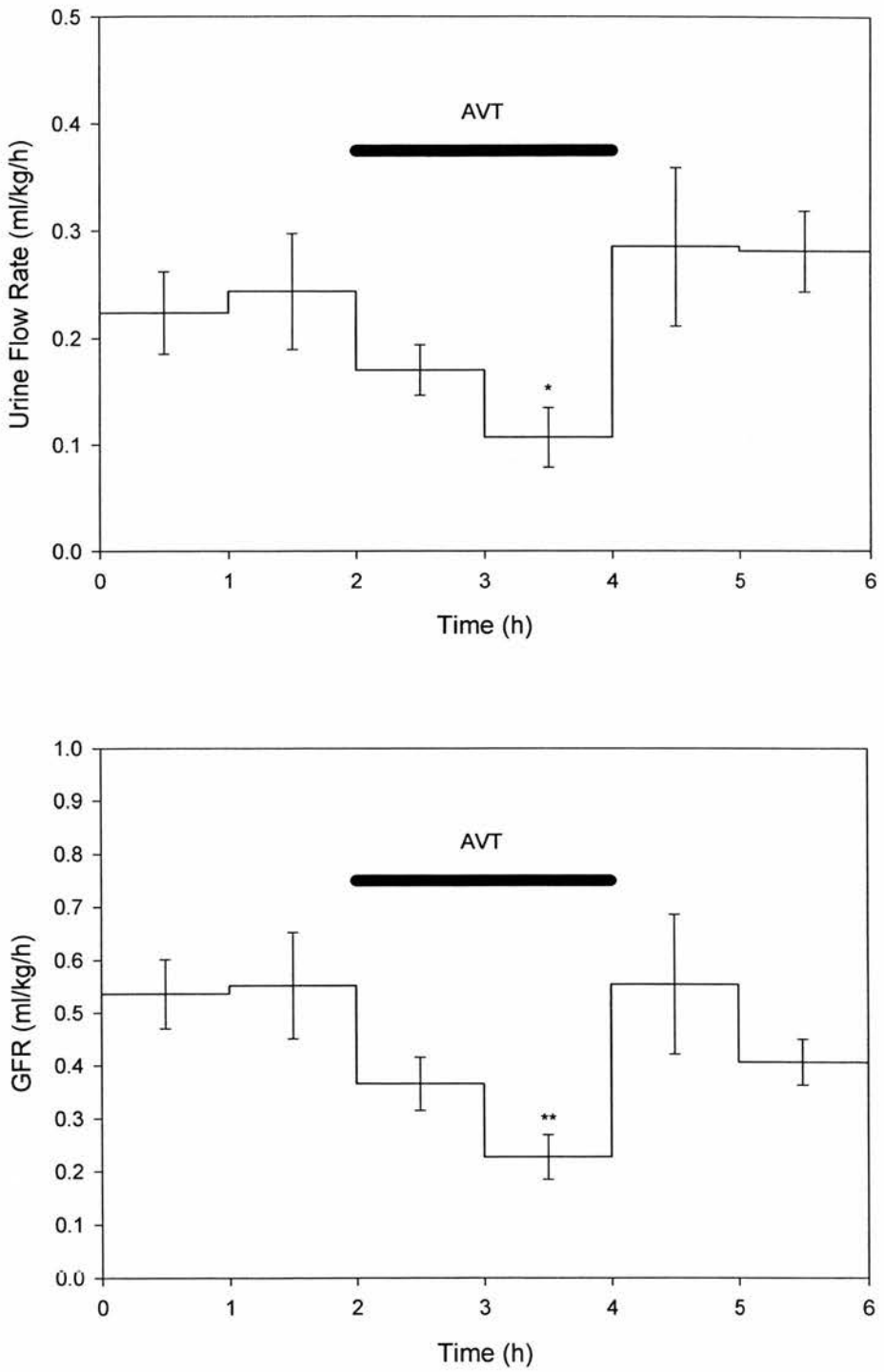

Fig. 2.8. Urine flow rate (A) and GFR (B) in the perfused trunk preparation of the dogfish (SW-acclimated fish). The two-hour AVT infusion phase (10^{-8} M) is marked by the black line. Values are means ± SEM, n = 5 at each timepoint; $*P<0.05$ and $**P<0.01$ indicate statistically significant difference from the renal clearance period immediately prior to administration of AVT.

2.3.5. Recovery of preparation after a single dose of AVT

Figure 2.8. (a & b) show the urine flow rate and GFR respectively in preparations perfused with 10^{-8} M AVT. Following an antidiuresis, which reached significance after 1 hour, both urine flow rate and GFR recovered to levels statistically indistinguishable from the clearance period immediately prior to treatment, on resumption of perfusion with hormone-free Ringer's solution.

2.3.6. Collection of urine for 30-minute clearances

Figure 2.9. shows the urine flow rate in preparations perfused with 10^{-9} M AVT, using 30 minute clearances rather than 1 hour clearances. A significant antidiuresis is induced, which becomes apparent during the second 30-minute collection period.

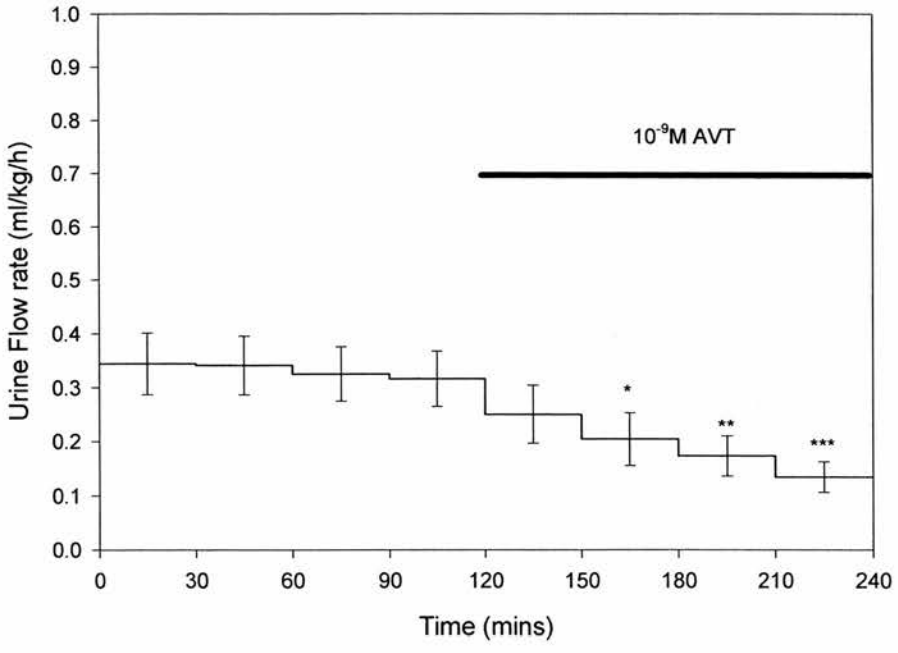

Fig 2.9. Urine flow rate of SW-acclimated *in situ* perfused dogfish kidneys perfused for 4 hours using 30-minute urine collections. The black line marks the two-hour AVT infusion phase. Values are means ± SEM. * $P<0.05$, ** $P<0.01$ and *** $P<0.005$ indicate statistically significant difference from final collection prior to AVT perfusion.

2.4. Discussion

2.4.1. In vivo renal function

S. canicula has previously been shown to successfully acclimate to dilute SW under laboratory conditions (Hazon and Henderson, 1984; Tierney *et al.*, 1998). In the present study dogfish were successfully acclimated to 70% SW for periods of 3 days, although they have previously been maintained in 70% SW for periods of a week or more at the Gatty Marine Laboratory (Tierney *et al.*, 1998). In order to reduce some of the problems involving *in vivo* renal studies, highlighted in Section 2.1., very long clearance periods of 12 hours were utilised. This allowed the accurate measurement of urine flow rate in various salinities.

Most marine elasmobranchs are slightly hyperosmotic to their surrounding SW environment (Smith, 1936). However, in the present study the dogfish were iso- or slightly hypo-osmotic to the sea water medium (Table 2.1.). This may be explained by the unfed state of the experimental animals as significant reductions in urea and osmolality have been reported in the unfed pyjama shark, *Poroderma africanum* (Haywood, 1973). Urine collection *in vivo* demonstrated a significant diuresis during acclimation to reduced salinity (Figure 2.3.). In agreement with previous studies (Hazon and Henderson, 1984; Tierney *et al.*, 1998), movement into dilute SW would be expected to cause an increase in extra cellular fluid (ECF) volume and decreases in plasma urea, sodium and chloride concentrations were observed, resulting in a significant decrease in plasma osmolality (Figure 2.4.). Plasma osmolality decreased in proportion to medium dilution with the animals becoming slightly hyperosmotic with environmental dilution. This is a similar effect to that seen in other elasmobranch

species such as the lip shark, *Hemiscyllium plagiosum*, the little skate, *Raja erinacea*, the Atlantic stingray *Dasyatis sabina*, and the yellow stingray, *Urolophus jamaicensis*, in which the plasma becomes progressively hyperosmotic with environmental dilution (Chan and Wong, 1977; De Vlaming and Sage, 1973; Goldstein and Forster, 1971; Sulikowski and Maginniss, 2001). It therefore appears that *S. canicula* follow a similar osmoregulatory strategy as these fully, or at least partially, euryhaline elasmobranch species.

In terms of urine composition, osmolality and sodium and chloride concentrations were all significantly reduced with environmental dilution whereas urine urea concentration remained unchanged (Figure 2.5.). However, when urine flow rate is taken into account the renal clearance of urea, sodium and chloride are all significantly elevated with environmental dilution (Figure 2.6.). An increase in the renal clearance of urea and sodium chloride on environmental dilution has also been observed in a number of other elasmobranch species (Goldstein and Forster, 1971; Goldstein *et al.*, 1968; Wong and Chan, 1977).

It would appear therefore that *S. canicula* employs a similar osmoregulatory strategy to other, more euryhaline elasmobranchs. This species may possess the capability to alter urine output and composition in order to control extracellular fluid volume. It is therefore of great interest to investigate the potential hormones involved in renal function, but the very long clearance periods used in the *in vivo* experiments did not allow the investigation of hormonal effects on renal function. It was therefore decided to develop an *in situ* perfused renal preparation.

2.4.2. Verification of the in situ perfused trunk preparation

The *in situ* perfused renal trunk preparation has been used in freshwater acclimated rainbow trout, *Onchorynchus mykiss* for the investigation of renal actions of AVT (Amer and Brown, 1995; Brown and Balment, 1997) and Ang II (Brown and Balment, 1997; Brown *et al.*, 2000). Due to anatomical and functional differences in the trout and dogfish kidney and urinary systems it was necessary to make several changes to the technique in order to adapt it for *S. canicula*. These differences are summarised in Table 2.3.

Amer & Brown (1995)	**Current Study**
FW acclimated Rainbow Trout (*Onchorynchus mykiss*)	SW or 85% SW acclimated Dogfish (*Scyliorhinus canicula*)
Bladder cannulated	Urinary papilla cannulated
Fish were not pithed to prevent movement.	Pithing was necessary to prevent movement of the trunk preparation and to avoid the dorsal aorta cannula becoming dislodged.
Heparin injected via a cannula into the caudal vasculature.	Heparin perfused via the dorsal aorta cannula. This technique had no subsequent detrimental effect on the preparation.
Basal urine flow rate relatively high allowing 15 minute urine clearance collections.	Urine flow rate significantly lower, and tended to be less predictable in SW. 1-hour clearance collection periods partly overcame this problem. In order to make urine flow more consistent a diuresis was induced by acclimating the fish to 85% SW. This salinity was chosen, because a significant diuresis was induced in the *in vivo* cannulated fish (Fig. 2.2.b), and also because dogfish can tolerate direct transfer from SW to 85% SW.

Table 2.3. Differences between the trout and dogfish perfused trunk preparations.

As urine flow rate is considerably higher in a fresh water teleost than in a marine elasmobranch, it was decided to investigate renal function in kidneys of fish acclimated to two salinities, SW and 85% SW. Eighty-five percent SW was chosen on the basis that

it appeared to be the threshold for a significant diuresis during environmental dilution *in vivo* (Fig. 2.3.) and because dogfish are capable of direct transfer from SW to 85% SW. It was also decided to attempt to measure renal parameters in fish acclimated to SW in addition to fish acclimated to 85% SW, in order to provide a comparison, despite the fact that urine flow rate was less consistent in SW.

In the absence of hormone, urine flow rate, GFR and TmG remained stable for at least 6 hours in fish acclimated to both SW and 85% SW (Figure 2.7.), indicating that this preparation remained viable for a sufficient period to investigate the actions of specific peptides on renal function. The values for urine flow rate and GFR were similar to those previously published for *S. canicula in vivo* (Brown and Green, 1987) and to the *in vivo* values reported in the present study. The values for U/P_{in}, U/P_{osm}, C_{osm}/GFR and C_{H20}/GFR also all remained stable for the duration of the experiment. However, the U/P inulin ratio for the *in situ* perfused preparation, and therefore the fraction of filtered water reabsorbed, in the present study was lower than previously reported for *S. canicula* (Brown and Green, 1987). They demonstrated a U/P inulin ratio of 3.5, resulting in 70% of the filtered volume of water being reabsorbed. This decreased U/P inulin ratio implies a reduced tubular reabsorption of water and may signify a somewhat reduced tubular capacity in the preparation (See Chapter 7). However, it should be noted that the U/P inulin ratio has been shown to be somewhat variable in elasmobranchs (Boylan, 1967).

The use of inulin to measure GFR depends on the assumption that inulin is freely filtered at the glomerulus and neither reabsorbed or secreted by the renal tubule (Levinsky and Levy, 1973). However, slight tubular reabsorption has been reported in

the urinary bladder and tubules of some teleost fish (Beyenbach and Kirschner, 1976). Although there have been no detailed studies of the handling of inulin by elasmobranch renal tubules or the urinary sinus, the present measurements may slightly underestimate GFR.

It is clear that the diuresis observed on transfer from SW to 85% SW *in vivo* (Fig. 2.3.) was also evident in the *in situ* perfused trunk preparation (Fig. 2.7.). This will be discussed further in Chapter 4 and Chapter 7. It is also clear that the urine flow rates *in vitro* are comparable to the urine flow rates in the *in vivo* study, as well as those quoted in previous studies (Brown and Green, 1987).

As preliminary studies had shown an antidiuretic effect on perfusion with Ang II and AVT it was decided, as part of the verification of the preparation, to ensure that the preparation would recover from a high dose of AVT. During perfusion of 10^{-8} M AVT there was a profound, antidiuretic effect, resulting in urine flow rate and GFR decreasing by approximately 50%. This decrease in urine flow rate and GFR became apparent after one hour of perfusion. Recovery from this antidiuresis occurred within 1 hour of perfusion with hormone-free Ringer's solution. This recovery provided further evidence of the viability of the renal preparation. However, despite this recovery, a recovery period was not routinely incorporated in subsequent preparations. In order to incorporate a recovery period the preparation would have to be run for a period in excess of 7 hours. However, after 6 hours of perfusion, collection of urine becomes much less reliable, and it was therefore decided not to routinely include a recovery period.

It was noted that on perfusion with hormone, despite a reduction in urine flow rate and GFR after 1 hour of perfusion with AVT, this reduction did not reach significance until the second hour of collection. Initial experiments during the protocol development employed 30-minute urine collections, and a significant antidiuresis was observed after 30 minutes of administration of 10^{-9} M AVT (Fig. 2.9.). However, this 30-minute urine collection did not routinely produce a sufficient volume of urine to complete all the required urine analysis and therefore 1-hour urine collection periods were employed in all subsequent studies.

The stability of the perfused trunk preparation and the potential as a tool for the investigation of renal function in the dogfish has therefore been established. The urine flow rate and GFR were comparable to the *in vivo* results reported in the present study and also to data reported for *S. canicula* in previous studies (Brown and Green, 1987). Urine flow rate and GFR remain stable for a sufficient period of time to allow the investigation of the effects of osmoregulatory hormones and the preparation recovers from a high dose of AVT. The renal effects of various osmoregulatory hormones will be investigated using this trunk preparation in the following chapters.

3

Effects of AVT on the perfused dogfish trunk

Wells *et al.* (2002) *Am J Physiol* **282**: R1636-R1642 (Appendix 5).

3.1. Introduction

The aim of following chapters was to use the *in situ* perfused dogfish renal preparation to examine the renal actions of various peptides that play a role in elasmobranch osmoregulation. In this chapter the renal role of arginine vasotocin (AVT) was investigated.

Arginine vasotocin (AVT) is one of a number of neurohypophysial peptides and is found in all non-mammalian vertebrates (Table 3.1.). Neurohypophysial peptides have been identified in all extant vertebrate classes, and AVT is the homologue of arginine vasopressin (AVP) in mammals. However, the physiological functions of these peptides remain obscure, particularly in fishes (Acher, 1996). AVT is synthesised in the magnocellular neurophysin cells which form a distinct preoptic nucleus in the hypothalamus (Meurling *et al.*, 1996). AVT is initially synthesised in the form of a precursor, linked to a neurophysin and is transported in secretory granules along the axis of the magnocellular neurons to the neurohypophysis, where it is stored until release into the circulation. At the point of secretion AVT is cleaved from the neurophysin.

As in all non-mammalian vertebrates, cartilagenous fish possess AVT. However, when teleost and elasmobranch fish are compared, a marked difference can be observed in the amount of AVT stored in the neurohypophysis (Acher, 1996). Despite this marked

Vasotocin/vasopressin family	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**		
Vasotocin	Cys	Tyr	Ile	Gln	Asn	Cys	Pro	Arg	Gly	(NH_2)	All nonmammalian vertebrates
Vasopressin	–	–	Phe	–	–	–	–	–	–		Mammals
Lysipressin	–	–	Phe	–	–	–	–	Lys	–		Pig, Macropodids, Didelphids, Peramelids
Phenypressin	–	Phe	Phe	–	–	–	–	–	–		Macropodids
Oxytocin-like hormones											
Oxytocin	–	–	–	–	–	–	–	Leu	–		Ratfish, placental mammals
Mesoticin	–	–	–	–	–	–	–	Ile	–		Lungfishes, nonmammalian tetrapods, marsupials
Isotocin	–	–	–	Ser	–	–	–	Ile	–		Bony fishes
Glumitocin	–	–	–	Ser	–	–	–	Gln	–		Rays
Aspargtocin	–	–	–	Asn	–	–	–	Leu	–		Spiny dogfish
Valitocin	–	–	–	–	–	–	–	Val	–		(*S. acanthias*)
Asvatocin	–	–	–	Asn	–	–	–	Val	–		Spotted dogfish
Phasvatocin	–	–	Phe	Asn	–	–	–	Val	–		(*S. canicula*)

Table 3.1. Structures of vertebrate neurohypophysial hormones. Residues identical with those of vasotocin are indicated by dashes (Acher 1996). (See Appendix 1. For amino acid abbreviations).

difference, it is difficult to interpret these results in relation to AVT secretion, as an apparent increase in the pituitary concentration of AVT could either represent an increase in the rate of synthesis or a decrease in the rate of secretion. Virtually all teleost fish investigated to date possess two neurohypophysial hormones, isotocin and vasotocin (Acher, 1996). This is in contrast to the elasmobranch fish where there are a great variety of neurohypophysial peptides. In addition to AVT, elasmobranch fish also possess a range of oxytocin-like peptides (Table 3.1. & Table 3.2.). In the case of *S. canicula*, phasvatocin and asvatocin are found, and occur in roughly equal molar amounts in the pituitary (Chauvet *et al.*, 1994). However, in *S. acanthias*, two different oxytocin-like peptides are found, aspargtocin and valitocin. The physiological role of these oxytocin like peptides is yet to be examined in an elasmobranch fish.

Subclass *Selachii* Order Pleurotremata	**Oxytocin-like hormones**	**Vasotocin/vasopressin family**
Squalus acanthias	Aspargtocin Valitocin	Vasotocin
Scyliorhinus canicula	Asvatocin Phasvatocin	Vasotocin
Order Hypotremata		
Raja clavata	Glumitocin	Vasotocin
Raja batis	Glumitocin	Vasotocin
Raja fullonica	Glumitocin	Vasotocin
Raja naevus	Glumitocin	Vasotocin
Subclass Bradyondonti Order Holocephali		
Hydrolagus colliei	Oxytocin	Vasotocin

Table 3.2. Distribution of neurohypophysial hormones chemically identified in elasmobranch fishes (adapted from Acher 1996).

3.1.1. Osmoregulatory effects

To date very little work on the osmoregulatory effects of AVT in elasmobranch fish has been completed. In mammals, renal urea reabsorption is controlled by vasopressin through an increase in the number of UT1 and UT2 urea transporters (Acher *et al.*, 1999; You *et al.*, 1993). It is possible that AVT operates in a similar way in elasmobranch fish (Acher *et al.*, 1999). A urea transporter homologous to the rat UT-A2 transporter has been cloned from the kidney of *S. acanthias* (Smith and Wright, 1999) and another from the euryhaline elasmobranch *D. sabina* (Janech *et al.*, 2001). It has been suggested that the low amount of AVT usually stored in the neurohypophysis may reflect a permanent secretion of AVT for the maintenance of plasma urea levels through renal urea reabsorption [Chauvet *et al.* (1971), quoted from Acher *et al.* (1999)]. However, Warne *et al.* (1994) indicated that circulating levels of AVT in *S. canicula* may be similar to those of teleosts. These measurements were complicated by the presence of phasvatocin and asvatocin in the plasma of *S. canicula*, and the potential for these to cross-react with the specific antiserum used to measure AVT. To date therefore, it has not been possible to provide a definitive accurate measurement of AVT in elasmobranch plasma.

In terms of teleost renal function, administration of AVT was first reported to have a diuretic effect (Chester-Jones *et al.*, 1969; Maetz *et al.*, 1964). Later, in the European eel, injections of low doses were found to be antidiuretic, with only high doses resulting in diuresis (Henderson and Wales, 1974). A sensitive radioimmonuassay for AVT has been developed suggesting that the high doses of AVT, which caused a diuresis, were supra-physiological (Balment *et al.*, 1993; Warne *et al.*, 1994). In the light of this work, the renal effects of AVT have been examined using an isolated perfused trunk

preparation of the rainbow trout, *O. mykiss* (Amer and Brown, 1995; Brown and Balment, 1997). AVT caused a dose-dependent decrease in urine flow rate, glomerular filtration rate and tubular transport maxima for glucose.

The action of AVT on elasmobranch gills also remains unclear. The gills appear to be largely impermeable to urea compared with the gills of most teleost fishes (Boylan, 1967; Smith, 1936). The precise underlying mechanism for this low permeability coefficient has been poorly studied. One possible mechanism that may confer low urea permeability to the elasmobranch gill is that some sort of physical barrier minimises urea loss across gill epithelia (See Section 1.7.). There is also evidence supporting the existence of urea transporters in elasmobranch gill (Section 1.7.). It is possible that AVT may control the number of these transporters in a manner similar to that postulated for the kidney.

3.1.2. Cardiovascular effects

The effect of AVT on blood pressure has been investigated in the lip shark (*Hemiscyllium plagiosum*) (Chan, 1977). AVT elevated the blood pressure in both the ventral and dorsal aorta, and this effect was thought to be derived from peripheral vasoconstriction (Chan, 1977). AVT has been shown to produce dose dependent contraction of isolated rings of a range of blood vessels in the trout, *O. mykiss* (Conklin *et al.*, 1999). It was concluded that this vasoconstriction was regulated by a smooth muscle AVT receptor. Similar AVT-mediated pressor responses have also been observed in other fish species (Bennett and Rankin, 1986; Chan, 1977; Pang *et al.*, 1983; Somlyo and Somlyo, 1968). In lower vertebrates these vasoconstrictor effects of

AVT have been reported to be entirely responsible for AVT-induced renal effects (Pang *et al.*, 1983).

It is perhaps surprising that AVT has not received more attention in terms of the investigation of renal function in elasmobranch fish, particularly in the light of a possible role for AVT in the renal reabsorption of urea. It is clear that AVT has a range of renal effects in teleost fish, and the aim of this chapter was to use the isolated perfused dogfish trunk preparation to examine the renal effects of AVT in an elasmobranch fish.

3.2. Materials and Methods

3.2.1. Animals

Dogfish were collected and maintained according to section 2.2.1.

3.2.2. Chemicals and Equipment

Arg^8-Vasotocin was purchased from Sigma (Sigma Chemical Company, Poole, Dorset). Peptide was made up to a concentration of 10^{-3} M using Milli-Q water and stored in 10 µl aliquots at –20° C until use.

3.2.3. In situ perfusion of the kidney

Kidneys were perfused according to section 2.2.4. and 2.2.5. Urine flow was allowed to stabilise for at least one hour before two, one-hour urine samples were collected into pre-weighed microcentrifuge tubes. AVT was then added to the perfusate at a concentration of either 10^{-9} M or 10^{-10} M and two further one-hour urine samples were collected. Comparisons of renal parameters were made between the last one-hour collection period immediately prior to addition of peptide to the perfusate and the final one-hour collection period during administration of peptide. Each fish therefore acted as it's own control.

3.2.4. Analysis and calculations

The analysis of urine and perfusate, and the calculations for the majority of renal parameters used in this chapter are described in section 2.2.7 to section 2.2.12. In addition, the rate of excretion (ER) of ions in the urine were calculated.

The rate of excretion of ions was calculated from the following equation:

$$ER_y = \dot{V} \times U_y \text{ (mmol/kg/h)}$$

Where $\dot{V}$ is urine flow rate, y is the ion of interest and U_y is the urinary concentration of that ion.

3.2.5. Statistical analysis

Comparisons were made between the final renal clearance period immediately prior to administration of AVT and the final clearance period during AVT administration. Paired *t*-tests were used to assess physiological changes (INSTAT).

3.3. Results

3.3.1. Perfusate flow rates

Table 3.3. shows the effect of AVT on perfusate flow rates. There was a significant decrease in perfusate flow rate during AVT perfusion in SW and 85% SW at both AVT concentrations.

	Control	10^{-9} M AVT	Control	10^{-10} M AVT
Perfusate flow rate (SW)	36.7 ± 5.1	21.0 ± 5.0*	26.7 ± 4.1	15.7 ± 1.9*
Perfusate flow rate (85% SW)	38.5 ± 3.9	23.3 ± 3.0*	25.0 ± 3.4	16.0 ± 3.4*

Table 3.3. Effect of AVT on perfusate flow rate. Values are mean ± S.E.M. from 6 fish in each group. *$P < 0.05$.

3.3.2. Effects of AVT on urine flow rate

Addition of 10^{-9} M AVT to the perfusate resulted in a significant antidiuresis in both SW and 85% SW-acclimated preparations (Figure 3.1.). Addition of 10^{-10} M AVT, however, resulted in a significant antidiuresis in SW but not in 85% SW (Figure 3.2.).

3.3.3. Glomerular effects

Addition of 10^{-9} M AVT to the perfusate resulted in a significant decrease in GFR in both SW and 85% SW-acclimated preparations (Figure 3.1). On perfusion with 10^{-10} M

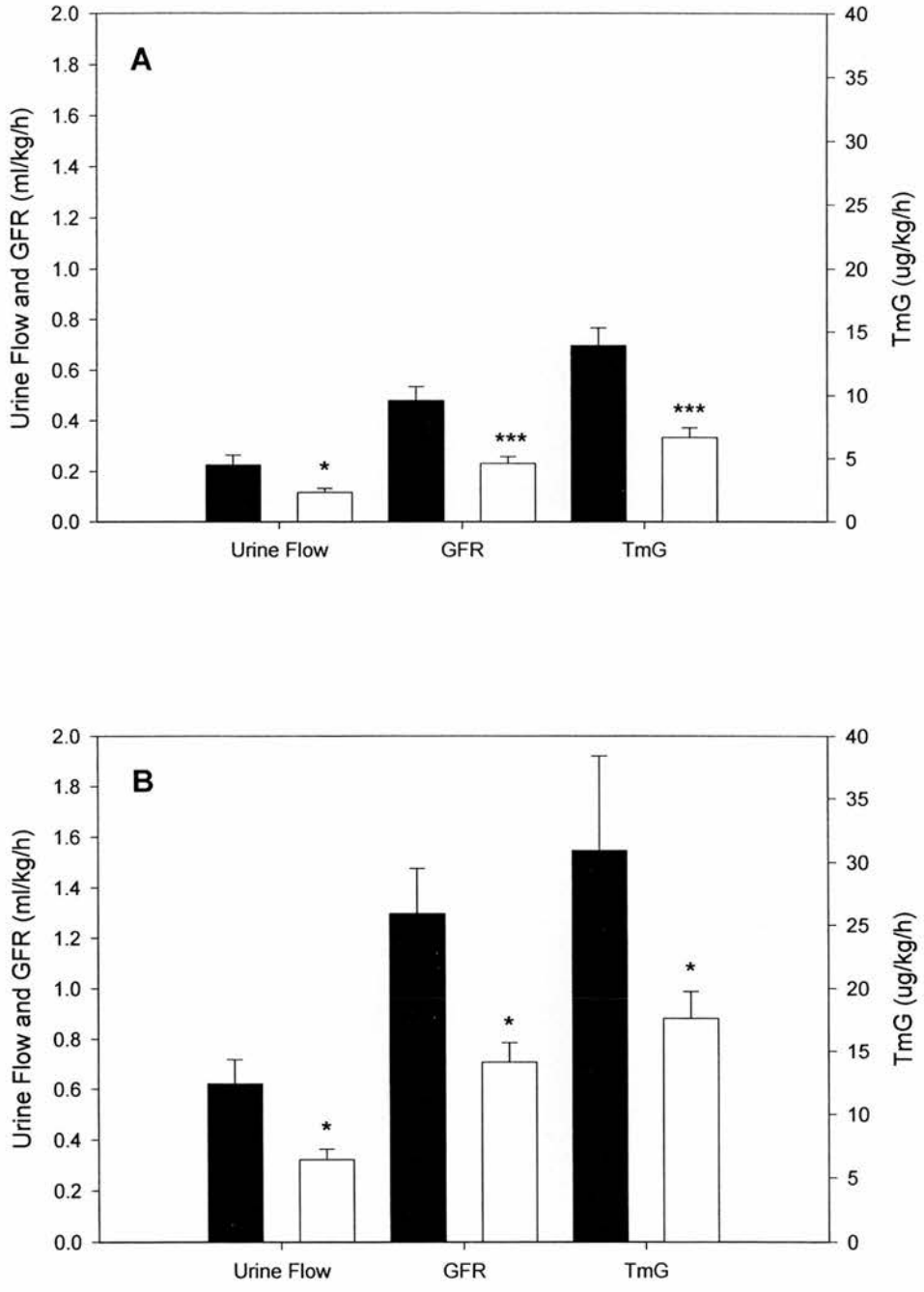

Fig. 3.1. Urine flow rate, GFR and TmG of *in situ* perfused dogfish kidney (10^{-9} M AVT). Comparisons are between the final renal clearance period immediately before AVT administration (solid bars) and for the final clearance period during AVT administration (open bars) in fish acclimated to SW (A) and 85% SW (B). Values are means ± SEM; n=6 in each group. $*P < 0.05$, and $***P < 0.005$.

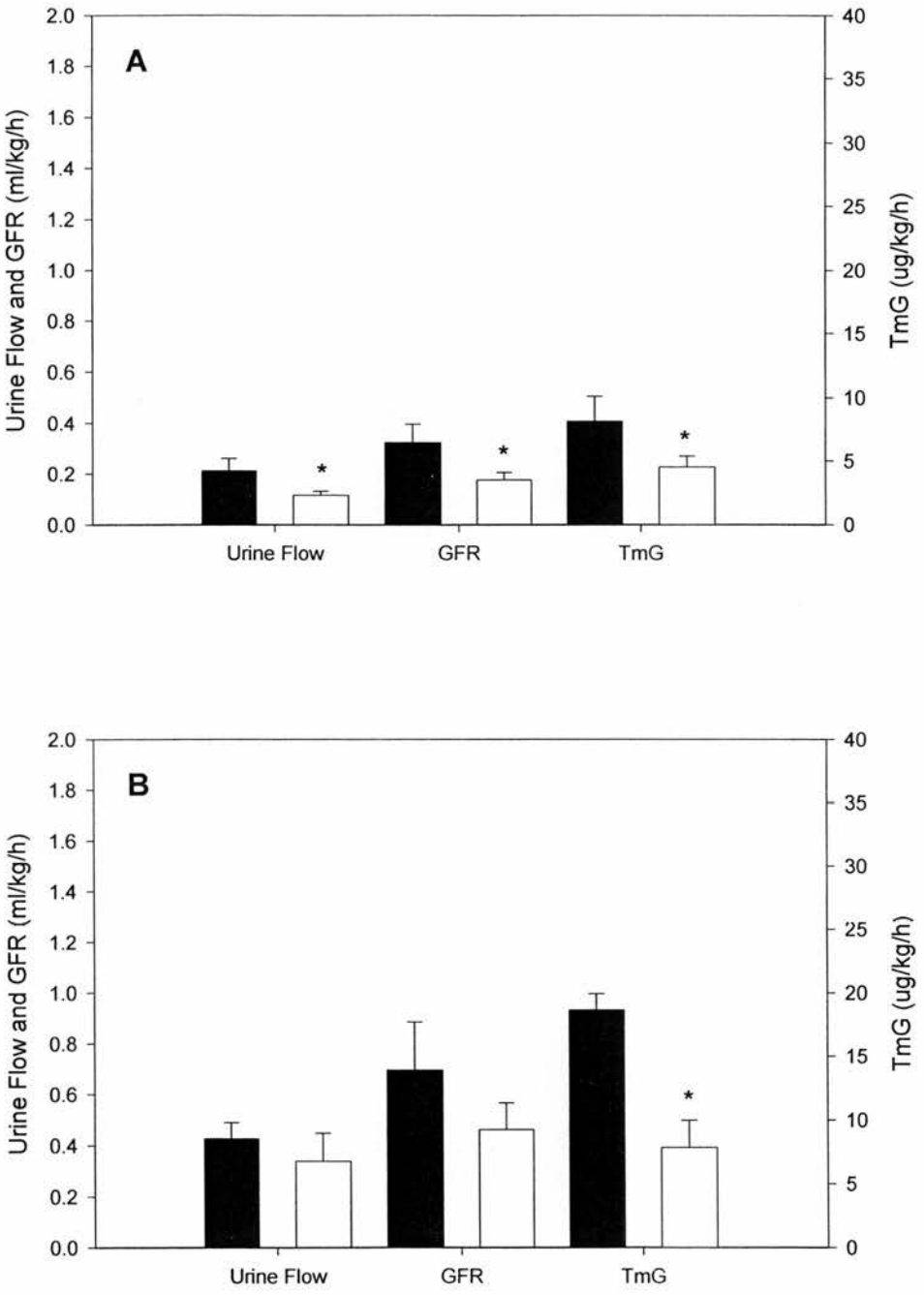

Fig. 3.2. Urine flow rate, GFR and TmG of *in situ* perfused dogfish kidney (10^{-10} M AVT). Comparisons are between the final renal clearance period immediately before AVT administration (solid bars) and for the final clearance period during AVT administration (open bars) in fish acclimated to SW (A) and 85% SW (B). Values are means ± SEM; n=6 in each group. *$P < 0.05$.

AVT, there was a significant decrease in GFR in SW, but no effect in 85% SW-acclimated preparations (Figure 3.2).

3.3.4. Functional Tubular Mass

Addition of 10^{-9} M and 10^{-10} M AVT to the perfusate caused a significant reduction in TmG values in both experimental salinities (Fig. 3.1 & 3.2).

	U/P_{in}	U/P_{osm}	C_{osm}/GFR	C_{H_2O}/GFR
Control (SW)	1.5 ± 0.1	0.994 ± 0.002	67.0 ± 3.9	0.4 ± 0.1
10^{-10} M AVT	1.5 ± 0.1	0.997 ± 0.002	68.9 ± 3.9	0.2 ± 0.1
Control (SW)	2.2 ± 0.1	0.997 ± 0.008	44.5 ± 2.2	0.2 ± 0.1
10^{-9} M AVT	2.0 ± 0.1	0.998 ± 0.006	50.1 ± 1.9	0.1 ± 0.1
Control (85% SW)	1.6 ± 0.4	0.998 ± 0.008	78.3 ± 13.8	0.7 ±0.6
10^{-10} M AVT	1.7 ± 0.3	0.985 ± 0.007	68.2 ± 11.1	1.1 ± 0.6
Control (85% SW)	1.9 ± 0.4	0.995 ± 0.004	51.5 ± 10.6	0.5 ± 0.3
10^{-9} M AVT	2.3 ± 0.2	0.994 ± 0.003	45.7 ± 4.7	0.3 ± 0.1

Table 3.4. Effect of AVT on tubular function in the *in situ* perfused trunk preparation. Values are mean ± S.E.M. from 6 fish in each group. U/P_{in}, urine/perfusate inulin concentration ratio; U/P_{osm}, urine/perfusate osmolality ratio; C_{osm}/GFR, relative clearance of osmolytes; C_{H2O}/GFR, relative free water clearance.

3.3.5. Tubular effects

Tubular function is summarised in Table 3.4. The mean urine/plasma inulin concentration ratio (U/P ratio) indicates that on average 44% of the filtered volume of water was reabsorbed by the renal tubule. Urine was slightly hypotonic to plasma with a

mean U/P_{osm} of 0.99. This resulted in a small relative free water clearance of less than 1%. The relative osmolar clearance demonstrates that on average approximately 59% of filtered osmolytes were excreted. Perfusion of AVT had no significant effect on any of the tubular parameters measured.

3.3.6. Effects on ion clearance and excretion

Addition of 10^{-9} M AVT to the perfusate resulted in a significant decrease in urea clearance and urea excretion, in fish acclimated to both SW and 85% SW (Fig 3.3.). However, the fractional excretion of urea remained unchanged following perfusion of 10^{-9} M AVT. Addition of 10^{-10} M AVT to the perfusate also resulted in a significant decrease in urea clearance and urea excretion, in fish acclimated to SW but there was no significant effect in fish acclimated to 85% SW (Fig. 3.4.). A similar pattern to that observed for urea was observed for chloride and sodium at both doses of AVT in both salinities. There was a significant decrease in clearance and excretion of these ions in all experimental groups apart from the 85% SW group perfused with 10^{-10} M AVT, in which no significant effects were observed (Figs 3.5.-3.8.). There were no significant effects on the fractional excretion of ions or urea in either salinity at either dose of AVT (Figs 3.3.-3.8.).

