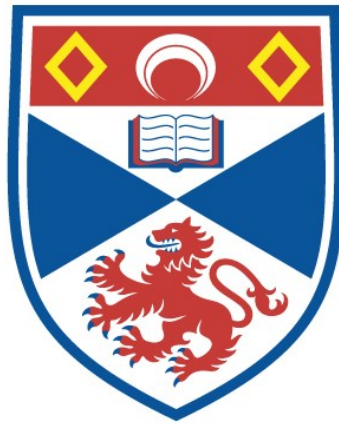# OSMOREGULATION IN GLASS EELS AND ELVERS OF THE EUROPEAN EEL, 'ANGUILLA ANGUILLA'

Lynne M. Birrell

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews

1999

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Osmoregulation in glass eels and elvers of the European eel, *Anguilla anguilla*

Lynne M. Birrell

Thesis submitted for the degree of Doctor of Philosophy
University of St Andrews

September 1998

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Abstract

Glass eels of the European eel migrate from coastal waters inland to freshwater as part of the catadromous lifecycle. The osmotic challenge faced at this time is augmented by their large surface area to volume ratio, and by the fact that the migration may only be completed after several attempts, due to the effects of tide and river flow.

Glass eels and elvers developed normally when maintained in waters of differing salinity over a six month period. Drinking rates increased with environmental acclimation salinity (from 0.072 ± 0.023 µl/g/h to 0.698 ± 0.099 µl/g/h in FW and SW respectively), and freshwater acclimated fish exhibited a rapid drinking response upon contact with seawater. These accounts of dipsogenic behaviour are similar to those previously reported for adult eels.

Results obtained from determinations of branchial $Na^+K^+ATPase$ activities were more equivocal. Only after nearly five months were activities higher in SW (508.52 ± 99.76 nmoles/5pairs gills/h) as compared to FW fish (151.65 ± 8.9 nmoles/5pairs gills/h). Following the transfer of FW acclimated fish to SW there was a trend towards increased $Na^+K^+ATPase$ activity after seven days post-transfer, which reached a significant peak after two months post-transfer. A transient increase in whole body cortisol content was noted following the transfer of fish from freshwater (388.02 ± 90.38 pg/g) to seawater (6268.44 ± 773.14 pg/g). However, it was not possible to ascertain that this was due to a direct effect of environmental salinity change. There were no clear changes in interrenal cell morphology between salinity groups, although the cells did appear reduced in size with time, regardless of environmental salinity. Total body Na^+ content increased with time, and was higher in SW (58.66 ± 1.66 µmoles/g) as compared to FW reared fish (44.85 ± 1.01 µmoles/g).

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Chapter 4 - Discussion

Abbreviations

ACE	Angiotensin converting enzyme
ACTH	Adrenocorticotropic hormone
AI	Angiotensin I
AII	Angiotensin II
ANOVA	Analysis of variance
ANP	Atrial natriuretic peptide
AQU	Aquaporin
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
AVT	Arginine vasotocin
BNP	Brain natriuretic peptide
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CFTR	Cystic fibrosis transduction regulator
cGMP	Cyclic guanosine monophosphate
CNP	C-type natriuretic peptide
cpm	Counts per minute
d	Day(s)
dpm	Disintigrations per minute
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
FITC	Fluorescein-5-isothiocyanate
FW	Freshwater
GFR	Glomerular filtration rate
GH	Growth hormone
h	Hour(s)
mRNA	Messenger ribosomal nucleic acid
OD	Optical density
Pi	Inorganic phosphate
PMSF	Phenylmethyl-sulfonyl fluoride
RAS	Renin angiotensin system
RIA	Radioimmunoassay
SDS	Sodium dodecyl sulphate
SE	Standard error of the mean
SNGFR	Single nephron glomerular filtration rate
SW	Seawater
TCA	Trichloroacetic acid
TMG	Tubular maximum for glucose

T_3	Triiodothryronine
T_4	Thyroxine
UI	Urotensin I
UII	Urotensin II
VIP	Vasoactive intestinal peptide
VNP	Ventricular natriuretic peptide

Chapter 1

Introduction

1.1 Osmoregulation

This thesis is concerned with osmoregulation in glass eels and elvers of the European eel, *Anguilla anguilla*. To introduce the subject, this chapter first looks at osmoregulation in teleost fish, and in particular adult fish, as the literature focuses on adult fish with very little information pertaining to larval and juvenile lifestages. The thesis will concentrate largely upon eel studies. It must be noted that the picture of osmoregulation in teleost fish has been formed having studied comparatively few species. Teleost fish number around 23,600 species and comprise 96% of known living fish and about half of all living vertebrates (Nelson, 1994). In terms of osmoregulation, the literature addresses very few of the total number of species, and concentrates largely on salmonids and to a lesser extent tilapia and anguillid eels. Studies largely focus on euryhaline species, such as the eel, which are in many ways non-typical teleosts.

This chapter will first give a brief outline of the osmoregulatory issues facing teleost fish in their different environments, and then each of the organ systems involved in maintaining ion and water homeostasis will be addressed in more detail. The hormonal control of the osmoregulatory processes described for adult fish are then explored. The lifecycle of the eel is described such that the osmoregulatory role of each of the organs and their endocrine control can be related to the various life-stages.

1.1.1 General Osmoregulation

The internal body fluids of most fish differ from the surrounding environmental medium in both osmotic strength and ionic composition. As the outer surface of the fish acts as a semi-permeable membrane (see page 2), there is the opportunity for substantial movements of water and ions, into and out of the fish by the processes of osmosis and diffusion respectively. Teleost fish maintain their body fluids within set limits, and are thus osmoregulators. Osmoconforming aquatic animals, e.g. the crab *Cancer pagarus*, are incapable of precise regulation of their body fluids and allow them to fluctuate together with any changes in the external medium. Although there may be considerable alterations in the ionic composition of the extracellular fluid, osmoconformers must still regulate at the interface between the extracellular and intracellular fluids in order to survive. Within the body of the fish there is further ion regulation at the cell-blood interface, as the parameters of the intracellular fluid are maintained within strict ionic and osmotic limits. There is a high intracellular concentration of K^+ (120-150 mM) as compared to that within the blood (2-10 mM), whilst in contrast the intracellular concentration of Na^+ is low (around 20 mM) whilst that of the blood is high (140-180 mM). The intracellular fluid is always isosmotic to the extracellular fluid, although much of the intracellular osmotic pressure is made up from large organic molecules, as opposed to the higher concentrations of inorganic ions that are found in the blood. The osmotic

pressure between the intracellular and extracellular fluids is usually balanced by controlling the levels of non-essential amino acids and metabolically inert molecules within the cell.

1.1.2 Outline of Osmoregulation in Teleost Fish

Teleost fish inhabit a wide range of aquatic environments from freshwater (FW) which may be < 0.1 mOsm/kg, to seawater (SW) which is approximately 1000 mOsm/kg, and may even live in solutions more concentrated than SW up to 2300 mOsm/kg. Whatever the concentration of the external environment the osmotic concentration of the blood is maintained between 250-500 mOsm/kg (Parry, 1966). Figures 1.1a and 1.1b summarize how teleosts osmoregulate with what are often substantial differences between their internal and external environments. The skin is relatively impermeable to both water and ions, being poorly vascularized and protected by a covering of scales and mucus (Fromm, 1968). The majority of passive ion and water fluxes occur across the gills, which are well supplied with blood and constitute a large surface area concurrent with their role in gas exchange. In FW (Fig. 1.1a) teleost fish are hyperosmotic to the environment, and will tend to gain water by osmosis, and lose salts by diffusion. They therefore must resist the passive loss of ions and accumulate salt, whilst at the same time produce copious volumes of dilute urine to remove the excess water. To account for the diffusional loss of ions, which is compounded by inevitable losses through urine production, ions are taken up into the blood via active transport at the gills, and are also absorbed from food by the gut. In SW on the other hand (Fig. 1.1b), teleosts are hypoosmotic to the environment, and thus face the opposite problem, an osmotic loss of water and a passive, diffusional gain of salt. Consequently they imbibe the surrounding SW to account for the water loss and produce only scant amounts of urine. As drinking also compounds the problem of salt loading, excess ions are excreted primarily by active transport at the gills, and to a lesser extent in the urine. A large proportion of the divalent ions imbibed with the SW are excreted with faeces and are not absorbed into the blood, the remainder are lost in the urine.

1.1.3 Stenohaline and Euryhaline Teleosts

Most teleosts are stenohaline and can tolerate only very small fluctuations in the salinity of their external environment, and as such they are limited to either FW or marine environments. A small number however are euryhaline and can withstand wide changes in the salinity of their surrounding medium. Euryhaline fishes may encounter extremes of salinity when they migrate between FW and SW to complete their life cycle such as the Atlantic salmon *Salmo salar*, or the European eel, *A. anguilla*. On the other hand fish which inhabit estuarine environments encounter water of differing salinities over a time

Fig. 1.1a: Summary of Ion Exchange and Osmoregulation in a FW Teleost

Modified from Jobling (1995a). In addition there will also be a passive loss of ions via the gills.

Fig. 1.1b: Summary of Ion Exchange and Osmoregulation in a SW Teleost

Modified from Jobling (1995a). In addition there will also be a passive gain of ions via the gills.

External environment: less than 5 mOsm/Kg

Entry of water via skin
and gills

Ions ingested
in food

Body Fluids:
around 300 mOsm/Kg

Active uptake of
ions over gills

Some loss of ions
via faeces

Copious production of
dilute urine leading to high water
loss, but also results in some
loss of ions
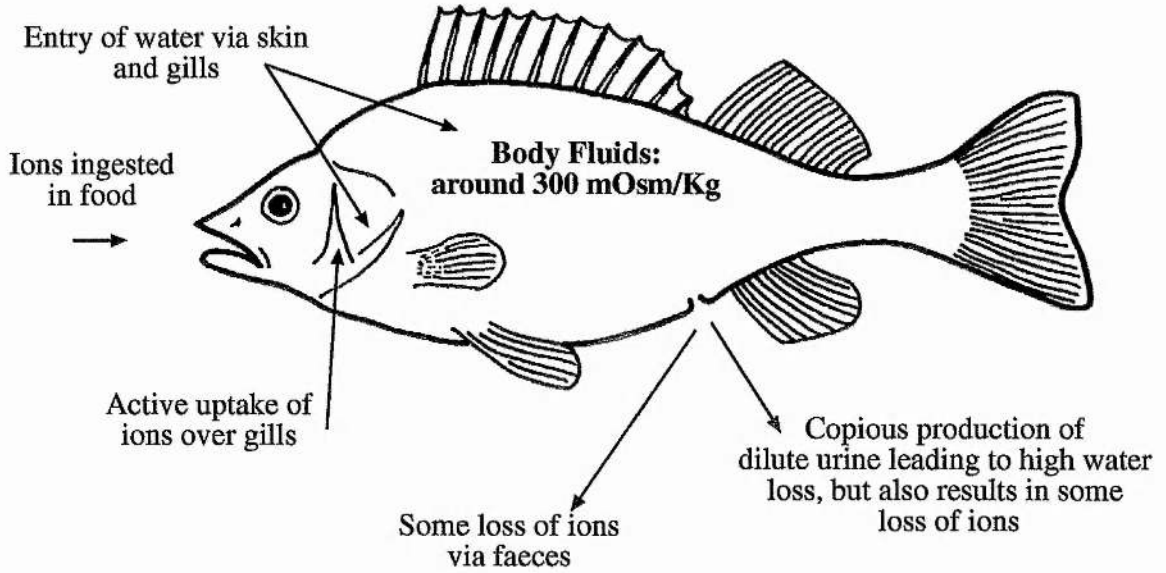
External environment: around 1000 mOsm/Kg

Water loss over the
skin and gills

Drinking of
seawater

Body Fluids:
around 400 mOsm/Kg

Active excretion of
monovalent ions (Na^+, Cl^-)
via chloride cells
in the gills

Ions, mostly divalent,
lost in faeces

Divalent ions (Mg^{2+}, SO_4^{2-})
and some water lost in scant urine
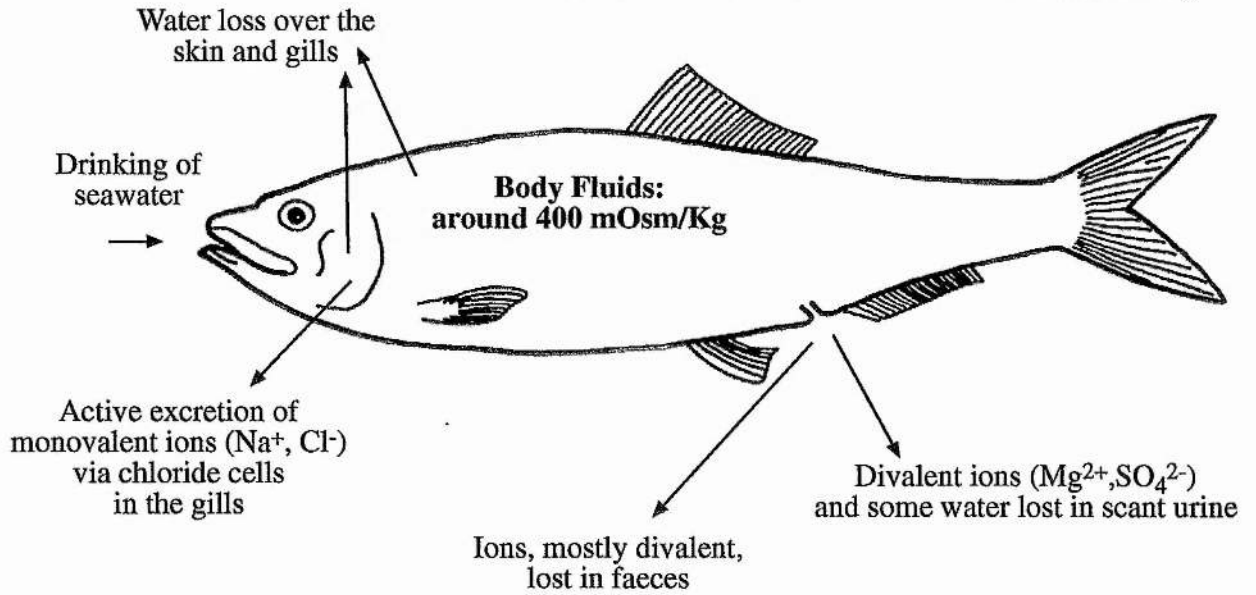
frame of days or even hours, moving between FW and SW, e.g. the killifish *Fundulus heteroclitus*. Euryhaline fish therefore face a complex suite of osmoregulatory problems and must be capable of adapting to their differing environments in order to survive, although they cannot be considered 'typical' teleosts.

Whether fish are stenohaline or euryhaline there must be integration and control of the osmoregulatory processes between the various organs and tissues involved. Although it was once thought that the hormonal and paracrine systems were separate and distinct, it has more recently been realised that various hormones act together with local autocrine and paracrine factors to produce a very complex communication network in order to achieve homeostasis. The control of osmoregulation by hormones is described in section 1.3.