3.3.7. Relationship between urine flow rate and GFR

Figure 3.9. shows the relationship between urine flow rate and GFR. There was a clear linear relationship both before and after perfusion with AVT in SW and 85% SW.

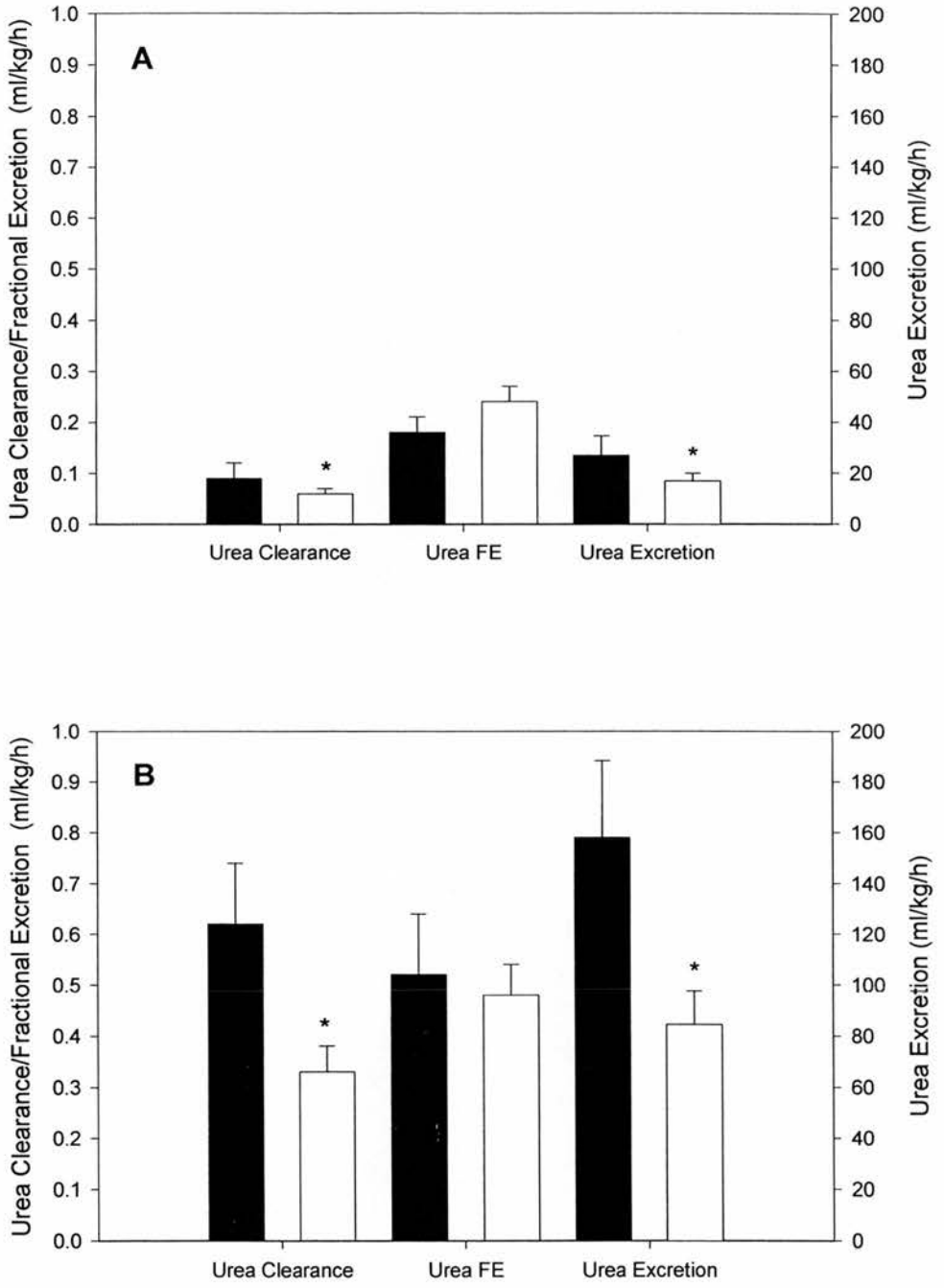

Fig. 3.3. Urea clearance, fractional excretion and excretion in the *in situ* perfused dogfish kidney (10^{-9} M AVT). Comparisons are between the final renal clearance period immediately before AVT administration (solid bars) and for the final clearance period during AVT administration (open bars) in fish acclimated to SW (A) and 85% SW (B). Values are means ± SEM; n=6 in each group. *$P < 0.05$.

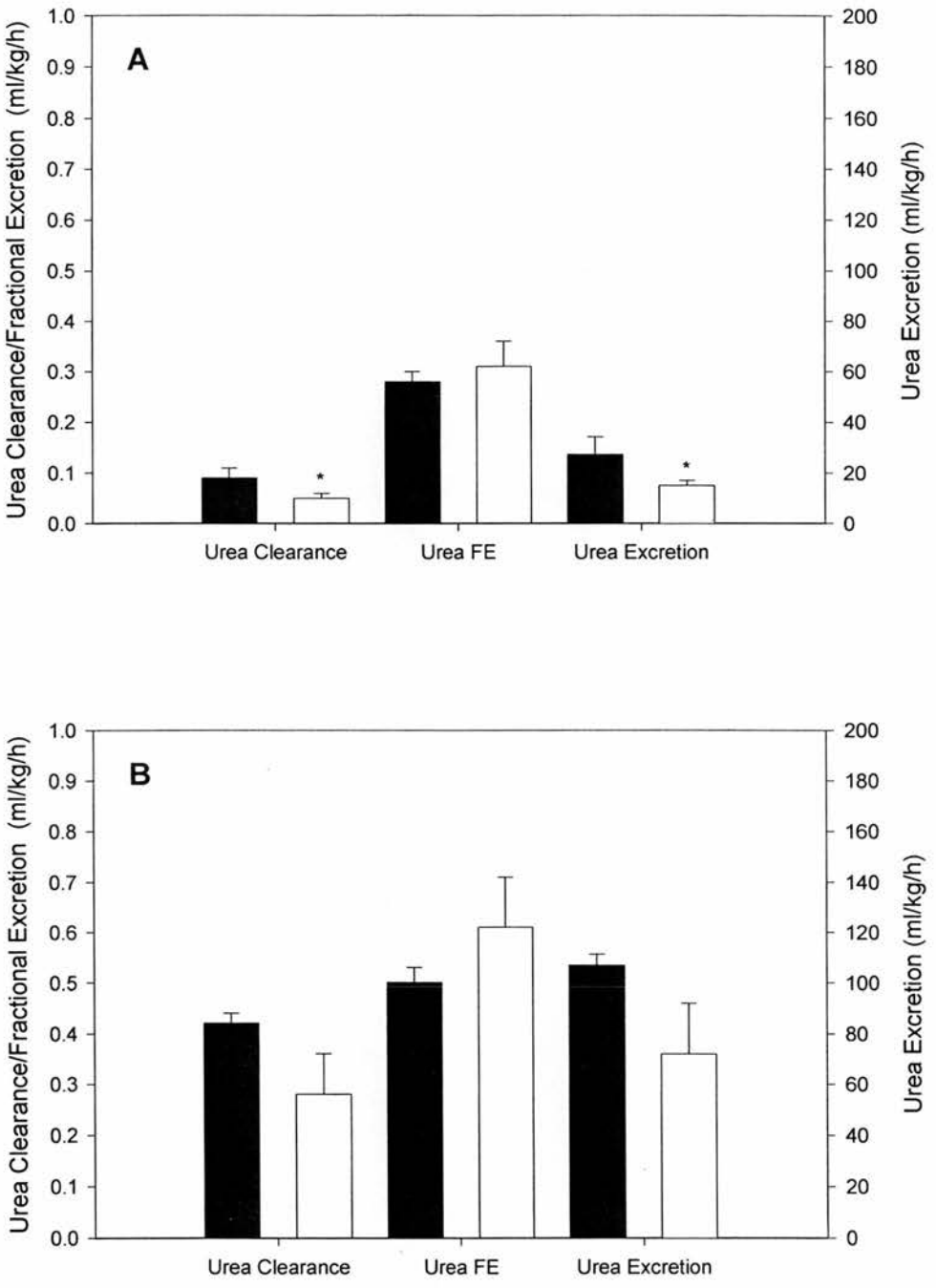

Fig. 3.4. Urea clearance, fractional excretion and excretion in the *in situ* perfused dogfish kidney (10^{-10} M AVT). Comparisons are between the final renal clearance period immediately before AVT administration (solid bars) and for the final clearance period during AVT administration (open bars) in fish acclimated to SW (A) and 85% SW (B). Values are means ± SEM; n=6 in each group. *$P < 0.05$.

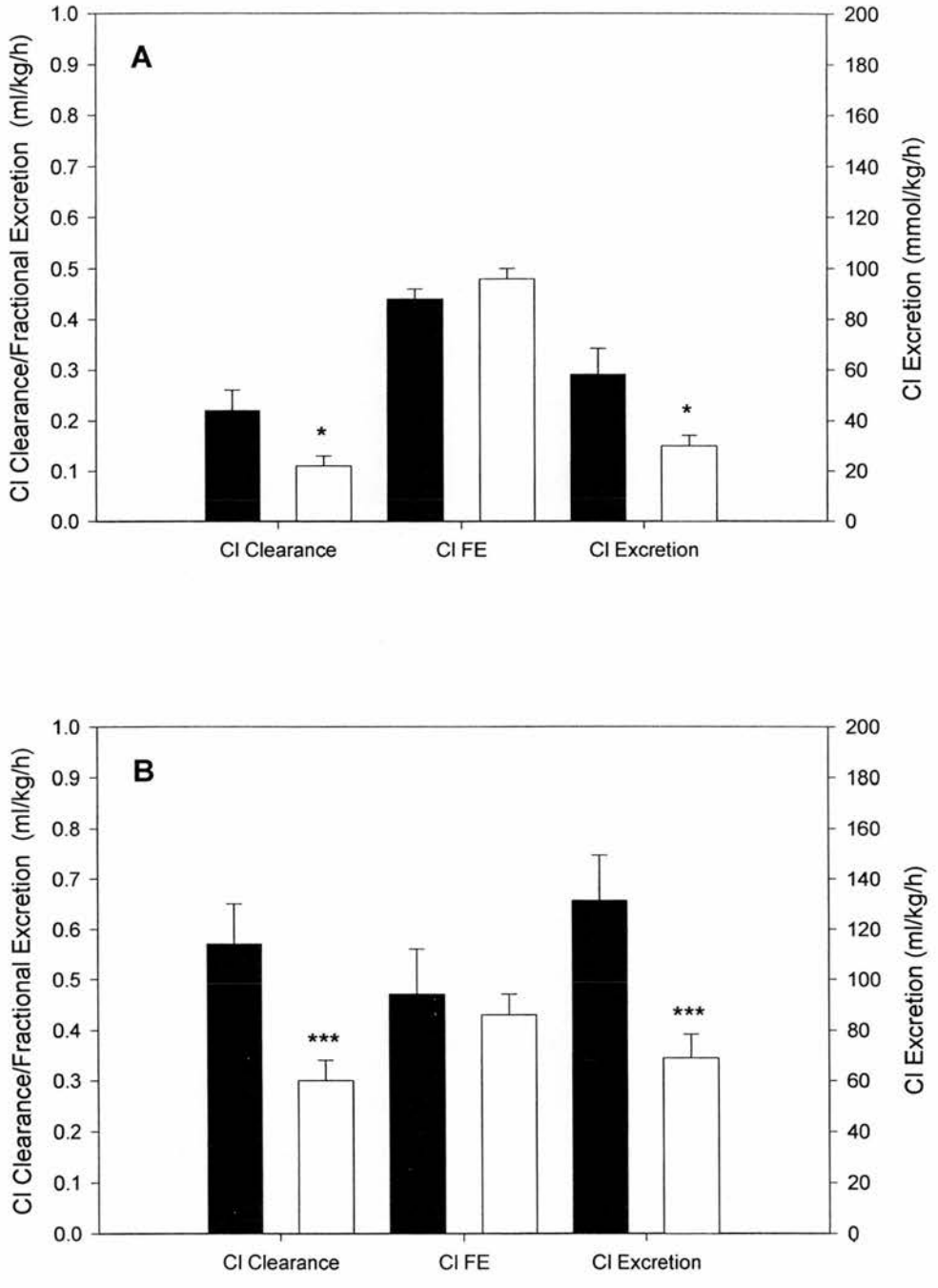

Fig. 3.5. Chloride clearance, fractional excretion and excretion in the *in situ* perfused dogfish kidney (10^{-9} M AVT). Comparisons are between the final renal clearance period immediately before AVT administration (solid bars) and for the final clearance period during AVT administration (open bars) in fish acclimated to SW (A) and 85% SW (B). Values are means ± SEM; n=6 in each group. *$P < 0.05$ and ***$P < 0.005$.

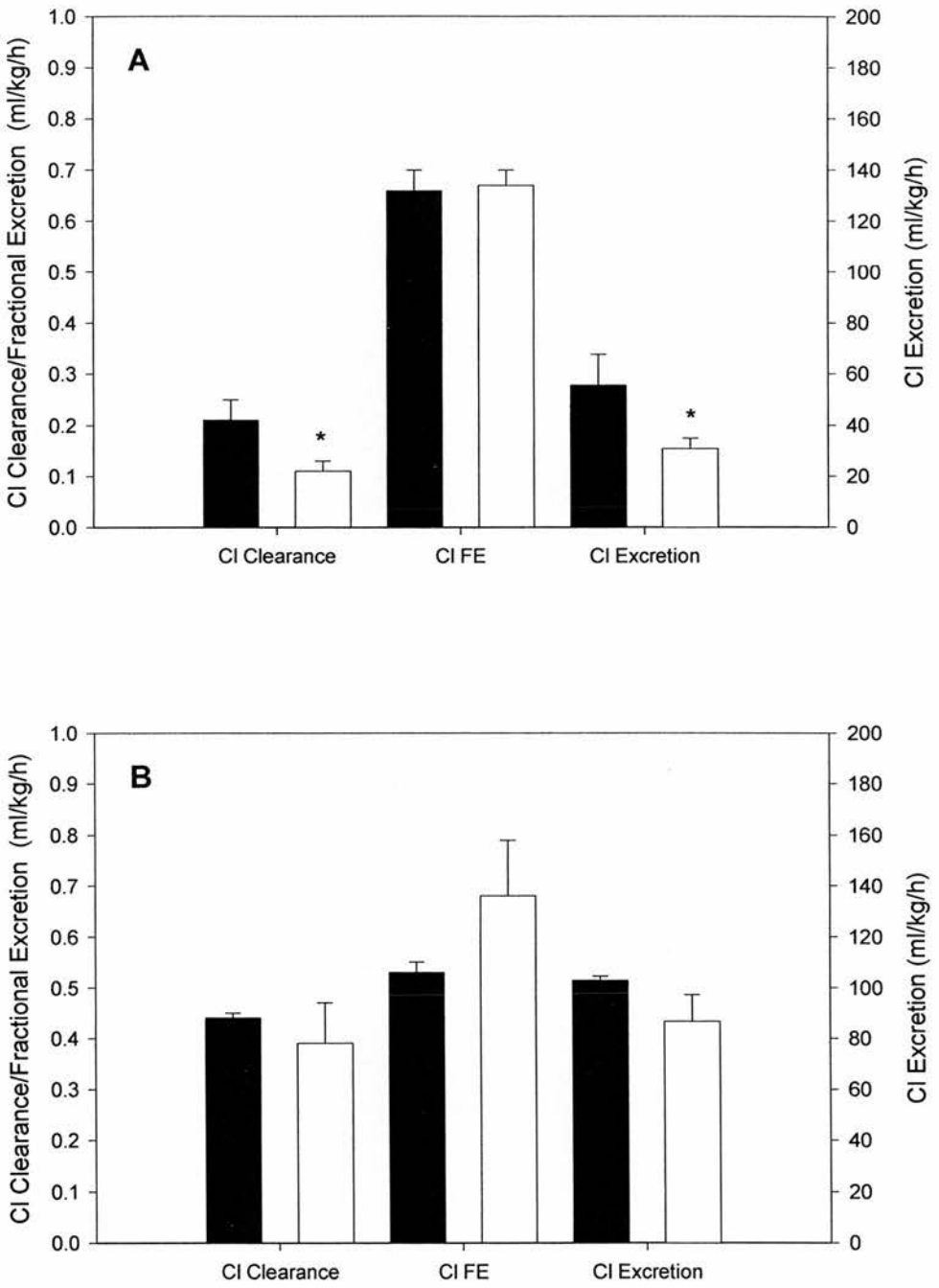

Fig. 3.6. Chloride clearance, fractional excretion and excretion in the *in situ* perfused dogfish kidney (10^{-10} M AVT). Comparisons are between the final renal clearance period immediately before AVT administration (solid bars) and for the final clearance period during AVT administration (open bars) in fish acclimated to SW (A) and 85% SW (B). Values are means ± SEM; n=6 in each group. *$P <$ 0.05.

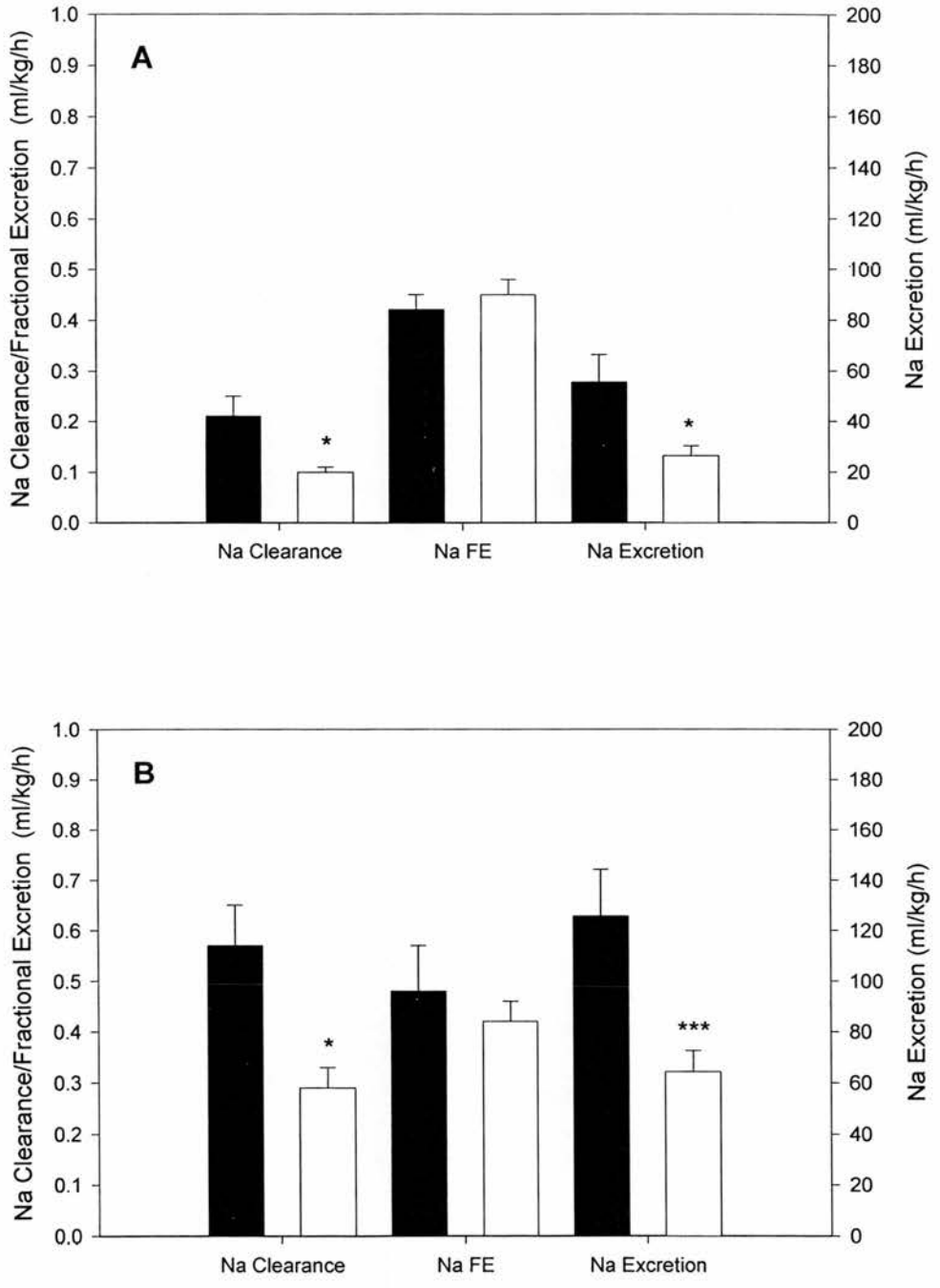

Fig. 3.7. Sodium clearance, fractional excretion and excretion in the *in situ* perfused dogfish kidney (10^{-9} M AVT). Comparisons are between the final renal clearance period immediately before AVT administration (solid bars) and for the final clearance period during AVT administration (open bars) in SW (A) and 85% SW (B). Values are means ± SEM; n=6 in each group. $*P < 0.05$ and $***P < 0.005$.

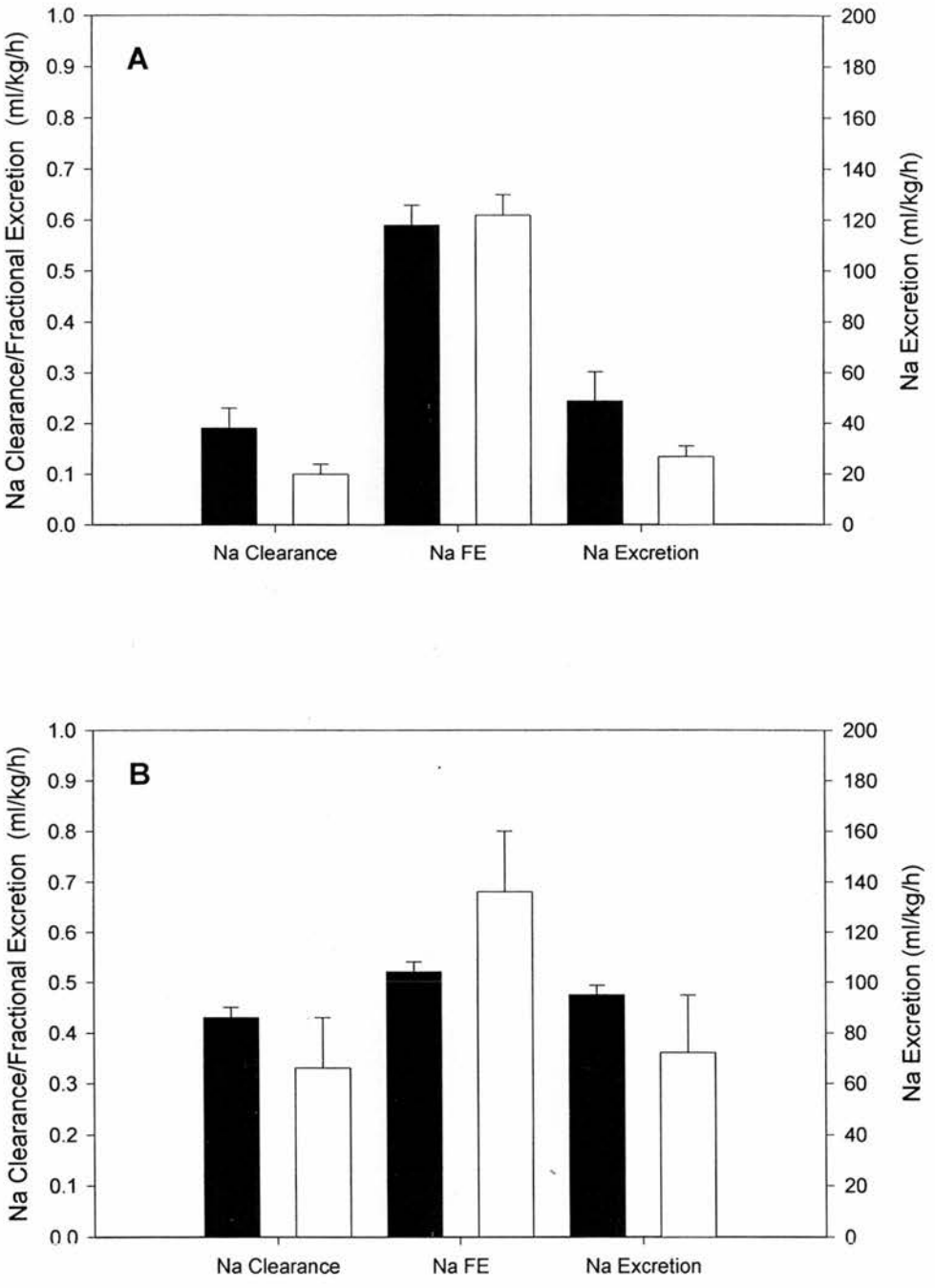

Fig. 3.8. Sodium clearance, fractional excretion and excretion in the *in situ* perfused dogfish kidney (10^{-10} M AVT). Comparisons are between the final renal clearance period immediately before AVT administration (solid bars) and for the final clearance period during AVT administration (open bars) in fish acclimated to SW (A) and 85% SW (B). Values are means ± SEM; n=6 in each group.

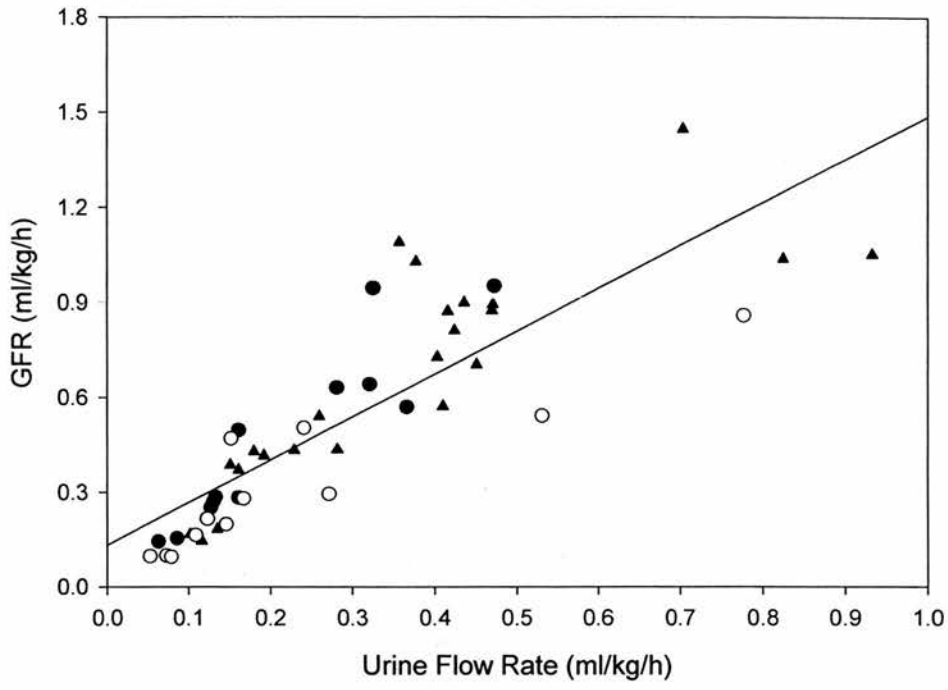

Fig. 3.9. Relationship between GFR and urine flow rate in the perfused kidney of the dogfish. $R_2 = 0.605$. Values are mean rates before perfusion of AVT (▲), and following perfusion with 10^{-9} M AVT (●) and 10^{-10} M AVT (○) in fish acclimated to both SW and 85% SW.

3.4. Discussion

The renal effects of AVT have been examined using an isolated perfused trunk preparation of the rainbow trout, *O. mykiss* (Amer and Brown, 1995; Brown and Balment, 1997). AVT caused a clear and immediate antidiuresis that largely reflected decreased glomerular filtration rates. In the same study, the tubular transport maxima for glucose was also decreased, implying reduced populations of filtering glomeruli.

The present study demonstrates that in the dogfish, there is a very similar renal action of AVT to that seen in the trout. Addition of 10^{-9} M AVT was found to have a profound, glomerular antidiuretic effect. An antidiuresis and reduction in GFR were observed in the first hour following administration of AVT, but this effect did not reach significance until the second hour after AVT administration. The reasons for this delay were discussed in section 2.4.2. A similar glomerular antidiuresis was observed following perfusion with 10^{-10} M AVT in fish acclimated to SW. However, despite a reduction in urine flow rate and GFR following perfusion with 10^{-10} M AVT in 85% SW, this reduction did not reach significance within 2 hours of perfusion. It is possible that given a slightly larger sample size that this may have been a significant effect. However, this phenomenon may also be a function of the increased basal urine flow in 85% SW. It may be the case that a higher threshold dose of AVT is required to initiate significant renal effects at the higher urine flow rates observed in 85% SW. This will be discussed further in Chapter 4 and Chapter 7.

AVT caused a significant decrease in perfusate flow rate at both 10^{-9} M and 10^{-10} M. A drop in perfusate flow rate was also observed in the perfused trout kidney on addition of

10^{-9} and 10^{-11} M AVT to the perfusate (Amer and Brown, 1995) and was attributed to a general vasoconstriction. Vasoconstriction has been demonstrated in the trout in response to AVT (Conklin *et al.*, 1999), and indeed, the vascular action of AVT has been proposed to be entirely responsible for the renal responses in lower vertebrates (Pang *et al.*, 1983).

Most teleost fish show linear relationships between urine flow and glomerular filtration rates, and this trend has also been observed in *S. canicula* (Brown and Green, 1987). This linear relationship suggests that extracellular fluid volume is principally regulated by changes in GFR. A linear relationship has also been demonstrated in the perfused dogfish trunk preparation both before and after perfusion with AVT (Figure 3.9.), providing further evidence that the perfused trunk preparation behaves in a manner similar to the whole animal.

Filtering populations of nephrons were assessed indirectly by measurement of TmG. Addition of 10^{-9} M AVT and 10^{-10} M AVT resulted in significant decreases in TmG. TmG in control preparations was stable (Figure 2.7.) so changes in TmG induced by addition of AVT to the perfusate are indicative of changes in the population of functional glomeruli. Recently the presence of glomerular bypass shunts was shown in the kidney of *S. canicula* (Brown and Green, 1992). This shunt arose from the afferent arteriole to join a peritubular network of capillaries and thereby offers the potential to vary the degree of glomerular perfusion and control the proportion of filtering glomeruli. It is possible that AVT may act at these shunts to decrease the population of filtering nephrons. However, interpretation of TmG data must be made with care as there is growing evidence that TmG may result from an alteration in SNGFR coupled

with altered tubular handling of sodium (Brown *et al.*, 1993). In addition, it is clear that there are relatively large differences in the absolute levels of TmG in control animals, before perfusion with AVT. In the light of these difficulties the filtering population of glomeruli will also be measured using a more direct method of visualising filtering glomeruli by perfusing with ferrocyanide (See Chapter 6).

U/P_{in} was unchanged during perfusion with AVT, indicating that AVT had no net effect on tubular water permeabilities. Relative clearance of osmolytes and free water clearance were also unchanged by the addition of AVT to the perfusate. The values for U/P_{osm} and C_{H_2O}/GFR closely matched *in vivo* values in *S. canicula* (Brown and Green, 1987). Therefore, AVT did not appear to affect tubular function, at least within the perfused renal preparation.

Perfusion of 10^{-9} M AVT caused a significant decrease in urea excretion and urea clearance, but had no significant effect on the fractional excretion of urea. This suggests that the decrease in renal urea excretion can be totally accounted for by the decrease in GFR since there was no change in the fractional excretion of urea. There was a qualitatively similar effect in SW at 10^{-10} M AVT but no significant effect on the clearance, excretion or fractional excretion of urea was observed following perfusion with 10^{-10} M AVT in 85% SW. This provides further evidence that this reduction in urea excretion was entirely due to the decrease in GFR, because there was a significant glomerular antidiuresis in SW, but not in 85% SW at 10^{-10} M AVT.

The patterns of clearance, excretion and fractional excretion of chloride and sodium were very similar to those observed for urea. Perfusion of 10^{-9} M AVT caused a

significant decrease in excretion and clearance of these ions, but had no significant effect on fractional excretion. There was a similar effect in SW at 10^{-10} M AVT but no significant effect on the clearance, excretion or fractional excretion was observed following perfusion with 10^{-10} M AVT in 85% SW.

In summary, addition of AVT to the perfusate resulted in a decrease in urine flow rate, GFR and TmG, driving a decrease in urea and sodium chloride clearance in both SW- and 85% SW-acclimated preparations. These data suggest that AVT induced a glomerular antidiuresis in *S. canicula*, perhaps in a manner similar to that previously reported in the trout.

4

Effects of Ang II on the perfused dogfish trunk

4.1. Introduction

The aim of this chapter was to investigate the renal role of angiotensin II (Ang II).

The renin-angiotensin system (RAS) (Fig. 4.1.) is an important endocrine system in vertebrates. Ang I, II and III have all been reported as bioactive components of the cascade but to date Ang II has been shown to exert the greatest physiological effects. In mammals, Ang II is known to be vasopressor, dipsogenic, a stimulant of adrenocortical secretion and to control glomerular and renal tubular function (Peach, 1977).

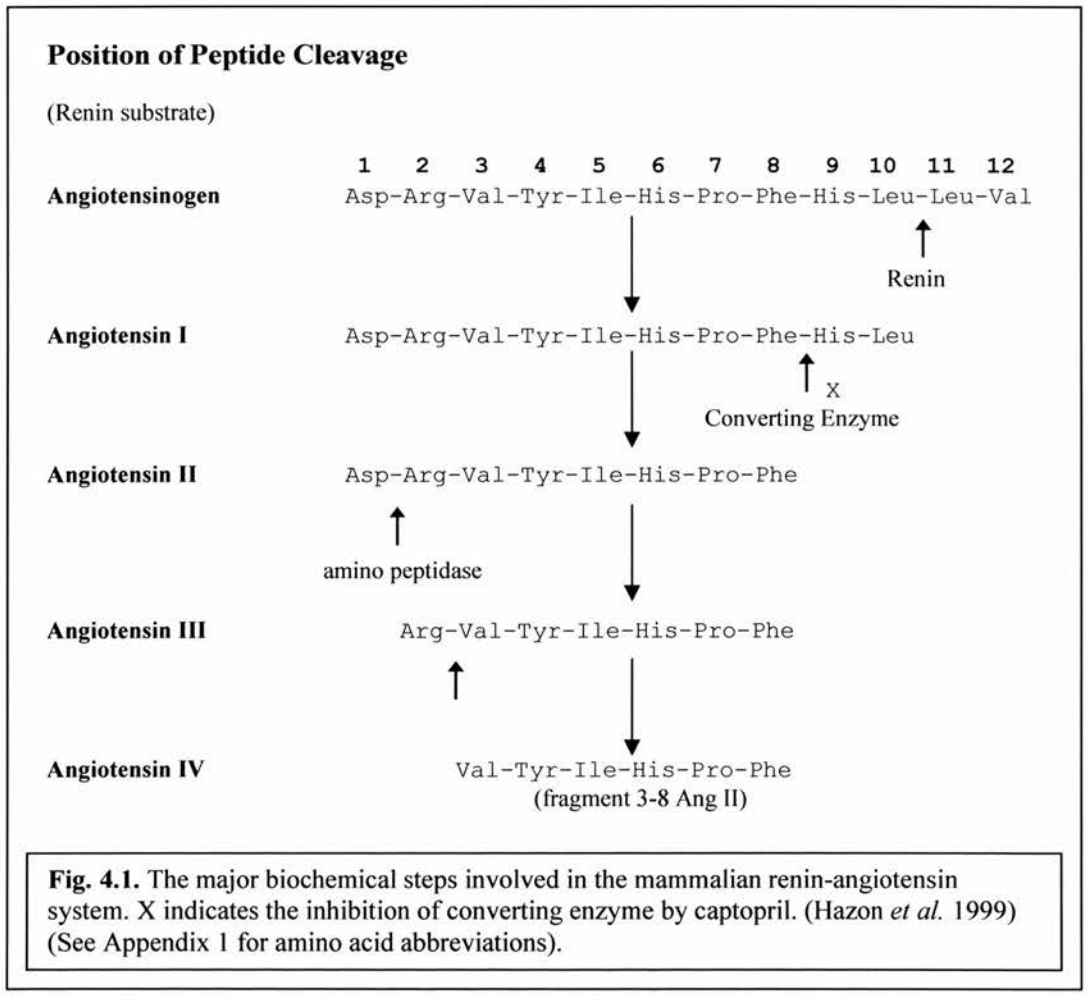

Fig. 4.1. The major biochemical steps involved in the mammalian renin-angiotensin system. X indicates the inhibition of converting enzyme by captopril. (Hazon *et al.* 1999) (See Appendix 1 for amino acid abbreviations).

During initial investigations into the presence of an elasmobranch RAS, no pressor-like activity was observed when crudely extracted elasmobranch kidneys were injected into an anaesthetised dog (Bean, 1942). Similarly, Nishimura *et al.* (1970) found no pressor-like activity when kidney extracts from four elasmobranch species, which had been pre-incubated with homologous plasma, were injected into the rat. In addition, anatomical studies suggested the lack of renin-containing, granulated, juxta-glomerular (JG) cells in elasmobranch fish (Capreol and Sutherland, 1968; Nishimura *et al.*, 1970) (See Section 1.2. & Figure 1.6.). It was therefore concluded that elasmobranch fish lacked the components of a functional RAS, and a RAS analogous to that found in mammals first appeared during the early evolution of bony fishes (Nishimura *et al.*, 1973).