1.2   Organs of Osmoregulation

As discussed earlier in section 1.1.2 and from figures 1.1a and 1.1b the main sites for osmoregulation in teleost fish are that of the gill, gut and kidney. The appropriate ion and water transporting activities, together with changes in permeability, must be put in place depending upon the osmotic and ionic nature of the surrounding environment.

1.2.1   The Osmoregulatory Role of the Gut and Drinking

1.2.1.1   Drinking

Smith (1930) first demonstrated that the SW acclimated American eel, *Anguilla rostrata,* and the marine sculpin, *Myoxocephalus octodecimspinosus,* drank the surrounding medium in order to replace water lost osmotically by dehydration. Drinking has been shown to increase with the salinity of the external environment such that SW acclimated fish drink at a greater rate then those acclimated to FW. It was believed that FW acclimated fish did not drink (Smith, 1930; Shedadeh and Gordon, 1969), however more recent evidence suggests that they do drink, albeit at a considerably lower rate than fish in SW (Maetz and Skadhauge, 1968; Gaitskell and Chester-Jones, 1971; Hirano, 1974; Malvin *et al.*, 1980; Carrick and Balment, 1983; Balment and Carrick, 1985; Perrott *et al.*, 1992; Tierney *et al.*, 1993; Balment *et al.*, 1995; Tierney *et al.*, 1995b). The physiological role of drinking in FW however remains unexplained (Maetz and Skadhauge, 1968; Gaitskell and Chester-Jones, 1971).

The transfer of euryhaline fish from FW to SW results in an increase in drinking. Hirano (1974) showed that drinking occurred immediately following transfer of FW acclimated Japanese eels, *Anguilla japonica* to SW, and stopped immediately when the fish were transferred back to FW. In *A. anguilla* there appears to be a two-phased

response to changes in drinking rate with transfer to SW. An initial increase in drinking occurred over the first two days post-transfer, and this was followed by a further increase at six days post-transfer, such that drinking rates stabilized between 7 and 14 days at a level significantly higher than the FW control (Tierney *et al.*, 1993). Similar two-phased drinking responses were found by Kirsch and Mayer-Gostan (1973) also working with *A. anguilla* and by Oide and Utida (1968) using *A. japonica*.

1.2.1.2 The Gut

In addition to absorbing nutrients from food during digestion, the gut has a considerable role to play in osmoregulatory homeostasis in teleost fish. In FW the gut is responsible for absorbing dietary Na^+ and Cl^- to help replace salts lost by diffusion to the dilute external environment. In the marine environment the gut absorbs water via solute-linked water transport to replace the water lost osmotically to the dehydrating medium. The additional salt load thus incurred is excreted primarily at the gills, and this will be discussed in section 1.2.2.

From observations *in vivo* it is clear that monovalent ions from the ingested SW are progressively absorbed as they travel along the length of the gut (Sharratt *et al.*, 1964a; Shedadeh and Gordon, 1969; Kirsch and Meister, 1982; Parmlee and Renfro, 1983). Monovalent ions and divalent ions are subject to quite different treatment once ingested by the fish. Most of the monovalent ions are absorbed by the gut, whilst the bulk of the divalent ions remain, and are subsequently excreted via the rectum (Smith, 1930; Hickman (Jr.), 1968; Parmlee and Renfro, 1983). In the southern flounder *Paralichthys lethostigma* for example, nearly 99%, 98% and 93% of the ingested Na^+, K^+ and Cl^- respectively are absorbed by the gut to be excreted at the gills (Hickman (Jr.), 1968). However, only around 10% and 15% of the divalent ions SO_4^{2-} and Mg^{2+} respectively, are absorbed by the gut to be excreted by the kidney, the majority remaining within the gut to be excreted in the faeces (Hickman (Jr.), 1968). About 20-30% of the ingested SW goes on ultimately to be excreted in the rectal fluid (Smith, 1930; Hickman (Jr.), 1968; Shedadeh and Gordon, 1969; Sleet and Weber, 1982; Parmlee and Renfro, 1983).

There are a number of studies which address the progressive processing of ingested fluid along the whole of the gut (Smith, 1930; Hickman (Jr.), 1968; Shedadeh and Gordon, 1969; Kristensen and Skadhauge, 1974; Skadhauge, 1974; Hirano and Mayer-Gostan, 1976; Kirsch and Meister, 1982; Sleet and Weber, 1982; Parmlee and Renfro, 1983), although it is more common for the different regions of the gut to be examined individually. Most of the work pertains to marine or SW acclimated fish where the ion transport activities are the most active. The alimentary tract in teleosts is divided into a number of distinct regions on the basis of morphology and histology, each having particular ion transporting properties corresponding to their specific role in

osmoregulation. The gut is divided into sections known as the oesophagus, stomach, anterior/middle intestine and the posterior intestine, although how discrete these sections are depends upon the species (Tesch, 1977).

(i)   <u>The Oesophagus</u>

The oesophagus of the eel has been well studied, however it is not typical to that of other fish, where further work is required. It is generally thought that the oesophagus in FW adapted eels plays no role in osmoregulation. Figure 1.2a outlines the important features of the oesophagus in a FW eel. In FW adapted *A. anguilla* the oesophagus was found to be impermeable to both water and Na+ and Cl- ions (Hirano and Mayer-Gostan, 1976). Throughout the length of the oesophagus in FW adapted *A. japonica* and *A. anguilla* there is a thick stratified epithelium with many mucous cells and a simple pattern of longitudinal folds (Yamamoto, 1978; Abaurrea-Equisoain and Ostos-Garrido, 1996). In FW the filament-rich cells possess many microridges (Yamamoto, 1978; Abaurrea-Equisoain and Ostos-Garrido, 1996), and the mucosal layer is thick and dense throughout its length (Abaurrea-Equisoain and Ostos-Garrido, 1996). Together these features are thought to act as a barrier to the passive diffusion of ions and water through the oesophageal epithelium of FW adapted eels.

Upon acclimation from FW to SW the impermeability of the oesophagus to water remains, however the oesophagus becomes selectively permeable to Na+ and Cl- (Skadhauge, 1969; Hirano and Mayer-Gostan, 1976; Nagashima and Ando, 1994). Progressive changes in the structure of the oesophageal epithelium from anterior to posterior accompany alterations in the mucous layer to contribute to the important role of the oesophagus as an osmoregulatory organ in SW (Fig. 1.2b) (Yamamoto, 1978; Humbert *et al.*, 1984; Cataldi *et al.*, 1988). In SW acclimated *A. japonica* the thick stratified epithelium, rich in mucous cells and containing apical cells with microridges at the beginning of the oesophagus, is progressively replaced by a more simple columnar epithelium containing many mitochondria-rich, microvillous, absorptive cells together with dilated intercellular spaces (Yamamoto, 1978). The underlying connective tissue layer, poorly vascularized at the anterior end, becomes highly vascularized and there is increased and more complicated folding which increases the epithelial surface area (Yamamoto, 1978). These features are indicative of areas with high rates of ion transport (Yamamoto, 1978). Similar findings were also recorded in *A. anguilla* (Laurent and Kirsch, 1975) and have also been noted in the euryhaline tilapia, *Oreochromis mossambicus* (formerly *Sarotherodon mossambicus*) (Cataldi *et al.*, 1988), although they have not been observed in rainbow trout, *Oncorhynchus mykiss* (formerly *Salmo gairdneri*) (MacLeod, 1978). The morphological changes generally associated with adaptation to SW are identifiable by three days post-transfer, and are fully complete by two weeks post-transfer from FW to SW in *A. japonica* (Yamamoto, 1978).

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Fig. 1.2a: Model of the Oesophagus in a FW Adapted Eel

Numbers refer to local concentrations of Cl⁻ ions (mM). Arrow length is proportional to the rate of local water flow. Note the dense mucus and thick stratified epithelium throughout the length of the oesophagus. Modified from Kirsch (1978).

Fig. 1.2b: Model of the Oesophagus in a SW Adapted Eel

Numbers refer to local concentrations of Cl⁻ ions (mM). Arrow length is proportional to the rate of local water flow. Note that the mucus changes from dense to fibrous, and that the epithelium changes from thick and stratified to a monostratified columnar epithelium with dilated intercellular spaces with movement down the oesophagus. Modified from Kirsch (1978).

0.1 mM Cl⁻ ← ←——————————————→ 0.1 mM Cl⁻

520 mM Cl⁻

The mucus lining the oesophagus, as well as helping to avoid mechanical damage and preventing bacterial invasion, plays an important role in maintaining ion and water balance in fish in SW (Humbert et al., 1984; Shephard, 1994). The presence of the mucous layer lining the oesophagus has been shown to be essential to prevent the absorption of water in the SW adapted eel (Kirsch, 1978). In the SW eel A. anguilla the mucous layer progressively decreases in density and thickness from the anterior to the posterior oesophagus (Fig. 1.2.b) (Humbert et al., 1984; Simonneaux et al., 1987a). In addition the mucous fibres are gradually rearranged from an irregular network to form channel-like structures that lie perpendicular to the oesophageal epithelium (Simonneaux et al., 1987a). It is thought that the mucous layer acts as an important diffusion barrier which allows ion absorption without direct contact of the ingested SW with the cells of the oesophageal epithelium (Kirsch et al., 1984; Simonneaux et al., 1987a). The fibrous mucus serves to channel the ingested SW down the centre of the lumen. As it is at its most dense nearest the epithelium the mucus creates a diffusion barrier, and results in a slower rate of fluid movement nearest to the epithelium (Kirsch et al., 1984). This results in the standing gradient of ions from the lumen to the epithelial cells. The ions then move down this concentration gradient to be absorbed by the blood and rapidly extruded at the gills, such that there is little disturbance of plasma ion levels (Kirsch et al., 1984).

Although Smith (1930) first proposed that the ingested SW was diluted in the stomach by fluid secretion, and that this was followed by solute-linked water transport in the intestine, the first step in the processing of the ingested SW is now widely accepted to be desalination in the oesophagus. This is true desalination where ions are removed from the imbibed SW, without dilution by the secretion of water (Hirano and Mayer-Gostan, 1976). Desalination occurs as a result of both passive and active transport of NaCl from the lumen (Hirano and Mayer-Gostan, 1976; Kirsch and Meister, 1982; Sleet and Weber, 1982; Parmlee and Renfro, 1983; Simonneaux et al., 1987a; Nagashima and Ando, 1994).

There is passive transport as Na^+ and Cl^- diffuse down their concentration gradients and out from the lumen through the oesophagus wall and into the blood. In addition, ions are actively absorbed in conjunction with the action of the $Na^+K^+ATPase$ sited in the lateral membranes of the absorptive cells (Simonneaux et al., 1987a). A model of ion absorption in the oesophagus is illustrated in figure 1.3. The arrangement of the circulatory system in the region of the oesophagus ensures that the absorbed ions are transported rapidly, via the heart, to the gills where they are excreted by the chloride cells (Parmlee and Renfro, 1983). Between 60-70% of the net Na^+ and Cl^- fluxes are active via coupled transport depending on $Na^+K^+ATPase$, and the remaining 30-40% are via simple diffusion (Parmlee and Renfro, 1983; Simonneaux et al., 1987a; Nagashima and Ando, 1994). Around half of the active component of Na^+ and Cl^- entry is thought to be explained by a $Na^+/H^+:Cl^-/HCO_3^-$ double exchanger on the apical membrane of the

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Fig. 1.3:   Proposed Model for Na$^+$ and Cl$^-$ Absorption by the Transporting Cells
of the Posterior Oesophagus of a SW Adapted Eel

Modified from Simmoneaux *et al.* (1987a), after Nagashima and Ando (1994). See text
for details.

Apical

Na+

H+

Cl-

HCO3-

H₂0   H₂0

Na+   K+   Cl-

H₂0

H₂0

Na+

Cl-

Basolateral
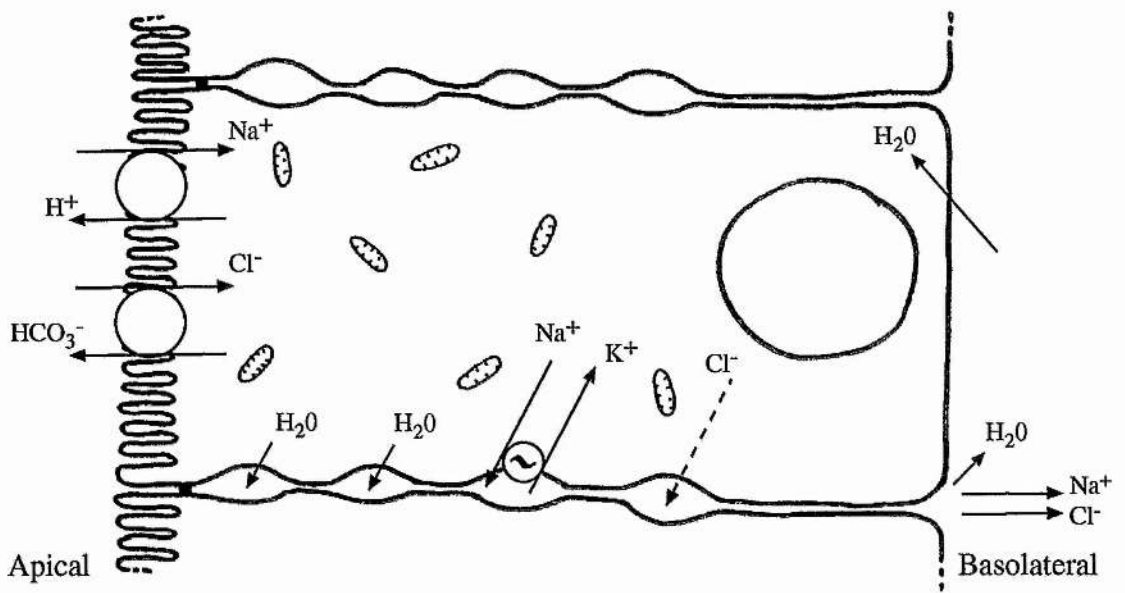
oesophageal cell (Nagashima and Ando, 1994). The other half of the active component remains to be explained (Nagashima and Ando, 1994).

The first few millimetres of the oesophagus after the changes in mucus and epithelium type, have been shown to be the most efficient in the desalination of the ingested SW in a number of SW teleosts including *A. anguilla* (Kirsch and Meister, 1982), and the desalination process has been shown to be complete by the time the fluid reaches the anterior intestine (Hickman (Jr.), 1968; Shedadeh and Gordon, 1969; Kirsch and Meister, 1982; Parmlee and Renfro, 1983). For example Parmlee and Renfro (1983) found that in the winter flounder, *Pleuronectes americanus* (formerly *Pseudopleuronectes americanus*), stomach fluid osmolality was 45% that of SW, and fluid from the intestine was isosmotic to plasma. The SW eel has been shown to ingest water almost continually rather than gulping intermittently, and the ingested water was observed to move along the oesophagus slowly (Hirano, 1974; Kirsch and Meister, 1982). Similarly sculpin, *Enopgrys bison*, were found to intermittently ingest small volumes of SW (Sleet and Weber, 1982). By drinking in a more or less continual manner the SW will pass over the epithelia of the oesophagus (and gut) slowly to allow progressive modification (Kirsch *et al.*, 1984).