There was however, some evidence at that time that elasmobranch fish possessed at least some renin-like activity. Henderson *et al.* (1981) reported that dogfish renal extracts, incubated with rat angiotensinogen, generated angiotensin-like pressor materials which elevated the blood pressure of nephrectomised rats. In addition, histological and electron microscopy studies identified granular peripolar cells with a macula densa in a number of elasmobranch species, demonstrating the presence of a JG apparatus (Lacy and Reale, 1989; Lacy and Reale, 1990; Reale and Lacy, 1987). These granules, which characterise JG cells, contain both renin and angiotensin in mammals, and their presence suggests renin activity (Lacy and Reale, 1990). The presence of a RAS in *S. canicula* was further supported by Uva *et al.* (1992) who found that a renal extract of *S. canicula* elevated arterial blood pressures of rats and dogfish. This renal extract also generated Ang I from both porcine and synthetic angiotensinogen, and the subsequent generation of Ang II was inhibited following the addition of the ACE

inhibitor captopril. Conclusive evidence that elasmobranch fish possess a RAS analogous to that in mammals was provided by Takei *et al.* (1993), who isolated and purified endogenous elasmobranch Ang I from the Japanese dogfish, *Triakis scyllia*. Table 4.1. shows the structure of Ang I from various vertebrates. There are several amino acid substitutions in elasmobranch Ang I in comparison with mammalian Ang I, but the most profound difference, particularly affecting the tertiary structure of the peptide, is the substitution of valine by proline at position 3. This unique structure may explain the lack of bioactivity when elasmobranch renal extracts, which had been pre-incubated with homologous plasma, were bioassayed in the rat (Nishimura *et al.*, 1970).

	Amino Acid Sequence									
	1	2	3	4	5	6	7	8	9	10
Common Structure	-	Arg	Val	Tyr	-	His	Pro	Phe	-	Leu
Species Variation										
Human, pig, rabbit, rat, horse and dog	Asp				Ile				His	
Bovine	Asp				Val				His	
Chicken	Asp				Val				Ser	
Turtle	Asp				Val				His	
Alligator	Asp				Val				Ala	
Bullfrog	Asp				Val				Asn	
Goosefish	Asn				Val				His	
Salmon	Asn				Val				Asn	
Eel	Asn				Val				Gly	
Elasmobranch	Asn		**Pro**		Ile				Gln	

Table 4.1. Angiotensin I amino acid sequence from various vertebrates (Takei *et al.* 1993) (See Appendix 1 for amino acid abbreviations).

4.1.1. Osmoregulatory effects

In higher vertebrates, it is recognised that a major function of the RAS is the endocrine control of water and ion balance and that the kidney is a principal site for the action of Ang II (Kobayashi and Takei, 1996). However, in addition to the kidneys, elasmobranchs along with other non-mammalian aquatic vertebrates, possess a range of organs involved in salt and water balance. Osmoregulation in elasmobranch fish

therefore involves an integrated response between the gills, intestine, rectal gland and kidney (Chapter 1). Given the presence of a RAS and the role that it plays in higher vertebrates, it is reasonable to predict that the RAS may influence the role and function of all these organs in elasmobranchs. However, the details of the endocrine control of these organs is not yet fully understood (Anderson *et al.*, 2001a).

To date, there have been no published reports on the renal actions of angiotensin II in elasmobranchs. The presence of JG cells and a macula densa in elasmobranchs (Lacy and Reale, 1989; Lacy and Reale, 1990) indicates that the RAS may be involved in the control of GFR as occurs in teleosts (Brown *et al.*, 1980). In addition, the specific binding of homologous ^{125}I-labelled Ang II to kidney membrane preparations from *T. scyllia* indicates the presence of renal Ang II receptors (Tierney *et al.*, 1997b). However, the exact location and mechanism of action of these receptors has yet to be established.

Specific Ang II binding has been reported in gill cell membrane preparations and branchial blood vessels of *T. scyllia* (Tierney *et al.*, 1997b). In the same investigation, displacement studies using the AT_2-type receptor antagonist CGP42112, indicated the presence of an AT_2-like Ang II receptor. However, the use of antagonists developed for mammalian studies is not ideal in lower vertebrates, where the specific structure of the receptor may differ. It is therefore clear that further research is required before the physiological role of Ang II in terms of gill function can be clarified.

A number of peptides have been implicated in rectal gland secretion rate (See Section 1.6.1.). However, the role of Ang II in rectal gland function has not been investigated to

any great extent. Preliminary studies have suggested a possible inhibitory role for Ang II (Anderson *et al.*, 1995b) in terms of rectal gland secretion. It has also been suggested that secretion from the rectal gland was closely related to blood flow through the secretory tissue (Shuttleworth and Thompson, 1983; Shuttleworth and Thompson, 1986). Recent investigations have demonstrated an increase in vascular resistance of the isolated perfused rectal gland of *S. canicula* following homogenous Ang II administration (Anderson *et al.*, 2001a). It is possible that the vasorelaxant actions of homologous CNP (see Chapter 5) (Bjenning *et al.*, 1992) coupled with the vasoconstrictor actions of Ang II (Hamano *et al.*, 1998) could combine to create a complex shunting system of blood flow to and from the secretory parenchyma of the rectal gland (Anderson *et al.*, 2001a). Specific binding of heterologous Ang II to the rectal gland of *S. canicula* and a nurse shark *Ginglymostoma cirratum*, has been reported (Galli and Cook, 1993; Masini *et al.*, 1994). More recently, binding of homologous ^{125}I-labelled Ang II was found to be most abundant in the capsular region of the rectal gland of *S. canicula,* perhaps indicating a role in controlling blood flow through the rectal gland (Hazon *et al.*, 1997a). Competitive binding in the capsular region showed greater affinity for the mammalian AT_2 receptor antagonist CGP42112 suggesting the presence of an AT_2-like receptor although it is difficult to draw conclusions from the use of mammalian receptor antagonists (see above).

In higher vertebrates, one of the primary functions of the RAS is its dipsogenic role (Kobayashi and Takei, 1996). Elasmobranchs were classically considered not to drink (Kobayashi *et al.*, 1983; Smith, 1936) and therefore there has been little work on the osmoregulatory role of the elasmobranch gut. However, drinking has been demonstrated in *S. canicula* by gut accumulation of radioisotopically labelled marker (Hazon *et al.*,

1989), and pharmacological manipulation of the endogenous RAS, using the smooth muscle relaxant Papaverine and the ACE-inhibitor captopril, implicated the RAS in the control of drinking in elasmobranchs. Furthermore, using the same technique, drinking rates of *S. canicula* have been shown to vary in response to changes in environmental salinity (Hazon *et al.*, 1997b). Using a technique of oesophageal cannulation to directly measure drinking rate in *T. scyllia* and *S. canicula* (Anderson *et al.*, 2001b), homologous Ang II has been shown to stimulate drinking in a dose dependant manner. This led the authors to suggest the presence of a brain Ang II receptor involved in the control of drinking in elasmobranch fish as was previously suggested in teleost fish (Cobb and Brown, 1992; Takei *et al.*, 1985). Tierney *et al.* (1997b) found specific Ang II binding in the brain of *S. canicula*. However, the position and mechanism of these Ang II receptors located in the brain of elasmobranchs remains to be determined (Tierney *et al.*, 1997b).

4.1.2. Cardiovascular effects

Vasopressor effects of heterologous angiotensins have been demonstrated in *Squalus acanthias* (Opdyke and Holcombe, 1976), where [Asp^1-Ile^5] Ang I and [Asp^1-Val^5] Ang II caused a sustained increase in mean arterial pressure. In addition, injection of the ACE-inhibitor SQ-20881 blocked the pressor response of heterologous Ang I but did not effect either resting blood pressure or the Ang II-mediated pressor response. Similarly, Hazon *et al.* (1989) reported vasopressor effects following intra-muscular injection of heterologous [Asp^1-Ile^5] Ang II in *S. canicula*. Furthermore, the recovery of mean arterial pressure following injection of Papaverine, was abolished following an injection of captopril, suggesting that this hypertension is associated with the activation of an endogenous RAS.

In mammals, there is a well-established effect of Ang II-mediated release of catecholamines from adrenal medulla cells (Peach, 1971). This effect has been investigated in elasmobranchs (Opdyke and Holcombe, 1976) and it was demonstrated that the α-adrenergic receptor blocker, phentolamine, completely blocked the heterologous [Asp^1-Val^5] Ang II-mediated pressor response in *S. acanthias*. In a further study, the same authors demonstrated that infusion of heterologous [Asp^1-Ile^5] Ang I and [Asp^1-Val^5] Ang II through the dogfish gut did not increase vascular resistance, although epinephrine significantly increased vascular resistance (Opdyke and Holcombe, 1978). Using a coeliac artery ring preparation from *S. acanthias*, Carrol (1981) observed no vasopressor activity from teleost [Asn^1-Val^5] Ang II, but a significant increase in resting tension was observed following the administration of epinephrine. In addition, the plasma levels of the catecholamines epinephrine and norepinephrine were significantly elevated in *S. acanthias* following the injection of [Asn^1-Val^5] Ang II (Opdyke *et al.*, 1981). These results strongly suggest that catecholamines may play a mediating role in the Ang II-induced pressor response in elasmobranch fish. However, it has also been postulated that an Ang II-like peptide and catecholamines may act through a common vascular receptor (Opdyke and Holcombe, 1976).

Hazon *et al.* (1995) investigated the vasoconstrictor effects of the mammalian Ang II antagonists [Sar^1-Ile^8]-Ang II and [Sar^1-Val^5-Ala^8]-Ang II in *T. scyllia*. [Sar^1-Val^5-Ala^8]-Ang II had no effect on basal arterial blood pressure whereas [Sar^1-Ile^8]-Ang II produced a dose dependant pressor response. Neither antagonist blocked the increase in blood pressure caused by subsequent injection of homologous Ang II. Takei *et al.*

(1993) showed that homologous dogfish angiotensin was more potently vasopressor in the dogfish compared to rat angiotensin by a factor of 22.6, with the reverse relationship occurring in the rat. These results indicate that vascular Ang II receptors in elasmobranchs differ from those found in other vertebrates, suggesting the co-evolution of Ang II receptors with the unique peptide structure of elasmobranch Ang II.

More recent studies employing homologous elasmobranch [Asn^1-Pro^3-Ile^5] Ang II have demonstrated that the pressor response induced following the injection of Ang II is at least in part independent of catecholamine release. Both noradrenaline and homologous Ang II elicited an increase in mean arterial pressure in *S. canicula* (Tierney *et al.*, 1997a). Pre-treatment with phentolamine completely abolished the noradrenaline response but after an initial delay did not affect the magnitude of the homologous Ang II pressor response. These results led the authors to suggest that the pressor action of Ang II appears to be a direct Ang II-mediated vascular response, but that both catecholamines and Ang II are required for the complete vasopressor effect (Tierney *et al.*, 1997a). Phentolamine was also shown to abolish the vasopressor effects of norepinephrine in three different isolated arterial ring preparations in *T. scyllia* (coeliac, branchial and ventral aorta) (Hamano *et al.*, 1998). In the same study, phentolamine had no effect on the vasopressor effect of homologous Ang II. This evidence contradicts the previous suggestion of catecholamines mediating the Ang II pressor response and provides additional evidence of the presence of specific Ang II vascular receptors in elasmobranchs. However, the results obtained from *S. canicula* (Tierney *et al.*, 1997a) contrast with recent studies in *S. acanthias* (Bernier *et al.*, 1999), in which the pressor effects of [Asn^1-Pro^3-Ile^5] Ang II were reported to be mediated through the release of catecholamines. Cardiovascular effects of adrenoreceptor blockade appear to differ

between the two species, indicating a species difference in the way in which homologous Ang II pressor response is controlled (Anderson *et al.*, 2001a).

Specific Ang II binding and ACE-like activity have been found in the heart and branchial vessels of elasmobranchs (Tierney *et al.*, 1997b; Uva *et al.*, 1992). In displacement studies the mammalian Ang II AT_2 receptor antagonist, CGP42112, slightly reduced the binding of ^{125}I-labelled homologous ANG II in branchial blood vessels of *T. scyllia*, indicating the possible presence of an AT_2-like receptor in this tissue (Tierney *et al.*, 1997b). ACE-like activity has been identified in the heart of *R. erinacea*, *S. acanthias* and *S. canicula* (Lipke and Olson, 1988; Uva *et al.*, 1992). In the light of this Cerra *et al.* investigated the specific binding of Ang II in the heart of *S. canicula*, which was found to be most dense in the outer layer of the conus arteriosus [Cerra *et al.* (unpublished data) – quoted from Anderson *et al.* (2001a)]. The conus arteriosus is principally concerned with regulating the pulsatile nature of blood flow from the ventricle and is thought to be under hormonal control [Satchell (1971), quoted from Anderson *et al.* (2001a)].

Although the true nature of Ang II receptors in the cardiovascular system of elasmobranchs has yet to be elucidated, it is clear that catecholamines and Ang II play a role in the regulation of blood pressure in elasmobranchs. However, results suggest that the RAS may not be concerned with regulating resting blood pressure, but rather play a crucial role during situations such as acute blood volume loss and/or decrease in blood pressure (Anderson *et al.*, 2001a).

4.1.3. Adrenocortical effects

The elasmobranch interrenal gland is homogeneous in nature and has little or no contamination from chromaffin or renal tissue (Fig. 1.10.). This allows a unique opportunity to study the control of corticosteroidogenesis, the major corticosteroid in elasmobranch fish being 1α-hydroxycorticosterone (1α-OH-B) (Henderson *et al.*, 1986; Idler and Truscott, 1966). The specific role of 1α-OH-B was investigated by Armour *et al.* (1993a; 1993b), who suggested a mineralocorticoid role in elasmobranchs. It was suggested that 1α-OH-B may act at specific renal and extrarenal sites to reduce the loss of plasma sodium, and receptors for 1α-OH-B have been identified in the rectal gland, kidney and gills of the ray, *Raja ocellata* (Idler and Kane, 1980; Moon and Idler, 1974).

A major role of Ang II in higher vertebrates is the retention of sodium primarily through the stimulation of adrenal steroid release (Kobayashi and Takei, 1996). Both homologous renal extracts and heterologous [Asn^1-Val^5] Ang II have been shown to stimulate 1α-OH-B secretion *in vivo* (Hazon and Henderson, 1984; Hazon and Henderson, 1985) and *in vitro* (Armour *et al.*, 1993a; O'Toole *et al.*, 1990). Homologous Ang II was also found to be potently steroidogenic in the perfused interrenal tissue of *S. canicula* (Anderson *et al.*, 2001a). It would therefore appear that the elasmobranch RAS plays a role in corticosteroidogenesis, as is the case in higher vertebrates (Kobayashi and Takei, 1996) and also an indirect role in sodium retention in hyperosmotically challenged elasmobranchs (Anderson *et al.*, 2001a).

Specific Ang II binding has been demonstrated in 10 different membrane preparations taken from *T. scyllia*, with the greatest specific binding being found in interrenal tissue (Tierney *et al.*, 1997b). Specific binding of homologous ^{125}I-labelled Ang II was also

found to be greatest in the interrenal gland of *S. canicula* (Hazon *et al.*, 1995). Displacement studies using the mammalian antagonists CGP142112 (AT_2 specific) and CV11974 (AT_1 specific) found low specificity with only a small discrimination to CV11974 (Hazon *et al.*, 1995). However, it is clear that the structure and mode of action of the homologous Ang II receptor must be elucidated before strong conclusions can be drawn from this work.

4.1.4. Tissue specific renin-angiotensin systems

There is increasing support in mammals that, in addition to the systemic RAS, there are local renin-angiotensin systems responsible for the generation of Ang II within a wide range of tissues (Henderson *et al.*, 1993). These tissues include the kidney, heart, adrenal glands, gonadal tissue, brain, pituitary and arterial wall (Danser, 1996; Dzau, 1993; Erdos and Skidgel, 1990; Hackenthal *et al.*, 1990; Lindpainter and Ganten, 1991; Taugner and Ganten, 1982)

Ang II generated in the circulation may be supplemented by Ang II generated in the tissues of the kidney. Renin and angiotensinogen are present in the mammalian kidney and ACE occurs in endothelia of the renal vasculature, in the glomerulus and in the proximal brush border (Erdos and Skidgel, 1990; Taugner and Ganten, 1982). The evidence for a mammalian intra-renal RAS has been reviewed recently by Bader et al. (2001). Angiotensinogen and angiotensinogen gene expression have been localised in proximal tubular cells (Darby and Sernia, 1995; Gomez et al., 1988; Ingelfinger et al., 1990). In addition, ACE is located in endothelial cells of the renal vasculature and on proximal tubular brush border membranes (Schultz et al., 1988). AT1 receptors are present on the luminal and basolateral membranes of tubules and on the blood vessels

(Allen et al., 1999). The total intrarenal Ang II concentration has been determined and found to be surprisingly high (Campbell et al., 1991), indicating that Ang II must be produced in high concentration in the kidneys themselves. Experiments utilizing AT1 blockers have shown that intrarenal blockade of Ang II causes substantial increases in renal blood flow, glomerular filtration rate and fractional sodium excretion (Cervenka et al., 1999).

In addition to the evidence for a mammalian intra-renal RAS, the existence of an intra-renal RAS in the rainbow trout, *O mykiss*, has been demonstrated recently (Brown *et al.*, 2000). The ACE inhibitor captopril was perfused through an *in situ* perfused trunk preparation and caused a profound glomerular diuresis and decrease in vascular resistance, consistent with an inhibition of the known vasoconstrictor and antidiuretic actions of Ang II (Brown *et al.*, 2000). Existence of the renal RAS in trout was further supported in the same study by evidence for angiotensinogen gene expression in the kidney. To date, there have been no studies on the possible existence of local renin-angiotensin systems in elasmobranch fish.

The aim of this chapter was to investigate the renal actions of Ang II. In addition, preliminary studies were carried out to investigate the possible presence of an intra-renal RAS in the dogfish.

4.2. Materials and Methods

4.2.1. Animals

Dogfish were collected and maintained according to section 2.2.1.

4.2.2. Chemicals and Equipment

Dogfish Ang II was synthesised by Dr Graham Kemp, Biomedical Sciences, University of St Andrews. The peptide was made up to a concentration of 10^{-3} M in Milli-Q water and stored in 10 µl aliquots at –20° C until use.

4.2.3. In situ perfusion of the kidney

Kidneys were perfused according to section 2.2.4. and 2.2.5. Urine flow was allowed to stabilise for at least one hour before two, one-hour urine samples were collected into pre-weighed microcentrifuge tubes. Ang II was then added to the perfusate at a concentration of either 10^{-9} M or 10^{-10} M and two further one-hour urine samples were collected.

4.2.4. Analysis and calculations

All analysis and calculations are described in section 2.2.7 to section 2.2.12. and section 3.2.4.

4.2.5. Statistical analysis

Comparisons were made between the final renal clearance period immediately prior to administration of Ang II and the final clearance period during Ang II administration. Paired *t*-tests were used to assess physiological changes (INSTAT).

4.3. Results

4.3.1. Perfusate flow rates

Table 4.2. shows the effect of Ang II on perfusate flow rates. There was a significant decrease in perfusate flow rate during Ang II perfusion in both SW and 85% SW at both concentrations of Ang II.

	Control	10^{-9} M Ang II	Control	10^{-10} M Ang II
Perfusate flow rate (SW)	30.6 ± 2.9	18.4 ± 4.2*	31.5 ± 4.7	17.6 ± 1.9*
Perfusate flow rate (85% SW)	34.2 ± 6.1	20.8 ± 3.4*	29.1 ± 2.6	18.0 ± 3.4*

Table 4.2 Effect of Ang II on perfusate flow rate. Values are mean ± S.E.M. from 6 fish in each group. *$P < 0.05$.

4.3.2. Effects of Ang II on urine flow rate

Addition of 10^{-9} M Ang II to the perfusate resulted in a significant antidiuresis in both SW and 85% SW-acclimated preparations (Figure 4.2.). Addition of 10^{-10} M Ang II, however, had no significant effect in either SW or 85% SW (Figure 4.3.).

4.3.3. Glomerular effects

Addition of 10^{-9} M Ang II to the perfusate resulted in a significant decrease in GFR in both SW and 85% SW-acclimated preparations (Figure 4.2.). Addition of 10^{-10} M Ang II, however, had no significant effect in either SW or 85% SW (Figure 4.3.).

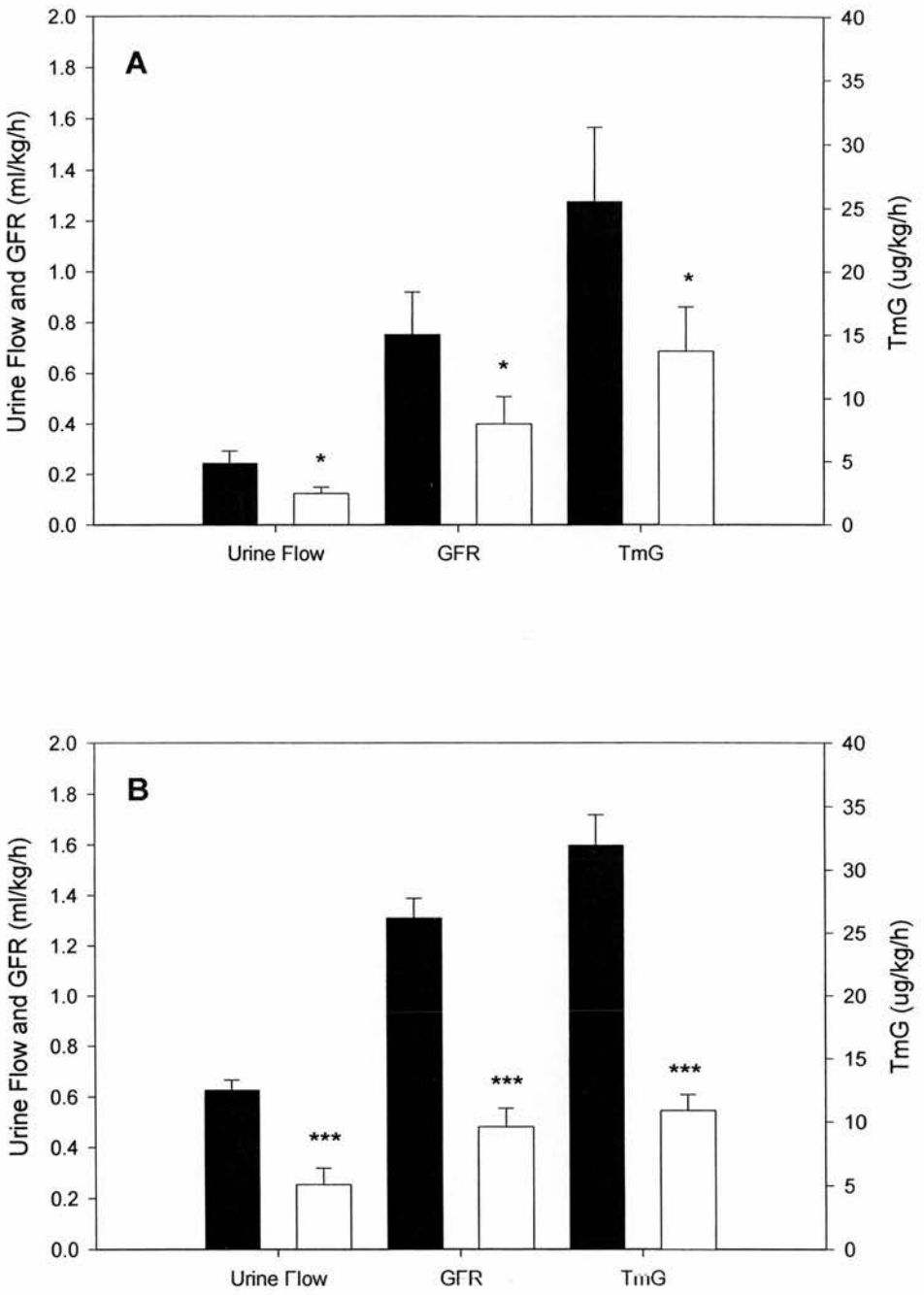

Fig. 4.2. Urine flow rate, GFR and TmG of *in situ* perfused dogfish kidney (10^{-9} M Ang II). Comparisons are between the final renal clearance period immediately before Ang II administration (solid bars) and for the final clearance period during Ang II administration (open bars) in SW (A) and 85% SW (B). Values are means ± SEM; n=6 in each group. $*P < 0.05$, and $***P < 0.005$.

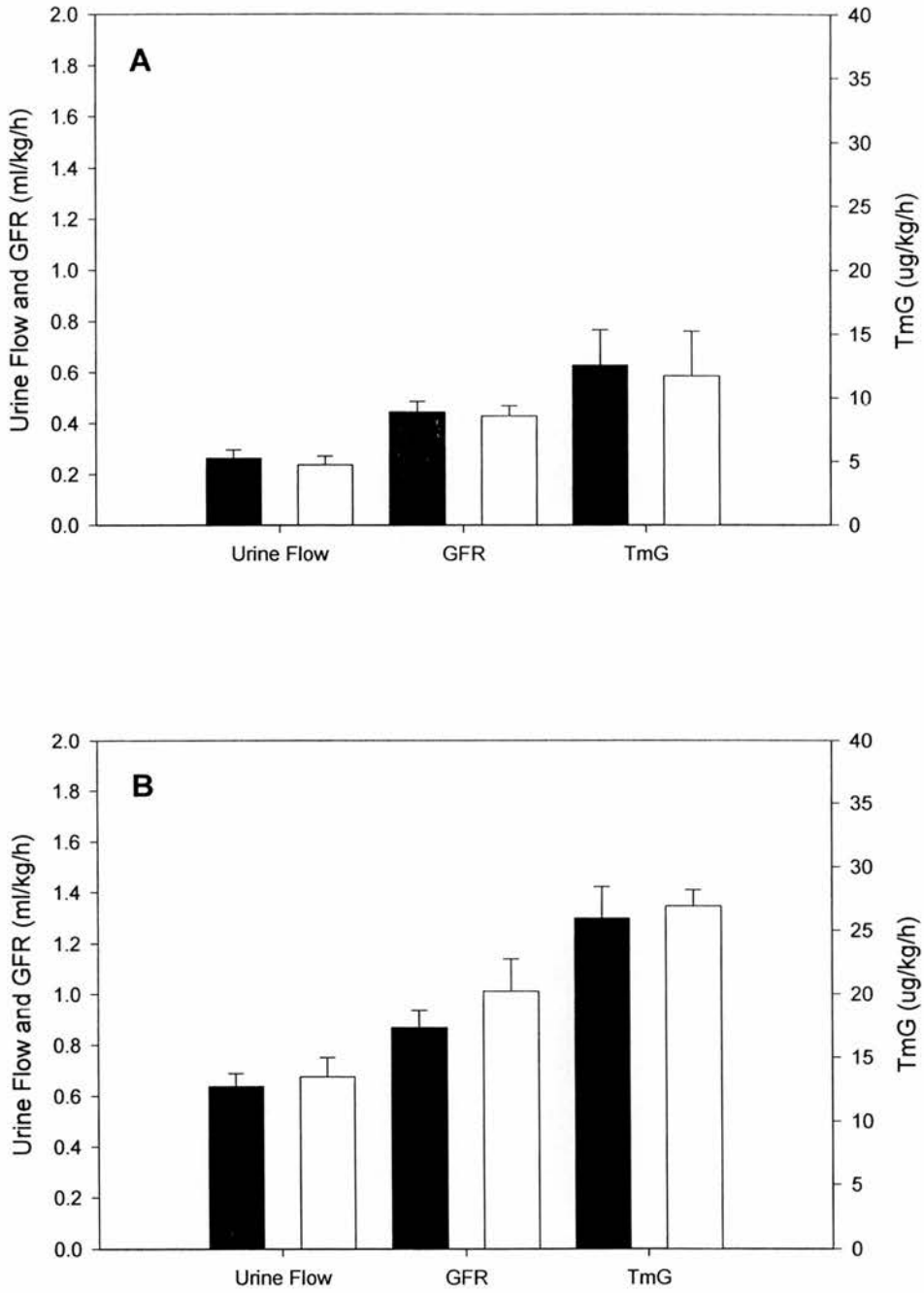

Fig. 4.3. Urine flow rate, GFR and TmG of *in situ* perfused dogfish kidney (10^{-10} M Ang II). Comparisons are between the final renal clearance period immediately before Ang II administration (solid bars) and for the final clearance period during Ang II administration (open bars) in SW (A) and 85% SW (B). Values are means ± SEM; n=6 in each group.

4.3.4. Functional tubular mass

Addition of 10^{-9} M Ang II to the perfusate resulted in a significant reduction in TmG values to both experimental salinites (Figure 4.2.). Addition of 10^{-10} M Ang II, however, had no significant effect in either SW or 85% SW acclimated preparations (Figure 4.3.).

	U/P_{in}	U/P_{osm}	C_{osm}/GFR	C_{H_2O}/GFR
Control (SW)	1.7 ± 0.2	0.995 ± 0.004	58.1 ± 11.1	0.01 ± 0.0
10^{-10} M Ang II	2.1 ± 0.3	0.996 ± 0.007	65.2 ± 9.9	0.01 ± 0.0
Control (SW)	1.7 ± 0.1	0.989 ± 0.002	43.2 ± 9.4	0.10 ± 0.0
10^{-9} M Ang II	1.5 ± 0.2	0.998 ± 0.004	42.4 ± 5.9	0.01 ± 0.0
Control (85% SW)	1.5 ± 0.2	0.994 ± 0.002	72.1 ± 2.2	0.10 ± 0.0
10^{-10} M Ang II	2.0 ± 0.3	0.997 ± 0.006	69.2 ± 4.8	0.01 ± 0.0
Control (85% SW)	1.6 ± 0.2	0.996 ± 0.005	52.3 ± 6.1	0.01 ± 0.0
10^{-9} M Ang II	2.2 ± 0.4	0.992 ± 0.009	50.4 ± 2.8	0.10 ± 0.0

Table 4.3. Effect of Ang II on tubular function in the *in situ* perfused trunk preparation. Values are mean ± S.E.M. from 6 fish in each group. U/P_{in}, urine/perfusate inulin concentration ratio; U/P_{osm}, urine/perfusate osmolality ratio; C_{osm}/GFR, relative clearance of osmolytes; C_{H2O}/GFR, relative free water clearance.

4.3.5. Tubular effects

Tubular function is summarised in Table 4.3. The mean U/P inulin concentration indicates that on average 43% of the filtered volume of water was reabsorbed by the renal tubule. Urine was slightly hypotonic to plasma with a mean U/P_{osm} of 0.99. This resulted in a small relative free water clearance of less than 1%. The relative osmolar clearance demonstrates that on average approximately 56% of filtered osmolytes were

excreted. Perfusion of Ang II had no significant effect on any of the tubular parameters measured.

4.3.6. Effects on ion clearance and excretion

Perfusion of 10^{-9} M Ang II resulted in a significant decrease in urea clearance and urea excretion in both SW and 85% SW (Fig. 4.4.). At this dose, the fractional excretion of urea was not significantly altered. The clearance and excretion of both chloride and sodium were also significantly reduced on perfusion with 10^{-9} M Ang II in both SW and 85% SW (Figs 4.6. & 4.8.). Perfusion of 10^{-10} M Ang II however, had no significant effect of the clearance, excretion or fractional excretion of urea, chloride or sodium (Figs. 4.5., 4.7. & 4.9.).

4.3.7. Relationship between urine flow rate and GFR

There was a clear linear relationship between urine flow rate and GFR both before and after perfusion with Ang II in both SW and 85% SW (Fig 4.10.).

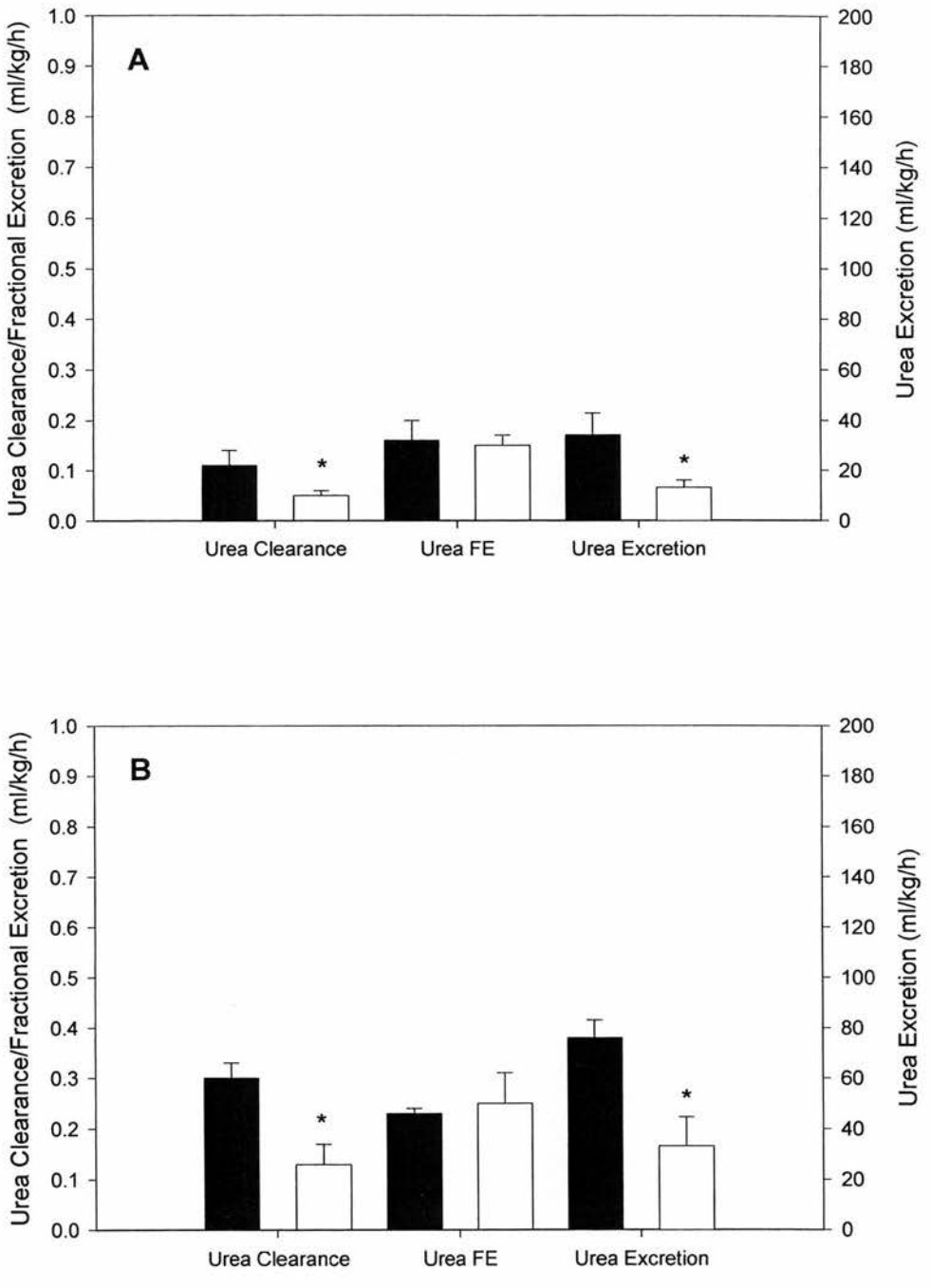

Fig. 4.4. Urea clearance, fractional excretion and excretion in the *in situ* perfused dogfish kidney (10^{-9} M Ang II). Comparisons are between the final renal clearance period immediately before Ang II administration (solid bars) and for the final clearance period during Ang II administration (open bars) in SW (A) and 85% SW (B). Values are means ± SEM; n=6 in each group. $*P < 0.05$.

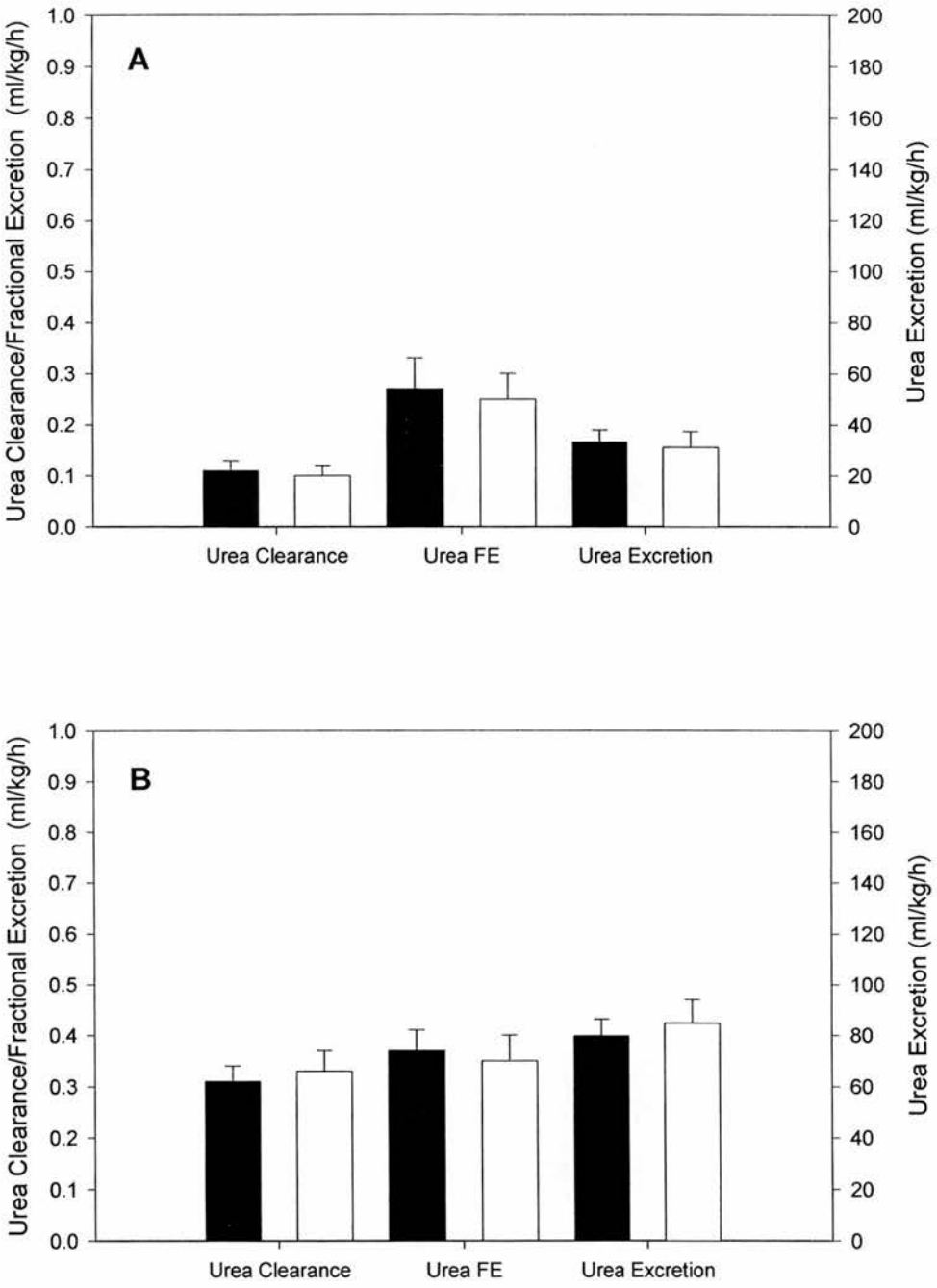

Fig. 4.5. Urea clearance, fractional excretion and excretion in the *in situ* perfused dogfish kidney (10^{-10} M Ang II). Comparisons are between the final renal clearance period immediately before Ang II administration (solid bars) and for the final clearance period during Ang II administration (open bars) in SW (A) and 85% SW (B). Values are means ± SEM; n=6 in each group.