(ii)   The Stomach

The stomach is thought to have only a minor role, if any in teleost osmoregulation. Hirano and Mayer-Gostan (1976) found that ion permeabilities in the stomach of FW and SW adapted *A. anguilla* were lower than that of the oesophagus of SW adapted fish. Water permeability however, was found to be higher than in the oesophagus (Hirano and Mayer-Gostan, 1976). The stomach may act to complement oesophageal processing, with further dilution via a small amount of salt absorption by passive diffusion together with water addition by osmosis (Hirano and Mayer-Gostan, 1976). This work by Hirano and Mayer-Gostan (1976) was carried out using isolated stomach sacs containing SW. However, Kirsch and Meister (1982) did not find such a dilution in their perfusion experiments, and they suggest that there is probably no dilution *in vivo*. Studies of the stomach as a possible site for osmoregulation in FW are lacking.

(iii)   The Intestine

As in the SW oesophagus, a number of morphological features of the intestinal epithelium are concurrent with a role in ion transport, and mucus may again play an important functional role (Simonneaux *et al.*, 1987b). The anterior part of the intestine has been shown to be the most efficient in terms of ion and water transport (Kirsch and Meister, 1982) and also possesses numerous tightly packed folds (Simonneaux *et al.*,

1988) which are larger in SW as compared to FW adapted fish (Kirsch *et al.*, 1984). These folds increase the surface area between the luminal solution and the blood, and they sustain ion and osmotic gradients to assist osmoregulation (Simonneaux *et al.*, 1988). Morphological changes in the epithelium of the intestine have been noted in rainbow trout, *O. mykiss*, transferred from FW to SW (Nonnotte *et al.*, 1986). Changes were similar to those described above for the oesophageal epithelium of *A. japonica*, including the distension of the intercellular spaces (Yamamoto, 1978; Nonnotte *et al.*, 1986). Alterations in morphology were most obvious two days post-transfer, however once one month has passed the morphological features were similar to FW acclimated fish, demonstrating that long term adaptation in the intestine is based upon synthesis of new protein and phospholipid rather than new cellular structures (Nonnotte *et al.*, 1986).

The mucous fibres show a regular structural organization in the intestine, running parallel to each other and perpendicular to the intestinal epithelium (Simonneaux *et al.*, 1987b). As the mucus acts as a diffusion barrier, the movement of ions around the area is limited, such that active ion absorption creates local ionic gradients within the mucous layer (Kirsch *et al.*, 1984; Simonneaux *et al.*, 1987a; Simonneaux *et al.*, 1987b; Shephard, 1994).

The intestinal epithelium, unlike that of the oesophagus, is permeable to water (Hirano and Mayer-Gostan, 1976; Parmlee and Renfro, 1983). There is an increase in water permeability during the adaptation of FW eels to SW (Skadhauge, 1969) and the intestinal absorption rate of NaCl increases with increasing salinity of the external environment (Oide and Utida, 1967; Maetz and Skadhauge, 1968; Skadhauge, 1969). By the time the ingested fluid reaches the anterior intestine of SW fish it is at least 50% of its original concentration or isosmotic to plasma, through the actions of the oesophagus, and to a lesser extent the stomach. Some workers have reported that the luminal fluid is more dilute than plasma by the time it reaches the intestine (Sharratt *et al.*, 1964a; Shedadeh and Gordon, 1969; Kirsch and Meister, 1982). However, Skadhauge (1969) found that water could be absorbed by *A. anguilla* intestine when the luminal fluid concentration was still higher then plasma, through the creation of local osmotic gradients. In the intestine NaCl is taken up by active transport with water following osmotically through the establishment of a water potential gradient (Smith, 1930; Oide and Utida, 1967; Skadhauge, 1969; Utida *et al.*, 1972; Skadhauge, 1974; Ando *et al.*, 1975; Hirano and Mayer-Gostan, 1976; Ando and Kobayashi, 1978; Ando, 1980; Ando, 1981; Ando, 1983; Ando, 1985; Ando *et al.*, 1986). This is known as solute-linked water flow (Skadhauge, 1969; Utida *et al.*, 1972). Intestinal water absorption rates in SW are in the region of 65-80% of the water ingested (Oide and Utida, 1968; Shedadeh and Gordon, 1969).

Although the mechanisms of salt and water absorption in the intestine are far from fully elucidated, a number of features have been identified by physiological experiments. Ion transporters have been identified through ion substitution experiments and the use of pharmacological inhibitors, in conjunction with Ussing chamber mounted tissue. A current model for intestinal ion transport across the teleost intestine (Loretz, 1995b) is shown in Fig. 1.4.

Upon acclimation to SW, intestinal $Na^+K^+ATPase$ activity has been shown to increase in *A. anguilla* (Jampol and Epstein, 1970) and *O. mykiss* (Colin *et al.*, 1985). Evidence for the presence of a basolaterally located $Na^+K^+ATPase$ has been provided by a number of studies (Ando and Kobayashi, 1978; MacKay and Janicki, 1979; Ando, 1981; Ramos and Ellory, 1981; Parmlee and Renfro, 1983; Ando, 1985; Ando *et al.*, 1986; Simonneaux *et al.*, 1987b; Trischitta *et al.*, 1989; Trischitta *et al.*, 1992b; Baldisserotto and Mimura, 1994; Marvão *et al.*, 1994; Nagashima and Ando, 1994). The $Na^+K^+ATPase$ on the basolateral membrane generates an inwardly directed electrochemical gradient for Na^+ by extruding Na^+ from the cell in exchange for K^+. As the $Na^+K^+ATPase$ has been demonstrated upon the lateral as well as the basal surface of the enterocyte, Na^+ is also transported into the lateral spaces between cells (Field *et al.*, 1978). Cl^- ions can then diffuse, via channels, down the electrochemical gradient from the cell into these lateral spaces, which may allow paracellular osmotic water flow (Field *et al.*, 1978; Nonnotte *et al.*, 1986).

The action of the $Na^+K^+ATPase$ results in a low concentration of Na^+ within the cell and a negative electrical potential thus generating a favourable electrochemical gradient for the influx of Na^+ across the luminal membrane. Na^+ enters the cell via a number of routes. The electrochemical gradient provides the driving force for $Na^+/K^+/2Cl^-$ cotransport into the cell across the apical membrane. There is a considerable body of evidence for the presence of an $Na^+/K^+/2Cl^-$ cotransporter upon the apical membrane of the enterocyte (Ando and Kobayashi, 1978; Ando, 1980; Musch *et al.*, 1982; Ando, 1983; Ando, 1985; Halm *et al.*, 1985b; Simonneaux *et al.*, 1987b; Trischitta *et al.*, 1989; Ando and Subramanyam, 1990; Trischitta *et al.*, 1992b; Baldisserotto and Mimura, 1994; Marvão *et al.*, 1994). However, it should be noted that the identification of $Na^+K^+2Cl^-$ cotransport has not been universal, as Howard and Ahearn (1988) found no evidence for it in the intestine of SW adapted tilapia, *O. mossambicus*. The entry of Na^+ may also be coupled to that of organic solutes e.g. glucose and amino acids such as alanine, cysteine, and proline (Cassano *et al.*, 1988). There is additional net Na^+ uptake via paracellular diffusion down the transepithelial electrochemical gradient that is generated by basolateral Cl^- channels. The Na^+ transported into the cell is actively transported out again at the other side into the blood via the action of the $Na^+K^+ATPase$ sited on the basolateral membrane.

Fig. 1.4: Proposed Model for Ion Transport by the Transporting Cells of the
Teleost Intestine

Transepithelial transport ultimately depends upon the $Na^+K^+ATPase$ on the basolateral
surface which creates a transmembrane electrochemical gradient for Na^+. Appropriate
electrochemical gradients drive coupled transmembrane ion movements, whilst channel
mediated ion flows occur down electrochemical gradients created via either active or
coupled transport. X represents non-electrolytes such as glucose or amino acids which
may be transported via Na^+ coupled mechanisms sited upon the apical surface of the
enterocyte. Active transport and cotransport are shown by solid lines whilst passive
diffusion is represented by dashed lines. The tight junction is depicted by a dashed line.
Modified from Loretz (1995b). See text for further details.

Apical   Basolateral
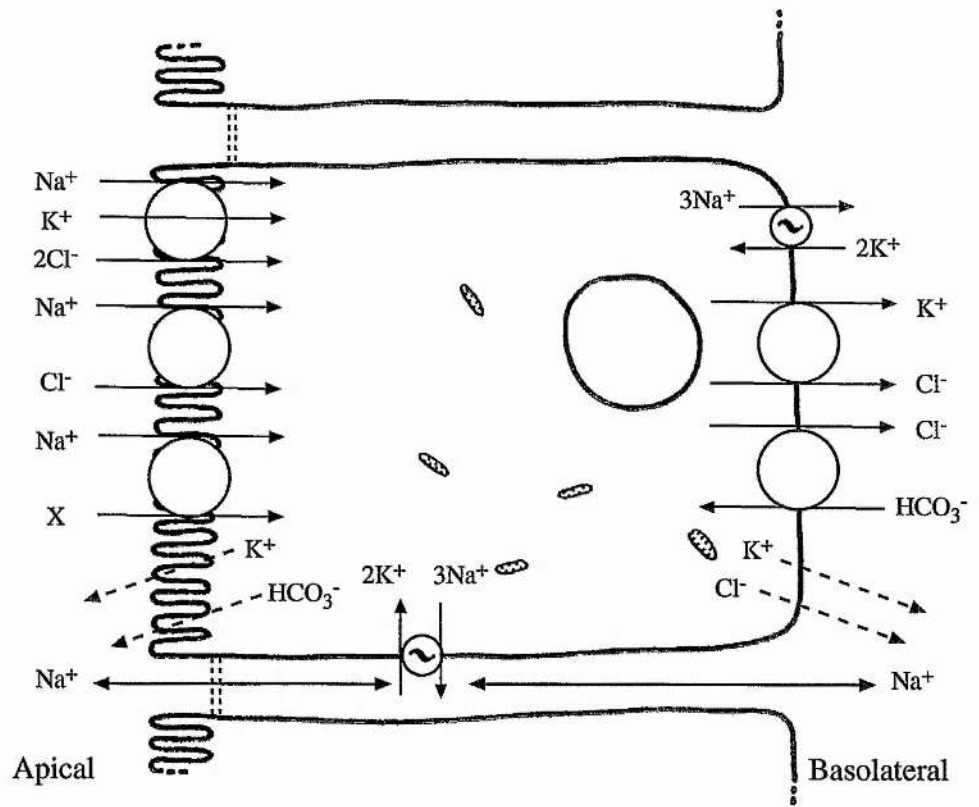
K$^+$ ions entering the enterocytes via the Na$^+$K$^+$ATPase or the Na$^+$/K$^+$/2Cl$^-$ cotransporter are secreted to the gut lumen for recycling via barium sensitive K$^+$ channels on the apical surface of the cells (Simonneaux et al., 1987b; Ando, 1990; Trischitta et al., 1992b; Baldisserotto and Mimura, 1994). The dispersion of these K$^+$ ions is inhibited by the mucous layer and this appears to be essential to K$^+$ recycling (Simonneaux et al., 1987b). The recycling of K$^+$ across the basolateral surface has also been proposed, partly via barium-sensitive channels (Simonneaux et al., 1988). The operation of the Na$^+$/K$^+$/2Cl$^-$ cotransporter together with the recycling of K$^+$ via the barium-sensitive channels would result in the accumulation of Cl$^-$ within the cells. The Cl$^-$ could then exit the cell via diffusion down the electrochemical gradient across the basolateral membrane (Trischitta et al., 1992a). The site of Cl$^-$ exit from the serosal side of the enterocyte has been little studied so far, however Halm et al. (1985a) demonstrated the existence of a Cl$^-$ conductance on the basolateral membrane of *P. americanus* enterocytes to account for some of the Cl$^-$ exit. The remainder is though to be via a K$^+$/Cl$^-$ cotransporter (Ando, 1983; Ando, 1985) also sited upon the basolateral surface (Halm et al., 1985a). The relative importance of the Cl$^-$ conductance and the postulated K$^+$/Cl$^-$ cotransporter has not been identified directly, however, Trischitta et al. (1992a) and Halm et al. (1985a) suggest that at least 30% of Cl$^-$ exit may be due to the Cl$^-$ conductance.

The presence of a number of other ion transporters has been proposed. There may also be an Na$^+$/Cl$^-$ cotransporter upon the apical surface (Halm et al., 1985b), although Trischitta et al. (1992b) found no evidence for it in *A. anguilla*. The presence of a Cl$^-$/HCO$_3$$^-$ exchanger has been proposed for both apical and basolateral surfaces of the enterocyte (Howard and Ahearn, 1988; Ando, 1990; Ando and Subramanyam, 1990). However, other studies have found no evidence for the presence of a Cl$^-$/HCO$_3$$^-$ exchanger on the apical surface (Trischitta et al., 1989; Trischitta et al., 1992b; Baldisserotto and Mimura, 1994; Marvão et al., 1994). A sodium dependent Cl$^-$/HCO$_3$$^-$ exchanger has also been proposed for the apical surface together with a Na$^+$/HCO$_3$$^-$ cotransporter upon the basolateral surface (Ando and Subramanyam, 1990). A Na$^+$/H$^+$ exchanger has been identified upon the basolateral surface of the enterocyte of the eel by some studies (Ando and Subramanyam, 1990; Baldisserotto and Mimura, 1994), although not by others (Schettino et al., 1992). Na$^+$/H$^+$ exchangers have been identified upon the apical surface of *O. mossambicus* enterocytes instead, where there appears to be dual exchange via Na$^+$/H$^+$:Cl$^-$/HCO$_3$$^-$ transporters rather than Na$^+$K$^+$2Cl$^-$ cotransport (Howard and Ahearn, 1988). The possibility of such dual exchange had previously been discussed by other workers (Halm et al., 1985b), however this Na$^+$/H$^+$ exchanger may be of the "house-keeper" type and have no role in osmoregulation, given that there is no difference in activity of the Na$^+$/H$^+$ exchanger between FW and SW adapted eels (Vilella et al., 1995). The transport of water across the cell membrane has been shown to be coupled to ion transport in some mammalian studies (Zeuthen, 1995). However, this may be due to alterations of the tight junctions to allow water through, rather than water

and ion cotransport *per se* (Madara, 1998). Clearly there is a possibility of water cotransport in teleost fish, however further research is required. In addition ten water channels (Aquaporins 0-9) have been identified in mammals, and two of the ten homologues have been demonstrated in teleost tissue using molecular techniques (Cutler, personal communication). In *A. anguilla* mRNA expression of AQU 3 in the gill has been shown to increase with transfer of the fish from SW to FW, whilst expression of AQU 1 in the intestine has been noted to dramatically increase in SW adapted fish (Cutler, personal communication). Furthermore, expression of AQU 1 is elevated in FW as compared to SW adapted eel kidney (Cutler, personal communication).