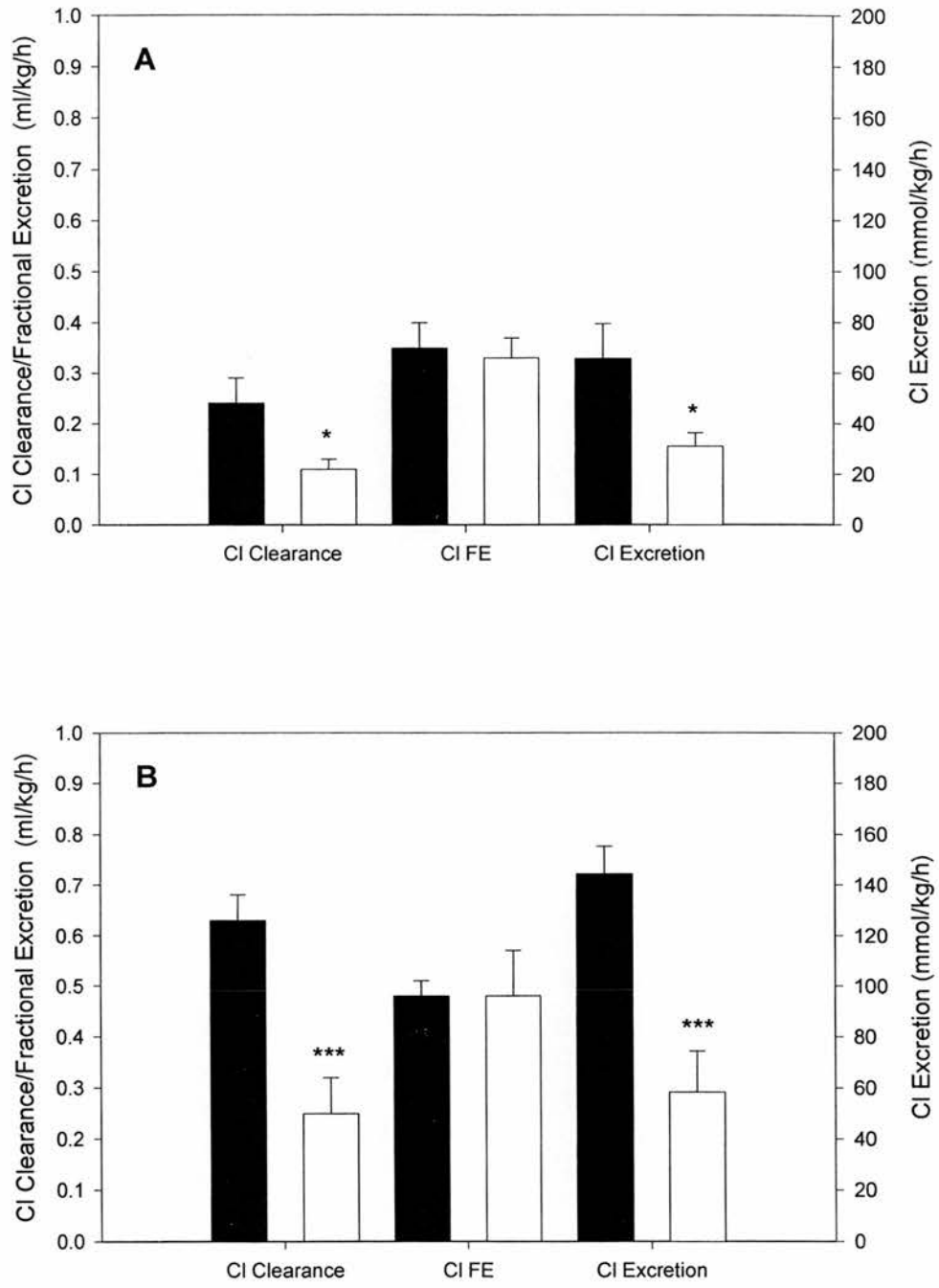

Fig. 4.6. Chloride clearance, fractional excretion and excretion in the *in situ* perfused dogfish kidney (10^{-9} M Ang II). Comparisons are between the final renal clearance period immediately before Ang II administration (solid bars) and for the final clearance period during Ang II administration (open bars) in SW (A) and 85% SW (B). Values are means ± SEM; n=6 in each group. $*P < 0.05$ and $***P < 0.005$.

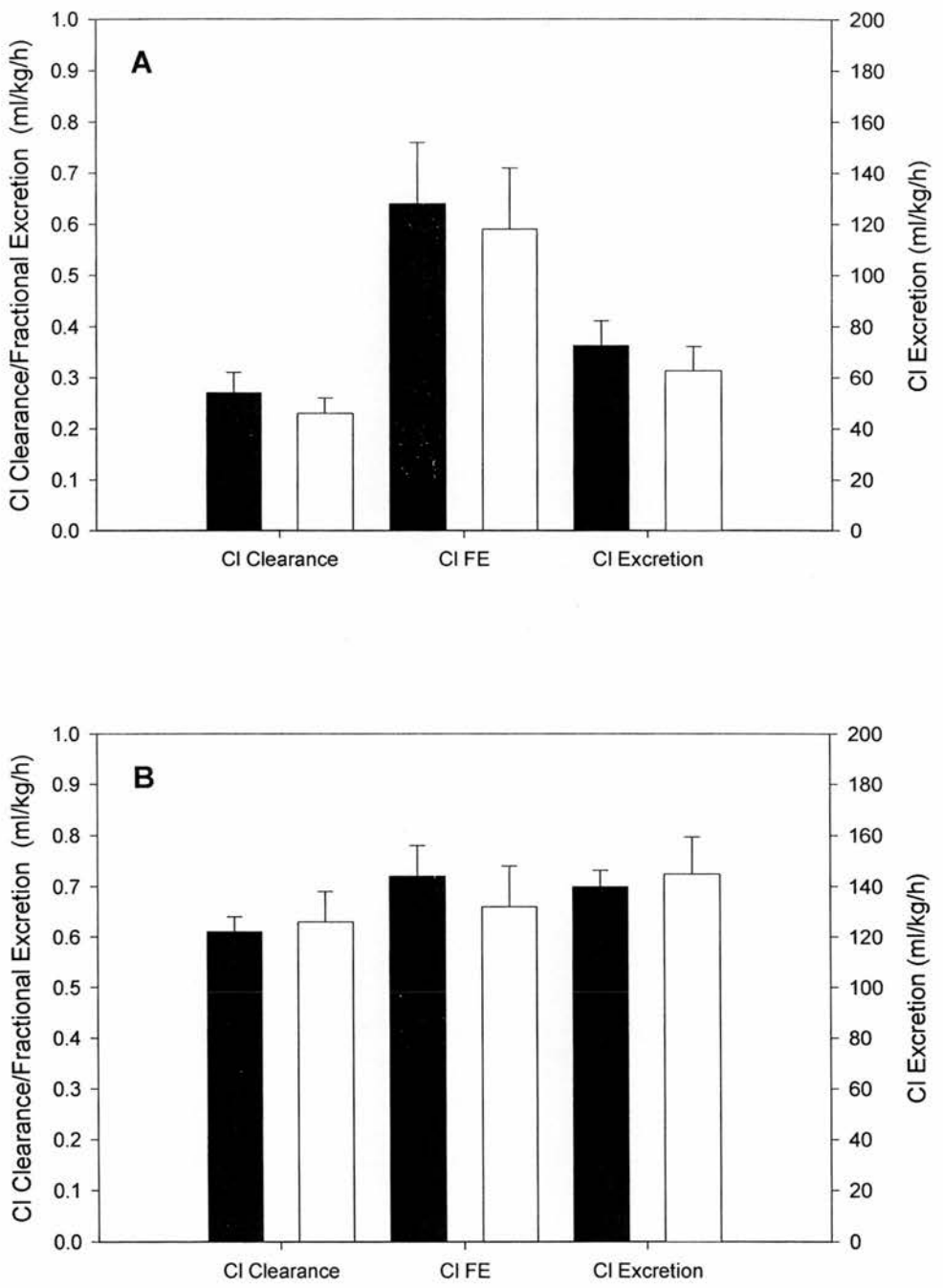

Fig. 4.7. Chloride clearance, fractional excretion and excretion in the *in situ* perfused dogfish kidney (10^{-10} M Ang II). Comparisons are between the final renal clearance period immediately before Ang II administration (solid bars) and for the final clearance period during Ang II administration (open bars) in SW (A) and 85% SW (B). Values are means ± SEM; n=6 in each group.

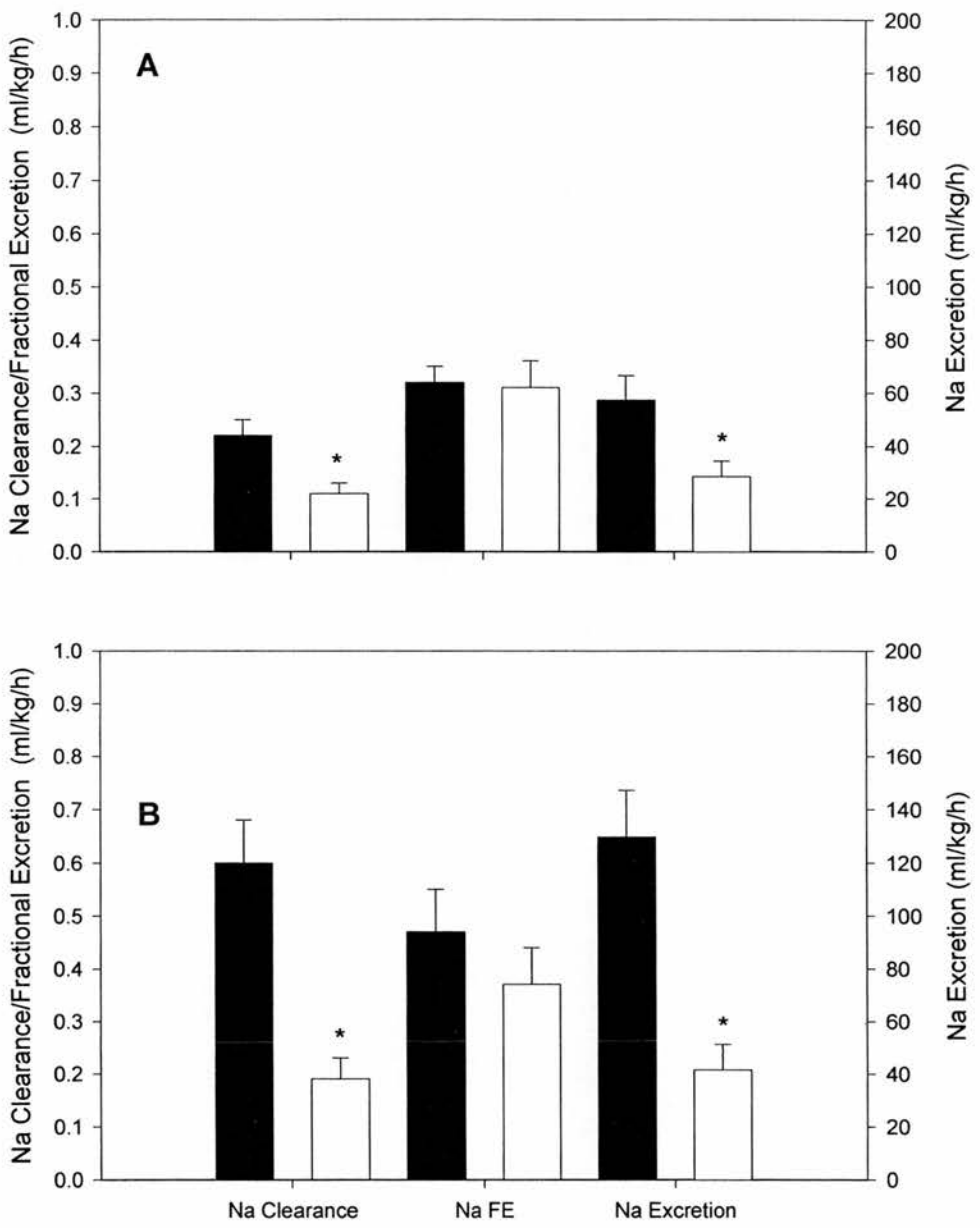

Fig. 4.8. Sodium clearance, fractional excretion and excretion in the *in situ* perfused dogfish kidney (10^{-9} M Ang II). Comparisons are between the final renal clearance period immediately before Ang II administration (solid bars) and for the final clearance period during Ang II administration (open bars) in SW (A) and 85% SW (B). Values are means ± SEM; n=6 in each group. *$P < 0.05$.

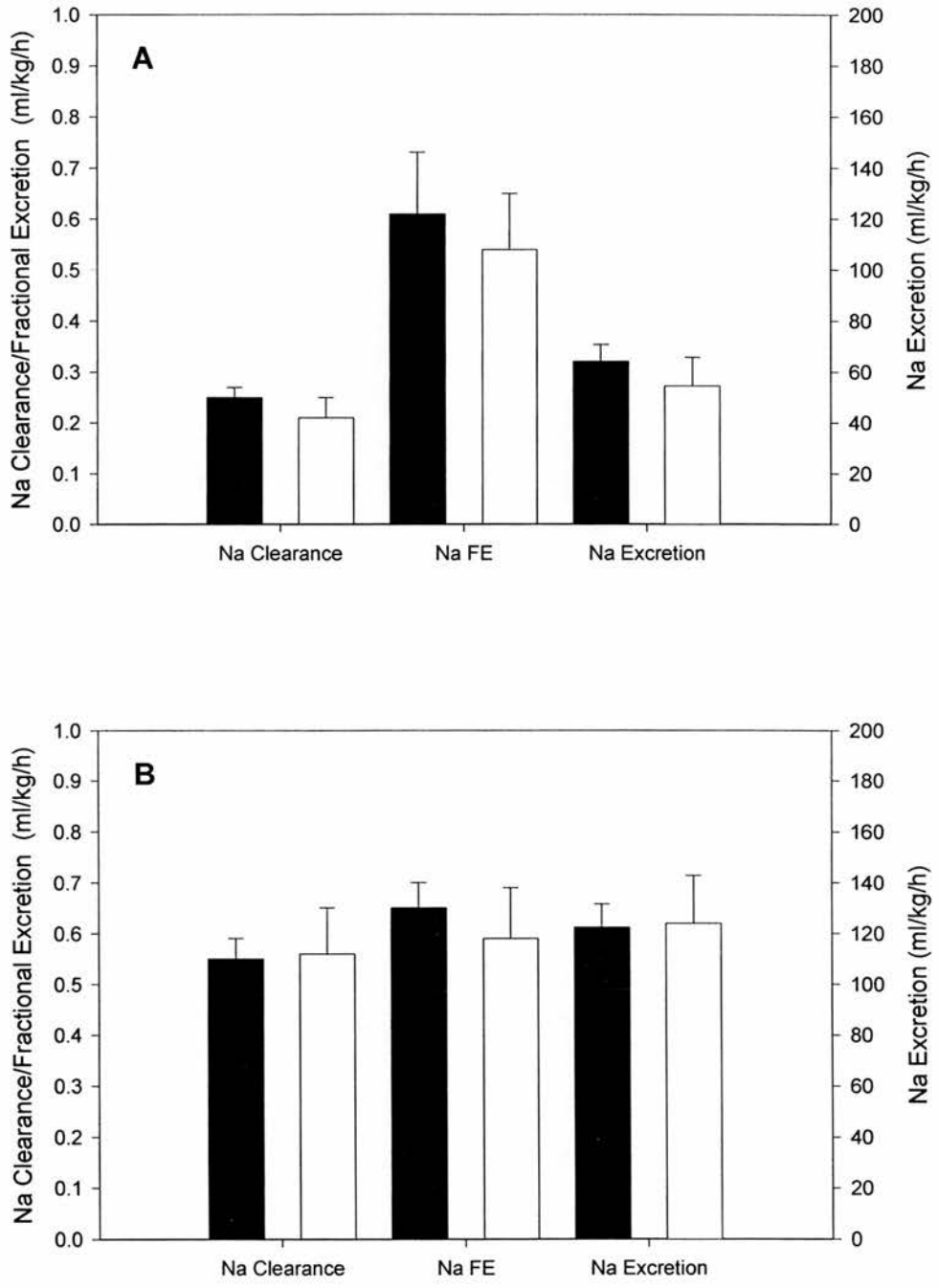

Fig. 4.9. Sodium clearance, fractional excretion and excretion in the *in situ* perfused dogfish kidney (10^{-10} M Ang II). Comparisons are between the final renal clearance period immediately before Ang II administration (solid bars) and for the final clearance period during Ang II administration (open bars) in SW (A) and 85% SW (B). Values are means ± SEM; n=6 in each group.

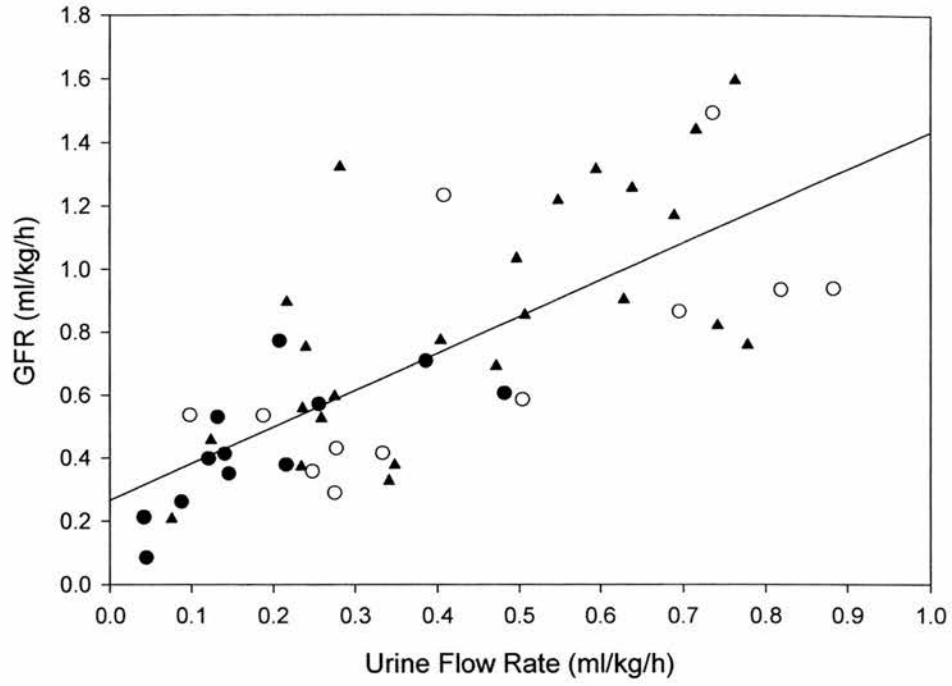

Fig. 4.10. Relationship between GFR and urine flow rate in the perfused kidney of the dogfish. $R_2 = 0.605$. Values are mean rates before perfusion of Ang II (▲), and following perfusion with 10^{-9} M Ang II (●) and 10^{-10} M Ang II (○) in both SW and 85% SW.

4.3.8. Effects of captopril on the perfused dogfish trunk

Table 4.4. shows the effect of captopril on perfusate flow rates. There was no significant effect on perfusate flow in either SW or 85% SW. Addition of 10^{-4}M captopril to the perfusate resulted in a significant diuresis in SW but had no significant effect in 85% SW (Fig. 4.11.). This diuresis in SW was accompanied by a significant increase in GFR and TmG. Tubular function is summarised in Table 4.5. All tubular parameters remained unchanged by the addition of captopril.

	Control	10^{-4}M captopril
Perfusate flow rate (SW)	33.6 ± 2.7	39.3 ± 3.1
Perfusate flow rate (85% SW)	35.3 ± 4.2	41.7 ± 3.3

Table 4.4. Effect of captopril on perfusate flow rate. Values are mean ± S.E.M. from 6 fish in each group.

	U/P_{in}	U/P_{osm}	C_{osm}/GFR	C_{H_2O}/GFR
Control (SW)	1.9 ± 0.3	0.99 ± 0.04	67 ± 6.8	0.5 ± 0.2
10^{-4}M Captopril	1.8 ± 0.3	0.99 ± 0.04	64 ± 10.2	0.7 ± 0.2
Control (85% SW)	1.6 ± 0.3	0.99 ± 0.04	71 ± 6.7	0.6 ± 0.2
10^{-4}M Captopril	1.4 ± 0.3	0.99 ± 0.03	65 ± 4.3	0.7 ± 0.3

Table 4.5. Effect of captopril on tubular function in the *in situ* perfused trunk preparation. Values are mean ± S.E.M. from 6 fish in each group. U/P_{in}, urine/perfusate inulin concentration ratio; U/P_{osm}, urine/perfusate osmolality ratio; C_{osm}/GFR, relative clearance of osmolytes; C_{H2O}/GFR, relative free water clearance.

Addition of 10^{-4}M captopril to the perfusate resulted in a significant increase in the clearance and excretion of both urea and chloride in SW, but had no effect on the fractional excretion of these ions. There was no significant effect on ion or urea excretion or clearance in 85% SW (Figs 4.12. & 4.13.).

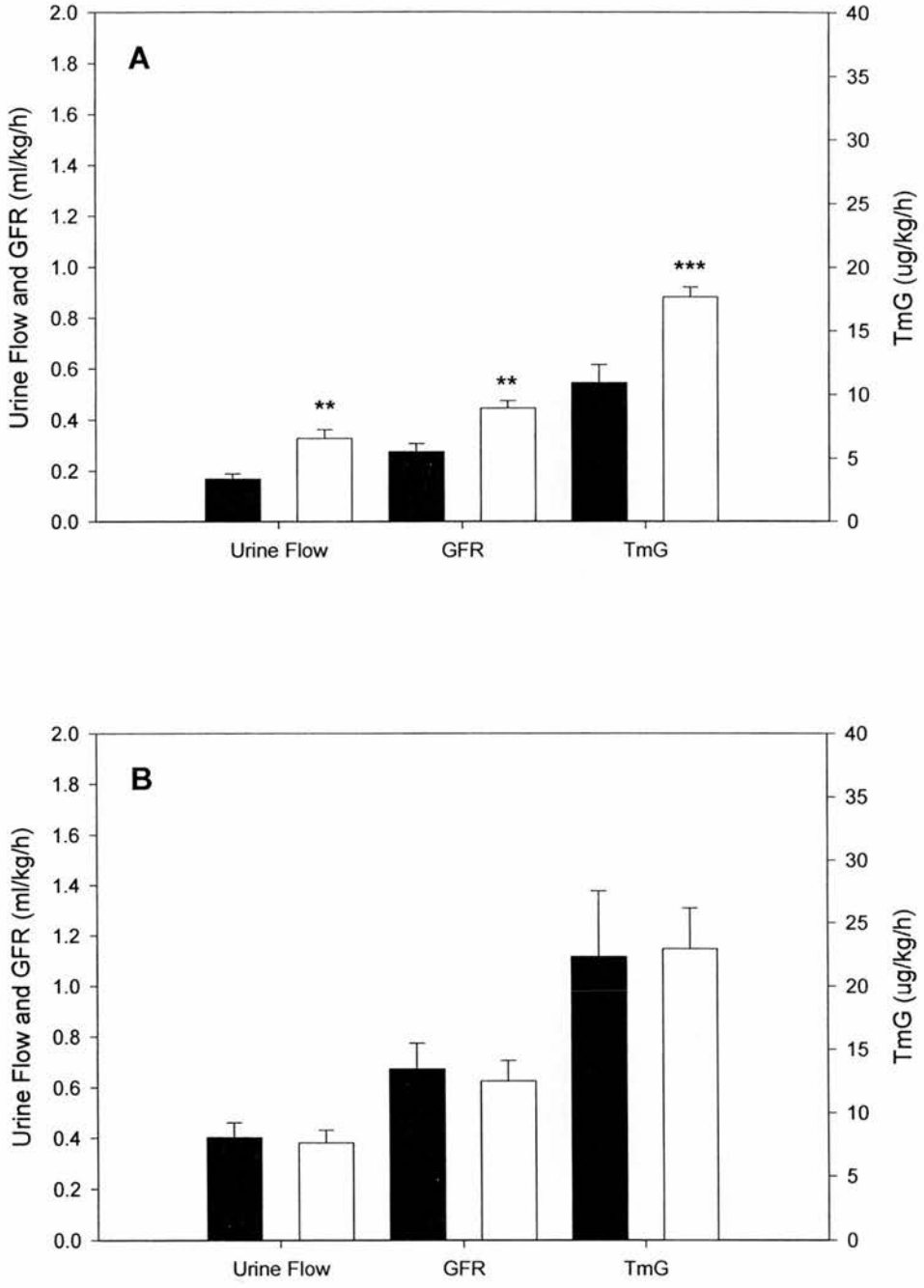

Fig. 4.11. Urine flow rate, GFR and TmG of *in situ* perfused dogfish kidney (10^{-4} M captopril). Comparisons are between the final renal clearance period immediately before captopril administration (solid bars) and for the final clearance period during captopril administration (open bars) in SW (A) and 85% SW (B). Values are means ± SEM; n=6 in each group. $^{**}P < 0.01$, and $^{***}P < 0.005$.

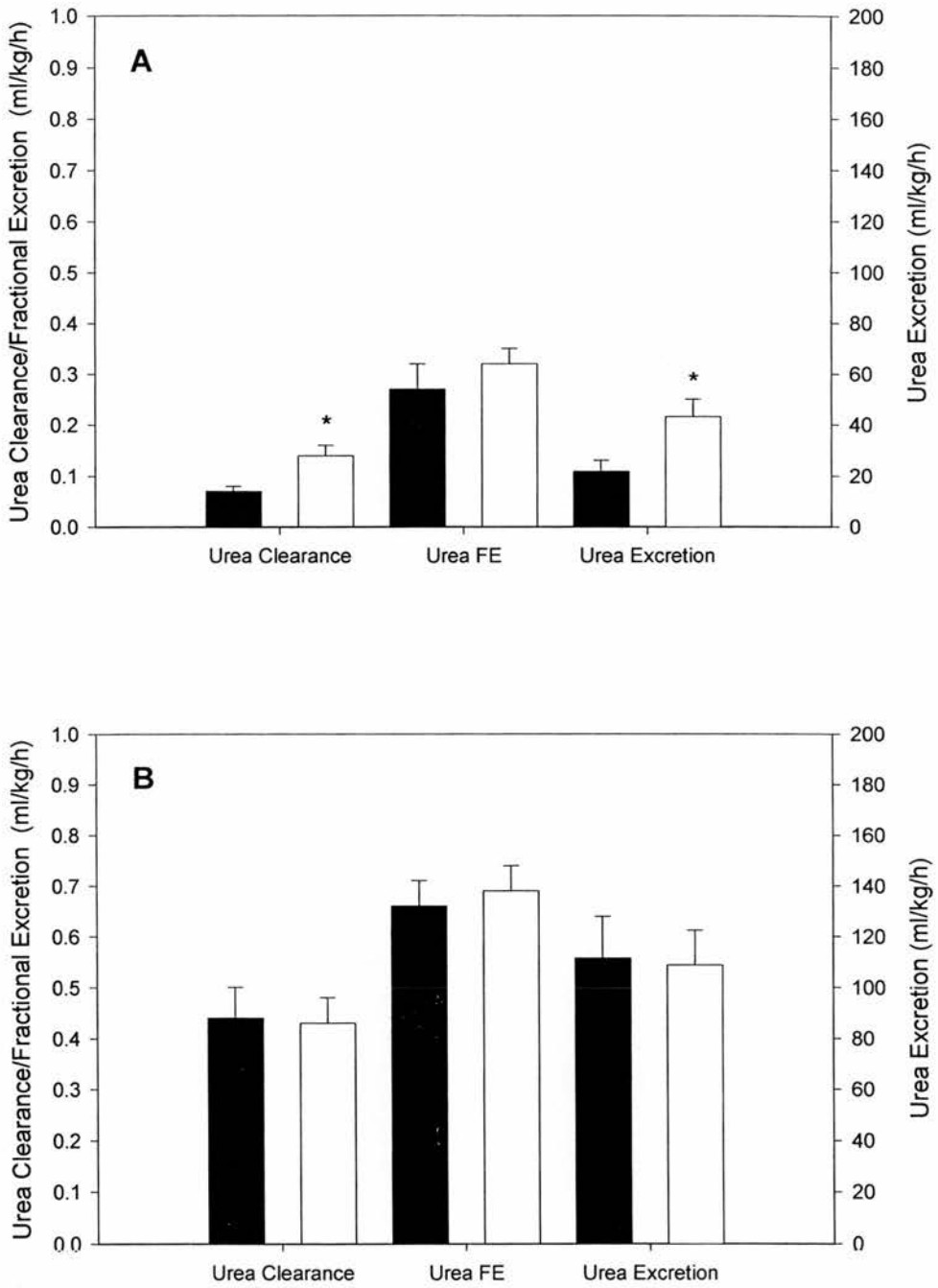

Fig. 4.12. Urea clearance, fractional excretion and excretion in the *in situ* perfused dogfish kidney (10^{-4} M captopril). Comparisons are between the final renal clearance period immediately before captopril administration (solid bars) and for the final clearance period during captopril administration (open bars) in SW (A) and 85% SW (B). Values are means ± SEM; n=6 in each group. $*P < 0.05$.

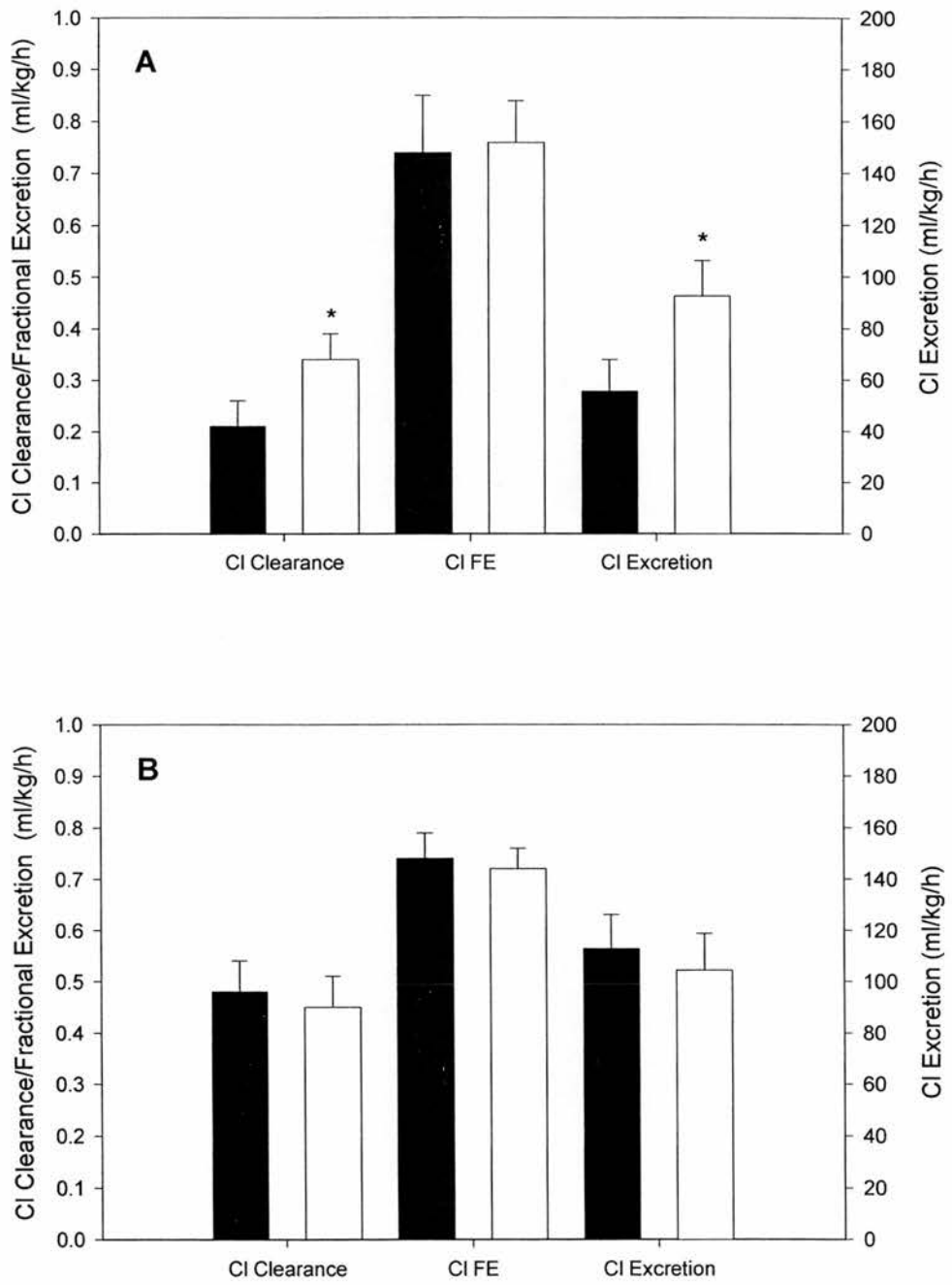

Fig. 4.13. Chloride clearance, fractional excretion and excretion in the *in situ* perfused dogfish kidney (10^{-4} M captopril). Comparisons are between the final renal clearance period immediately before captopril administration (solid bars) and for the final clearance period during captopril administration (open bars) in SW (A) and 85% SW (B). Values are means ± SEM; n=6 in each group. *$P < 0.05$.

4.4. Discussion

In the anaesthetised rainbow trout, *O mykiss*, intravenous infusion of Ang II had an antidiuretic action, reducing urine flow rate and glomerular filtration rate (Brown *et al.*, 1980). In addition, Ang II reduced the filtering population of glomeruli. Ang II has also been shown to have antidiuretic actions in the *in situ* perfused trout trunk preparation (Brown and Balment, 1997; Dunne and Rankin 1992).

The present study has demonstrated that in the dogfish perfused trunk preparation there is a similar renal action of Ang II to that seen in the trout. Ang II appears also to affect renal function in a similar manner to that described for AVT (Chapter 3). Like AVT, 10^{-9} M Ang II caused a profound glomerular antidiuresis in both SW and 85% SW. The threshold for renal effects of Ang II therefore appeared to lie somewhere between 10^{-9} M and 10^{-10} M, as 10^{-10} M Ang II had no significant effect on any of the renal parameters investigated.

Ang II caused a significant decrease in perfusate flow rate at both 10^{-9} M and 10^{-10} M (Table 4.2.). This drop in perfusate flow rate was of a similar order of magnitude to that seen for AVT (Table 3.3). Like AVT, Ang II has been shown to have a vasoconstrictor action (Bernier *et al.*, 1999; Hazon *et al.*, 1995; Takei *et al.*, 1993; Tierney *et al.*, 1997a), and the reduction of perfusate flow by Ang II is probably due to vasoconstriction. Perfusate flow rate is a crude measure of the total vascular resistance of the preparation, and vasoconstrictor actions of hormones will effect the vasculature at a number of different sites. It could be expected that these would include both major arteries and the afferent or efferent arterioles of the glomeruli. However, the

vasocontrictor effect of Ang II on the vasculature alone is not enough to explain the renal effects, as significant renal responses were not observed on perfusion with 10^{-10} M Ang II, despite a significant reduction in perfusate flow at this dose. It would appear that 10^{-10} M Ang II is below the threshold dose for renal effects in both SW and 85% SW. This may reflect a variation in the sensitivity of receptors at the glomeruli as opposed to those in the general circulation. It is interesting to note that on perfusion with captopril there was a significant renal diuresis in SW, but no associated significant increase in perfusate flow rate. This provides additional evidence that total vascular resistance alone cannot explain the renal haemodynamic effects observed. This will be discussed further in Chapter 7.

Addition of 10^{-9} M Ang II caused a significant decrease in TmG, indicating a decrease in the population of filtering glomeruli. As discussed in the previous chapter, AVT may act at the glomerular bypass shunts to vary the degree of glomerular perfusion and control the proportion of active glomeruli. It is likely that Ang II could affect the degree of glomerular perfusion by a similar mechanism. The filtering population of glomeruli will be measured using a more direct method of visualising filtering glomeruli by perfusing with ferrocyanide (See Chapter 6).

Ang II appeared to have no net effect on tubular water permeabilities as U/P_{in} was unchanged by perfusion with Ang II. In addition, relative clearance of osmolytes and relative free water clearance were unchanged by addition of Ang II to the perfusate.

Perfusion of 10^{-9} M Ang II caused a significant decrease in the clearance and excretion of urea, chloride and sodium. There was no significant effect on the fractional excretion

of these ions however, suggesting that the decrease in renal excretion of ions can be totally accounted for by the decrease in GFR. 10^{-10} M Ang II had no significant effect on the renal clearance, excretion or fractional excretion of these ions.