Again, as in the oesophagus, little work has been done using FW fish or euryhaline fish in FW. However the enterocytes are thought to absorb Na^+ and Cl^- from the ingested food to assist in balancing the ion losses to the hypoosmotic environment. Similar ion transporting processes to those described for SW fish above may be in place in FW fish too. The $Na^+/K^+/2Cl^-$ cotransporter, the basolateral ouabain-sensitive $Na^+K^+ATPase$, and apical barium-sensitive K^+ channels have been found in the FW, as well as the SW *A. anguilla* intestine (Trischitta *et al.*, 1989; Trischitta *et al.*, 1992b), although not in that of FW adapted *O. mossambicus* (Groot and Bakker, 1988).

The approach largely used to date, where electrophysiological characteristics are ascertained together with the application of pharmacological agents, has perhaps only limited physiological significance to the whole animal. In addition, almost all of this work has been carried out on a very small number of species, namely *A. japonica*, *A. anguilla*, *P. americanus*, and goby (*Gillichthys mirabilis*), although primarily using *A. japonica*. As some of the results obtained by Ando and his co-workers on *A. japonica* have not been confirmed by studies of the other species, it may be that species differences exist. There is still a wealth of information to be elucidated pertaining to ion and water transport in teleost fish intestine. Furthermore, there are differences in ion and water transporting capabilities down the length of the intestine (Colin *et al.*, 1985) that require further investigation.

More recently molecular biological techniques have been used in the study of ion transporting mechanisms in a number of tissues including the intestine. These molecular techniques can potentially identify the expression of various transporting molecules in euryhaline species associated with changes in external salinity. The $Na^+K^+ATPase$ has been studied to considerable extent. The $Na^+K^+ATPase$ molecule exists as two subunits: α and β, each of which may be expressed as a number of isoforms. Table 1.1 shows the isoforms of the $Na^+K^+ATPase$ subunits that have been identified in mammals and teleosts. It is of note that some of the $Na^+K^+ATPase$ subunit isoforms are novel to teleosts.

Na⁺K⁺ATPase subunit isoforms	Localization	
	Mammals	Teleosts
$\alpha 1$	Most tissues	Most tissues except muscle
$\alpha 2$	Brain, muscle	
$\alpha 3$	Brain	Brain, gill, intestine and kidney
$\alpha 4$	Testes	
$\beta 1$	Most tissues	Most tissues except muscle
$\beta 2$	Brain, muscle	
$\beta 3$	Brain	Brain
$\beta 233$		Brain, gut, gill, kidney
$\beta 179$, $\beta 185$, $\beta 185b$		Brain

Tab. 1.1 Na⁺K⁺ATPase subunit isoforms identified in mammals and teleosts. A β subunit isoform (βbl) has also been identified from amphibian bladder (Cutler *et al.*, 1995a; Cutler *et al.*, 1995b; Cutler *et al.*, 1996; Cutler *et al.*, 1997 and Cutler, personal communication).

There are high levels of expression of the $\alpha 1$, $\alpha 3$, $\beta 1$ and $\beta 233$ isoform subunits of the Na⁺K⁺ATPase in FW acclimated eels, and these have been found to increase in mid-gut samples of SW as compared to FW acclimated eels (Cutler *et al.*, 1996). The $\beta 3$ subunit isoform is thought to play little role in osmoregulation as no expression was found in the gill, gut and kidney (Cutler *et al.*, 1997). An absorptive Na⁺/K⁺/2Cl⁻ cotransporter isoform has also been shown to increase in expression in SW as compared to FW adapted eel intestine (Cutler, personal communication). A number of other ion transporters have also been identified in eel intestinal tissue by molecular techniques including Na⁺/Cl⁻, K⁺/Cl⁻ and Na⁺/HCO₃⁻ cotransporters and Cl⁻/HCO₃⁻ and Na⁺/H⁺ exchangers (Cutler, personal communication). Whether the ion transporters proposed as a result of electrophysiological studies function in osmoregulation, or simply have a "house-keeping" role remains to be determined by molecular techniques.

1.2.2 The Osmoregulatory Role of the Gill

The NaCl absorbed across the gut from SW ingested by fish in the marine environment is actively excreted by specialized chloride cells in the gill, opercular epithelium and skin. These chloride cells were discovered by Keys and Willmer (1932) not long after the publication of Smith's keystone paper in 1930 where he identified the head region as the source of salts secreted to the external medium. These chloride cells of a can be found in both SW and FW fish, including *A. anguilla* (Keys and Willmer,

1932). Although sometimes sceptically referred to as "mitochondria-rich cells" it is now known that they actively excrete NaCl by transporting Cl⁻ ions from blood to medium in hyperosmotic environments, see Zadunaisky (1984) for a review. Strictly speaking these cells in FW should still be referred to as "mitochondria-rich cells" as there is continuing debate as to their role in ion absorption in FW (although they will continue to be called chloride cells in this thesis).

Figure 1.5 outlines the basic morphology of the teleost gill. The gills of teleosts consist of several branchial arches each bearing primary lamellae (gill filaments) from which radiates the secondary lamellae (gill lamellae). These lamellae are covered with two different epithelial surfaces; the filament (or primary) epithelium and the lamellar (or secondary) epithelium which cover the primary and secondary lamellae respectively. The chloride cell is one of at least four differentiated cell types to be found within the branchial epithelia of teleost fish together with neuroepithelial cells, mucous cells and pavement or respiratory cells (Laurent and Dunel, 1980; Laurent, 1984; Pisam and Rambourg, 1991; Perry and Laurent, 1993). Chloride cells are generally more numerous in the multilayered filament epithelium which is mainly supplied by arterial blood. Chloride cells are sited primarily in the interlamellar regions of the filament epithelium, and at the base of the lamellar epithelium at its junction with the filament epithelium (Laurent, 1984). The multilayered filament epithelium can thus be considered largely the area of ion balance (Laurent, 1984). Chloride cells appear more erratically within the lamellar epithelium, depending upon species and the salinity of the environment (Laurent, 1984; Perry, 1997). The bilayered lamellar epithelium contains mainly pavement cells and can thus be considered primarily the site of respiration. There are considerable species differences as to the sites of chloride cells, in trout they tend to be found on both the filament and lamellar epithelia, whilst in the eel they are largely confined to the filament epithelium (Laurent, 1984). For more details on the general characteristics of the branchial epithelia see Laurent and Dunel (1980) and Laurent (1984). Figure 1.6a is a schematic diagram showing the major cell types present on the gill epithelia together with the blood channels.

Upon transfer of euryhaline fish from FW to SW, there is an increase in the size (Pisam, 1981; Pisam et al., 1987) and number (Utida et al., 1971; Thomson and Sargent, 1977; Foskett et al., 1981) of chloride cells. In addition, the number of chloride cells has been shown to increase proportional to the salinity of the external environment (Utida et al., 1971). High levels of Na⁺K⁺ATPase activity can be found in the chloride cells of teleost fish implicating them in active ion transport (Hootman and Philpott, 1979; Karnaky (Jr.), 1986; McCormick, 1990b). Na⁺K⁺ATPase activities tend to be higher in stenohaline marine teleosts than in FW teleosts (Kamiya and Utida, 1969; Jampol and Epstein, 1970), and increase following adaptation of a number of euryhaline teleost

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Fig. 1.5:   Basic Morphology of the Teleost Gill

The gills of a teleost consist of several gill arches on either side of the head (normally four) and these are held beneath the operculum or gill cover. Each gill arch carries two rows of gill filaments (primary lamellae) and each filament carries a series of thin, plate-like lamellae (secondary lamellae). Within these lamellae the blood flows in a direction opposite to that of the water which flows between the lamellae. Modified from Randall (1968).

Operculum

4 Gill arches

1 Gill arch

Gill filaments
(primary lamellae)

Gill lamellae (secondary lamellae)

Artery

Vein

Gill filament
(primary lamella)

Water
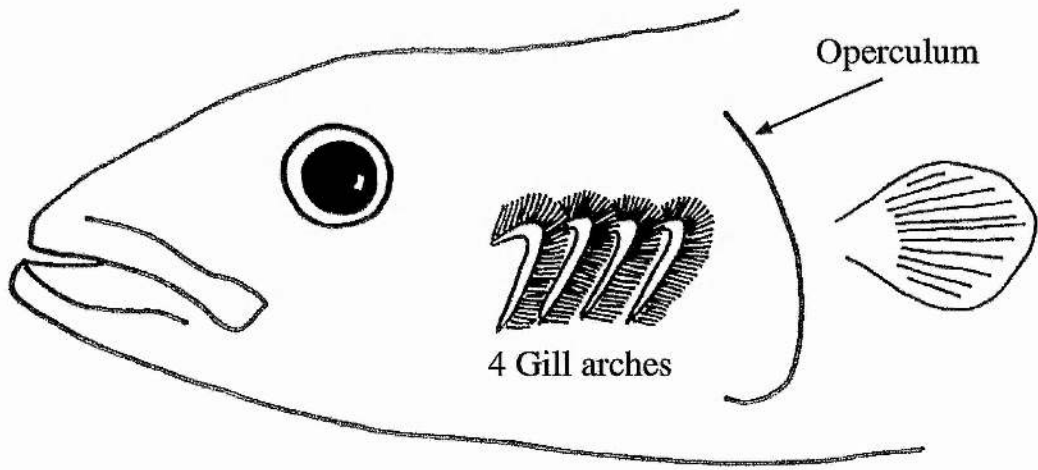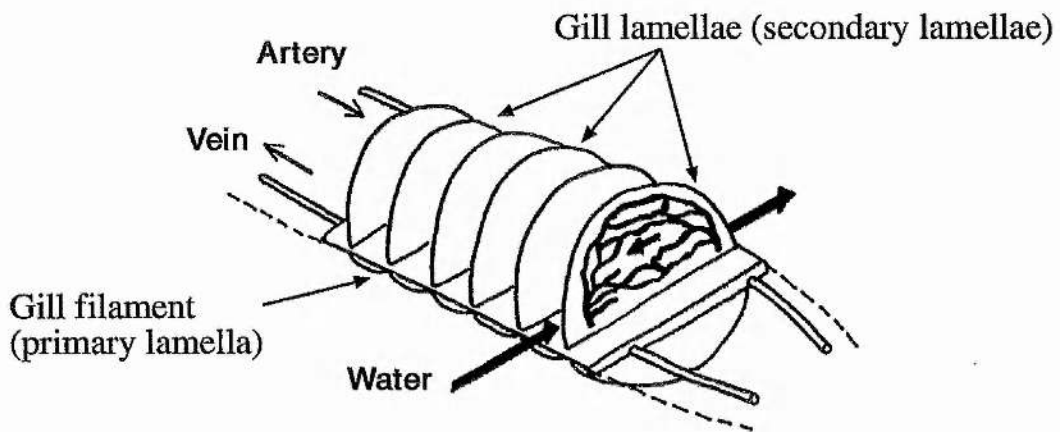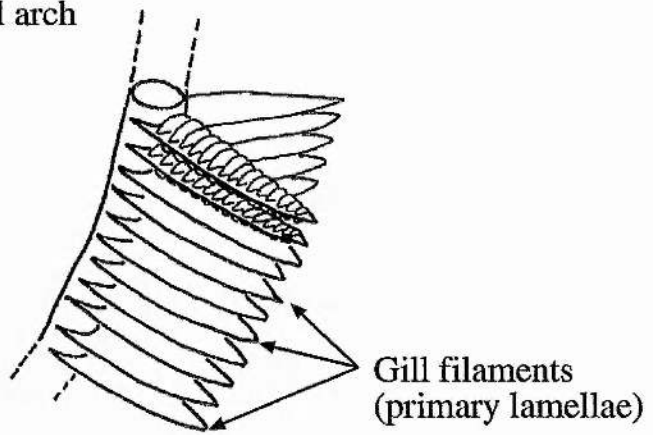
Fig. 1.6a: Schematic Representation of the Gill Lamellae

Cross-section of the secondary lamellae on the gill filament (primary lamella) showing the blood channels and some of the cell types present within the gill epithelia. Modified from Jobling (1995b).

Fig. 1.6b: Schematic Diagram of a Gill Chloride Cell

A gill chloride cell together with neighbouring pavement cells and an accessory cell. The typical ultrastructural features of a chloride cell are depicted. Multistranded tight junctions are represented by a number of solid lines, whilst 'leaky' tight junctions are shown by a pair of dotted lines. Modified from Karnaky (Jr.) 1986. See text for details.

Blood channel

Pillar cell

Mucous cell

Pavement cell

Chloride cells

Gill lamellae (secondary lamellae)