The possible presence of an intra-renal RAS was investigated by perfusing the trunk preparation with 10^{-4}M captopril, as previously described in the trout (Brown *et al.*, 1995; Brown *et al.*, 2000). Captopril caused a significant increase in urine flow, GFR and TmG in SW-acclimated trunk preparations, in the absence of angiotensinogen synthesis by the liver, or ACE conversion of Ang I by the gills. It may therefore be assumed that the renal effects of captopril are caused solely by the inhibition of intra-renal Ang II formation. It is possible that, like trout (Brown *et al.*, 2000), the dogfish may possess an intra-renal RAS, which is distinct from the systemic RAS. However, care must be taken in interpreting these results. Danser (1996) defined 3 situations when the term *local RAS* may exist.

1. The RAS components required for Ang II synthesis, i.e. renin, angiotensinogen and ACE are synthesised *in situ*, thus allowing local Ang II generation to occur independently of the circulating RAS;
2. The RAS components are not synthesised *in situ*, but taken up from the circulating blood, thus allowing local RAS synthesis to occur only when RAS components are available in the blood;
3. A combination of 1. and 2.

When applying definition 2 or 3, it may be more applicable to speak of a system generating Ang II locally, instead of a local RAS (Danser, 1996). It is clear that in the present study, definitions 2 and 3 are not possible as the Ringer's solution circulating through the preparation did not contain any RAS components. However, conclusive

proof of a complete intra-renal RAS in the dogfish would require evidence of all the RAS components within the kidney.

An interesting point to be noted is that captopril had no significant effect on renal function in the *in situ* perfused trunk preparations acclimated to 85% SW. This may suggest that the intra-renal RAS is only activated in SW-acclimated trunk preparations. When the conversion of Ang I to Ang II is blocked by captopril, the urine flow rate then becomes similar to that of 85% SW-acclimated preparations. This may explain the fact that the diuresis observed in 85% SW *in vivo*, is maintained *in vitro* (Chapter 2). The urine flow rate in the SW-acclimated renal trunk prep may be reduced by the activation of an intra-renal RAS but this effect may be abolished in the 85% SW model. This may reflect a permanent activation of the intra-renal RAS in SW *in vivo* in order to maintain a reduced urine flow rate and therefore maintenance of urea and ions within the plasma.

It should be noted that an additional action of ACE is the control of Bradykinin (BK) degradation (Gardiner *et al.*, 1993). BK is an active component of the kallikrein-kinin system (Fig 7.1.), and in mammals, generation of BK results in vasodilation, increased vascular permeability and stimulation of renal electrolyte secretion (Conlon, 1999). It is therefore possible that renal effects following administration of captopril may be due to an inhibition of Ang II production, in combination with an increase in BK levels. However, attempts to generate BK in the plasma of *S. canicula* have been unsuccessful to date (Conlon, 1999).

In summary, perfusion of the dogfish trunk preparation with 10^{-9} M Ang II resulted in a decrease in urine flow rate, GFR and TmG, coupled with a decrease in the clearance and

excretion of urea and sodium chloride. These effects were apparent in both SW and 85% SW, as was the case with the same dose of AVT. In addition, preliminary evidence of an intra-renal RAS has been presented. This is the first study in which a local RAS has been demonstrated in an elasmobranch fish. These data suggest that Ang II induced a glomerular antidiuresis in *S. canicula*, perhaps in a manner similar to that previously reported in the trout.

5

Effects of CNP on the perfused dogfish trunk

5.1. Introduction

The aim of this chapter was to investigate the renal role of C-type natriuretic peptide (CNP). CNP is a member of the natriuretic peptide (NP) family first identified in the brain of pig and teleost fish (Price *et al.*, 1990; Sudoh *et al.*, 1990; Takei *et al.*, 1990). The NP system consists of at least 4 types of hormones, atrial NP (ANP), brain or B-type NP (BNP), C-type NP (CNP) and ventricular NP (VNP) (Takei, 2000) (Fig. 5.1.). Natriuretic peptides are multifunctional hormones, involved in the regulation of fluid volume and composition (Loretz and Pollina, 2000). In higher vertebrates ANP and BNP are circulating hormones secreted from the heart, whereas CNP is basically a neuropeptide (Takei, 2000). ANP, BNP and CNP occur commonly in all tetrapods, but VNP replaces BNP in teleost fish. Attempts to identify and characterise NPs in several species of shark have yielded only CNP isohormones, suggesting that CNP is the primary, if not the only NP in elasmobranch fish (Schofield *et al.*, 1991; Suzuki *et al.*, 1991; Suzuki *et al.*, 1992; Suzuki *et al.*, 1994; Takano *et al.*, 1994). More recently, Kawakoshi *et al.* (2001) were only able to clone CNP cDNAs from *T. scyllia*, using primers that can amplify all piscine NPs. This strongly suggests that CNP is the only NP present in elasmobranchs. In the light of this, earlier work utilising heterologous peptides needs to be interpreted with caution.

Atrial (A-type) natriuretic peptide (ANP)

Eel	SKSSSPCFGGKLDRIGSYSGLGCNSRK-NH_2
Rainbow trout	SKAVSGCFGARMDRIGTSSGLGCSPKRRS
Bullfrog	SSDCFGSRIDRIGAQSGMGC-GRRF
Frog	APRSMRRSSDCFGSRIDRIGAQSGMGC-GR-F
Man	SLRRSSCFGGRMDRIGAQSGLGCNSFRY
Rat	SLRRSSCFGGRIDRIGAQSGLGCNSFRY

Brain (B-type) natriuretic peptide (BNP)

Bullfrog	SNCFGRRIDRIDSVSGMGCNGSRNRYP
Chicken	MMRDSGCFGRRIDRIGSLSGMGCNGSRKN
Pig	SPKTMRDSGCFGRRLDRIGSLSGLGCNVLRRY
Dog	SPKMMHKSGCFGRRLDRIGSLSGLGCNVLRKY
Ox	GPKMMRDSGCFGRRLDRIGSLSGLGCNVLRRY
Man	SPKMVQGSGCFGRKMDRISSSSGLGCKVLRRH
Hamster	NSKKMHNSGCFGQRIDRIGSFSRLGCNVLKRY
Rat	SQDSAFRIQERLRNSKMAHSSSCFGQKIDRIGAVSRLGCDGLRLF

C-type natriuretic peptide (CNP)

Spotted dogfish[1]	GPSRGCFGVKLDRIGAMSGLGC
Spiny dogfish	GPSRSCFGLKLDRIGAMSGLGC
Eel	GWNRGCFGLKLDRIGSLSGLGC
Killifish	GWNRGCFGLKLDRIGSMSGLGC
Bullfrog I	GYSRGCFGVKLDRIGAFSGLGC
Bullfrog II	GTSKGCFGLKLDRIGAMSGLGC
Newt	GQSSGCFGLKLDRIGSMSGLGC
Snake	GAAKGCFGLKLDRIGTMSGLGC
Chicken	GLSRSCFGLKLDRIGSMSGLGC
Man	GLSKGCFGLKLDRIGSMSGLGC
Platypus	GLSKGCFGLKLDRIGSTSGLGC

Ventricular natriuretic peptide (VNP)

Eel	KSFNSCFGTRMDRIGSWSGLGCNSLKNGTKKKIFGN
Rainbow trout	KSFNSCFGNRIERIGSWSGLGCNNVKTGNKKRIFGN

Fig. 5.1. Amino acid sequences of all natriuretic peptides identified to date. The amino acids residues identical in the same group are shaded. The brackets below each group of peptides show the position of disulphide bonds. [1]*Triakis scyllia*, *Scyliorhinus canicula* and *Lamna ditroptis* have the same sequence. Adapted from Takei (2000) (See Appendix 1 for amino acid abbreviations).

The 115-amino acid proCNP (CNP-115) can be extracted from the hearts of *S. canicula* and *T. scyllia*, and it is largely this form that circulates in the bloodstream (Suzuki *et al.*, 1991; Suzuki *et al.*, 1992). A processed shorter form (CNP-22) is isolated from the brain and may function as a neuropeptide (Suzuki *et al.*, 1992). In elasmobranch fish, two patterns of secretory cell processing occur. Circulating CNP is derived from cardiac secretion of the prohormone, whereas neurotransmitter or neuromodulator CNP in the brain is cleaved from the prohormone. Since dogfish GC-coupled receptors display low ligand selectivity (Sakaguchi and Takei, 1998), it is possible that the prohormone directly binds to the receptor without being processed to a mature form.

5.1.1. Osmoregulatory effects

In elasmobranch fish, synthetic mammalian ANP (atriopeptin II) is antidiuretic and anti-natriuretic in *S. acanthias* (Benyajati and Yokota, 1990). This renal antidiuresis and anti-natriuresis was associated with a drop in mean aortic pressure and glomerular filtration rate. The renal effect of atriopeptin II was temporally dissociated from the vasodepressor effect by a period of at least one hour (Benyajati and Yokota, 1990). However, it is possible that the renal effects in this study are a secondary rather than a direct effect. The drop in blood pressure may in fact have caused a release of vasopressor factors such as Ang II, and this possible release of Ang II may also have caused the antidiuresis observed. However, following volume-expansion in 90% SW, the renal effect of atriopeptin II is reversed to diuresis and natriuresis (Benyajati and Yokota, 1987). The authors suggested that the actions of atriopeptin II depend upon the hydrational status of the animal and that this effect may be modulated by catecholamines.

Although CNP binding was found to be low in the kidney of *T. Scyllia* (Sakaguchi and Takei, 1998), CNP-stimulated cGMP production was significant. The authors postulated that the low receptor population may have been due to the localisation of these receptors in the glomeruli relative to the large size of the dogfish kidney.

Due to the hyperosmotic plasma of elasmobranch fish, the gills are the major site of osmotic water influx. The net flux will be determined by a combination of the permeability of the gill epithelium and the blood flow profile of the gill which would determine the amount of plasma available for molecular exchanges at the epithelium (Donald *et al.*, 1997). The effects of circulating catecholamines on the branchial circulation are well established (Nilsson and Holmgren, 1988), and it could be expected that circulating CNP could have important actions on vascular and epithelial effector tissues that effect branchial exchanges and contribute to blood volume homeostasis (Donald *et al.*, 1997). Bjenning *et al.* (1992) established that dogfish CNP-22 is a profound vasodilator during *in vitro* studies on the branchial artery of *S. canicula.* Donald *et al.* (1997) found the presence of a GC-linked natriuretic peptide receptor, as well as a receptor with homology to the mammalian and eel natriuretic peptide receptor-C (NPR-C) and eel NPR-D. GC-coupled and -uncoupled receptors have also been identified in the gill cells of *T. scyllia* using homologous ^{125}I-CNP binding and cGMP assay (Sakaguchi and Takei, 1998), suggesting that the gills are an important site for NP action.

The role of NPs in rectal gland function has been extensively studied. Initial studies indicated that chloride secretion rate was increased by atriopeptin, both *in vivo* and *in vitro* from the perfused rectal gland of *S. acanthias* (Solomon *et al.*, 1985). It was later

suggested that the effect of atriopeptin was mediated by local secretion of vasoactive intestinal polypeptide (VIP) from the nerve terminals innervating the gland (Silva *et al.*, 1987). However, the addition of rat ANP to cultured rectal gland cells from *S. acanthias* increased chloride secretion to the apical side of the culture, with a concomitant increase in cGMP (Karnaky *et al.*, 1991), indicating that ANP acts directly on the rectal gland secretory epithelium. More recently, endogenous CNP was found to be a much more potent stimulator of salt secretion in the isolated perfused rectal gland of *S. acanthias* (Solomon *et al.*, 1992). In the rectal gland of *S. acanthias* two types of receptor have been identified, guanylate cyclase (GC)-coupled and uncoupled receptors (Gunning *et al.*, 1993). Similar GC-coupled and uncoupled receptors were recently identified in *T. scyllia* (Sakaguchi and Takei, 1998). CNP appears to act on the rectal gland by stimulating Na^+K^+ATPase activity, since stimulation is inhibited by HS142-1, a specific inhibitor for tetrameric NP receptors (Gunning *et al.*, 1997).

The effects of homologous CNP administration on drinking rate in *S. canicula* have been investigated using an oesophageal cannulation technique to directly measure drinking rate (Anderson *et al.*, 2001c). A bolus injection of CNP had no effect on drinking rate. However, when Ang II and CNP were co-administered, the increase in drinking rate induced by Ang II alone (See Section 4.1.1.) was significantly reduced, perhaps indicating a potential inhibitory role for CNP in the control of drinking (Anderson *et al.*, 2001c).

5.1.2. Cardiovascular effects

Bjenning *et al.* (1992) established that dogfish CNP-22 is a profound vasodilator *in vivo* and *in vitro* in *S. canicula*. *In vitro* studies on the ventral aorta of *S. acanthias* confirmed

this vasodilator effect and established that elasmobranch smooth muscle is very sensitive to dogfish CNP-22. (Evans *et al.*, 1993). A significant drop in mean dorsal aortic pressure was also observed in *S. acanthias* following intra-arterial injection of synthetic atriopeptin II (Benyajati and Yokota, 1990). More recently, in *S. acanthias* the response to injection of homologous CNP was biphasic, with a pressor response immediately followed by a sustained depressor response (McKendry *et al.*, 1999). The injection of homologous CNP also elicited a marked and sustained increase in circulating noradrenaline levels (McKendry *et al.*, 1999). Circulating adrenaline levels were not altered, suggesting that homologous CNP elicited catecholamine release exclusively from noradrenaline-containing chromaffin cells (Fig. 1.10.) (McKendry *et al.*, 1999). Furthermore, these circulating catecholamines may contribute to the cardiovascular effects of NPs in elasmobranchs.

Binding of homologous CNP was found to be most abundant in the outer layer of the conus arteriosus of *Scyliorhinus stellaris* (Cerra *et al.*, 1995). This may suggest a functional antagonism between CNP and Ang II, which also shows specific binding in the conus arteriosus (see section 4.1.2.), in controlling the pulsatile activity of this vessel.

In conclusion, the renal effects of homologous CNP have been poorly studied in elasmobranch fish and still require a great deal of further investigation. There are many difficulties involved in the interpretation of *in vivo* hormonal effects, due to possible paracrine/endocrine interactions. Therefore, perhaps particularly in the case of CNP, a perfused trunk preparation is the best model for initial studies into the role of this

peptide in renal function. The aim of this chapter was to utilise the *in situ* perfused dogfish trunk preparation to investigate the renal effects of CNP.

5.2. Materials and Methods

5.2.1. Animals

Dogfish were collected and maintained according to section 2.2.1.

5.2.2. Chemicals and Equipment

CNP was kindly donated by Prof. Yoshio Takei, Ocean Research Institute, University of Tokyo. Peptide was made up to a concentration of 10^{-3} M in Milli-Q water and stored in 5 µl aliquots at –20° C until use.

5.2.3. In situ perfusion of the kidney

Kidneys were perfused according to section 2.2.4. and 2.2.5. Urine flow was allowed to stabilise for at least one hour before two, one-hour urine samples were collected into pre-weighed microcentrifuge tubes. CNP was then added to the perfusate at a concentration of either 10^{-9} M or 10^{-10} M and two further one-hour urine samples were collected.

5.2.4. Analysis and calculations

All analysis and calculations are described in section 2.2.7. to section 2.2.12. and section 3.2.4.

5.2.5. Statistical analysis

Comparisons were made between the final renal clearance period immediately prior to administration of CNP and the final clearance period during CNP administration. Paired *t*-tests were used to assess physiological changes (INSTAT).

5.3. Results

5.3.1. Perfusate flow rates

Table 5.1. shows the effect of CNP on perfusate flow rates. There was a slight increase in perfusate flow rate that did not reach significance during the perfusion period. This effect occurred at both concentrations of CNP in both SW and 85% SW.

	Control	10^{-9} M CNP	Control	10^{-10} M CNP
Perfusate flow rate (SW)	34.6 ± 4.2	39.5 ± 2.9	32.2 ± 4.2	38.1 ± 3.6
Perfusate flow rate (85% SW)	35.7 ± 3.6	40.9 ± 4.5	30.9 ± 5.1	37.4 ± 4.1

Table 5.1. Effect of CNP on perfusate flow rate. Values are mean ± S.E.M. from 6 fish in each group.

5.3.2. Effects of CNP on urine flow rate

Addition of 10^{-9} M CNP to the perfusate resulted in a significant diuresis in both SW- and 85% SW-acclimated preparations (Fig. 5.2.). Addition of 10^{-10} M CNP had no significant effect in either salinity (Fig 5.3.).

5.3.3. Glomerular effects

Addition of 10^{-9} M CNP to the perfusate resulted in a significant increase in GFR in both SW and 85% SW-acclimated preparations (Fig. 5.2.). Addition of 10^{-10} M CNP, however, had no significant effect in either SW or 85% SW (Fig. 5.3.).

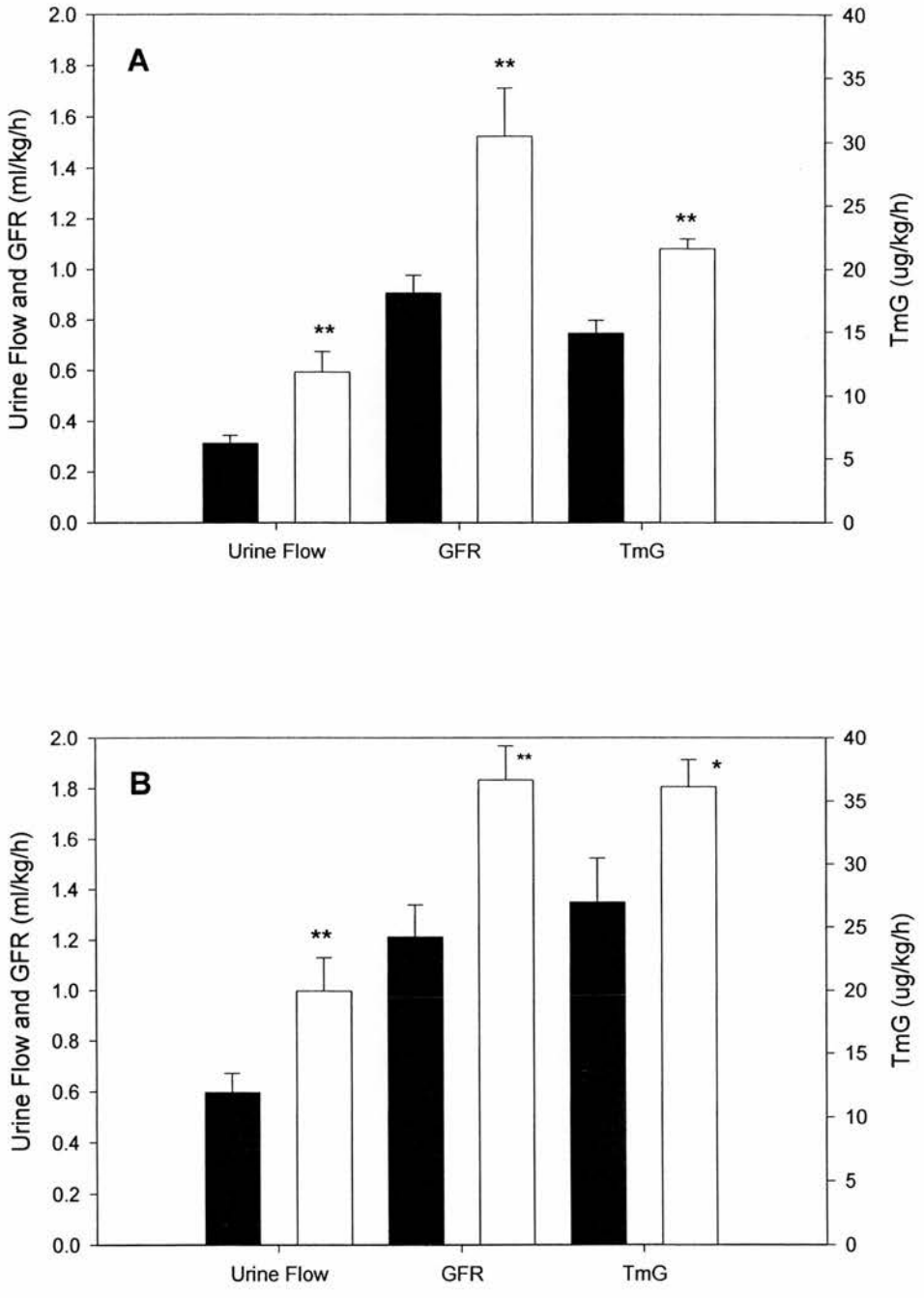

Fig. 5.2. Urine flow rate, GFR and TmG of *in situ* perfused dogfish kidney (10^{-9} M CNP). Comparisons are between the final renal clearance period immediately before CNP administration (solid bars) and for the final clearance period during CNP administration (open bars) in SW (A) and 85% SW (B). Values are means ± SEM; n=6 in each group. $*P < 0.05$, and $**P < 0.01$.

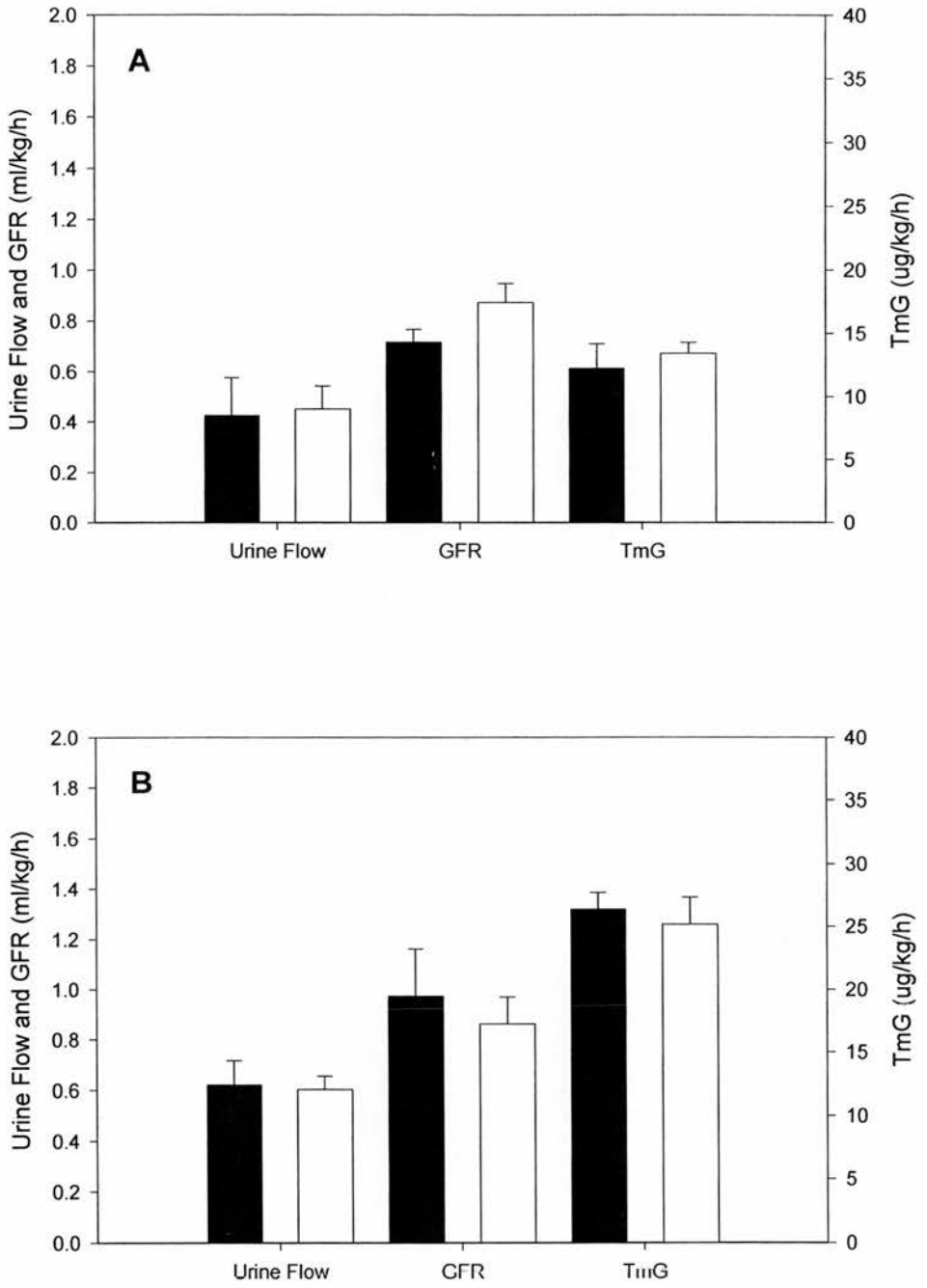

Fig. 5.3. Urine flow rate, GFR and TmG of *in situ* perfused dogfish kidney (10^{-10} M CNP). Comparisons are between the final renal clearance period immediately before CNP administration (solid bars) and for the final clearance period during CNP administration (open bars) in SW (A) and 85% SW (B). Values are means ± SEM; n=6 in each group.

5.3.4. Functional tubular mass

Addition of 10^{-9} M CNP to the perfusate resulted in a significant increase in TmG values in experimental salinities (Fig. 5.2.). Addition of 10^{-10} M CNP, however, had no significant effect in either SW or 85% SW (Fig. 5.3.).

	U/P_{in}	U/P_{osm}	C_{osm}/GFR	C_{H_2O}/GFR
Control (SW)	2.1 ± 0.4	0.972 ± 0.023	56.9 ± 9.0	0.4 ± 0.9
10^{-10} M CNP	2.1 ± 0.3	0.990 ± 0.003	51.2 ± 10.6	0.5 ± 0.1
Control (SW)	2.3 ± 0.4	0.994 ± 0.006	41.4 ± 3.7	0.3 ± 0.1
10^{-9} M CNP	2.4 ± 0.4	0.988 ± 0.004	45.8 ± 10.2	0.6 ± 0.3
Control (85% SW)	1.7 ± 0.4	0.991 ± 0.006	61.9 ± 9.8	0.2 ± 0.1
10^{-10} M CNP	1.7 ± 0.2	0.993 ± 0.005	63.8 ± 10.2	0.1 ± 0.2
Control (85% SW)	1.7 ± 0.2	0.994 ± 0.004	58.1 ± 8.6	0.1 ± 0.2
10^{-9} M CNP	1.8 ± 0.3	0.993 ± 0.004	60.2 ± 5.3	0.1 ± 0.1

Table 5.2. Effect of CNP on tubular function in the *in situ* perfused trunk preparation. Values are mean ± S.E.M. from 6 fish in each group. U/P_{in}, urine/perfusate inulin concentration ratio; U/P_{osm}, urine/perfusate osmolality ratio; C_{osm}/GFR, relative clearance of osmolytes; C_{H2O}/GFR, relative free water clearance.

5.3.5. Tubular effects

Tubular function is summarised in Table 5.2. The mean U/P inulin concentration indicates that on average 48% of the filtered volume of water was reabsorbed by the renal tubule. Urine was slightly hypotonic to plasma with a mean U/P_{osm} of 0.99. This resulted in a small relative free water clearance of less than 1%. The relative osmolar clearance demonstrates that on average approximately 55% of filtered osmolytes were

excreted. Perfusion of CNP had no significant effect on any of the tubular parameters measured.

5.3.6. Effects on ion clearance and excretion

Perfusion of 10^{-9} M CNP resulted in a significant increase in urea clearance and urea excretion in both SW and 85% SW (Fig. 5.4.). At this dose, the fractional excretion of urea was not significantly altered. The clearance and excretion of both chloride and sodium were also significantly reduced on perfusion with 10^{-9} M CNP in both SW and 85% SW (Figs 5.6. & 5.8.). Perfusion of 10^{-10} M CNP however, had no significant effect of the clearance, excretion or fractional excretion of urea, chloride or sodium (Figs. 5.5., 5.7. & 5.9.).

5.3.7. Relationship between urine flow rate and GFR

There was a clear linear relationship between urine flow rate and GFR both before and after perfusion with CNP in both SW and 85% SW (Fig 5.10.).

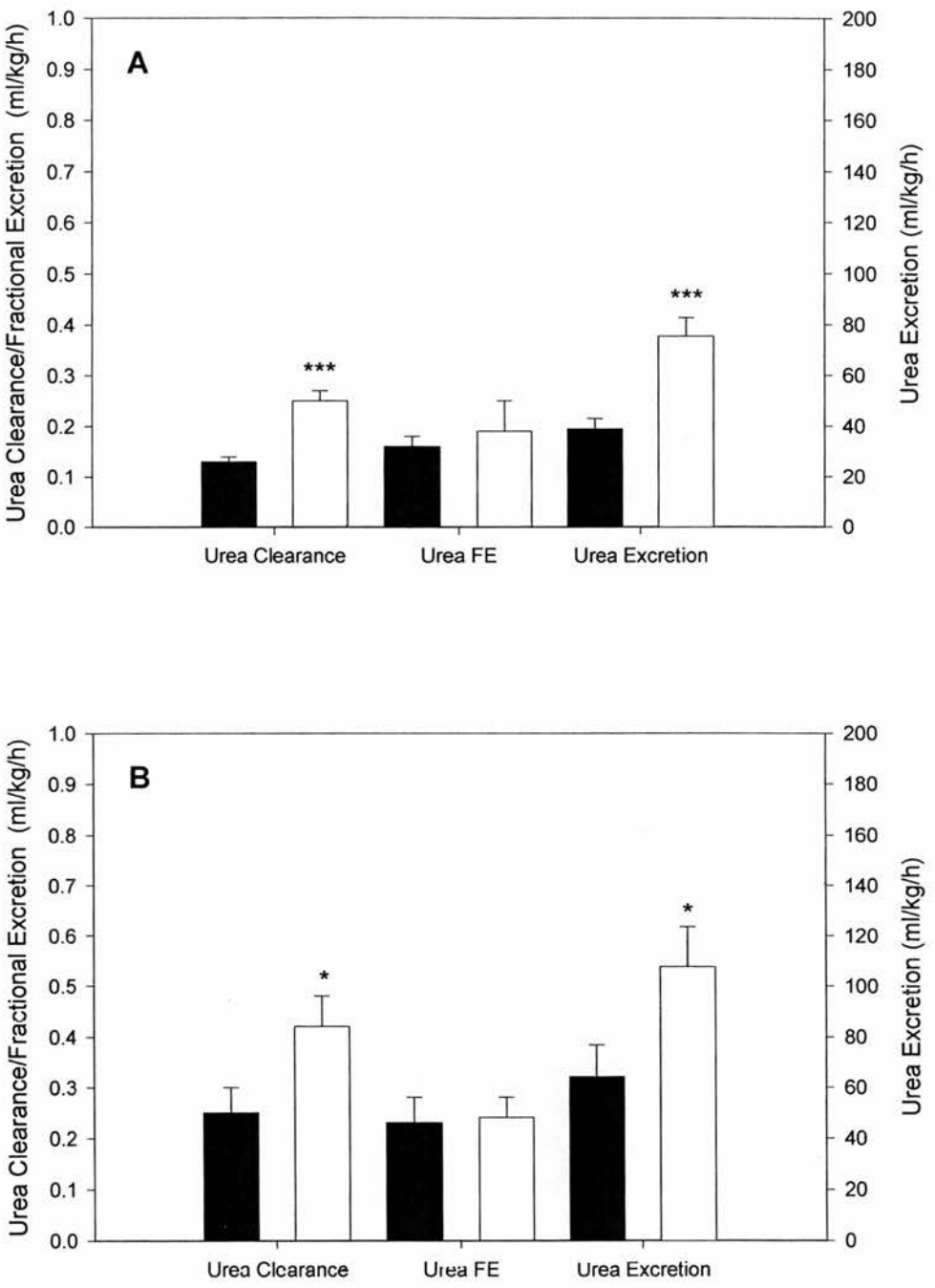

Fig. 5.4. Urea clearance, fractional excretion and excretion in the *in situ* perfused dogfish kidney (10^{-9} M CNP). Comparisons are between the final renal clearance period immediately before CNP administration (solid bars) and for the final clearance period during CNP administration (open bars) in SW (A) and 85% SW (B). Values are means ± SEM; n=6 in each group. $*P < 0.05$ and $***P < 0.005$.

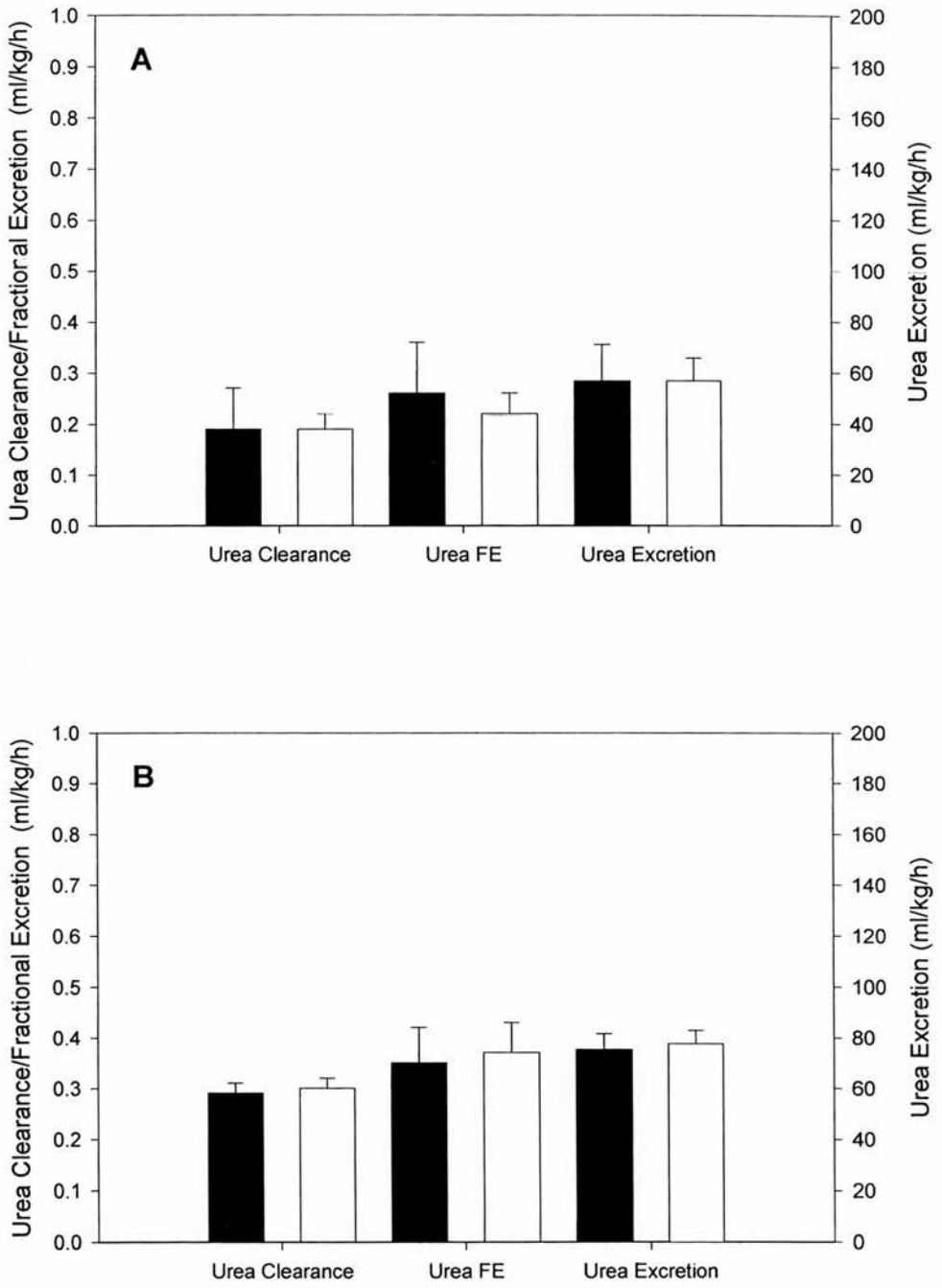

Fig. 5.5. Urea clearance, fractional excretion and excretion in the *in situ* perfused dogfish kidney (10^{-10} M CNP). Comparisons are between the final renal clearance period immediately before CNP administration (solid bars) and for the final clearance period during CNP administration (open bars) in SW (A) and 85% SW (B). Values are means ± SEM; n=6 in each group.

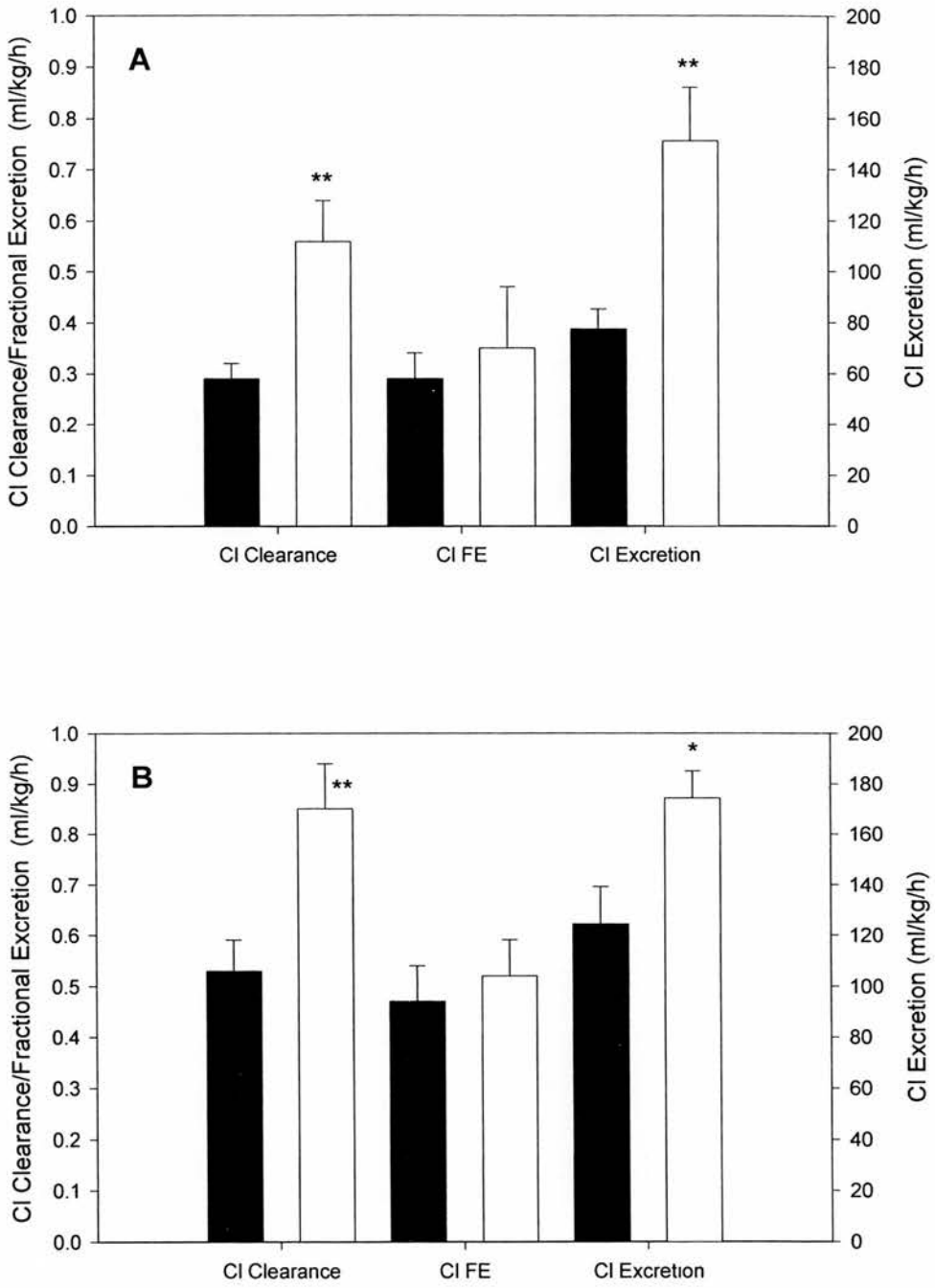

Fig. 5.6. Chloride clearance, fractional excretion and excretion in the *in situ* perfused dogfish kidney (10^{-9} M CNP). Comparisons are between the final renal clearance period immediately before CNP administration (solid bars) and for the final clearance period during CNP administration (open bars) in SW (A) and 85% SW (B). Values are means ± SEM; n=6 in each group. $*P < 0.05$ and $**P < 0.01$.

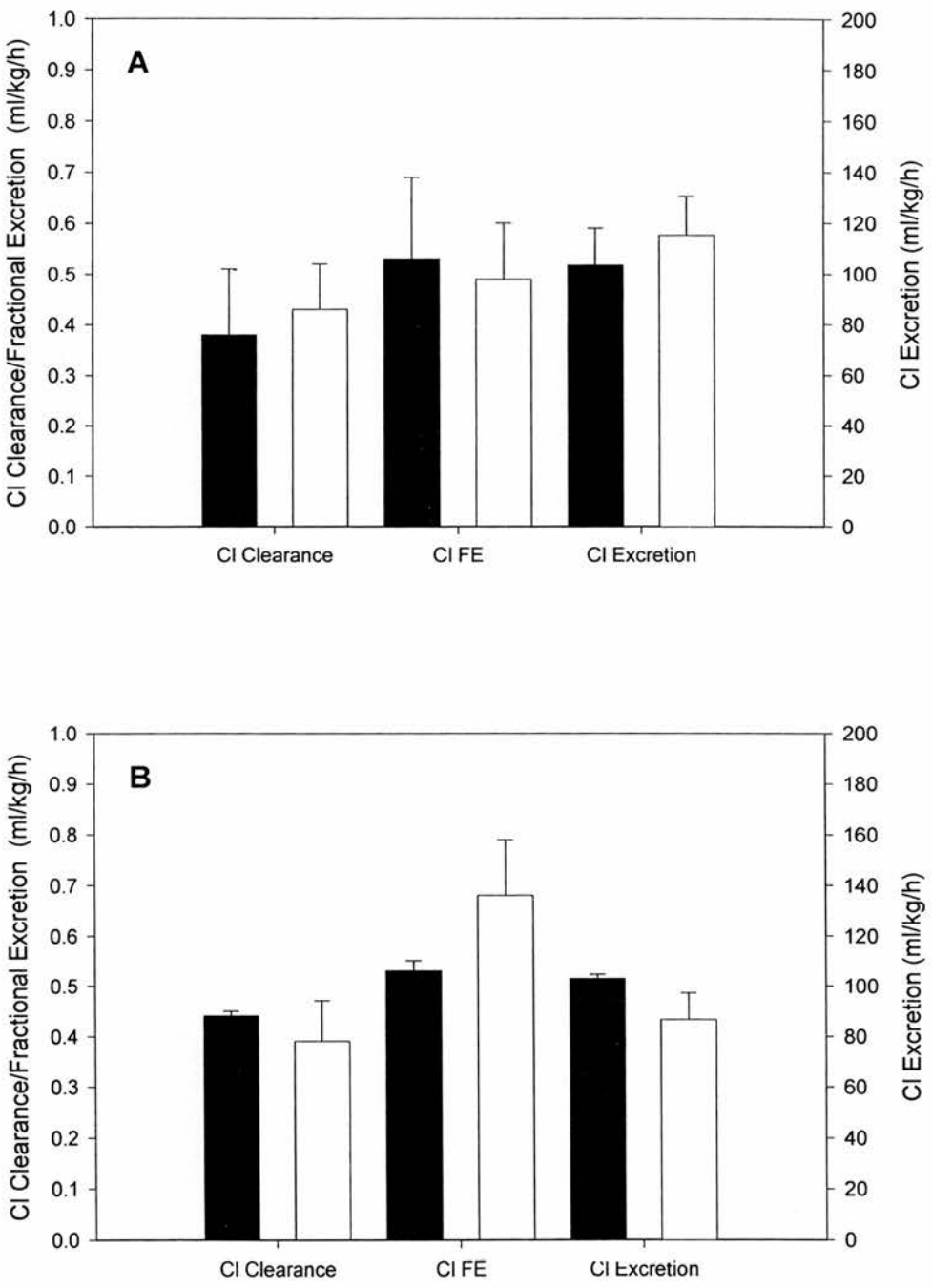

Fig. 5.7. Chloride clearance, fractional excretion and excretion in the *in situ* perfused dogfish kidney (10^{-10} M CNP). Comparisons are between the final renal clearance period immediately before CNP administration (solid bars) and for the final clearance period during CNP administration (open bars) in SW (A) and 85% SW (B). Values are means ± SEM; n=6 in each group.

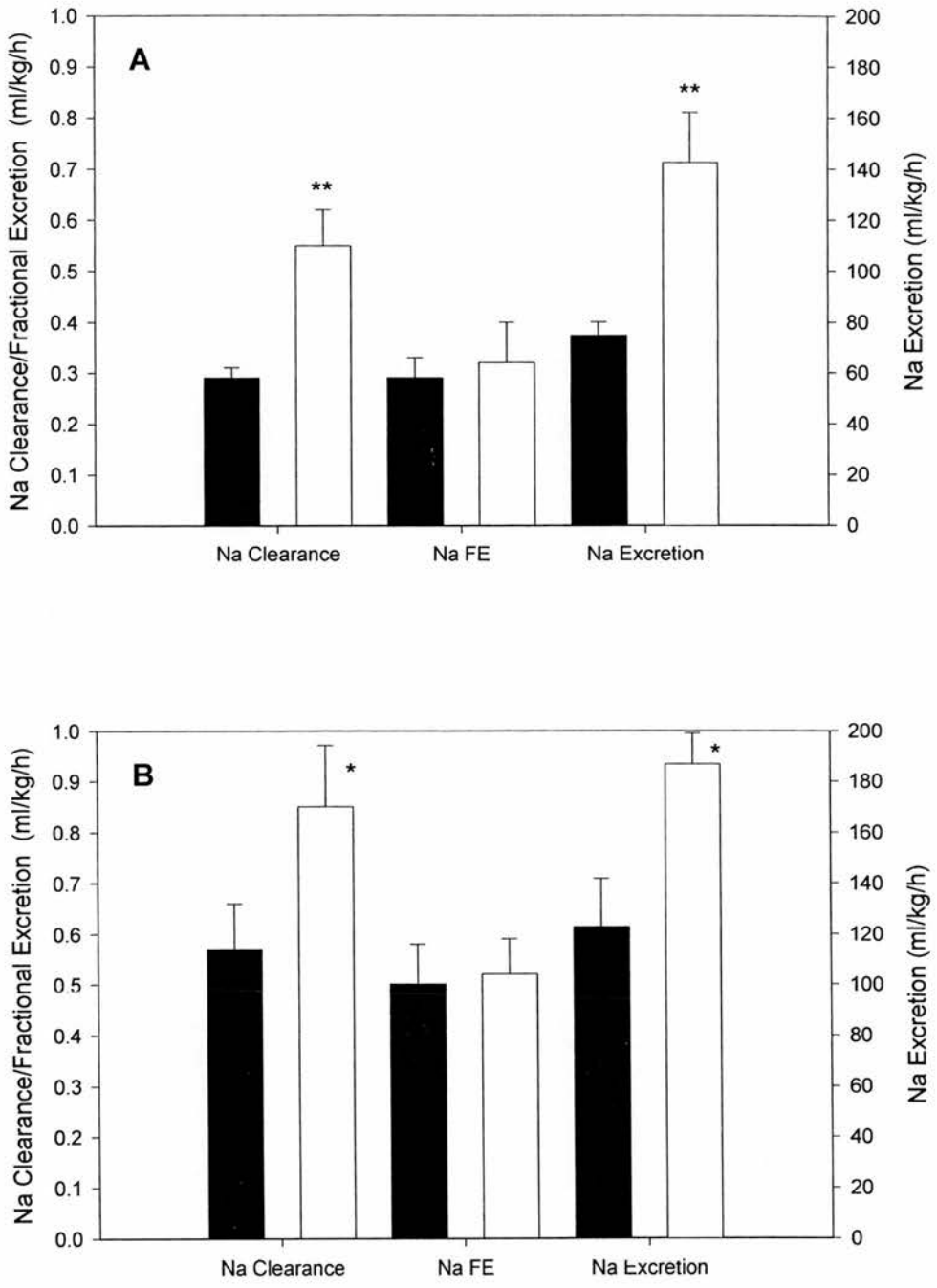

Fig. 5.8. Sodium clearance, fractional excretion and excretion in the *in situ* perfused dogfish kidney (10^{-9} M CNP). Comparisons are between the final renal clearance period immediately before CNP administration (solid bars) and for the final clearance period during CNP administration (open bars) in SW (A) and 85% SW (B). Values are means ± SEM; n=6 in each group. *P < 0.05 and **P < 0.01.

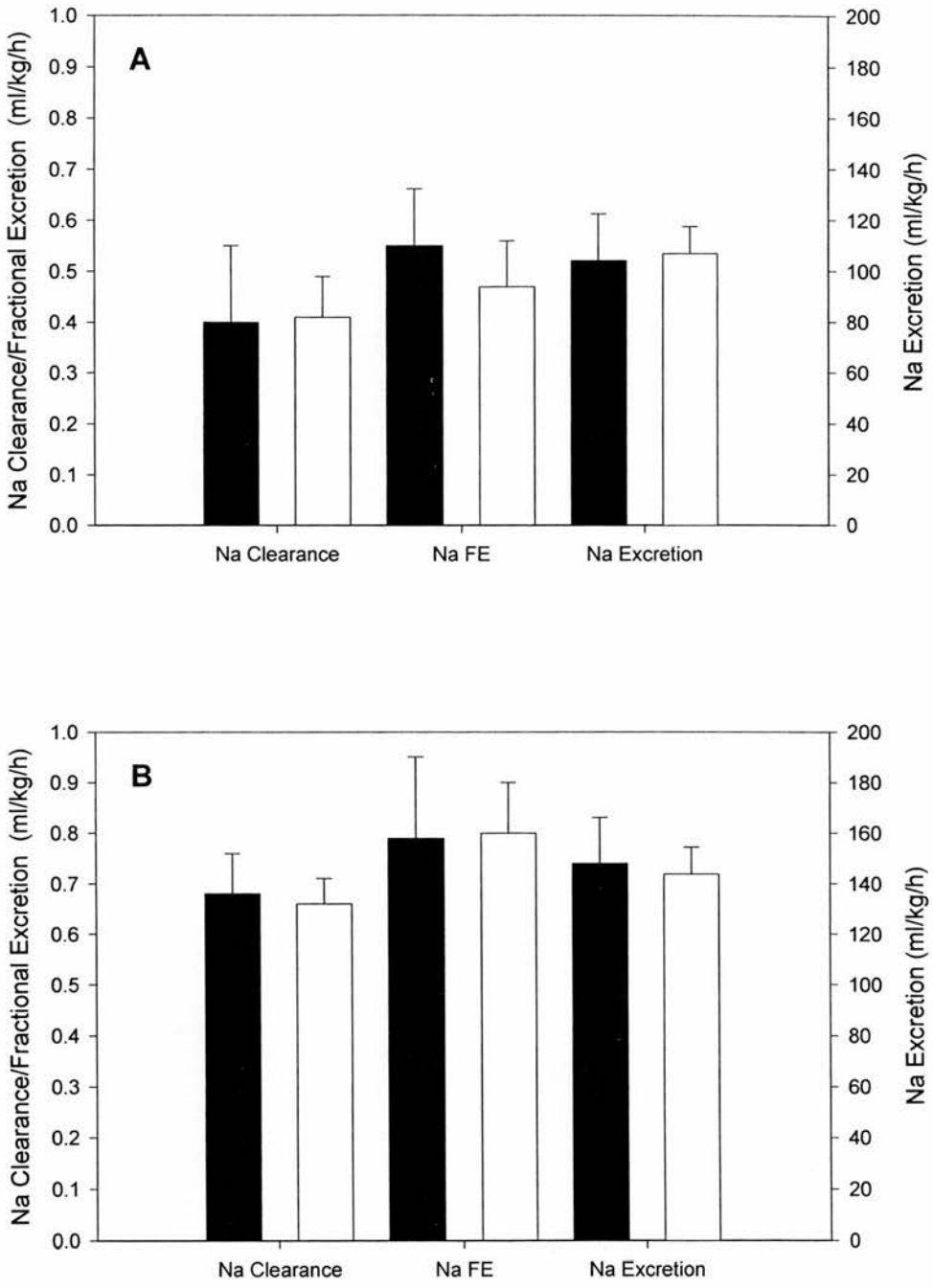

Fig. 5.9. Sodium clearance, fractional excretion and excretion in the *in situ* perfused dogfish kidney (10^{-10} M CNP). Comparisons are between the final renal clearance period immediately before CNP administration (solid bars) and for the final clearance period during CNP administration (open bars) in SW (A) and 85% SW (B). Values are means ± SEM; n=6 in each group.

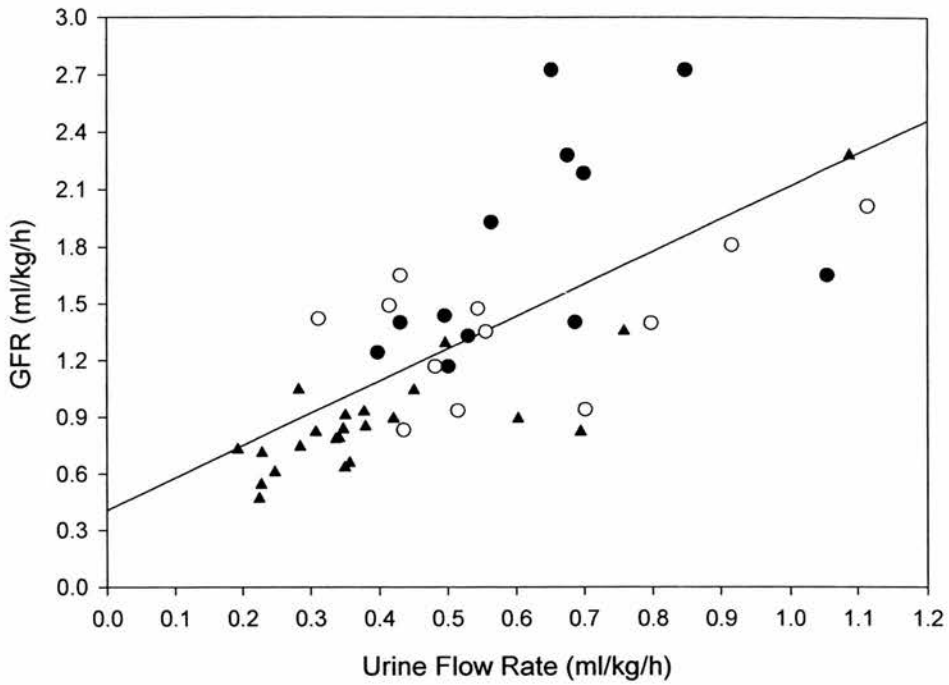

Fig. 5.10. Relationship between GFR and urine flow rate in the perfused kidney of the dogfish. $R_2 = 0.461$. Values are mean rates before perfusion of CNP (▲), and following perfusion with 10^{-9} M CNP (●) and 10^{-10} M CNP (○) in both SW and 85% SW.

5.4. Discussion

Synthetic mammalian ANP caused a glomerular antidiuresis and anti-natriuresis in *S. acanthias* (Benyajati and Yokota, 1990). However, this effect was reversed to diuresis and natriuresis when the fish were volume expanded in 90% SW.

In the present study, CNP caused a slight increase in perfusate flow rate at both doses, but this increase in perfusate flow rate did not reach significance. As discussed in Section 5.1., CNP has a profound vasodilatory effect in dogfish (Bjenning *et al.*, 1992), so it is perhaps surprising that there was no significant rise in perfusate flow. However, in the light of the possible presence of an intra-renal RAS (Chapter 4), one might expect this tissue specific system to be activated by vasodilatory effects of CNP, thereby reducing or completely negating any effects on perfusate flow rate. If this is the case, interpretation of the results for CNP must be undertaken with caution.

10^{-9} M CNP caused a profound glomerular diuresis in both SW and 85% SW. However, 10^{-10} M CNP had no significant effect on any of the renal parameters measured so the threshold dose for this diuretic effect apparently lies between 10^{-9} M and 10^{-10} M. In the light of the possible presence of an intra-renal RAS, and the fact that the vasodilatory effects of CNP would probably activate such a system, one might expect an even greater diuresis and increase in GFR at 10^{-9} M CNP. It is also possible that renal effects of 10^{-10} M CNP are in fact masked by an interaction with Ang II. In order to investigate this possibility, it would be necessary to perfuse the renal preparation with a combination of CNP and captopril. However, problems in fish supply and pressure on the limited supply of peptide have made this impossible in the present study. It is therefore clear

that, despite the advantages of using an *in situ* perfused trunk preparation, there are still additional intra-renal endocrine influences occurring in the kidney. Interpretation of CNP results obtained using the perfused trunk preparation, may be further complicated by the results of a recent study examining interactions between NPs and catecholamine release (McKendry *et al.*, 1999). Bolus injection of homologous CNP in *S. acanthias, in vivo*, elicited a marked and sustained increase in circulating noradrenaline levels. The authors suggested that CNP elicited catecholamine release from noradrenaline-containing chromaffin cells and, due to the close association of chromaffin bodies to the dogfish kidney (See Figure 1.10.), there is scope for the release of noradrenaline within the dogfish renal preparation. The mechanism by which CNP elicits catecholamine release is as yet unknown, but circulating catecholamines may contribute to the cardiovascular effects of CNP (McKendry *et al.*, 1999). In terms of the present study, noradrenaline release has not been measured, but clearly this phenomenon may have influenced the perfusion flow rate and therefore the renal effects of CNP.

Addition of 10^{-9} M CNP to the perfusate caused a significant increase in TmG indicating an increase in the population of filtering glomeruli. TmG was increased by 45% and 34% in SW and 85% SW respectively, indicating a comparable increase in the filtering population of glomeruli. However, Brown & Green (1987) reported that 94% of glomeruli are perfused and filtering in control animals in SW. In the light of this the increase in GFR cannot be fully explained by increases in the filtering population of glomeruli and therefore an effect on SNGFR must be assumed. This will be discussed further in Chapter 7. However, it is still possible, given the effects of CNP on the vasculature, that CNP and ANG II/AVT may act antagonistically on controlling blood flow to the glomeruli, and therefore alter the population of filtering glomeruli. The

effects of CNP, AVT and Ang II will be investigated further using a more direct method of visualising filtering glomeruli with ferrocyanide (See Chapter 6).

CNP appeared to have no net effect on tubular water permeabilities as U/P_{in} was unchanged by perfusion with peptide. In addition, relative clearance of osmolytes and relative free water clearance were unchanged by addition of CNP to the perfusate.

Perfusion of 10^{-9} M CNP caused a significant increase in the clearance and excretion of urea, chloride and sodium. There was no significant effect on the fractional excretion of these ions or urea however, suggesting that the increase in renal excretion of ions and urea can be totally accounted for by the increase in GFR. 10^{-10} M CNP had no significant effect on the renal clearance, excretion or fractional excretion of these ions. These data, coupled with U/P_{in} and the relative clearance of osmolytes and free water suggest that, like AVT and Ang II, CNP has no significant effect on tubular function in the perfused trunk preparation.

In summary, perfusion of the dogfish trunk preparation with 10^{-9} M CNP resulted in an increase in urine flow rate, GFR and TmG, coupled with an increase in the clearance and excretion of urea and sodium chloride. These effects were apparent in both SW and 85% SW, in disagreement with previous studies in *S. acanthias in vivo* whereby a diuresis and increase in GFR on perfusion with atriopeptin was only observed in dilute SW. It would appear therefore that CNP has the opposite renal action to both AVT and Ang II. Together these peptides may play an important role in the renal function of the dogfish.

6

Renal Haemodynamics

6.1. Introduction

In previous chapters the effects of various peptides on the dogfish kidney have been examined. The filtering population of nephrons has been assessed indirectly, using the tubular transport maxima for glucose as an indicator of the functional tubular mass of the whole kidney. However, TmG has been shown to be proportional to GFR and there is growing evidence to suggest that variations in TmG may also result from changes in SNGFR and altered tubular handling of sodium (Brown *et al.*, 1993). In addition TmG, prior to the addition of peptide, has proved to be somewhat variable in the present study. It was therefore decided to investigate the filtering population of glomeruli directly, using the ferrocyanide technique (Hanssen, 1958).

Sodium ferrocyanide has been demonstrated to be excreted solely by glomerular filtration without apparent tubular reabsorption or secretion, and may therefore be considered a suitable indicator for histochemical demonstration of glomerular filtration (Hanssen, 1958). The ferrocyanide technique was originally developed in the mouse kidney, allowing the investigator to examine the distribution of the filtrate among the nephron population by comparing the amount of precipitated ferrocyanide in individual nephrons. This technique involves the infusion of a saturated solution of ferrocyanide through the kidney for a short period. Blood (or saline) flow is then stopped, and the kidney is rapidly snap frozen and transferred to ferric chloride for freeze substitution at

–20°C. This causes the precipitation of ferrocyanide as Prussian blue, which can then be visualised in the glomeruli and neck segment of the renal tubule following microdissection (See Fig. 6.1.).

Previously, the ferrocyanide technique has been used to assess the filtering population of glomeruli in renal studies on the trout, *O. mykiss* (Amer and Brown, 1995; Brown and Balment, 1997). This allowed the authors to categorise glomerular populations into three functional categories.

1. Perfused and filtering
2. Perfused but non-filtering
3. Non-perfused

The population of filtering nephrons in the kidney of elasmobranch fish has been poorly studied. In the only study in an elasmobranch fish to date, the population of filtering nephrons, and changes in the population following infusion of adrenaline was examined in *S. canicula* (Brown and Green, 1987). The authors observed the same categories of functional glomeruli that are described above. In the case of *S. canicula* the majority of glomeruli (94%) were found to be perfused and filtering, with only 4% arterially perfused but not filtering and 2% non-perfused. Infusion of adrenaline caused a significant diuresis, but this was coupled by a decrease in the filtering population of glomeruli, with an associated increase in the proportion that were non-perfused.

The purpose of this chapter is to assess the perfused trunk preparation in both SW and 85% SW acclimated dogfish in terms of the ferrocyanide method. Initially, the filtering population of nephrons was determined in control preparations that received no peptide.

This allowed a comparison with previous studies performed *in vivo* as well as allowing comparison with the effects of two hours of perfusion with AVT, Ang II or CNP.

6.2. Materials and Methods

6.2.1. Animals

Dogfish were collected and maintained according to section 2.2.1.

6.2.2. In situ perfusion of the kidney

Kidneys were perfused according to section 2.2.4. and 2.2.5. Urine flow was allowed to stabilise for at least one hour before two, one-hour urine samples were collected into pre-weighed microcentrifuge tubes. Peptide was then added to the perfusate at a concentration of 10^{-9} M and two further one-hour urine samples were collected. Urine flow rate was determined gravimetrically and comparisons were made between the last one-hour collection period immediately prior to addition of peptide to the perfusate and the final one-hour collection period during administration of peptide to ensure that the hormonal effects were consistent with those reported in previous chapters. In addition, control animals were perfused with Ringer's solution alone for an equivalent period. The kidneys from control animals were then treated in an identical manner to those which were perfused with hormone. At the end of the perfusion period a freshly prepared solution of 30% (wt/vol) sodium ferrocyanide (sodium hexacyanoferrate (II) – Fisher Chemical Co.) in dogfish Ringer's solution was infused via a fine aortic cannula at 3 ml/min for 40 seconds. The kidney was then immediately removed and snap frozen by immersion in isopentane, which had been pre-cooled in liquid nitrogen. The kidney was then chopped into pieces of less than $2mm^3$, and placed in a pre-cooled solution of ferric chloride (60g hydrated ferric chloride dissolved in 95 ml ethanol and 5 ml concentrated hydrochloric acid). This freeze substitution reaction was allowed to proceed for 18 hours at -20°C. The renal tissue was then macerated in 20% hydrochloric

acid at 37°C for 3-4 hours and subsequently stored at room temperature in a dilute solution of ferric chloride (0.2% ferric chloride and 1% acetic acid in Milli-Q water). Kidneys were processed within one month of removal from the animal.

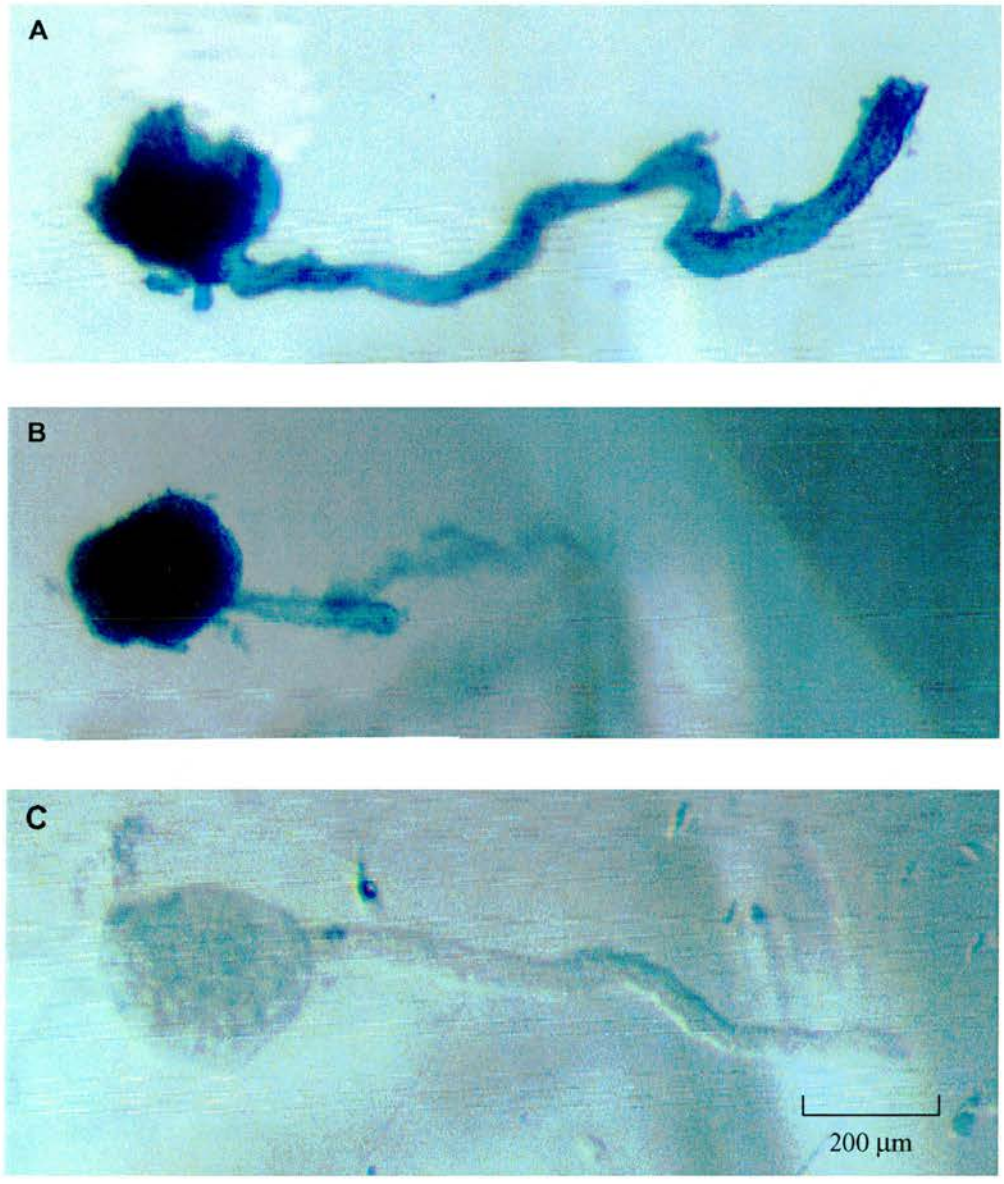

Fig. 6.1. Glomerular types showing precipitated Prussian blue. A, arterially perfused and filtering (P&F) with Prussian blue precipitate in the glomerulus and neck segment, B, arterially perfused but not filtering (PNF) with Prussian blue precipitate in the glomerulus but not in the neck segment and C, non-perfused (NP) with no associated Prussian blue in either the glomerulus or the neck segment.

Microdissection of nephrons was performed in glycerol under a binocular microscope (Leica Wild M8, Leica Microsystems Ltd, UK), using glass teasing needles pulled from glass haematocrit capillary tubing. Individual glomeruli, complete with a section of renal tubule, were dissected free and categorised according to the presence or absence of Prussian blue. If Prussian blue was present both in the glomerulus and the neck segment of the renal tubule the glomerulus was categorised as perfused and filtering (P&F) (Fig. 6.1.A.). If Prussian blue was present in the glomerulus but not in the neck segment, the glomerulus was categorised as perfused, but not filtering (PNF) (Fig. 6.1.B.). If Prussian blue was absent from both the glomerulus and the neck segment, then they were categorised as non-perfused (NP) (Fig. 6.1.C.)

Glomeruli were classified until the proportions of glomeruli in each classification reached constancy. This usually required the classification of between 120-160 glomeruli per kidney.

6.2.3. Data and statistical analysis

The proportions of glomeruli that fell into each category were expressed as a percentage of the total number of glomeruli counted for that kidney. In each case values were expressed as a mean of 5 or 6 fish ± SEM. Prior to statistical analysis data were arcsin transformed and unpaired *t*-tests were used to assess differences in the proportions of glomeruli in each state between SW and 85% SW. One-Way ANOVA followed by Tukey's post hoc test was used to assess changes between basal states and the states after 2 hours perfusion with peptide. Differences between hormonal groups were also assessed using one-way ANOVA followed by Tukey's post hoc test. Due to time

constraints and pressure on fish numbers, the population of filtering glomeruli was only investigated at a dose of 10^{-9} M for each peptide.

6.3. Results

6.3.1. Verification of the renal effects of peptide

In order to ensure that the hormonal effects of AVT, Ang II and CNP were comparable with those reported in Chapters 3-5, urine flow rate was measured in all trunk preparations. The effects of all three peptides on urine flow, in both SW and 85% SW, are demonstrated in Figs. 6.2.-6.4. In the case of AVT and Ang II (Figs. 6.2. & 6.3.) an antidiuresis, consistent with that seen in Chapters 3 and 4 was observed in both salinities. In the case of CNP (Figs. 6.4.) a diuresis, consistent with that seen in Chapter 5 was observed.

6.3.2. Distribution of glomerular types in control preparations

The proportion of glomerular types observed in renal preparations which did not receive peptide is shown in Figure 6.5.A. (SW) and Figure 6.5.B. (85% SW). In SW-acclimated animals, 86% of glomeruli were perfused and filtering; 10% perfused but not filtering and 4% non-perfused. There was a similar effect in 85% SW-acclimated animals, where 83% of glomeruli were perfused and filtering; 15% perfused but not filtering and 2% non-perfused. There was no significant difference between the perfused and filtering or the non-perfused groups between SW and 85% SW. However, there was a small, but significant difference between the perfused but not filtering groups in SW and 85% SW.

6.3.3. Distribution of glomerular types in preparations perfused with hormone

Figure 6.6. shows the distribution of glomeruli in preparations perfused with 10^{-9} M AVT in SW (A) and 85% SW (B). A very similar pattern of perfusion was observed in both SW and 85% SW. There was a significant difference between AVT perfused

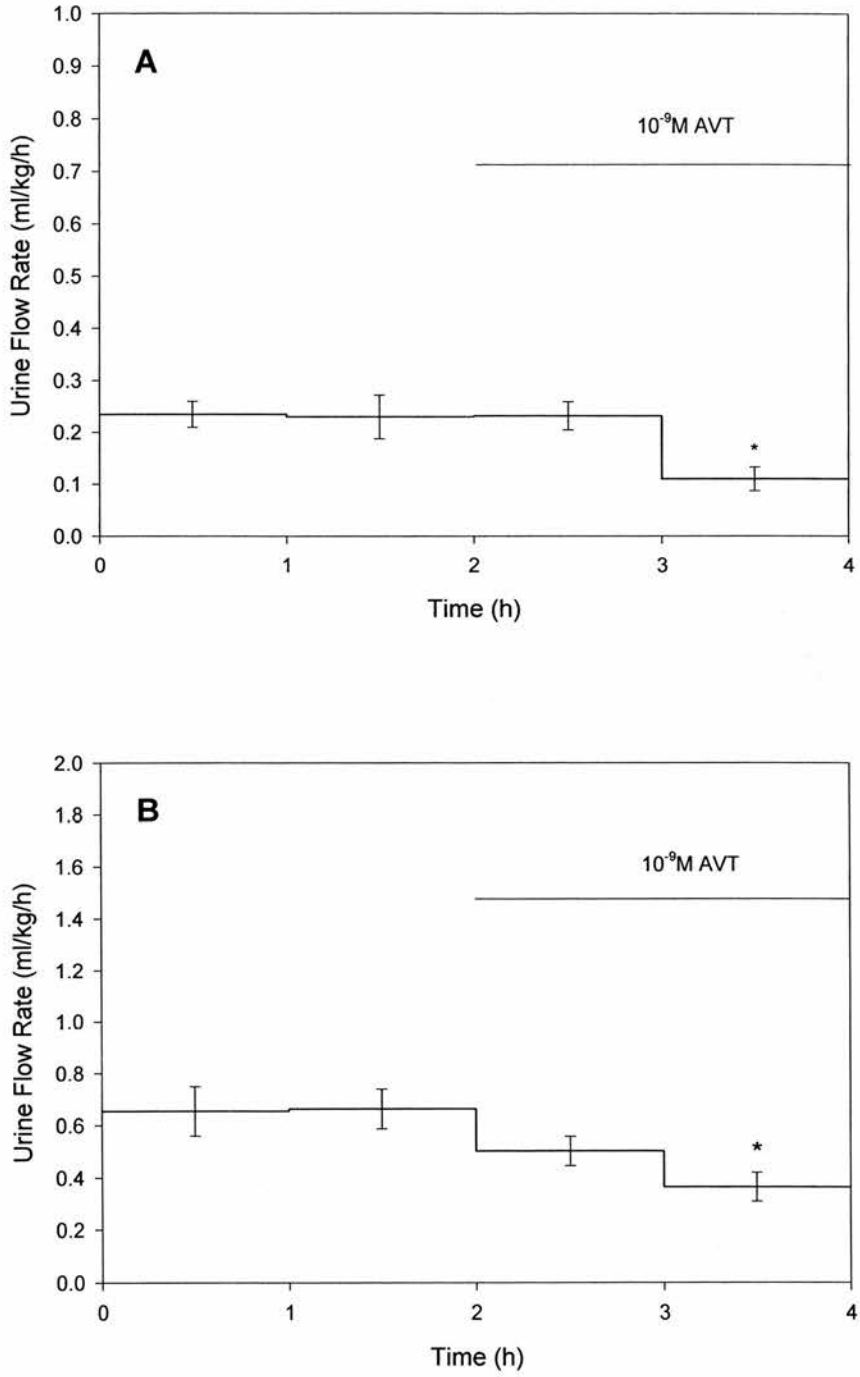

Fig. 6.2. Urine flow rate of *in situ* perfused dogfish kidney (10^{-9} M AVT). Comparisons are between the final renal clearance period immediately before AVT administration and for the final clearance period during AVT administration in SW (A) and 85% SW (B). Values are means ± SEM; n=5 in each group. *$P <$ 0.05

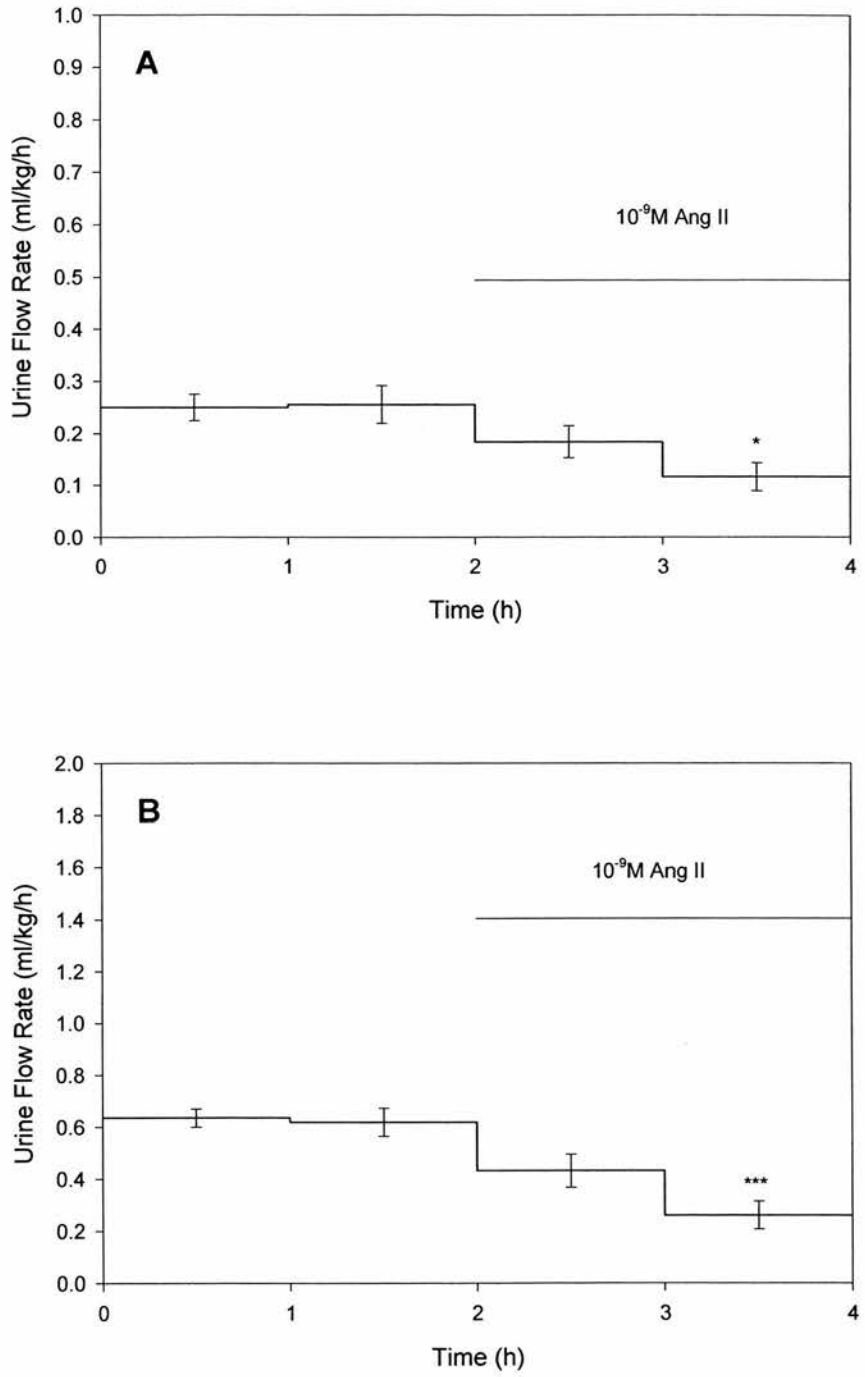

Fig. 6.3. Urine flow rate of *in situ* perfused dogfish kidney (10^{-9} M Ang II). Comparisons are between the final renal clearance period immediately before Ang II administration and for the final clearance period during Ang II administration in SW (A) and 85% SW (B). Values are means ± SEM; n=5 in each group. *$P <$ 0.05, ***$P < 0.005$.