Basal blood channel

Cartilage

Apical

Golgi

Vesiculo-tubular system

Nucleus

Mitochondria

Tubular system

Basolateral
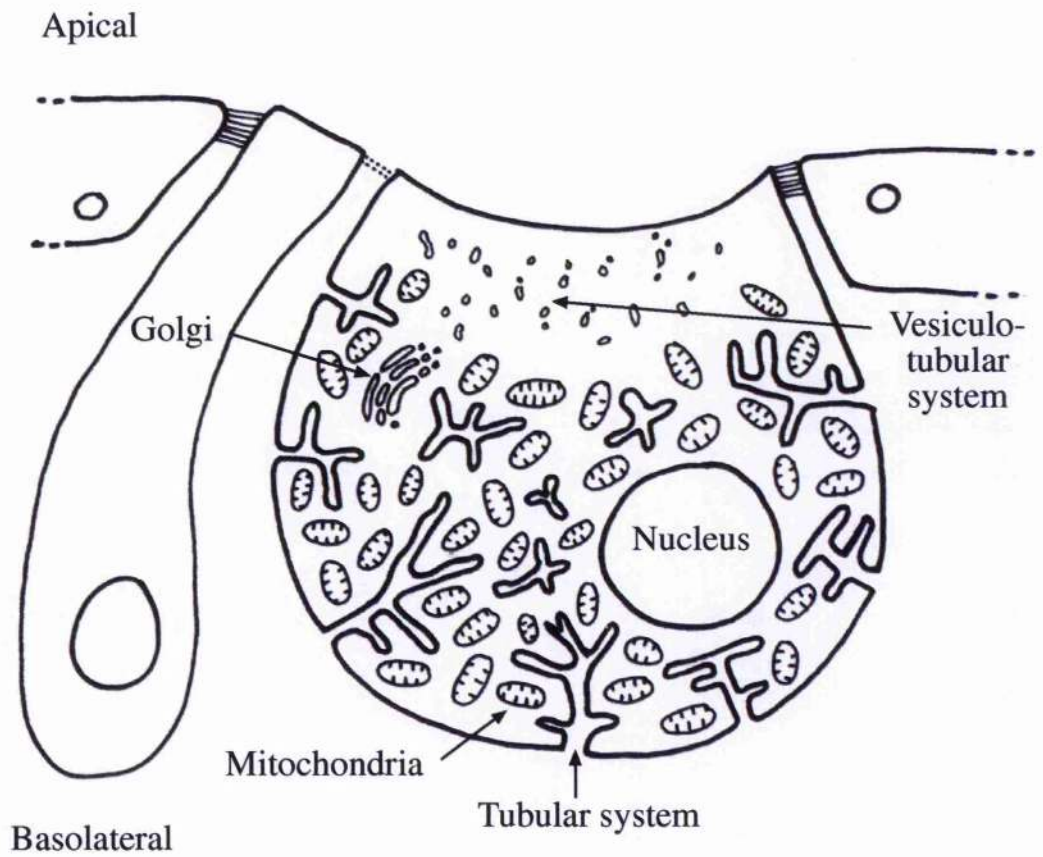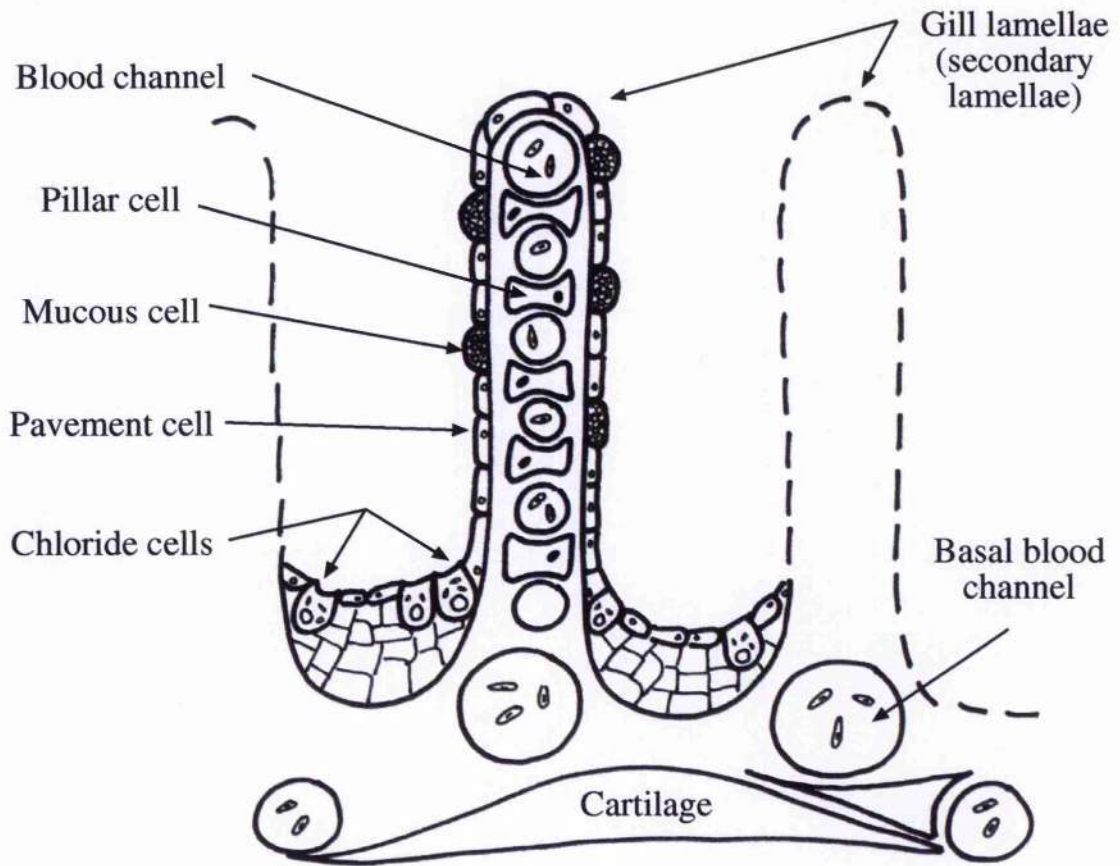
species from FW to SW including: *A. anguilla* (Motais, 1970; Milne *et al.*, 1971; Sargent *et al.*, 1975; Thomson and Sargent, 1977; Mayer-Gostan and Lemaire, 1991); *A. japonica* (Kamiya and Utida, 1968; Utida *et al.*, 1971; Ho and Chan, 1980); *A. rostrata* (Jampol and Epstein, 1970); *F. heteroclitus* (Epstein *et al.*, 1967) and *O. mykiss* (Kamiya and Utida, 1969). In addition, Na$^+$K$^+$ATPase activities have been shown to increase with increasing salinity of the external environment (Utida *et al.*, 1971; Butler and Carmichael, 1972).

However, Kirschner (1969) found no significant differences in branchial Na$^+$K$^+$ATPase activity between FW and SW adapted *A. anguilla* or flounder, *Platichthys flesus*. Similarly, no difference was found in branchial Na$^+$K$^+$ATPase activity between FW and SW adapted tilapia *O. mossambicus* (Verbost *et al.*, 1994). Madsen *et al.* (1994) found no difference in branchial Na$^+$K$^+$ATPase activity in striped bass, *Morone saxatilis*, between FW and SW adapted specimens, although they did note an increase in chloride cell size. Similar results were also observed in *O. mossambicus*, where in addition, chloride cell number was noted to decreased upon adaptation to SW (van der Heijden *et al.*, 1997).

The complex nature of the gill makes it difficult to study chloride cells by electrophysiological methods, however considerable progress was made after the discovery of models for SW gill chloride cells in the opercular (Karnaky (Jr.) and Kinter, 1977; Foskett and Scheffey, 1982; Zadunaisky, 1984) and other skin epithelia (Marshall, 1977) of SW adapted teleosts such as *F. heteroclitus* Although these species may not be considered 'typical' teleosts. The chloride cells in these epithelia were identical, in terms of morphology, to those in the gills, and as the opercular epithelium was flat and much less complex than that of the gills it could be used in Ussing chamber experiments. Although the opercular epithelium and skin have been used as models for ion transport in the gills it must be emphasized that chloride cells in these tissues also contribute in their own right to osmoregulation in teleost fish (Marshall, 1995).

Chloride cells were definitively identified as the site of net transepithelial Cl$^-$ extrusion by using the vibrating probe technique over the opercular epithelium of SW adapted *O. mossambicus* (Foskett and Scheffey, 1982). Non-chloride cells were not involved in net electrogenic ion transport and serve as impermeable barriers to passive ion transport (Foskett and Scheffey, 1982). A number of techniques are now used to identify chloride cells, including the use of fluorescent markers (Karnaky (Jr.), 1986; Li *et al.*, 1995) and histochemical (McCormick, 1990b; Li *et al.*, 1995) and immunocytochemical (Lee *et al.*, 1996) methods. A review of modern microscopical methods employed in chloride cell study is provided by van der Heijden and Morgan (1997).

Chloride cells exhibit a number of morphological features representing adaptations to their role as ion transporting cells (Fig. 1.6b). Chloride cells are large columnar cells with numerous mitochondria, they have a large ovoid nucleus, an

extensive tubular system continuous with the basolateral membrane, and exhibit exposure of the apical and basolateral surfaces to the external environment and the blood respectively (Laurent and Dunel, 1980; Pisam, 1981; Zadunaisky, 1984; Karnaky (Jr.), 1986; Perry *et al.*, 1992).

In FW the apical crypts of the chloride cells tend to be relatively flat broad and shallow (Sardet *et al.*, 1979; Laurent and Dunel, 1980; Pisam *et al.*, 1990), whilst in SW they are deeply invaginated and considered the site of ion excretion (Foskett *et al.*, 1981). The cytoplasm of chloride cells contains a highly developed system of anastamosing tubules that is continuous with the basolateral membrane (Sardet *et al.*, 1979; Pisam, 1981). This tubular system fills most of the cytoplasm except for the Golgi area and a narrow band just below the apical surface (Pisam, 1981). The abundance of mitochondria is a well recognised feature of chloride cells (Karnaky (Jr.) *et al.*, 1976) and they are found closely associated with the tubular system (Pisam, 1981; Pisam *et al.*, 1987). As noted above, chloride cells have high $Na^+K^+ATPase$ activity (Kamiya, 1972; Sargent *et al.*, 1975) and this was later localized to the basolateral tubular system (Karnaky (Jr.) *et al.*, 1976; Silva *et al.*, 1977; Hootman and Philpott, 1979), for a review see de Renzis and Bornancin (1984).

Pavement cells are joined to each other, and to chloride cells by deep, occluding, tight, multistranded junctions (Sardet *et al.*, 1979; Laurent and Dunel, 1980; Sardet, 1980; Karnaky (Jr.), 1986; Pisam *et al.*, 1990). It is assumed that these tight junctions have high electrical resistance and that the channels between adjacent pavement cells and each other or with chloride cells are relatively impermeable to ions (McDonald *et al.*, 1989). Chloride cells are generally described as existing singly in FW although they may be found in multicellular complexes (Karnaky (Jr.), 1986; Pisam *et al.*, 1990). In FW these groups of chloride cells are linked by deep, narrow, apical junctions, similar to those between the chloride cells and neighbouring pavement cells (Pisam *et al.*, 1990). The FW gill epithelia is therefore considered to be "tight" with respect to the transport of ions and other solutes (Sardet, 1980).

In SW however, the chloride cells largely exist as multicellular complexes with each other, and also with another type of cell, the accessory cell, where together they participate in the formation of apical crypts (Sardet *et al.*, 1979; Hootman and Philpott, 1980; Laurent and Dunel, 1980). The accessory cells have interdigitations with adjacent chloride cells in the apical region (Sardet *et al.*, 1979; Karnaky (Jr.), 1986; Pisam and Rambourg, 1991). These accessory cells are smaller than chloride cells proper, but contain numerous mitochondria and a highly developed, basolaterally derived tubular system, albeit less extensive (Hootman and Philpott, 1979; Sardet *et al.*, 1979; Laurent and Dunel, 1980). These multicellular groups of chloride cells and accessory cells in SW are linked by single stranded, shallow "leaky" tight junctions (Sardet *et al.*, 1979).

Mucous cells may play an indirect role in ion and water balance in the gill by creating a micro-environment rich in ions (Pisam *et al.*, 1980), although the effect of mucus on ion transport processes has been little studied in the gill as compared to the gut.

Generally, it is now thought that two distinct types of chloride cell (α and β) exist in FW adapted fish gills, whilst in SW adapted fish gills there is only one type of chloride cell (α), together with the smaller accessory cells (Pisam *et al.*, 1987; Pisam *et al.*, 1990; Pisam and Rambourg, 1991; Pisam *et al.*, 1993). These accessory cells were identified much earlier (Hootman and Philpott, 1980; Sardet, 1980). Unusually accessory cells have also been identified in FW adapted rainbow trout *O. mykiss*, although their function in this environment remains to be established (Pisam *et al.*, 1989). The two sub-types of chloride cell were identified on the basis of their morphology, location and apical surface topography and a brief outline of their features is given below (Pisam *et al.*, 1987; Pisam and Rambourg, 1991; Perry and Laurent, 1993; Pisam *et al.*, 1993; Pisam *et al.*, 1995).

In FW the α cell is located at the base of the secondary lamellae and is lighter and more elongate than the β cell. The α cell tends have its basal membrane in close contact with the pillar capillary and hence the arterial blood supply. The mitochondria, tubular system and endoplasmic reticulum are evenly distributed throughout the cytoplasm, with the exception of a narrow region just below the apical surface. There is a poorly developed vesiculotubular system sited just below the apical surface and the apical surface is slightly depressed to form shallow apical cavities. These α cells are generally considered the precursors of the chloride cells of SW adapted teleosts (Pisam *et al.*, 1987; Pisam and Rambourg, 1991; Perry and Laurent, 1993; Pisam *et al.*, 1993; Pisam *et al.*, 1995). The FW β cell is located in interlamellar region of the primary epithelium facing the central venous sinus, and is darker and more ovoid than the α cell. The mitochondria are more concentrated along the unevenly distributed tubular system, and the endoplasmic reticulum is more conspicuous in the β cell. The vesiculotubular system is more developed and the apical surface is flattened and hardly depressed (Pisam *et al.*, 1987; Pisam and Rambourg, 1991; Perry and Laurent, 1993; Pisam *et al.*, 1993; Pisam *et al.*, 1995). The function of β cells in FW remains little understood at present.

Upon SW transfer the β cells show signs of degeneration by two/three days post-transfer (Pisam *et al.*, 1987; Wendelaar-Bonga and Meij, 1989; Pisam and Rambourg, 1991). The α cells in SW on the other hand become darker and increase in both size (hypertrophy) and number (Yoshikawa *et al.*, 1993), the tubular system and vesiculotubular systems become even more well developed and there is an increase in the number of mitochondria (Pisam, 1981).

Pisam *et al.* (1988) have shown that structural transformations to the SW form of chloride cells as described above, occur during smoltification of *S. salar* whilst still in FW prior to entry to SW (Pisam *et al.*, 1988). Similar work has also shown that chloride cell size and number, and ultrastructural changes occur during the silvering process in *A.*

anguilla, whilst the eels are still in FW (Fontaine *et al.*, 1995). Although both *Oreochromis niloticus* and *O. mossambicus* show the ultrastructural changes associated with transfer to SW (large multicellular complexes with adjacent accessory cells and apical interdigitations, complete with "leaky" tight junctions) *O. niloticus* is not fully euryhaline and does not survive well in SW (Cioni *et al.*, 1991). This suggests that differences in their euryhaline capacities are due to functional differences in ion transporting capabilities (Cioni *et al.*, 1991).

Little work has been done on the two types of chloride cell in the eel. However a re-examination of a previous study (Shirai and Utida, 1970), shows what can now be interpreted as α and β chloride cells, which also show the typical changes in morphology associated with transfer from FW to SW. There is however still some controversy over whether the α, β and accessory cells are distinct cell types or are in fact different developmental stages of a single cell type. It has been shown that chloride cells undergo apoptosis and that different developmental stages of chloride cells show differing ultrastructural features (Wendelaar-Bonga and Meij, 1989), suggesting that the so called α and β chloride cell types represent cells at different stages of development of a single cell type rather than two distinct ones (Perry, 1997).

The model of ion transport in SW teleosts initially described by Silva *et al.* in 1977 is still considered the working model today, and is illustrated in figure 1.7a. The presence of the various ion transporters have been proposed on the basis of ion substitution experiments and the use of pharmacological agents to inhibit various aspects of ion transport.

The $Na^+K^+ATPase$ located on the basolateral surface provides the driving force for ion secretion. The $Na^+K^+ATPase$ acts to accumulate Na^+ ions in the tubular system which then travel to the external environment via the leaky paracellular route (Silva *et al.*, 1977; Karnaky (Jr.), 1980; Karnaky (Jr.), 1986). Cl^- ions enter via the $Na^+/K^+/2Cl^-$ cotransporter on the basal surface whose action is driven by the favourable Na^+ gradient created by the $Na^+K^+ATPase$ on the same membrane (Marshall, 1981; Degnan, 1984; Zadunaisky, 1984; Degnan, 1985; Zadunaisky *et al.*, 1995; Flik *et al.*, 1997). The Cl^- accumulated within the cell then exits down its electrochemical gradient via Cl^- channels in the apical crypt (Marshall *et al.*, 1995; Zadunaisky *et al.*, 1995). The apical Cl^- channel is little understood a present, however it is thought to resemble the cystic fibrosis transmembrane conductance regulator (CFTR) (Marshall *et al.*, 1995), the channel defective in individuals suffering from the genetic disease cystic fibrosis (Fuller and Benos, 1992; Riordan, 1993). K^+ ions are recycled to the basolateral side through barium sensitive K^+ channels (Degnan, 1985), and may additionally exit via the paracellular route in a similar way to the Na^+ ions, see Zadunaisky (1984). There has also been suggestion of the presence of basolaterally located Na^+/H^+ and Cl^-/HCO_3^-

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Fig. 1.7a: Model of Ion Transport in the Gills of SW Adapted Teleosts

The 'leaky' tight junctions between chloride cells and each other and with accessory cells are depicted by a pair of dotted lines. Deep multistranded tight junctions between chloride cells and pavement cells and between pavement cells and each other are depicted by a number of solid lines. Active transport and cotransport are shown by solid lines whilst passive diffusion is represented by dashed lines. Details are given in the text. Modified from Marshall (1995).