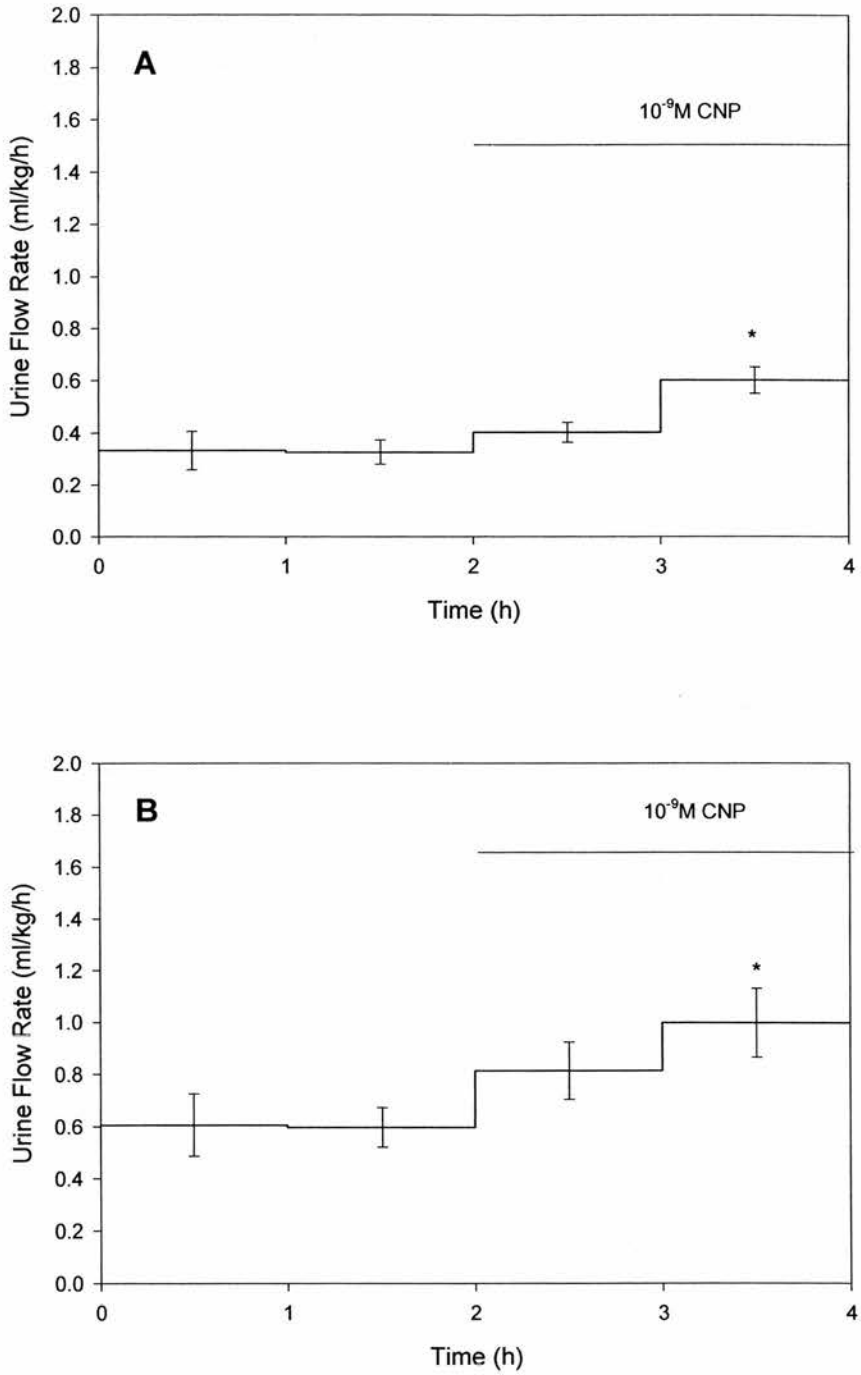

Fig. 6.4. Urine flow rate of *in situ* perfused dogfish kidney (10^{-9} M CNP). Comparisons are between the final renal clearance period immediately before CNP administration and for the final clearance period during CNP administration in SW (A) and 85% SW (B). Values are means ± SEM; n=5 in each group. *$P < 0.05$.

preparations and basal preparations in all three glomerular states in both SW and 85% SW (Fig. 6.6.). This was due to a significant drop in the percentage of glomeruli that were perfused and filtering and an associated rise in the proportions that were perfused but not filtering and non-perfused. In preparations acclimated to both SW and 85% SW the majority of glomeruli were arterially perfused but not filtering.

Figure 6.7. shows the distribution of glomeruli in preparations perfused with 10^{-9} M Ang II in SW (A) and 85% SW (B). The pattern of perfusion in preparations from fish acclimated to SW and 85% SW is similar but a significantly greater proportion of glomeruli were perfused and filtering in 85% SW following 2 hours of perfusion with 10^{-9} M Ang II. This was associated with a significantly reduced proportion of glomeruli that were perfused but not filtering. There was a significant difference between AVT perfused preparations and basal preparations in all three glomerular states in both SW and 85% SW (Figure 6.7.). This was due to a significant drop in the percentage of glomeruli that were perfused and filtering. However, despite there being no significant difference between the proportions of glomeruli that were perfused and filtering when the effects of Ang II and AVT are compared, there was a significant difference between the proportions that were perfused but not filtering and non-perfused (Table 6.1. and 6.2.). In the case of Ang II the majority of glomeruli are non-perfused.

Figure 6.8. shows the distribution of glomeruli in preparations perfused with 10^{-9} M CNP in SW (A) and 85% SW (B). A very similar pattern of perfusion was observed in both SW and 85% SW with only a slight difference in the proportion of glomeruli that were perfused but not filtering. In SW–acclimated preparations there was no significant difference between basal preparations and those perfused with CNP (Fig. 6.8.). Like the

basal preparations the vast majority of glomeruli are perfused and filtering in both SW and 85% SW. However, in 85% SW–acclimated preparations there was a slightly higher proportion of glomeruli that were perfused and filtering when compared with the basal states, associated with a slightly lower proportion that were perfused and not filtering. These differences were statistically significant (Fig. 6.8.). The proportions of glomeruli in all three states in both SW and 85% SW were significantly different from the patterns for both AVT and Ang II with the exception of the perfused non filtering group in 85% SW which is statistically indistinguishable from the corresponding group perfused with Ang II (Table 6.1.and 6.2.).

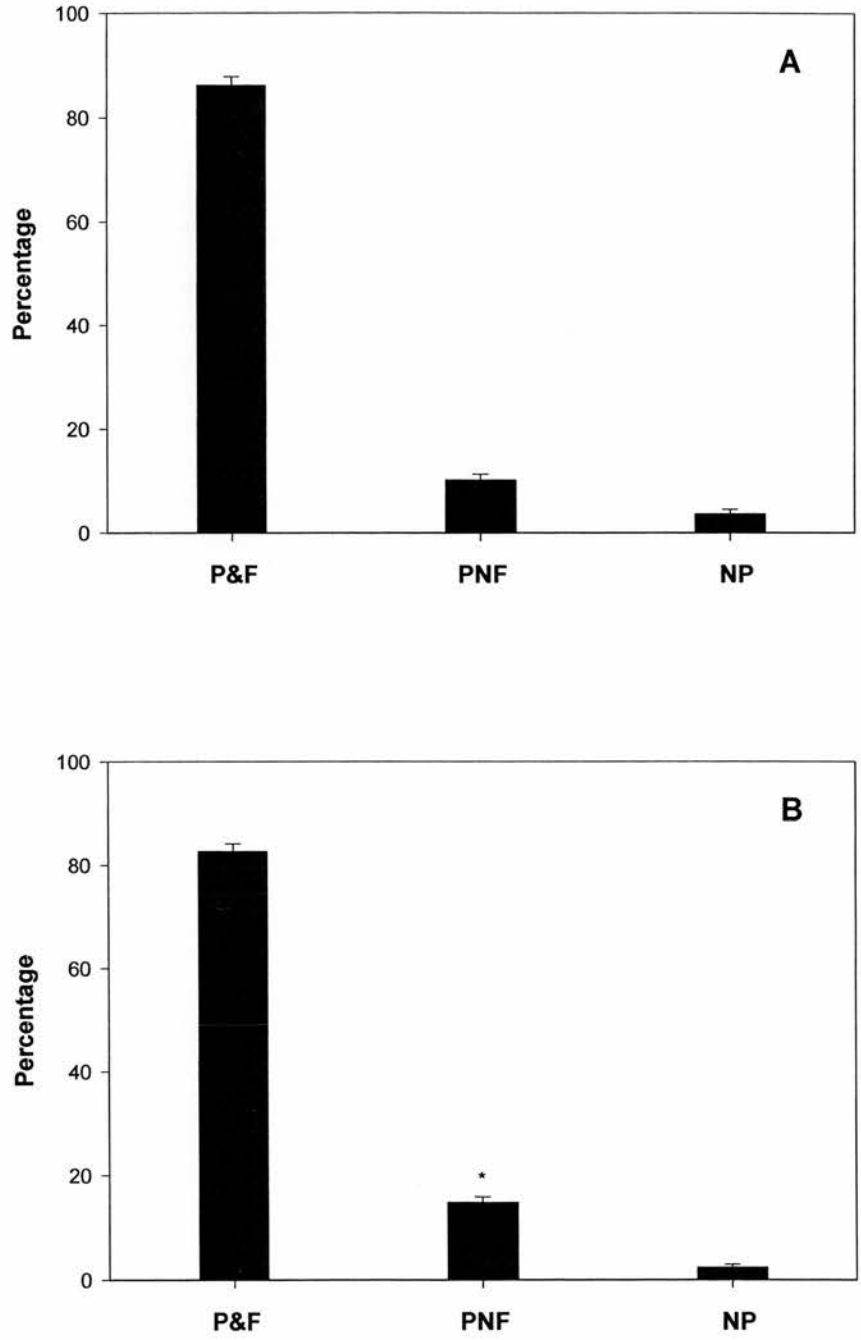

Fig. 6.5. Distribution of the three types of glomeruli occurring in the dogfish kidney in the absence of peptide in fish acclimated to SW (A) and 85% SW (B); arterially perfused and filtering (P&F), arterially perfused but not filtering (PNF) and non-perfused (NP). Values are mean percentages ± SEM; n=5 in each group. *$P < 0.05$, represents significant difference between SW and 85% SW.

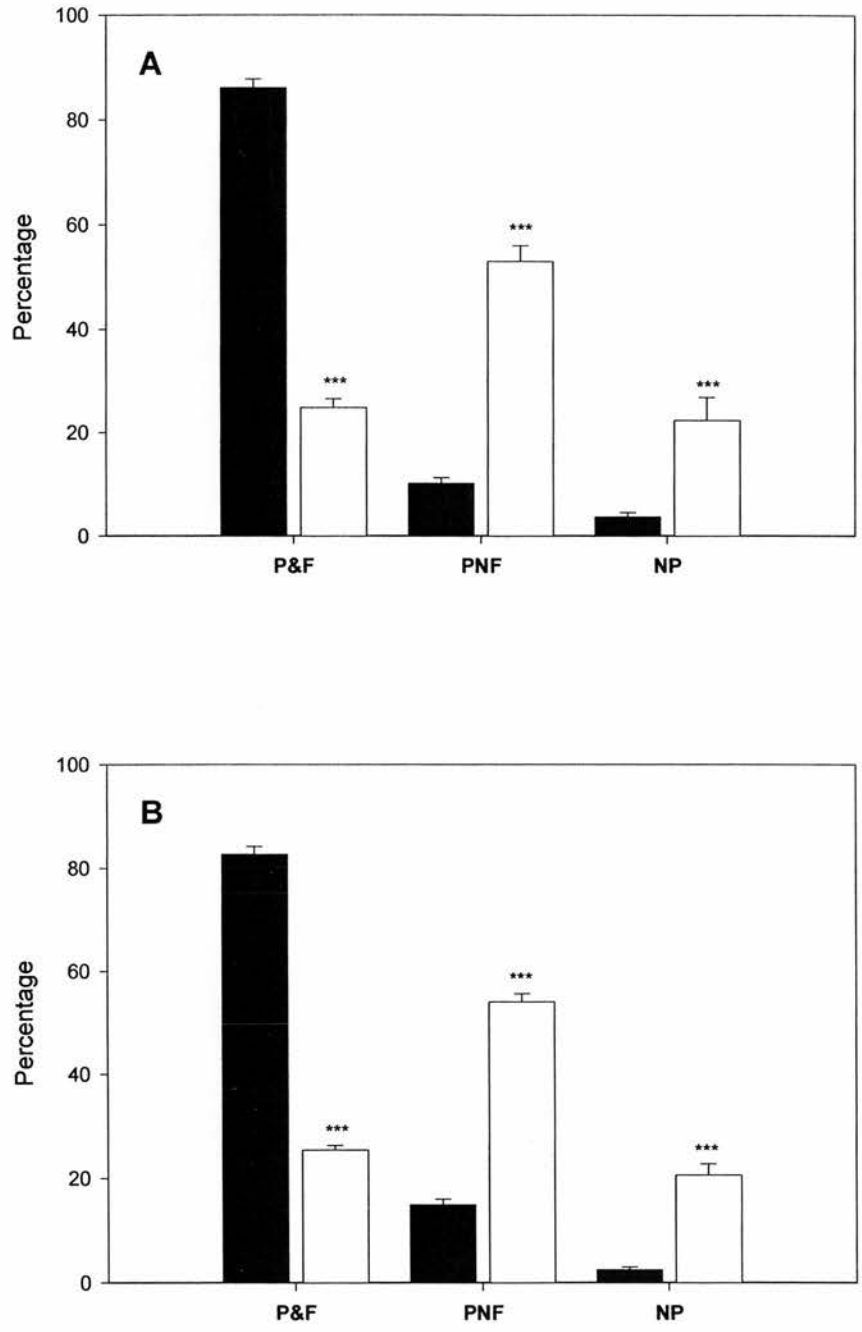

Fig. 6.6. Distribution of the three types of glomeruli occurring in the dogfish kidney in fish acclimated to SW (A) and 85% SW (B). Comparisons are between the control state (solid bars) and following 2 hours of perfusion of 10^{-9} M AVT (open bars); arterially perfused and filtering (P&F), arterially perfused but not filtering (PNF) and non-perfused (NP). Values are mean percentages ± SEM. ***$P < 0.005$ represents significant difference between the control and experimental groups.

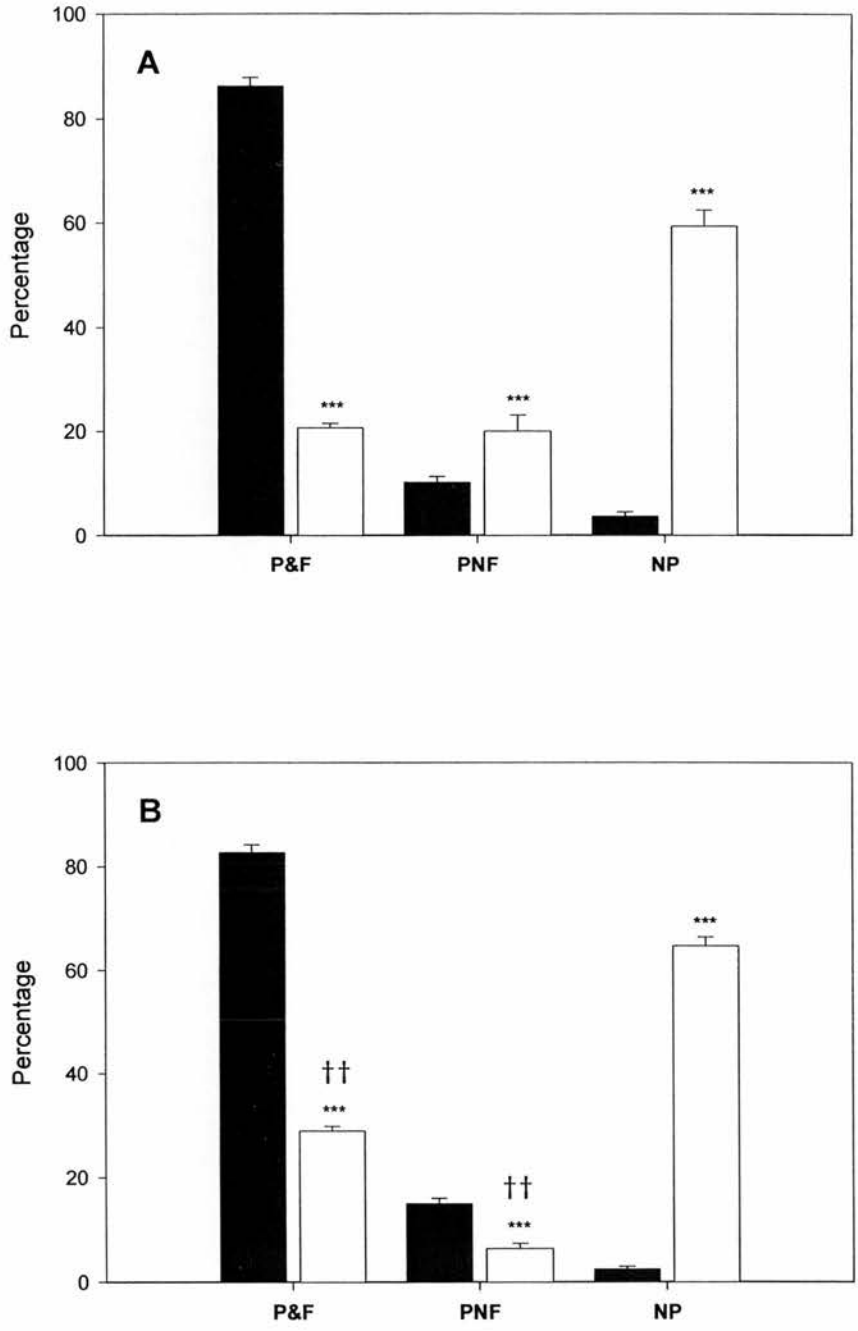

Fig. 6.7. Distribution of the three types of glomeruli occurring in the dogfish kidney in fish acclimated to SW (A) and 85% SW (B). Comparisons are between the control state (solid bars) and following 2 hours of perfusion of 10^{-9} M Ang II (open bars); arterially perfused and filtering (P&F), arterially perfused but not filtering (PNF) and non-perfused (NP). Values are mean percentages ± SEM. ***$P < 0.005$ represents significant difference between the control and experimental groups. ††$P < 0.01$ represents significant difference between SW & 85% SW.

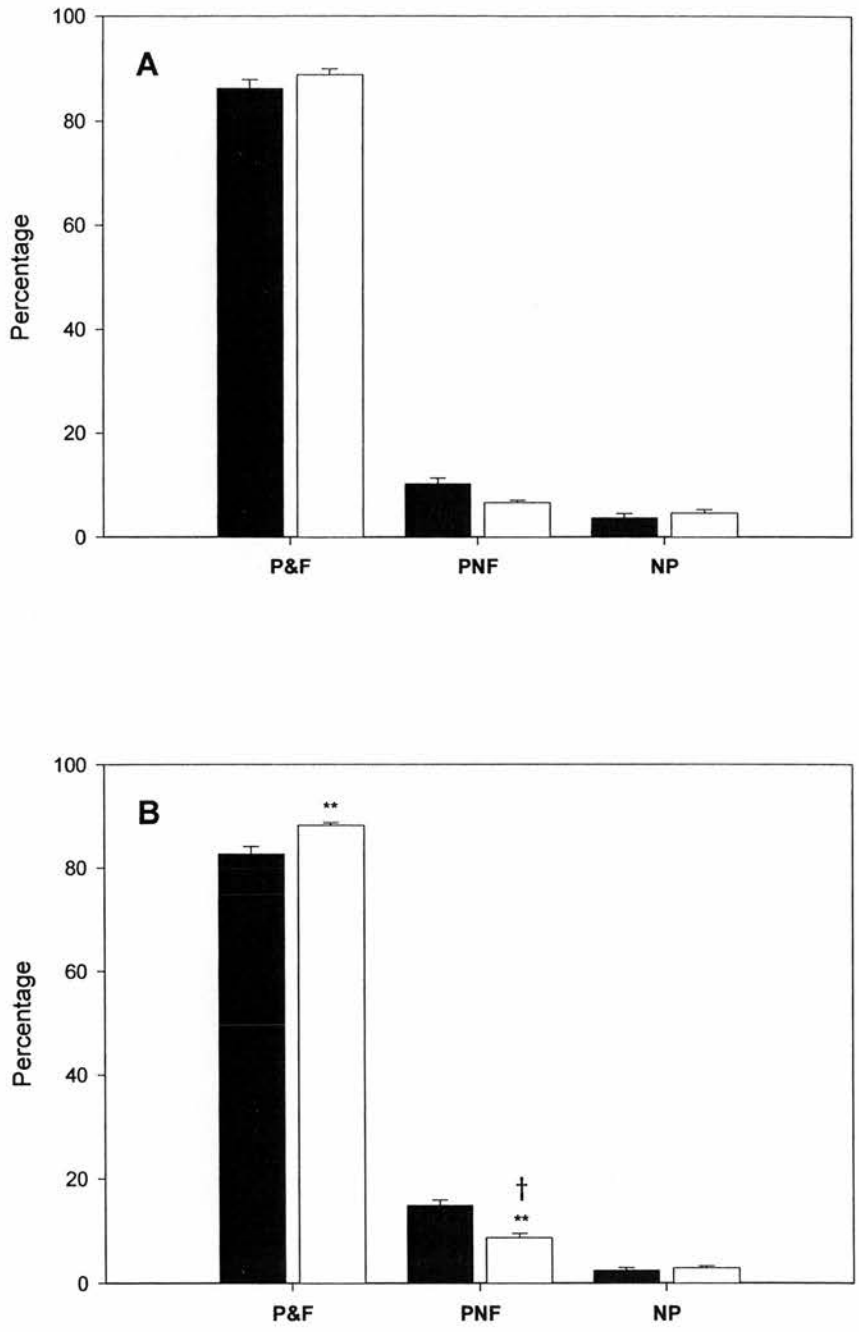

Fig. 6.8. Distribution of the three types of glomeruli occurring in the dogfish kidney in fish acclimated to SW (A) and 85% SW (B). Comparisons are between the control state (solid bars) and following 2 hours of perfusion of 10^{-9} M CNP (open bars); arterially perfused and filtering (P&F), arterially perfused but not filtering (PNF) and non-perfused (NP). Values are mean percentages ± SEM. ***$P < 0.005$ represents significant difference between the control and experimental groups. †$P < 0.05$ represents significant difference between SW & 85% SW.

		AVT			Ang II			CNP		
		P&F	PNF	NP	P&F	PNF	NP	P&F	PNF	NP
AVT	P&F	-	-	-	NS	-	-	***	-	-
	PNF	-	-	-	-	***	-	-	***	-
	NP	-	-	-	-	-	***	-	-	***
Ang II	P&F	NS	-	-	-	-	-	***	-	-
	PNF	-	***	-	-	-	-	-	**	-
	NP	-	-	***	-	-	-	-	-	***
CNP	P&F	***	-	-	***	-	-	-	-	-
	PNF	-	***	-	-	**	-	-	-	-
	NP	-	-	***	-	-	***	-	-	-

Table 6.1. Patterns of statistical significance between groups of perfused trunk preparations in SW. $*P < 0.05$, $**P < 0.01$ and $***P < 0.005$ represent statistical differences between groups.

		AVT			Ang II			CNP		
		P&F	PNF	NP	P&F	PNF	NP	P&F	PNF	NP
AVT	P&F	-	-	-	NS	-	-	***	-	-
	PNF	-	-	-	-	***	-	-	***	-
	NP	-	-	-	-	-	***	-	-	***
Ang II	P&F	NS	-	-	-	-	-	***	-	-
	PNF	-	***	-	-	-	-	-	NS	-
	NP	-	-	***	-	-	-	-	-	***
CNP	P&F	***	-	-	***	-	-	-	-	-
	PNF	-	***	-	-	NS	-	-	-	-
	NP	-	-	***	-	-	***	-	-	-

Table 6.2. Patterns of statistical significance between groups of perfused trunk preparations in 85% SW. $***P < 0.005$ represent statistical differences between groups.

6.4. Discussion

The ferrocyanide technique was first evaluated in the mouse kidney (Hanssen, 1958). Whilst there was some diffusion of ferrocyanide or Prussian blue into the cells, the majority of the ferrocyanide was retained within the nephron during preparation of the specimens. This diffusion affected the ferrocyanide to the same degree in all nephrons, therefore this method was considered suitable for examining the occurrence and distribution of ferrocyanide among different nephron populations (Hanssen, 1958). The ferrocyanide technique has been used to investigate the filtering population of glomeruli in the trout (Amer and Brown, 1995; Brown *et al.*, 1980) and the dogfish (Brown and Green, 1987).

The filtering population of glomeruli has previously been examined *in vivo* in the dogfish, and the majority of glomeruli were found to be perfused and filtering (Brown and Green, 1987). This is in contrast to the freshwater-acclimated rainbow trout in which only 45% of the glomeruli appeared to be filtering in the control animals (Brown *et al.*, 1980). However, in an *in situ* perfused trunk preparation of the rainbow trout, 77% of glomeruli were filtering in control animals (Amer and Brown, 1995). This was attributed in part to the lack of colloids in the physiological saline *in vitro*, but mainly due to the fact that numerous additional hormones, with potential effects on renal function, are present *in vivo*.

The present study demonstrates that, in control animals, a similar pattern is observed in the *in situ* perfused trunk preparation, to that previously described *in vivo* (Brown and Green, 1987). However, the proportion of glomeruli that were perfused and filtering in

SW (86%) was slightly lower than that observed *in vivo* (94%) (Brown and Green, 1987). It is still clear that in both SW and 85% SW–acclimated preparations the vast majority of glomeruli are filtering. There was no significant difference in the proportion of filtering nephrons between SW and 85% SW in control preparations. It would therefore appear that the diuresis observed following acclimation to reduced salinity (Chapter 2) is not due to an increase in the proportion of filtering nephrons. This suggests an increase in SNGFR in order to maintain increased urine flow in reduced salinity environments.

Perfusion of the *in situ* renal preparation with 10^{-9} M AVT caused a significant antidiuresis, as was observed in Chapter 3. This antidiuresis was associated with a significant reduction in the proportion of filtering glomeruli to 25% in preparations from fish acclimated to both SW and 85% SW. This reduction in filtering glomeruli agrees with the reduction in the functional tubular mass, measured using the transport maxima for glucose. However, the magnitude of this reduction was greater when the filtering population was measured directly (70% reduction, as opposed to approximately 50%) (Fig. 3.1.). As urine flow rate and GFR were also only reduced by approximately 50% (Fig. 3.1.) this would suggest that some other mechanism must be occurring to maintain urine flow rate. It is likely that there is an increase in SNGFR in the remaining filtering glomeruli, in order to maintain urine flow rate. SNGFR was not measured in the present study, and indeed there is only one study in which SNGFR has been measured directly in an elasmobranch (Brown and Green, 1987).

Following two hours of perfusion with AVT the majority of glomeruli were perfused but not filtering, but the proportion of glomeruli that were non-perfused was also

elevated. The majority of these non-perfused glomeruli tended to occur in blocks of unperfused (and therefore unstained) kidney. Similarly, when dogfish were examined *in vivo*, non-arterially perfused glomeruli tended to occur in patches of unperfused kidney (Brown *et al.*, 1993) leading the authors to suggest that local renal vasoconstriction in these regions may prevent blood from perfusing the glomeruli. This may reflect the operation of glomerular bypass shunts (Brown and Green, 1992).

Perfusion of the *in situ* renal preparation with 10^{-9} M Ang II also caused a significant antidiuresis of a similar magnitude to that observed in Chapter 4. This antidiuresis was associated with a decrease in the filtering population of glomeruli to 21% in SW-acclimated preparations and 29% in 85% SW-acclimated preparations, measured using the transport maxima for glucose (Fig. 4.2.). However, as was the case with AVT, the magnitude of this reduction was greater when the filtering population was measured directly (76% reduction in the filtering population as opposed to 49% reduction in TmG in SW and 65% reduction in the filtering population as opposed to 60% reduction in TmG in 85% SW.). This would again suggest an increase in SNGFR as urine flow and GFR were reduced by similar proportions to TmG.

Despite a similar effect on urine flow and glomerular filtration rate following perfusion of 10^{-9} M AVT and Ang II, there was a marked difference between the perfusion patterns between these two peptides. Following perfusion with AVT the majority of glomeruli were perfused but not filtering, whereas following perfusion with Ang II the majority of glomeruli were non-perfused. The proportion of glomeruli that were perfused and filtering was statistically indistinguishable between the two peptides in both salinites. The reasons for this disparity in perfusion patterns remains unclear, but if

we assume that the reduction in filtering glomeruli is due to renal vasoconstriction it may reflect a disparity between the vasoconstrictor actions of these two peptides at this concentration. It may be that Ang II is a more potent vasoconstrictor than AVT and therefore has the potential to increase the proportion of glomeruli that are non-perfused. It is also possible that this difference in perfusion pattern may reflect different populations of glomeruli, some of which are sensitive to AVT and others which are sensitive to Ang II. Variation in the receptor sites for these peptides between the two populations may result in the different patterns of perfusion observed. This will be discussed further in Chapter 7.

Perfusion of the *in situ* renal preparation with 10^{-9} M CNP caused a significant diuresis, as was observed in Chapter 5. In SW-acclimated preparations this diuresis was not associated with any significant change in the proportion of filtering glomeruli. However, in 85% SW-acclimated preparations there was a slight, but significant, rise in the proportion of filtering glomeruli. When functional tubular mass was measured using the tubular transport maxima for glucose there was an increase in the functional tubular mass of 31% and 25% in SW and 85% SW respectively (Fig. 5.2). The TmG increased by an equivalent proportion to the increases in urine flow rate and GFR (Fig. 5.2.) suggesting a substantial increase in SNGFR in order to maintain urine flow rate. It therefore appears that the diuresis observed during perfusion with CNP can not be explained solely by an increase in the population of filtering nephrons. The renal actions of CNP are complicated, however, by the possible presence of an intra-renal RAS (Chapter 4). It is possible that perfusion of CNP, in the absence of Ang II may indeed cause an increase in the population of filtering glomeruli, as indeed was the case in 85% SW. However, it is clear that since the vast majority of glomeruli in control preparations

are filtering, increases in urine flow cannot be explained by glomerular recruitment alone, as the capacity to increase the filtering proportion of glomeruli is limited. It has previously been suggested that an increase in GFR following infusion of adrenaline, was caused by recruitment of resting glomeruli (Deetjen and Boylan, 1968). In the light of this work, this would appear unlikely. In fact, when adrenaline was infused in *S. canicula*, it caused a diuresis, but the diuresis was associated with a reduction in the population of filtering glomeruli rather than an increase (Brown and Green, 1987) suggesting that SNGFR was increased.

In conclusion, assuming that the values for TmG in previous chapters are accurate, it is clear that overall SNGFR must change in order to explain the differences between TmG and the direct measure of the filtering population of nephrons. This is most evident in the case of CNP, where a large increase in GFR and TmG are not reflected in comparable changes in the population of filtering nephrons. Indeed, it would be impossible to increase the population of filtering nephrons by a sufficient amount to account for the increase in urine flow rate and GFR. This result has serious implications for the use of TmG as a measure of the functional tubular mass in elasmobranch fish. Previous work in *S. canicula* (Brown and Green, 1987) supports the assertion that SNGFR can be elevated to maintain urine flow rate, even in the face of a decrease in the filtering population of glomeruli. It would be interesting to examine the filtering population of glomeruli following perfusion of these peptides at a lower dose of 10^{-10} M. Unfortunately it was not possible to do so in the course of this study due to time constraints and pressure on the numbers of available fish.

7

General Discussion

Scyliorhinus canicula is a widely used experimental species that adapts well to captive conditions. *S. canicula* is classically regarded as a stenohaline marine species although it has been shown to successfully acclimate to dilute seawater in the laboratory (Hazon and Henderson, 1984; Tierney *et al.*, 1998). Female dogfish migrate to shallow waters in early winter where they deposit their egg cases and are joined by males in April or May (Wheeler, 1969). It is likely that dogfish encounter brackish water whilst in coastal waters and as such would require an ability to acclimate to less saline conditions. Indeed dogfish have been observed in brackish water during tracking studies (Simms, D.W. pers. comm.).

Initial experiments were carried out *in vivo*, in order to provide a baseline for the subsequent *in vitro* work. It is apparent that *S. canicula* employs a similar osmoregulatory strategy to that of many other elasmobranchs, including more euryhaline species. Under normal circumstances, the rectal gland functions to remove sodium and chloride from the elasmobranch fish (Burger and Hess, 1960). There would therefore appear to be no necessity to remove sodium and chloride via the kidney. However, the overall significance of the rectal gland in terms of osmoregulation was challenged by Burger (1965), who examined the relative roles of the rectal gland and kidney in *S. acanthias.* Following removal of the rectal gland for 14, 17, or 21 days, it was observed that without a rectal gland and receiving no food, dogfish can maintain normal plasma osmolality and chloride with no associated rise in urinary osmolality and

chloride (Burger, 1965). In the same study however, removal of the rectal gland was associated with a diuresis, and therefore an increase in the renal clearance of salt. However, it was not clear whether this renal effect was a direct, hormone-mediated action, or simply a consequence of volume loading, thereby invoking a diuresis resulting in an associated natriuresis. In addition, force-feeding or injection of hypertonic saline in animals in which the rectal gland had been removed, resulted in a slight increase in plasma sodium and a considerable increase in muscle sodium content (Chan *et al.*, 1967). However, these authors concluded that, in the face of a salt load, the gills, rectal gland and kidney seem to contribute jointly in order to excrete excess salt.

In the present study, following transfer to reduced salinity there was a significant increase in the renal clearance of sodium and chloride in addition to urea. The rectal gland was intact and presumably functioning, so this renal clearance of ions suggests that the kidney may also play a role in the clearance of ions during acclimation to reduced salinity in addition to the rectal gland. Similar results, whereby an increase in the renal clearance of sodium chloride and urea was observed, have previously been reported (Goldstein and Forster, 1971; Goldstein *et al.*, 1968; Wong and Chan, 1977). However, in these studies it was not clear if the increase in sodium chloride clearance is simply a product of the diuretic effect of acclimation to reduced salinity. In the present study, results from the perfused trunk preparation suggest that this effect was due to an increase in GFR.