Fig. 1.7b: Model of Ion Transport in the Gills of FW Adapted Teleosts

The deep multi-stranded tight junctions between chloride cells and pavement cells and between pavement cells and each other are depicted by a number of solid lines. Active transport and cotransport are shown by solid lines whilst passive diffusion is represented by dashed lines. This is now generally considered an older model, see text (page 26) for updated details. Modified from Marshall (1995).

Apical

Cl⁻   K⁺   Na⁺

K+

Na⁺ K⁺ 2Cl⁻

3Na⁺

2K⁺

Basolateral

Apical   Cl⁻   Na⁺ H⁺ (NH₄⁺)

HCO₃⁻

K⁺

Cl⁻

2K⁺ 3Na⁺

Basolateral

Apical

Cl^- K^+ Na^+

$K+$

Na^+ K^+ $2Cl^-$

$3Na^+$

$2K^+$

Basolateral

Apical Cl^- Na^+ H^+ (NH_4^+)

HCO_3^-

K^+

Cl^-

$2K^+$ $3Na^+$
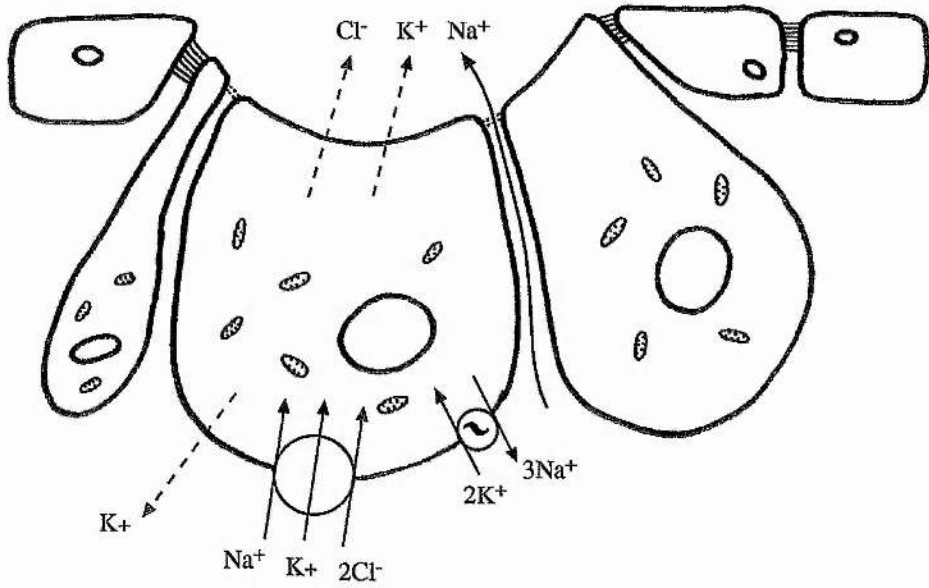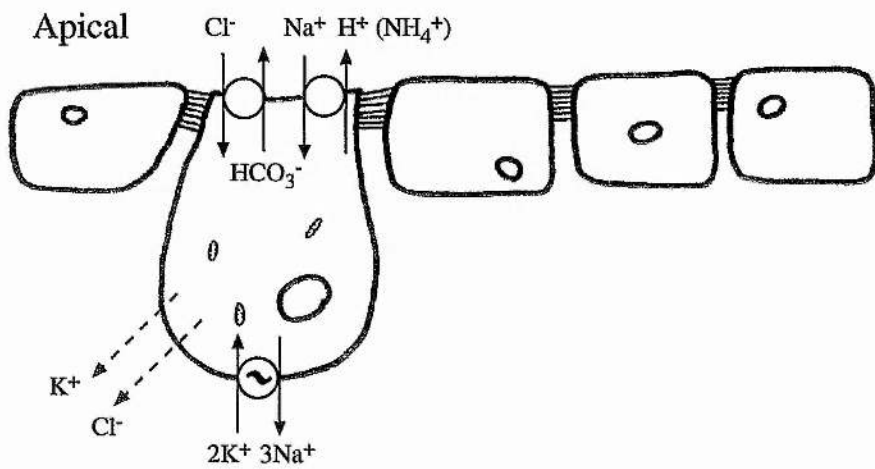
Basolateral

exchangers (Zadunaisky *et al.*, 1995), and a Na+/Cl⁻ cotransporter (Flik *et al.*, 1997). However, the Cl⁻/HCO$_3$⁻ exchanger is not thought to be involved in the hyperosmotic response (Zadunaisky *et al.*, 1995).

Proliferation of chloride cells and an amplification of their apical surfaces has also been observed in artifically prepared low NaCl FW water, as compared to natural FW (Perry and Laurent, 1989). The area of exposed chloride cells is significantly correlated with the rate of ion uptake in a number of FW teleosts (Perry *et al.*, 1992), supporting the idea that chloride cells are the site of ionic uptake in FW (Evans, 1980). This proliferation of chloride cells in FW occurs on both filament and lamellar epithelia and may (Perry and Laurent, 1993; Bindon *et al.*, 1994), or may not (Laurent and Hebibi, 1989) impede gas transfer. The increase of chloride cell number in ion poor water (low Na⁺, Cl⁻ and Ca^{2+}) as compared to FW results from initial differentiation followed by true proliferation through mitosis in the filament epithelium (Laurent and Dunel-Erb, 1994). However, in the lamellar epithelium chloride cells are recruited via differentiation and migration from the filament epithelium (Laurent and Dunel-Erb, 1994).

The gills are the site of a number of exchange processes in teleost fish. They are involved in respiratory gas exchange, ion exchange and also in the excretion of nitrogenous waste products. These processes do not occur in isolation to each other but appear to be coupled. In FW, fish can achieve an advantageous arrangement where unwanted waste is removed in exchange for sought after ions. The coupling of ion homeostasis, acid-base balance and gas exchange functions are complex. For detailed discussions see Perry and Laurent (1993), Goss *et al.* (1995) and Perry (1997). It must be noted that the nature of the transepithelial movements of Na⁺ and Cl⁻ across the branchial epithelia of teleost gills in FW fish has not been fully elucidated and are a matter of some conflict at present.

The opercular epithelium acts as a good model for the study of chloride cells in SW. The requirement for a similar model of ion transport in FW has long been recognised (Karnaky (Jr.) and Kinter, 1977; Zadunaisky, 1984). Unfortunately such a successful model for the study of Na⁺ and Cl⁻ fluxes in FW chloride cells has yet to be discovered. Generally the numbers of chloride cells disappear in the operculum and skin when fish are adapted to FW (Foskett *et al.*, 1981). However, more recently, Marshall *et al.* (1997) have found more encouraging results using FW adapted *F. heteroclitus* opercular epithelia. Efforts are also being concentrated on the culture of branchial epithelial cells *in vitro*, and although only pavement cells have been successfully cultured to date, short-term primary cultures have enabled patch clamp experiments on chloride cells (Marshall *et al.*, 1995; Pärt and Bergstrom, 1995; Wood and Pärt, 1997).

The role of chloride cells in FW is currently a matter of some debate. Figure 1.7b illustrates an older model for ion transport in the FW fish gill. Distinct types of chloride cells with varying apical surface topographies have been identified in *O. mossambicus* adapted to various hypotonic environments (Lee *et al.*, 1996). Some studies suggest that chloride cells are involved in the active uptake of both Na^+ and Cl^- (Laurent *et al.*, 1985; Laurent and Perry, 1990; Perry *et al.*, 1992), whilst other work suggests that the chloride cells are the site of Cl^- uptake only, and that the uptake of Na^+ is via the pavement cells (Goss *et al.*, 1992a; Goss *et al.*, 1992b; Laurent and Dunel-Erb, 1994; Morgan *et al.*, 1994). It is still assumed that the chloride cell is the site of Cl^- uptake in FW. Current evidence using pharmacological inhibitors (Morgan and Potts, 1995), correlation analyses of chloride cell surface area and Cl^- transport (Perry and Laurent, 1989; Laurent and Perry, 1990; Perry *et al.*, 1992), and *in situ* hybridization techniques (Sullivan *et al.*, 1996) still suggest that only the chloride cells are involved in the uptake of Cl^-.

There are conflicting reports as to changes in $Na^+K^+ATPase$ activity in fish acclimated to various FW environments. Mayer-Gostan and Naon (1992) found a decrease in activity in *A. anguilla* acclimated to distilled water, although Butler and Carmichael (1972) found increased activity in *A. rostrata*. It has been the proposed that Cl^- uptake occurs via an electroneutral Cl^-/HCO_3^- exchanger (Perry *et al.*, 1981; Goss *et al.*, 1992a; Marshall, 1995), however there may be insufficient chemical gradients of both Cl^- and HCO_3^- for this to work (Perry, 1997). It has been suggested that the earlier discounted suggestion of the involvement of an apical membrane anion-dependent ATPase (de-Renzis and Bornancin, 1984) ought to be re-examined (Perry, 1997). There is also indirect evidence to suggest another Cl^-/HCO_3^- exchanger for the basal membrane of the pavement cells (Lin and Randall, 1995). Current models depict the exit of Cl^- from the cell to the blood via basolateral Cl^- channels although they have not been identified (Marshall, 1995).

In FW Na^+ is transported from the environment to the blood whilst H^+ is moved in the opposite direction. However, there is considerable controversy over the transport processes achieving this result, and Na^+ uptake may be coupled with NH_4^+ excretion (Payan, 1978), H^+ excretion (Heisler, 1984) or indeed both (Wright and Wood, 1985). For many years it was thought that Na^+ uptake in FW was via an electroneutral Na^+/H^+ exchanger (Fig. 1.7b) however it became apparent that the chemical gradients across the gill epithelia could not support this proposed exchanger (Perry, 1997). Avella and Bornancin (1989) postulated another mode of Na^+ entry in FW fish. Na^+ is taken up by an electrogenic proton pump ($H^+ATPase$) which uses ATP to pump H^+ to one side of the epithelium which then drives the absorption of Na^+ via a Na^+ conductive channel (Avella and Bornancin, 1989). There is a growing body of evidence in support of this method of Na^+ entry (Lin and Randall, 1991; Potts, 1994; Sullivan *et al.*, 1995; Sullivan *et al.*, 1996). However, the site of this $H^+ATPase$ is currently a matter of some debate. Using

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antibody-immunofluorescence microscopy the H^+ATPase has been associated to the apical regions of both the chloride cells and pavement cells in some studies (Lin *et al.*, 1994) whilst in others it has been localized to only the apical regions of the pavement cells (Sullivan *et al.*, 1995; Sullivan *et al.*, 1996; Perry, 1997). For more details on this proton pump see Lin and Randall (1995).

More recently there has been work on the molecular biology of genes coding for the proteins involved in ion transport in the gill, for a review see Cutler *et al.*, (1996). Na^+K^+ATPase expression has been the most extensively studied to date. The Na^+K^+ATPase consists of two functional units - α and β, each of which may exist as a number of isoforms *in vivo*, see table 1.1 in section 1.2.1.2 iii.

When changes in expression and Na^+K^+ATPase activity were investigated in FW adapted *A. anguilla* transferred to SW, a three phased response was discovered (Luke *et al.*, 1994). Immediately upon transfer there was an increase in branchial Na^+K^+ATPase activity within the first 6 hours which then declined over the following 18 hours to resemble levels associated with the FW-FW control fish. There was no increase in $\alpha 1$ or $\beta 1$ subunit mRNA expression over this time period (Luke *et al.*, 1994). Three days post-transfer there was a further gradual and more sustained increase in Na^+K^+ATPase activity which was paralleled by an increase in both $\alpha 1$ and $\beta 1$ subunit mRNA expression, with maximum Na^+K^+ATPase activity levels noted after three weeks adaptation (Luke *et al.*, 1994). In long-term acclimated fish the Na^+K^+ATPase activity levels and the expression of both $\alpha 1$ and $\beta 1$ subunits had decreased to a level similar to, but still significantly higher than, the FW acclimated control group (Luke *et al.*, 1994). The regulation of Na^+K^+ATPase is clearly complex. The up-regulation of Na^+K^+ATPase activity immediately post-transfer to SW may be by recruitment of pre-assembled pumps into the plasma membrane, or through activation of existing pumps via phosphorylation/dephosphorylation mechanisms (Luke *et al.*, 1994). The reduction of Na^+K^+ATPase activity in long-term adapted SW fish may reflect changes in other ion transporting systems or be through permeability changes (Luke *et al.*, 1994).

In addition $Na^+/K^+/2Cl^-$, K^+/Cl^- and Na^+/Cl^- cotransporters, Cl^-/HCO_3^- and Na^+/H^+ exchangers and the CFTR-like channel have also been identified in the gill by molecular methods (Cutler, personal communication).

1.2.3 The Osmoregulatory Role of the Kidney and Urinary Bladder

In addition to the gut and gills, the kidney and urinary bladder play important roles in osmoregulation in teleost fish. The bladder does not simply act as a urine storage organ, but does in fact act to modify the urine. As noted in section 1.1.2, fish in FW must have high rates of urine flow in order to eliminate the osmotic water influx faced in

the dilute environment, whilst still retaining as much NaCl as possible. In SW on the other hand, urine flow rates are reduced in order to conserve water given the dehydrating environment, and the kidney functions primarily to excrete Mg^{2+} and SO_4^{2-} ions (Hickman (Jr.) and Trump, 1969).

1.2.3.1 The Kidney

The teleost kidney can generally be divided into two regions; the head kidney and the trunk kidney. In eels these two sections can be distinguished upon external examination, although this is not possible for all species e.g. herring. The trunk kidney is the site of osmoregulatory processes in the kidney. For details of the structure, function and vasculature of the kidney see (Hickman (Jr.) and Trump, 1969).

The teleost kidney exists of a number of units called nephrons. Each nephron begins with a Malpighian body which is the site of ultrafiltration. The Malpighian body is enclosed by the glomerulus - a basket of capillaries leading from a small artery. Fluid (glomerular filtrate) is forced out through the walls of the glomerular capillaries by the blood pressure and enters the tubule that leads from the Malpighian body. It is in the tubules of the nephron that modification of the glomerular filtrate takes place. In the first or proximal tubule, solutes such as glucose and salt are reabsorbed. This process of modification is continued in the second or distal tubule. Fish lack the loop of Henle present in mammalian and avian kidneys, therefore urine can never be more concentrated than plasma, and excess salt must be excreted by an extra-renal route i.e. by the gills (Hickman (Jr.) and Trump, 1969). The distal tubules join to form collecting ducts which pass the urine to the bladder from which it is discharged to the exterior at intervals (Hickman (Jr.) and Trump, 1969).