The present study is the first in which an *in situ* perfused renal preparation has been successfully developed in an elasmobranch fish. This preparation was verified in terms of stability of renal parameters, recovery to basal levels following addition of hormone,

and it remained viable for a sufficient period of time to allow the investigation of the renal effects of osmoregulatory hormones (Chapter 2). The colloid oncotic pressure of the physiological saline was not raised by the addition of colloids, similarly to the *in situ* perfused trout trunk preparation (Amer and Brown, 1995). The chosen pressure head was within the physiological range for these animals, but the effective hydrostatic pressure in the glomeruli would have been elevated due to the reduced colloid oncotic pressure. This may have resulted in an increase in SNGFR, thereby causing an increase in the rate of tubular fluid. This effect may explain the apparent lack of tubular effects in the dogfish renal preparation. However, it should bee noted that the protein concentration in the plasma of the skate, *R. oscillata*, was measured to be between one-third to one-half as great as in human plasma (Dill *et al.*, 1932). In addition, in the *in situ* perfused trout trunk preparation, lack of colloids was suggested as a possible factor in the increased proportion of filtering glomeruli *in vitro*, when compared with previous studies *in vivo* (Amer and Brown, 1995; Brown *et al.*, 1980). However, the proportion of filtering glomeruli in the *in situ* perfused dogfish trunk preparation, was actually slightly lower than that previously reported *in vivo* (Brown and Green, 1987). Finally, no significant oedema was observed throughout the entire 6 hour experimental period, as was the case in the *in situ* perfused trout trunk preparation (Amer and Brown, 1995), also suggesting that the lack of colloids was not a significant factor in the dogfish trunk preparation.

The present study suggests a renal role for AVT, Ang II and CNP, in terms of the control of glomerular filtration rate during acclimation to varying environmental salinity. As dogfish move from SW into dilute SW there would be an increase in ECF volume associated with a diuresis, an increase in GFR and an increase in the renal

clearance of ions and urea. This resulted in the observed decrease in plasma osmolality and may suggest a possible role for CNP, which caused a glomerular diuresis and an increase in the clearance of ions and urea in the *in situ* perfused trunk preparation. Conversely, as dogfish move from dilute SW back into SW there would be a decrease in ECF volume, associated with an antidiuresis, a decrease in GFR and a decrease in the clearance of ions and urea. This resulted in the observed increase in plasma osmolality and may suggest a possible role for AVT or Ang II, which both resulted in these effects in the *in situ* perfused trunk preparation (See Fig. 7.4.). The activation of the RAS during movement from dilute SW back into SW has already been observed in the dogfish, in terms of an increase in the plasma concentration of Ang II, accompanied by an increase in drinking rate (Anderson *et al.*, 2002). It would be extremely interesting in the future to examine the plasma concentrations of AVT and CNP, in order to investigate the hypothesis that the plasma concentration of these peptides changes in response to environmental salinity acclimation. It is therefore possible that these peptides play a role in maintaining the plasma hyperosmotic to the surrounding media during environmental salinity acclimation, by acting on the kidney, with AVT/Ang II acting antagonistically against CNP.

The filtering population of glomeruli has been measured in the present study using two methods; indirect measurement of the functional tubular mass by measurement of the transport maxima for glucose and direct measurement using the ferrocyanide technique. Direct measurements of changes in the proportion of filtering glomeruli give a much more reliable and complete assessment of functional glomerular states (Amer and Brown, 1995). It is also apparent, that measurements of functional tubular mass using TmG in the dogfish gave questionable results. This was particularly apparent when

TmG was measured following perfusion of CNP. The majority of glomeruli in the dogfish kidney are found to be perfused and filtering both *in vivo* (Brown and Green, 1987), and *in vitro*, in the present study, when measured using the ferrocyanide method. However, when TmG was measured following perfusion of 10^{-9} M CNP (Fig. 5.2.), an increase in functional tubular mass of 31% was observed. It is clearly impossible for the filtering population of glomeruli to increase by this amount when the basal state is that 86% of the glomeruli are perfused and filtering. Indeed, following perfusion of 10^{-9} M CNP in SW there was no significant change in the proportion of filtering nephrons (Fig. 6.8.). The precise reasons behind this discrepancy are unknown. This observation clearly has serious implications for the use of TmG as a measure of the functional tubular mass in elasmobranch fish. It would be extremely interesting to investigate this discrepancy further, and to look at the effects of additional doses of these hormones in the future.

Changes in SNGFR, whilst not directly measured in the present study, are suggested to explain the anomalies found in GFR and the proportion of filtering nephrons. If no changes in SNGFR occur it is possible to work out a theoretical average GFR. This can be achieved by using a mean SNGFR value of 9.5nl/min, as obtained for the dogfish by Brown and Green (1987). When coupled with an average of 1344 glomeruli in the kidneys of female dogfish (Brown and Green, 1992), a theoretical maximal GFR of 1.055 ml/kg/h can be calculated, assuming that all glomeruli were perfused and filtering and there was no change in SNGFR. This value takes into account a median fish mass of 725 g as the range of fish size was 600-850 g (Brown and Green, 1992). However, following infusion of adrenaline a GFR of 1.59 ml/kg/h was measured (Brown and Green, 1987) clearly suggesting an increase in SNGFR. Indeed, an increase in SNGFR

was measured following infusion of adrenaline, in the same study. Data from the present study also imply a change in SNGFR so it would appear that both *in vivo* and *in vitro*, changes in urine flow rate involve not only changes in the filtering population of glomeruli, but also changes in SNGFR.

Tissue specific renin-angiotensin systems have been demonstrated in a range of tissues in mammalian species and also in the kidney of *O. mykiss* (Brown *et al.*, 2000; Danser, 1996; Dzau, 1993; Erdos and Skidgel, 1990; Hackenthal *et al.*, 1990; Lindpainter and Ganten, 1991; Taugner and Ganten, 1982). This is the first study in which the possible presence of an intra-renal RAS has been demonstrated in an elasmobranch fish. Further work would be necessary to conclusively prove the existence of an intra-renal RAS in the dogfish, and this would require the provision of evidence of all the RAS components within the kidney. Good biochemical evidence for renal renin and renal ACE have been demonstrated previously (Uva *et al.*, 1992). However, molecular techniques should allow the identification of the particular genes for all these RAS components in the elasmobranch kidney. For example, evidence for renal angiotensinogen gene expression in the teleost, *O. mykiss*, was recently described (Brown *et al.*, 2000). The presence of an intra-renal RAS may have important implications for the interpretation of data, both *in vivo* and *in vitro*. For example, in the present study, the effects of CNP must be interpreted in the light of this intra-renal RAS, and it is possible that the observed diuretic and natriuretic effects may be somewhat reduced by activation of such a system. In addition, the interpretation of results obtained *in vivo* must be made with caution. For example, the drop in blood pressure following infusion of atriopeptin in *S. acanthias* (Benyajati and Yokota, 1990) could have caused the activation of an intra-renal RAS, thereby resulting in the anti-diuresis which was attributed to atriopeptin.

The fact that captopril had no significant effect on renal function in trunk preparations acclimated to 85% SW raises the possibility that the intra-renal RAS is only activated in SW-acclimated trunk preparations and may therefore reflect a continuous renal secretion of Ang II in SW. However, an additional action of ACE is the control of Bradykinin (BK) degradation (Gardiner *et al.*, 1993). BK is an active component of the kallikrein-kinin system (Fig 7.1.), and in mammals, generation of BK results in vasodilation, increased vascular permeability and stimulation of renal electrolyte secretion (Conlon, 1999). BK has also been shown to be potently anti-dipsogenic in the eel (Takei *et al.*, 2001). It is therefore possible that renal effects following administration of captopril may be due to an inhibition of Ang II production, in combination with an increase in BK levels. However, attempts to generate BK in the plasma of *S. canicula* have been unsuccessful to date (Conlon, 1999).

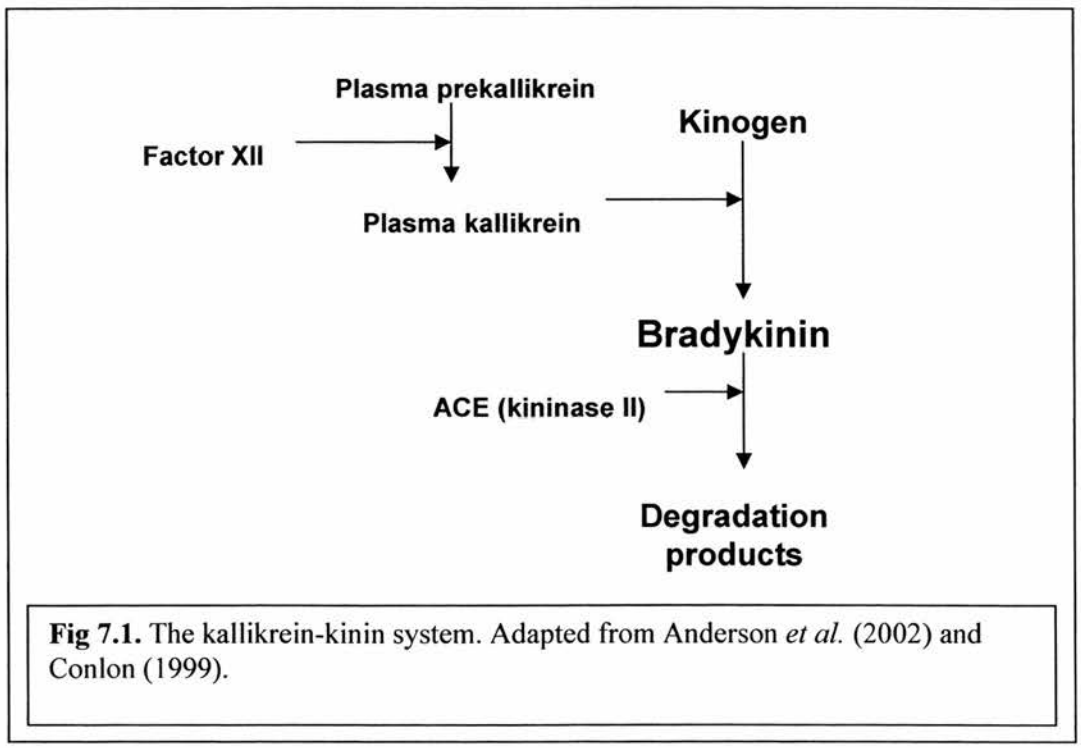

Fig 7.1. The kallikrein-kinin system. Adapted from Anderson *et al.* (2002) and Conlon (1999).

In Chapter 3, 4 and 5 TmG data appeared to suggest that AVT, Ang II and CNP all have effects on the filtering population of glomeruli, and, as previously demonstrated *in vivo*, a variable percentage of glomeruli were either perfused, but not-filtering or non-perfused. The differences between these physiological states remain unclear (Amer and Brown, 1995; Brown and Green, 1987), but in order to explain these differences it is necessary to consider the possible mechanisms involved. Vasoactive effects of AVT, Ang II and CNP have been suggested to account for the changes in physiological states of glomeruli. However, the precise site of this vasoconstriction/vasodilation remains unclear. There are at least three possible sites of action for these peptides. Firstly, these hormones could simply have an effect on the overall vasculature of the preparation. In the case of AVT and Ang II, this would be expected to cause a general vasoconstriction, resulting in a drop in perfusate flow rate. This could then influence the net filtration pressure in the glomeruli, and indeed, may result an increased number of glomeruli which were perfused, but non-filtering (See Section 1.2.). In the case of CNP, a general vasodilation could occur, possibly having the opposite effect, although, as previously discussed, this effect could be complicated by the activation of an intra-renal RAS.

A second possible site of action could be at the afferent arterioles of the glomeruli, whereby the hormones under test would act on the afferent arterioles, possibly allowing the diversion of blood through the glomerular bypass shunts described by Brown and Green (1987). This would be expected to influence the degree of glomerular perfusion and therefore the population of filtering glomeruli. In the case of AVT/Ang II this would be likely to increase the proportion of glomeruli that were non-perfused. The effect of CNP however would be likely to be the dilation of these afferent arterioles, thereby increasing the proportion of perfused and filtering glomeruli, as was the case in

85% SW (Fig. 6.8.). It was initially anticipated that an investigation into the possible presence of glomerular Ang II receptors would comprise an additional chapter in the present study. However, unexpected problems in attaining a reliable supply of dogfish towards the end of this study made this work impossible. Glomerular Ang II receptors have been identified in the rainbow trout (Brown *et al.*, 1990; Cobb and Brown, 1993; Cobb and Brown, 1994) and an aim of this additional chapter would have been to isolate glomeruli by graded sieving as described in these studies. The presence of glomerular receptors would then have been investigated by an angiotensin binding assay in membrane fractions prepared from the glomerular isolate, as described by Tierney *et al.* (1997b). Preliminary experiments have showed that a reliable glomerular isolate can be achieved for the dogfish kidney, so this would be a viable and important study for future work. Similarly it would be extremely interesting to investigate the possibility of glomerular receptors for AVT and CNP. The presence of glomerular receptors for CNP has previously been suggested (Sakaguchi and Takei, 1998).

Thirdly, it is also possible that these hormones may act on the renal tubule after filtration. We have preliminary evidence that at least Ang II is filtered or secreted into the renal tubule, as Ang II has been measured in dogfish urine by radioimmunoassay (Fig 7.2.). It is also interesting to note that Ang II was present in the urine, prior to perfusion of Ang II through the preparation (Fig 7.2.). This may provide additional evidence for an endogenous intra-renal RAS generating Ang II within the *in situ* perfused preparation. Ang II is a small peptide and it is therefore highly likely that any peptide present in the urine is filtered at the glomeruli. It is therefore possible that there are intra-tubular receptor sites for Ang II and also possibly for AVT and CNP, although the molecular weight of CNP is significantly greater than that of Ang II or AVT. AVT

has been implicated in the control of the number of urea transporters in the kidney

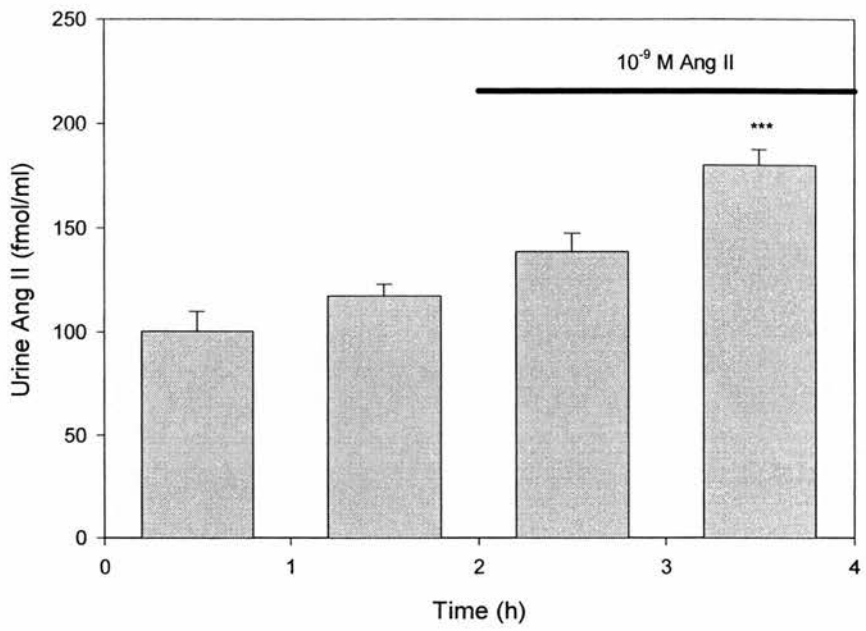

Fig 7.2. Urine Ang II concentration before and after perfusion of 10^{-9} M Ang II. Values are means ± SEM (n = 5). *** ($P < 0.005$) indicate statistically significant difference from the final renal clearance prior to administration of Ang II (one-way ANOVA followed by Tukey's Post Hoc Test.

(Acher *et al.*, 1999) and it is possible that this may involve intra-tubular receptor sites. Indeed, V_1-type AVT receptors have been proposed to be present on the luminal surface of the collecting duct and the luminal surface of the thick ascending limb of the mammalian renal tubule (Ando and Asano, 1993; Burgess *et al.*, 1994). However, it should be noted that no tubular effects were observed in the in situ perfused renal trunk preparation for any of the peptides investigated.

In addition to the various potential sites of action of these peptides, it is also possible that there are distinct populations of glomeruli within the dogfish kidney with varying

sensitivity to the peptides under investigation in this study. This may explain the discrepancies between the proportion of glomeruli that were perfused and non-filtering, and non-perfused when the renal effects of AVT and Ang II are compared (Chapter 6). Following perfusion with AVT, the majority of glomeruli were perfused but non-filtering (Fig. 6.6.), whereas following perfusion with Ang II the majority of glomeruli were non-perfused (Fig. 6.7.). It is therefore possible that a large proportion of glomeruli in the dogfish kidney are Ang II-sensitive, with specific receptor sites for Ang II. Perfusion with Ang II might be expected to cause vasoconstriction at the afferent arterioles, thereby causing blood to be redirected through the glomerular bypass shunts. This would account for the large proportion of glomeruli that were non-perfused. A specific glomerular effect for Ang II, rather than an effect on the general circulation is also suggested by the fact that captopril had no significant effect on perfusate flow rate (Table 4.4.) but had a significant effect on renal function. Following the same hypothesis, it is possible that only a small proportion of glomeruli are AVT-sensitive, possessing specific receptor sites for AVT. If this were the case, it would be expected that the renal effects of AVT would be brought about partly by a vasoactive effect on a small proportion of the afferent arterioles of the glomeruli but mainly by a general vasoconstriction on the entire vasculature of the preparation. This would not be expected to have such a large effect on the population of perfused glomeruli as that proposed for Ang II. However, this effect may have resulted in the large proportion of glomeruli that were perfused but not filtering, because the drop in perfusate flow might have been expected to result in insufficient net filtration pressure at the glomeruli. In the case of CNP, the increase in the proportion of glomeruli that were perfused and filtering could be explained by a small proportion of CNP-sensitive glomeruli which would increase the proportion of perfused and filtering glomeruli, as occurred in 85% SW.

A similar mechanism might be used to explain the discrepancies between renal effects of these peptides and the effects on perfusate flow rate highlighted in Sections 3.4. and 4.4. Perfusate flow rate in the *in situ* perfused dogfish trunk preparation is a crude measure of the total vascular resistance of the preparation. The significant decrease in perfusate flow rate for AVT (Table 3.3.) and Ang II (Table 4.2.) may reflect a general vasoconstriction of the vasculature throughout the entire preparation. However, renal effects were not perfectly coupled to effects on perfusate flow rate. On perfusion with 10^{-10} M AVT, significant renal effects were observed in SW, but not in 85% SW (Fig. 3.2.). If the renal effects of AVT are due mainly to a general vasoconstriction, rather than a specific glomerular effect, as hypothesised above, it would be expected that the renal effects would mimic the effects on perfusate flow rate. It is possible that the apparent lack of a renal effect at 10^{-10} M AVT in 85% SW-acclimated preparations may in fact be a type-2 error due to an insufficient number of experimental animals. Given a slightly higher n it is possible that a significant antidiuresis would also have been observed at 10^{-10} M AVT in 85% SW-acclimated preparations. It would be necessary to investigate this possibility in the future in order to draw any further conclusions about the renal effects of AVT in reduced salinity.

On perfusion with Ang II, the renal responses appear to be dissociated from the effects of perfusate flow, as there are no renal responses at 10^{-10} M Ang II (Fig. 4.3.). If we follow the same hypothesis discussed above, this may be because a large proportion of the decrease in GFR is due to the direct action of Ang II on receptors at the glomeruli or the afferent arterioles. This would be expected to result in a large proportion of non-perfused glomeruli in SW following perfusion with 10^{-9} M Ang II. It is therefore

possible that 10^{-10} M Ang II falls below the threshold dose required to activate these receptors, thereby accounting for the difference in renal responses between 10^{-9} M and 10^{-10} M Ang II. This hypothetical mechanism is summarised in Fig. 7.3. Clearly further work is required to confirm these hypotheses. An initial starting point would be the examination of the patterns of glomerular perfusion following administration of 10^{-10} M peptide through the *in vitro* renal preparation.

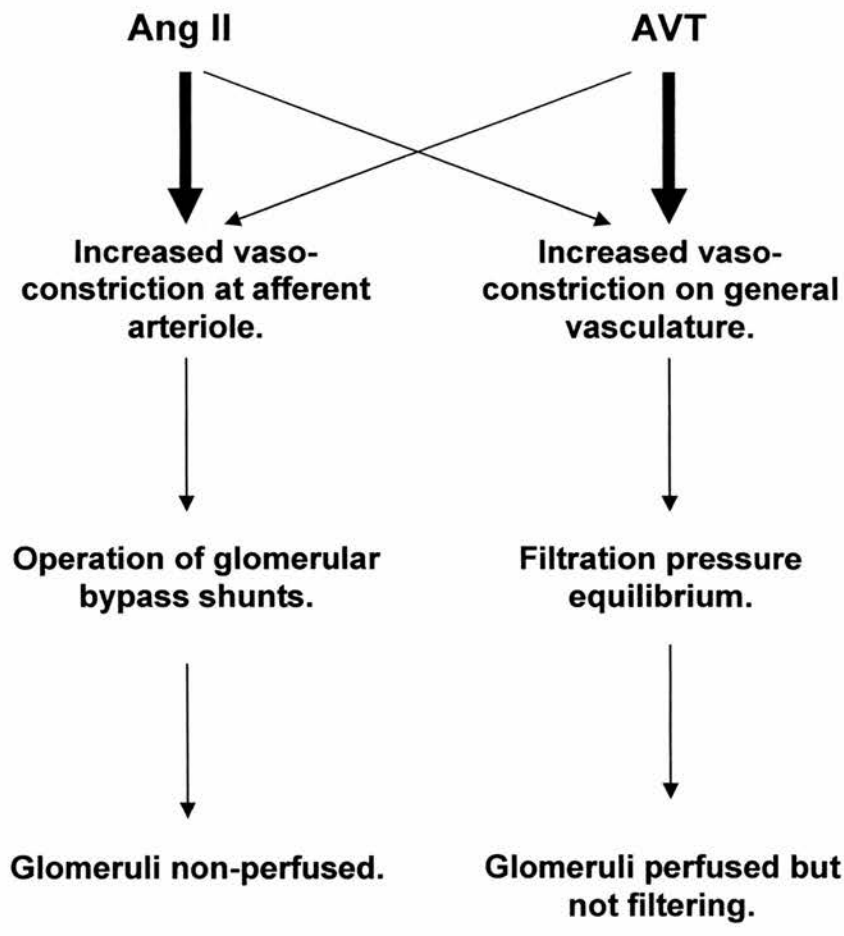

Fig 7.3. Hypothetical mechanisms for the renal haemodynamic effects of Ang II and AVT.

In the future is would be interesting to investigate some other factors which would be expected to change during environmental salinity acclimation. For example, extracellular fluid volume would be expected to change *in vivo* and this could perhaps be mirrored in the perfused renal preparation by altering the perfusion pressure. Combinations of changes in perfusion pressure and hormonal factors could provide an extremely interesting perspective on the role of osmoregulatory hormones during acclimation to reduced salinity, or movement from reduced salinity back to SW. Another important area for future work would be an investigation of the specific location of receptors for the peptides investigated in the present study. Initially, peptide-binding assays could be employed on glomerular isolates, as suggested earlier to investigate the presence of glomerular receptors. However, this would be unlikely to identify whether the receptors were located within the glomeruli themselves, or in fact located on the afferent of efferent arterioles, close to the glomeruli. This level of definition would require the use of *in situ* hybridisation techniques using specific probes to visualise the site of gene expression. The receptor protein itself could then be visualised using immunohistochemistry, whereby a specific antibody raised against the receptor protein could be used to visualise the specific receptor site.

The *in situ* perfused trunk preparation for an elasmobranch fish has therefore been developed, but there are clearly some limitations to this technique. Due to the low urine flow rate it is not possible to incorporate a recovery period and only one hormone can be perfused at a time. There is also potential for other local endocrine systems to be activated, and preliminary evidence of a renal RAS has been presented. In addition, there is potential to the release of urotensins, endothelins and catecholamines. It is also possible that the necessary lack of colloids may have resulted in ain increased SNGFR

and possibly reduced tubular function. However, despite these limitations the present study has provided an insight into the possible renal effects of AVT, Ang II and CNP, in terms of renal function during environmental salinity acclimation. The successful development of an *in situ* perfused trunk preparation for an elasmobranch fish has proven to be an extremely valuable tool for the investigation of specific renal effects, and the data presented has added to the overall picture of elasmobranch osmoregulation. The strength of an *in situ* perfused trunk preparation is that it allows the investigator to keep all renal parameters standard whilst only altering one factor at a time and this has allowed the clarification of the specific renal effects of AVT, Ang II and CNP. These renal effects have been summarised in Fig 7.4. in terms of an overall hypothesis of renal function and volume maintenance in an elasmobranch fish.

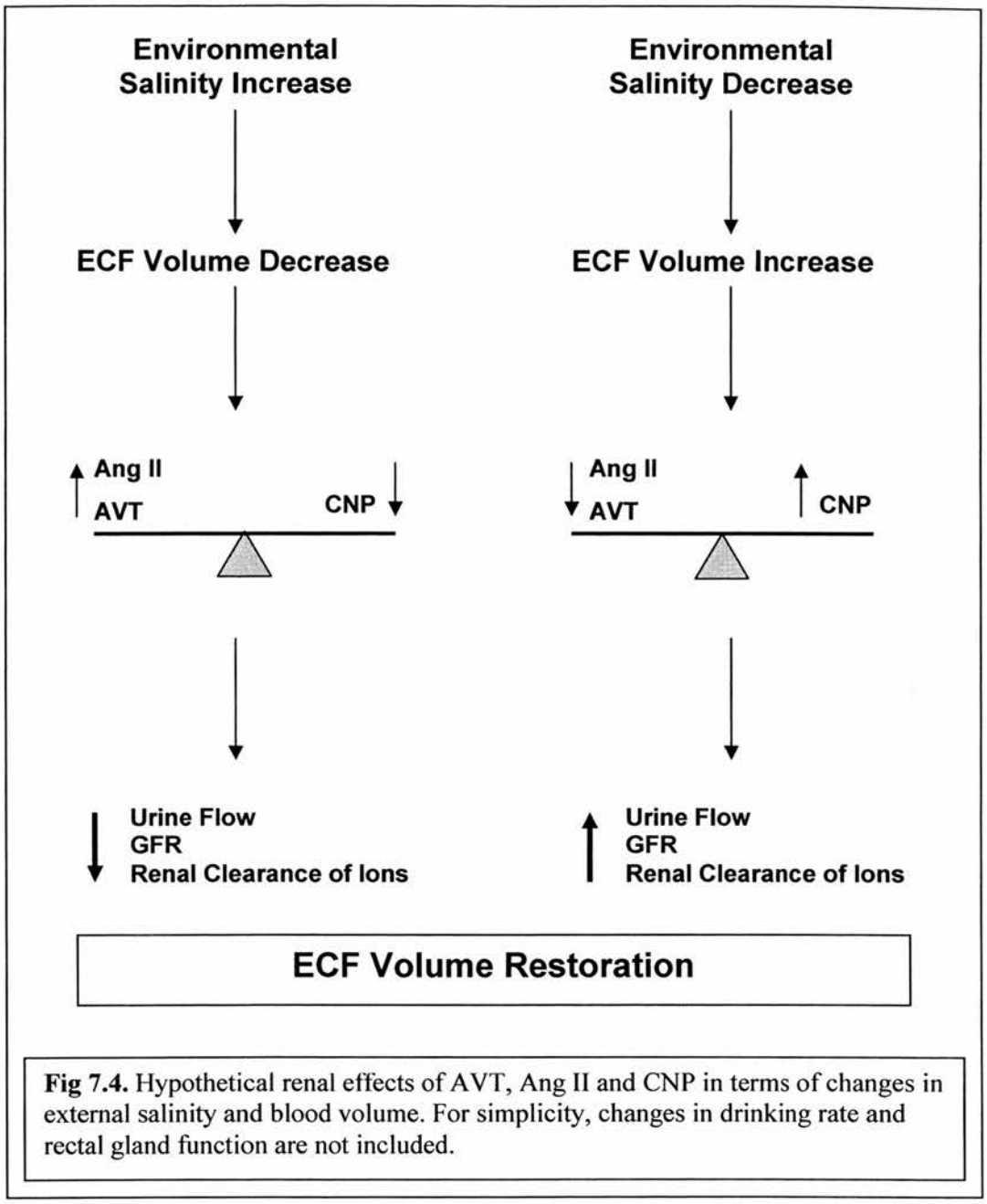

Fig 7.4. Hypothetical renal effects of AVT, Ang II and CNP in terms of changes in external salinity and blood volume. For simplicity, changes in drinking rate and rectal gland function are not included.

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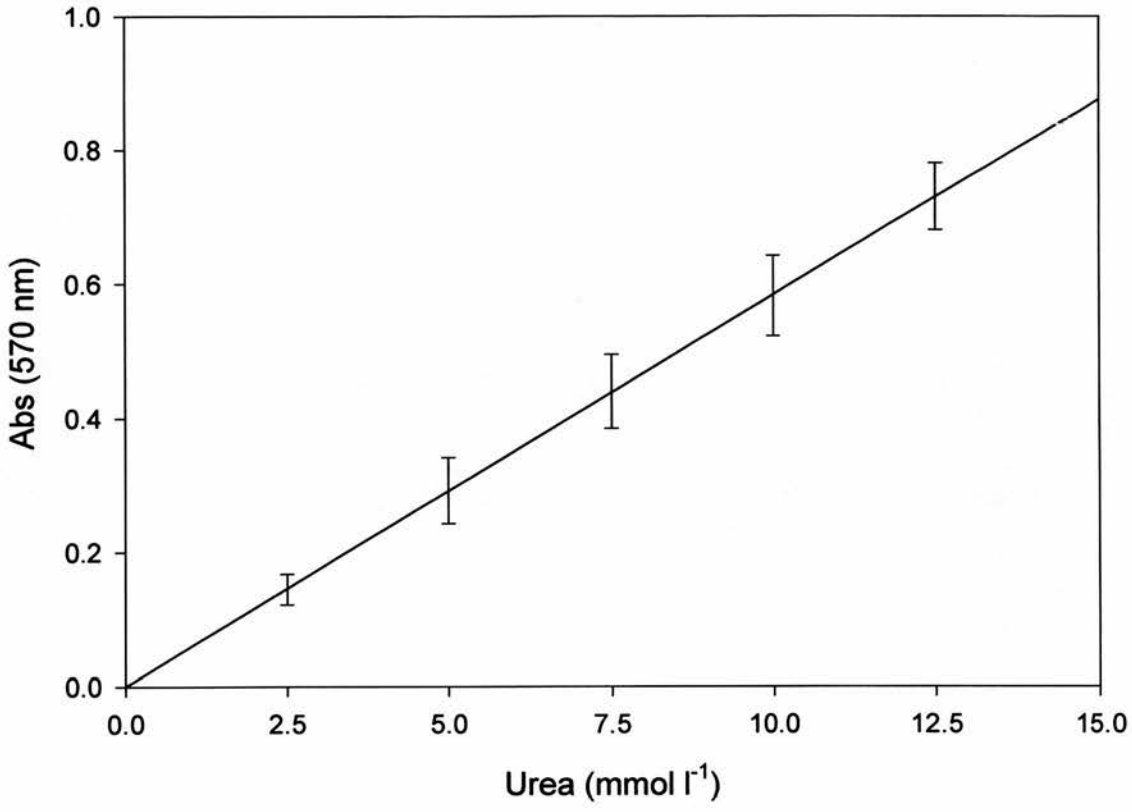
Appendix 1

Amino acid abbreviations

Amino Acid	Abbreviations	
	3-letter	1-letter
alanine	Ala	A
arginine	Arg	R
asparagine	Asn	N
aspartic acid	Asp	D
cysteine	Cys	C
glutamic acid	Glu	E
glutamine	Gln	Q
glycine	Gly	G
histidine	His	H
isoleucine	Ile	I
leucine	Leu	L
lysine	Lys	K
methionine	Met	M
phenylalanine	Phe	F
proline	Pro	P
serine	Ser	S
threonine	Thr	T
tryptophan	Trp	W
tyrosine	Tyr	Y
valine	Val	V

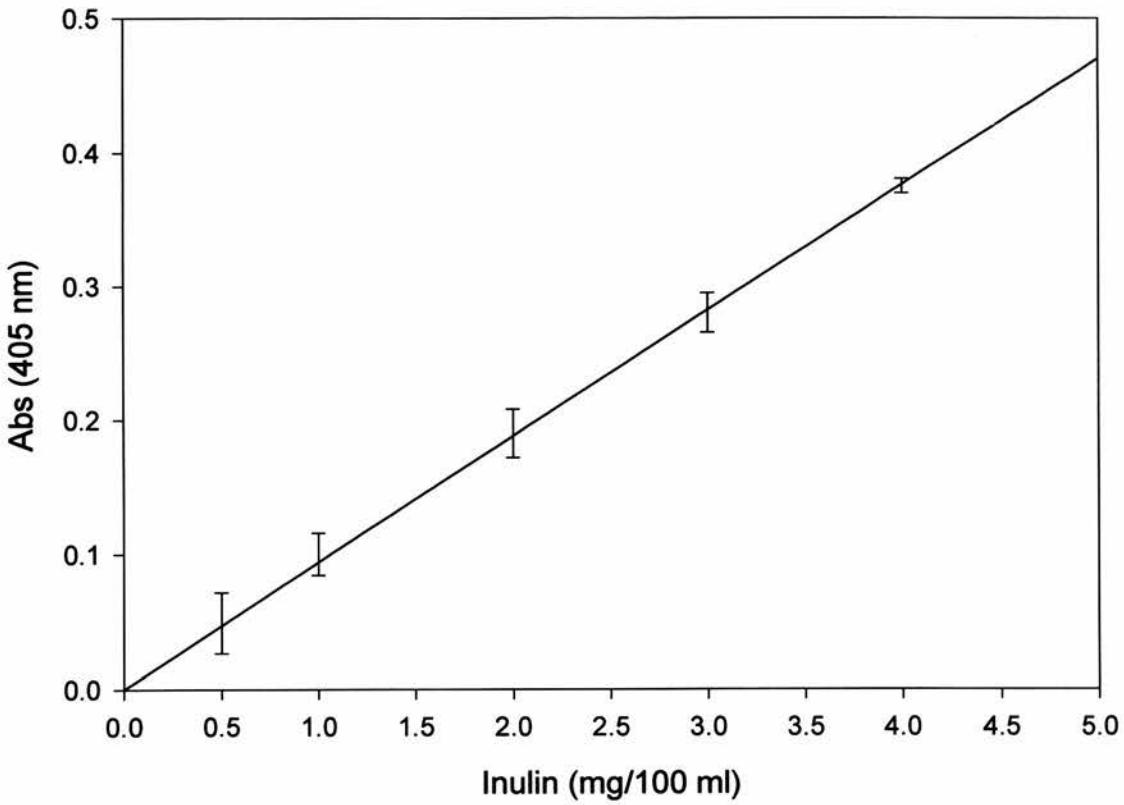
Appendix 2

Typical urea standard curve



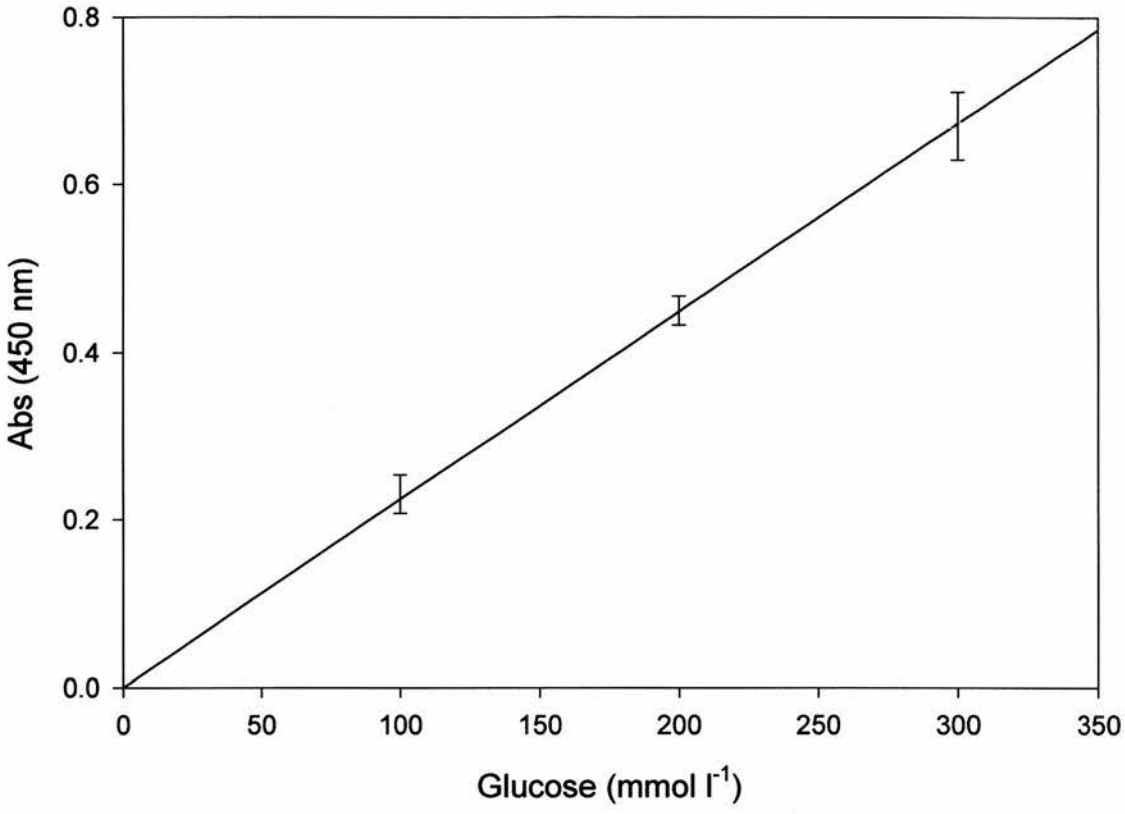
Appendix 3

Typical inulin standard curve



Appendix 4

Typical glucose standard curve



Appendix 5

Publications arising from this thesis

Wells, A., Anderson, W. G. and Hazon, N. (2002). Development of an *in situ* perfused kidney preparation for elasmobranch fish: action of arginine vasotocin. *Am. J. Physiol.* **282,** R1636-R1642.