In most fish, in addition to the processes of filtration and absorption, urine is also produced by secretion. Aglomerular fish (around 30 species), including the goosefish, *Lophius piscatorius*, and the toadfish, *Opsanus tau*, however must rely purely on tubular secretion to produce urine (Hickman (Jr.) and Trump, 1969). The initial filtrate contains all the products in the plasma except those of large molecular size such as proteins. Many of the filtered products such as glucose, amino acids and vitamins are valuable and are reabsorbed rather than lost in the urine. The filtration-reabsorption method of urine production ensures that new and perhaps undesirable substances in the blood are removed. With secretion only methods of urine production, specialized secretory mechanisms must be evolved to cope with each new substance encountered. Secretion only, aglomerular kidneys tend therefore to be found only in marine fish which live in a more stable environment. The presence of a secretion only kidney in many Antarctic fishes ensures that the highly valuable, small molecular weight antifreeze glycoproteins are conserved without having to perform the energetically expensive task of reabsorption.

In SW, excretion by the kidney is the main route of efflux of ingested Mg^{2+} and SO_4^{2-}, whilst it plays little role in the excretion of the ingested monovalent ions Na^+, Cl^- and K^+, which are extruded by the gills (Hickman (Jr.) and Trump, 1969). In SW the gut is perhaps more important in ion balance whilst the kidney is more important in FW fish. Although the urine of FW fish is quite dilute it still accounts for a significant component of ion loss to the order of 33% of the unidirectional Na^+ efflux in *A. anguilla* (Maetz, 1971).

Changes in glomerular filtration rate (GFR) allow teleosts to live in environments of different salinity. Marine fish tend to have GFRs of about one tenth that of FW species, although there are exceptions (Sharratt *et al.*, 1964b; Oide and Utida, 1968; Hickman (Jr.) and Trump, 1969; Schmidt-Nielsen and Renfro, 1975; Brown *et al.*, 1980). GFR and urine flow rates are higher in FW as compared to SW adapted eels (Sharratt *et al.*, 1964b; Gaitskell and Chester-Jones, 1971), and decrease within a few hours post-transfer from FW to SW (Oide and Utida, 1968; Sokabe *et al.*, 1973). Although urine flow is low in SW there is still a considerable loss of water to the order of 4% of the volume ingested in *P. lethostigma* (Hickman (Jr.), 1968). Conversely fish in ion poor water such as deionized water show increased urine production rates and GFR (Parwez *et al.*, 1994)

Much of the filtered water is reabsorbed by marine teleosts following the active transport of NaCl (Hickman (Jr.) and Trump, 1969). There is a basolaterally located Na^+K^+ATPase which is increased in *A. anguilla* renal cells adapted to SW as compared to FW (Zonno *et al.*, 1994; Vilella *et al.*, 1995). In addition, there is a Na^+/K^+ exchanger on the apical membrane which is also increased in SW (Zonno *et al.*, 1994; Vilella *et al.*, 1995). This would result in higher rates of NaCl reabsorption in SW and hence greater rates of water reabsorption by solute-linked water transport.

The first proximal tubule is thought to be the site of isosmotic reabsorption of NaCl (Hickman (Jr.) and Trump, 1969) and may also function in divalent ion secretion. Urine production by secretion at the proximal tubule has been observed in glomerular marine and euryhaline fish as well as aglomerular fish (Beyenbach, 1982; Cliff and Beyenbach, 1992), see Beyenbach (1995) for a review. There is thought to be a greater reliance on tubular secretion in SW as compared to FW fish (Hickman (Jr.) and Trump, 1969; Beyenbach, 1986), and this is reflected by a greater proportion of secretory proximal tubules in SW (Cliff and Beyenbach, 1992). The renal proximal tubules have high transepithelial water permeability and fluid movement into the tubule occurs by solute-linked water transport following the movement of the divalent ions. The active transport pathways that must exist within the teleost proximal tubule cells for the secretion of, for example Mg^{2+} have not been fully identified (Beyenbach, 1986; Cliff and

Beyenbach, 1992; Renfro, 1995). Urine production can thus be affected by the number of filtering glomeruli and the number of secretory proximal tubules.

As noted above, SW adapted fish have urine production rates and glomerular filtration rates around 10% that of FW adapted fish, and this is associated with a reduction in the proportion of filtering glomeruli (Brown *et al.*, 1980). There is a linear relationship between urine production rate, glomerular filtration rate and the renal tubular maximum for glucose (TMG) (Chester-Jones *et al.*, 1969). As TMG is a measure of the maximum ability to reabsorb glucose and hence an indicator of the functional tubular mass, this suggests that there is a lower proportion of filtering nephrons in SW. In addition, Brown *et al.* (1980) have visualized different populations of nephrons within the teleost kidney using the ferricyanide/prussian blue perfusion technique, and have demonstrated more directly that glomerular intermittency plays an important role in changes of urine output in teleost fish. In the euryhaline rainbow trout, *O. mykiss*, adaptation to SW results in a reduction in overall GFR. However, there is an increase in the single nephron glomerular filtration rate (SNGFR) of those nephrons which do filter (Brown *et al.*, 1980). Three types of nephron were identified in rainbow trout *O. mykiss* kidney (Brown *et al.*, 1980); filtering (F) - where prussian blue was found in the glomerular vessels and the tubular lumina, non-filtering (NF) - where prussian blue was only found in the glomerular vessels, and non-perfused (NP) - where there was no prussian blue associated with the nephron. In FW and SW the kidneys contained the same number of non-filtering glomeruli (40%). In FW there were many more filtering glomeruli as compared to SW acclimated trout (45% and 5% respectively). The percentage of non-perfused tubules was 13% in FW but 51% in SW. It must be noted that the non-perfused nephrons may still be functional by secretion (Schmidt-Nielsen and Renfro, 1975).

1.2.3.2 The Urinary Bladder

The urinary bladder in teleost fish acts as an accessory to the role of the kidney. As the urine is retained within the bladder for some time before being expelled its ionic composition is modified by the ion transport processes occurring within it. Curtis and Wood (1991) have shown that urine is maintained within the bladder of FW rainbow trout for about 30 minutes. There is considerable absorption of ions over this time, such that ureteral urine is approximately 10mM NaCl whilst urine released voluntarily by the fish is only 2 to 3 mM (Curtis and Wood, 1991). The FW urinary bladder has a much lower permeability to water than that of the SW bladder, such that the reabsorption of salts is accompanied by minimal reabsorption of water (Demarest, 1984). In FW NaCl is taken up by electroneutral transport processes which may be coupled (Fossat and Lahlou, 1979), partially coupled (Demarest, 1984) or uncoupled via paired anion and cation

exchangers (Loretz and Bern, 1980; Marshall, 1986). There is no evidence for the presence of a $Na^+/K^+/2Cl^-$ cotransporter in teleost bladder (Marshall, 1986). Although the reabsorption of ions accounts for only about 4% of those reabsorbed by the kidney, this reabsorption by the bladder results in a reduction of urinary NaCl losses by 40% (Curtis and Wood, 1991).

The SW urinary bladder has lower resistance to water than the FW urinary bladder (Loretz and Bern, 1980) such that water reabsorption can be coupled to ion uptake in solute-linked water transport (Utida et al., 1972; Renfro, 1975). Greater rates of water absorption are achieved in fish acclimated to high salinity as compared to FW (Demarest, 1984) thus conserving water in hyperosmotic environments (Howe and Gutknecht, 1978; Loretz and Bern, 1980). The SW bladder reabsorbs NaCl and water and excretes a urine that is rich in divalent ions such as Ca^{2+} and Mg^{2+} and SO_4^{2-}, and has an increased osmolality than in FW (Loretz and Bern, 1980). Howe and Gutknech (1978) have estimated that reabsorption of water from the toadfish bladder reduces the volume of SW to be imbibed by around 10%, thus saving on the amount of active transport that must be carried out at the gut and gill.

A basolaterally located $Na^+K^+ATPase$ has been identified in the urinary bladder by ouabain binding autoradiography (Renfro et al., 1976), and $Na^+K^+ATPase$ activity has been reported to be higher after transfer of starry flounder, Platichthys stellatus, from SW to FW (Utida et al., 1974). K^+ is recycled via a basolateral barium sensitive K^+ channel (Dawson and Frizzell, 1989), and there is also strong evidence of a Na^+/Cl^- cotransporter (Renfro, 1977; Gamba et al., 1993). Most passive ion fluxes are thought to occur via the cellular pathway rather than paracellularly (Stokes, 1988) and there is evidence to suggest the presence of basolateral Cl^- channels thus allowing the reabsorbed Cl^- to exit across the basolateral membrane and into the blood (Dawson and Frizzell, 1989; Chang and Loretz, 1993).

There is considerable variation in results from studies of urinary bladders which may reflect the osmoregulatory strategies of individual species i.e. euryhaline, stenohaline FW or marine (Marshall, 1995). Further work employing a wider range of species is clearly required before the overall importance of the bladder in osmoregulation can be fully evaluated.

1.3   Hormonal Control of Osmoregulatory Processes

It is of great importance that the gill, gut and renal osmoregulatory systems discussed in section 1.2 are integrated in both FW and SW fish. Integration and control of these systems must be of even more importance in euryhaline fish that move between these environments, in order to ensure that the appropriate ion and water transporting capabilities are employed depending upon the salinity of the external environment.

The control of drinking is primarily mediated through the renin angiotensin system (RAS) in both FW and SW (Carrick and Balment, 1983). Hormones shown to stimulate ion transport in the intestine include cortisol, urotensin II (UII) and catecholamines, whilst those shown to inhibit intestinal ion transport include prolactin, atrial natriuretic peptide (ANP) and vasoactive intestinal peptide (VIP) (Collie and Hirano, 1987). Cortisol, and prolactin appear to be the primary agents affecting osmoregulation at the gill, although actions for catecholamines, the urotensins, VIP and glucagon have also been suggested (Foskett et al., 1993; McCormick, 1995). The main hormones implicated in teleost renal function are arginine vasotocin (AVT) and the RAS (Brown and Balment, 1997). Obviously any changes in systemic blood pressure will indirectly affect renal function, hence complicating the interpretations concluded from studies employing vasoactive hormones such as angiotensin II (ANG II) and AVT. Water reabsorption at the urinary bladder is primarily controlled by the combined actions of prolactin and cortisol (Bentley, 1987) This section will address each of these hormones, and their actions at each of the osmoregulatory organs in turn.

Some of the responses to osmotic challenge, such as immediate burst drinking (Hirano, 1974; Okawara et al., 1987) and the rapid Cl⁻ secretion via gill chloride cells (Zadunaisky et al., 1995) are not mediated by hormones. The RAS is not thought to be involved in the immediate burst drinking response observed in eels upon transfer from FW to SW (Okawara et al., 1987). When FW A. japonica were transferred to SW they began to drink immediately although there were no increases in plasma ANG II until after two hours post-transfer (Okawara et al., 1987). The immediate burst drinking response upon transfer to SW is thought to be stimulated by Cl⁻ ions in the environment which are detected by halide sensitive receptors in the buccal cavity (Hirano, 1974).

There is evidence to suggest that the rapid signal to stimulate Cl⁻ secretion by the gills upon encountering higher salinities is the increased osmolality of the plasma (Zadunaisky et al., 1995; Zadunaisky, 1996; Zadunaisky, 1997). This elevation of plasma osmolality induces chloride cell shrinkage and an increased secretion of Cl⁻ via activation of the $Na^+/K^+/2Cl^-$ cotransporter, the Na^+/H^+ exchange mechanism and the opening of Cl⁻ channels. The proposed Cl^-/HCO_3^- exchanger however was not stimulated by increases in plasma osmolality (Zadunaisky et al., 1995; Zadunaisky, 1996; Zadunaisky, 1997) .

1.3.1 Renin Angiotensin System

The renin angiotensin system (RAS) is thought to play an important role in the control of drinking in teleost fish. All the components of the RAS as described in mammals have been identified in fish, although their biological actions have not been fully elucidated. Renin initiates the cascade by acting on renin substrate to form angiotensin I (ANG I) which is then converted to angiotensin II (ANG II) by the

angiotensin converting enzyme (ACE). The cascade then continues to angiotensins III and IV, mediated by peptidases. In fish, ANG I has no known biological action *per se* (Olson, 1992), and is rapidly converted to ANG II, the main biologically active component of the RAS. ANG III is not dipsogenic in eels (Hirano and Hasegawa, 1984), and has been shown to elicit cardiovascular pressor actions (Butler and Oudit, 1995). However, further work is required to establish whether these other angiotensins have a similar role in fish as they do in mammals.

Plasma renin activity is higher in SW as compared to FW adapted eels (Sokabe *et al.*, 1973; Henderson *et al.*, 1976). In addition, plasma renin activity has been shown to decrease upon transfer from SW to FW, and then to increase again when the reverse transfer was carried out (Sokabe *et al.*, 1973; Henderson *et al.*, 1976). Increases in plasma renin are also associated with the transfer of salmon smolts to SW (Smith *et al.*, 1991). Plasma ANG II undergoes a transient increase in concentration during adaptation of euryhaline species such as *A. anguilla* from FW to SW, reaching a peak after 6 days post-transfer (Balment *et al.*, 1993; Tierney *et al.*, 1993).

As discussed above, the burst drinking reflex when an eel is transferred from FW to SW is not controlled by the RAS. Tierney *et al.* (1993) have shown that increases in drinking post-transfer of FW eels to SW are paralleled by significant increases in the circulating levels of ANG II in the plasma only after two days post-transfer. The second phase of drinking however is mediated by the RAS which is stimulated as a result of decreased blood pressure or volume. Although it has been established that hypotension induces a drinking response, the underlying method of action has not been fully elucidated. There is evidence to suggest that baroreceptors or volume receptors may be located proximal to the branchial artery and play a role in the control of drinking, as decreases and increases in blood pressure result in the initiation and inhibition of drinking (Hirano and Hasegawa, 1984). ANG II has a vasoconstricting effect which acts to restore blood pressure (Nishimura and Sawyer, 1976; Takei *et al.*, 1979; Gray and Brown, 1985; Perrott and Balment, 1990).

Numerous studies have reported the dipsogenic effects of ANG II in fish by administering exogenous angiotensins or by stimulation of the endogenous RAS (Takei *et al.*, 1979; Malvin *et al.*, 1980; Carrick and Balment, 1983; Kobayashi *et al.*, 1983; Hirano and Hasegawa, 1984; Balment and Carrick, 1985; Beasley *et al.*, 1986; Perrott *et al.*, 1992; Tierney *et al.*, 1993; Balment *et al.*, 1995; Tierney *et al.*, 1995a; Tierney *et al.*, 1995b; Fuentes *et al.*, 1996b). As might be expected, administration of homologous peptides results in more marked dipsogenic responses. In some FW and SW stenohaline fish injected ANG II was found to have no effect (Kobayashi *et al.*, 1983; Beasley *et al.*, 1986). However, injection of ANG II into FW fish which were euryhaline or can tolerate brackish water did stimulate the dipsogenic response and the same was true for SW fish which could survive in brackish water (Kobayashi *et al.*, 1983). It seems that ANG II

does not stimulate drinking in truly stenohaline species inhabiting either FW or SW but does provoke a dipsogenic response in fish that may encounter waters of higher salinity than that they are accustomed to (Kobayashi *et al.*, 1983). However, Perrott *et al.* (1992) did manage to stimulate drinking in stenohaline marine species with ANG II.

There is an important link between blood pressure and drinking in teleost fish. Papaverine is a smooth muscle relaxant, and it has been shown to stimulate drinking. It is thought that this stimulation occurs by inducing hypotension and activation of the endogenous RAS (Carrick and Balment, 1983; Balment and Carrick, 1985; Perrott *et al.*, 1992; Tierney *et al.*, 1993; Tierney *et al.*, 1995a; Tierney *et al.*, 1995b). The injection of papaverine results in increased levels of circulating ANG II, and this stimulation of the RAS can be inhibited by administering captopril which inhibits the ACE thus preventing the conversion of ANG I to ANG II (Balment and Carrick, 1985; Perrott *et al.*, 1992; Tierney *et al.*, 1993; Balment *et al.*, 1995; Tierney *et al.*, 1995a; Tierney *et al.*, 1995b). A similar stimulation of drinking has been shown in *S. salar* alevins using sodium nitroprusside to induce hypotension and drinking, which could then be inhibited using another ACE inhibitor, enalapril (Fuentes *et al.*, 1996b). However, it must be noted that Beasley *et al.* (1986) could not attenuate the drinking response with a converting enzyme inhibitor in the marine fish *M. octodecemspinosus* and *P. americanus*.

Although the involvement of the RAS in drinking is now clear, the administration of ACE inhibitors such as captopril to FW adapted fish does not result in a decrease in the already low basal drinking rate (Perrott *et al.*, 1992; Tierney *et al.*, 1993; Tierney *et al.*, 1995b). This suggests that another hormone system may play a role in regulating drinking in FW teleosts.

Binding of ANG II has been noted in brain and may be involved in the control of drinking and or blood pressure (Cobb and Brown, 1992). ANG II is thought to act on the central nervous system at the level of the medulla oblongata in *A. japonica* (Takei *et al.*, 1979). This reflects the less complex mechanisms employed in fish (as compared to mammals and birds where ANG II acts on the forebrain) which are continually surrounded by water such that they do not have to feel thirst and then find water as terrestrial vertebrates do (Takei *et al.*, 1979). ANG II has also been shown to act directly on the brain via AT1 receptors to increase blood pressure and heart rate (LeMevel *et al.*, 1994).

Although there is no direct evidence, indirect evidence suggests that the RAS may be involved in controlling water absorption the gut. Stimulation of the RAS provokes a dipsogenic response and this is accompanied by a decrease in plasma osmolality in FW fish (Balment and Carrick, 1985; Tierney *et al.*, 1995b). ANG II receptors have also been identified in both whole plasma membrane and brush-border membrane preparations from the intestine of the eel, *A. anguilla* (Marsigliante *et al.*, 1994).

Administration of ANG II results in antidiuresis in *O. mykiss*, the reduction in urine flow rate largely being due to a reduction in GFR (Brown *et al.*, 1980; Gray and Brown, 1985). Angiotensin administration effects a reduction in the number of filtering nephrons, as determined by the reduced TMG and by the ferrocyanide technique to visualise functioning glomeruli (Brown *et al.*, 1980; Gray and Brown, 1985). An antidiuretic function for ANG II, at least in the trout, is consistent with elevated plasma levels of ANG II post-transfer from FW to SW, and the role of ANG II in the dipsogenic response to hyperosmotic media. Administration of ANG II in eel however had been found to have the opposite effect, causing diuresis (Nishimura and Sawyer, 1976). However, the circulating levels of ANG II in this study probably induced an increase in basal blood pressure which represented a pharmacological rather than physiological effect (Brown and Balment, 1997). In order to circumvent complications due to vasoactive events, a perfused kidney preparation for trout has been developed more recently (Brown and Balment, 1997). Using this technique physiological doses of ANG II have shown antidiuretic actions (Dunne and Rankin, 1992) which can be counteracted to some extent via coincident vasopressor actions (Gray and Brown, 1985). ANG III has also been demonstrated to have an antidiuretic effect in the perfused trout kidney preparation, although this was not as marked as that of ANG II (Brown *et al.*, 1995).

In addition to the systemic RAS, the presence of a local renal RAS has been identified in trout (Brown *et al.*, 1995). This local RAS appears to be active only under conditions of low perfusate pressure and could, as with the systemic RAS, have its effects abolished by the converting enzyme inhibitor captopril (Brown *et al.*, 1995).

ANG II receptors have been located in the glomeruli and proximal tubules of teleost kidney, amongst a number of other tissues (Cobb and Brown, 1992). The specific binding of ANG II was generally found to be similar between FW and SW adapted rainbow trout, (Cobb and Brown, 1992). However, binding by the proximal tubules was higher in FW as compared to SW trout, although there was no significant difference in binding at the glomerulus (Cobb and Brown, 1992). Characterization studies suggest that the ANG II receptors found in the trout glomerulus are novel (Cobb and Brown, 1993). Marsigliante *et al.* (1994) have demonstrated the presence of two different subtypes of ANG II receptor in SW eel liver, although only one subtype was found to exist upon renal tubular brush border membranes.

Specific binding of ANG II has also been demonstrated in the smooth muscle and epithelial lining of the bladder (Cobb and Brown, 1992), although its effects on ion transport processes are undocumented.

In addition to stimulating drinking and causing glomerular antidiuresis, ANG II has been shown to increase cortisol secretion (Perrott and Balment, 1990) and increase the release of catecholamines (Carroll and Opdyke, 1982).

1.3.2 Cortisol and Interrenal Tissue

Cortisol is a steroid hormone secreted from the interrenal tissue which is sited at the anterior part of the kidney near the heart, known as the "head kidney". The interrenal tissue consists of cubical cells arranged in cords or loops and these tend to be associated with the posterior cardinal vein and/or its branches (Chieffi and Botte, 1963; Chester-Jones and Mosley, 1980). Interrenal tissue in *A. anguilla* may occur around the anterior cardinal vein (Chester-Jones and Mosley, 1980). The interrenal cells mingle to a varying extent with the other cells of the head kidney such as the chromaffin, pigment and haematopoietic cells (Chester-Jones and Mosley, 1980). The patterns of distribution of the interrenal cells and the degree of intermingling with other cell types depends upon species (Chester-Jones and Mosley, 1980). Hypophysectomy results in varying degrees of atrophy of the interrenal tissue, where the cells collapse and disintegrate (Chavin and Kovacevic, 1961; Chester-Jones and Mosley, 1980) and there is clear evidence to suggest control by adrenocorticotropic hormone (ACTH) (Rasquin, 1951; Mahon *et al.*, 1962; Hanke and Jones, 1966; Babiker and Rankin, 1978; Chester-Jones and Mosley, 1980).

There are conflicting reports relating interrenal histology with variations in environmental salinity (Henderson and Garland, 1980). Transitory stimulation of the interrenal cells following transfer of eels from FW to SW has been reported, whilst other studies found reduced interrenal cells (Henderson and Garland, 1980). Eels long-term acclimated to SW have shown stimulation of the interrenal cells or no effect (Hanke and Jones, 1966; Hanke *et al.*, 1969). In some species there may even be greater interrenal activity in FW as compared to SW fish (Henderson and Garland, 1980). For more details of the structure and function of the interrenal tissue in teleost fish see Chester-Jones and Mosley (1980) and Henderson and Garland (1980).

Plasma cortisol has been shown to increase upon transfer of FW *A. rostrata* to SW and remains above FW levels for around a week post-transfer (Forrest (Jr.) *et al.*, 1973b). However, once full adaptation has taken place there is no difference in plasma cortisol levels between FW and SW adapted fish (Forrest (Jr.) *et al.*, 1973b; Tierney, 1993; Balm *et al.*, 1995). Metabolic clearance and production rates for cortisol are however elevated in SW as compared to FW eels (Leloup-Hatey, 1974; Nicols and Weisbart, 1985). Moreover, in fish transferred from FW to SW there is an increase in cortisol production rate with a decrease in production following transfer in the opposite direction (Henderson and Garland, 1980).

Administration of cortisol has been shown to enhance hypoosmoregulatory ability and reduce mortality rates post-transfer to SW in a range of species including *A. rostrata* (Epstein *et al.*, 1971; Forrest (Jr.) *et al.*, 1973a) and a number of salmonids (Madsen, 1990a; Madsen, 1990b; Madsen, 1990c; Sakamoto *et al.*, 1993; Fuentes *et al.*, 1996a). Hypophysectomy however results in a reduced ability to osmoregulate in SW

primarily through the loss of the cortisol secretetalogue ACTH (Butler, 1966). Interrenalectomy of SW eels results in reduced osmoregulatory abilities, reflected by very high plasma Na^+ and Cl^- concentrations, which can be reversed with the administration of cortisol (Mayer *et al.*, 1967) .

Cortisol has been shown to stimulate drinking in FW rainbow trout and Atlantic salmon presmolts post-transfer to SW, although there was no stimulatory effect whilst the fish were still in FW (Fuentes *et al.*, 1996a) .

The rate of intestinal fluid uptake is enhanced by cortisol treatment (Cornell *et al.*, 1994), and the rate of ion and water transport is also augmented by administration of ACTH, in a dose dependent manner (Hirano and Utida, 1968). An increase in intestinal ion and water absorption is necessary for the survival of fish transferred from FW to SW. Hypophysectomy of SW eels results in an impairment of osmoregulation (Butler, 1966), with low rates of ion and water absorption characteristic of the FW intestine (Hirano, 1967; Hirano and Utida, 1968). Treatment of hypophysectomized eels with cortisol results in a restoration of the high rates of intestinal ion and water absorption typical of a SW fish (Hirano, 1967).

Changes in intestinal fluid absorption during the parr-smolt transformation in Atlantic salmon have been shown to be positively correlated with changes in plasma cortisol concentration (Veillette *et al.*, 1995). Cortisol has been shown to increase intestinal $Na^+K^+ATPase$ activity (Epstein *et al.*, 1971; Madsen, 1990c; Cornell *et al.*, 1994; Veillette and Young, 1997), and there may also be an increase in osmotic permeability (Ando, 1974). The site of cortisol action has not been elucidated (Collie and Hirano, 1987), however there is evidence to suggest that cortisol may enhance the sensitivity of the eel intestine to adrenaline by increasing catecholamine receptor expression (Ando and Hara, 1994).

Cortisol has been shown to stimulate branchial $Na^+K^+ATPase$ activity in a number of species (Pickford *et al.*, 1970b; Epstein *et al.*, 1971; Forrest (Jr.) *et al.*, 1973a; Madsen, 1990a; Cornell *et al.*, 1994). In addition to an effect on branchial $Na^+K^+ATPase$ activity, cortisol has been shown to affect the structure and development of chloride cells resulting in an increased number (Foskett *et al.*, 1981; Madsen, 1990c) and size of chloride cells (Madsen, 1990c; Bindon *et al.*, 1994). Stimulation of gill $Na^+K^+ATPase$ and maintenance of SW type chloride cell morphology and size by cortisol has also been detected *in vitro* (McCormick and Bern, 1989; McCormick, 1990a). However, it is thought that other factors may also be involved in stimulating chloride cell proliferation *in vivo* (McCormick, 1995). Specific cortisol receptors have been located in the gills of a few species of teleost including *A. rostrata* (Sandor *et al.*, 1984; Chakrabotri *et al.*, 1987).

Hypophysectomy is accompanied by reduced gill $Na^+K^+ATPase$ activities which can be restored by cortisol treatment, in a number of species including *A. rostrata* (Butler and Carmichael, 1972). ACTH has been shown to stimulate branchial $Na^+K^+ATPase$ activities in FW juvenile Atlantic salmon (Lacy and Reale, 1995), however treatment with cortisol showed no such effect (Lacy and Reale, 1995). A lack of effect of cortisol treatment on branchial $Na^+K^+ATPase$ activity has also been noted for coastal cutthroat trout (*Oncorhynchus clarki clarki*) parr (Morgan and Iwama, 1996).

Treatment of *G. mirabilis* with cortisol has been shown to elicit an increase in Na^+ and water absorption in the bladder which can be antagonized by prolactin (Doneen and Bern, 1974; Doneen, 1976).

Cortisol has been shown to inhibit prolactin release and alter thyroid hormone levels in salmonids during smoltification (Redding *et al.*, 1986). Factors controlling the release of cortisol are complex. In addition to ACTH, cortisol secretion by the interrenal tissue of both FW and SW fish has been shown to be stimulated by ANP (Kloas *et al.*, 1994) and ANG II, UI and UII (Arnold-Reed and Balment, 1994). AVT and catecholamines may also modify cortisol secretion *in vitro* (Schreck *et al.*, 1989; Hazon and Balment, 1998).

SW adaptation of *O. mossambicus* results in stimulation of the interrenal cells where they undergo hyperplasia and release more cortisol (Balm *et al.*, 1995). Plasma ACTH is similar in both FW and SW adapted tilapia and sensitivity of the interrenal to ACTH is the same (Balm *et al.*, 1995). This suggests that cortisol production occurs at a greater rate in SW as compared to FW adapted tilapia due to the hyperplasia and hypertrophy of the interrenal tissue which accompanies SW adaptation (Balm *et al.*, 1995).

The role of cortisol in FW adaptation has been less well studied. However, cortisol may also play a role in the adaptation of SW fish transferred to FW (Perry *et al.*, 1992; Laurent and Dunel-Erb, 1994). Laurent and Perry (1990) have observed that cortisol treatment increased the influx of Na^+ and Cl^- ions, and increased chloride cell number and chloride cell surface area exposed to the external environment in FW rainbow trout (see page 21). Similar results have been obtained for other species including *A. anguilla* (Perry *et al.*, 1992). Cortisol has also been shown to increase gill $H^+ATPase$, the proton pump implicated by some studies in ion uptake in FW (Lin and Randall, 1993).

1.3.3 Growth Hormone

Growth hormone (GH) is produced by the pars distalis of the pituitary. There is little evidence to support a role for GH in ion and water homeostasis in eels, and the function of GH in the eel is thought to be in regulating growth (Marchelidon *et al.*, 1996). GH receptors in the eel are located mainly in the liver, and there are no specific binding sites in gill tissue (Hirano, 1991). In addition, neither plasma GH concentrations nor metabolic clearance rates were found to alter with the transfer of *A. japonica* from FW to SW (Kishida and Hirano, 1988; Duan and Hirano, 1991).

However, in addition to its action in growth promotion, GH has been implicated in osmoregulation of salmonids, where it increases salinity tolerance, see Sakamoto *et al.* (1993) for a review. A role for GH in adaptation to the hyperosmotic environment in salmonids is suggested by the fact that increases in plasma GH concentration are concomitant with fish undergoing the parr-smolt transformation (Prunet *et al.*, 1989). In addition, plasma GH and metabolic clearance rates increase upon transfer from FW to SW (Prunet *et al.*, 1989; Madsen, 1990b; Madsen, 1990c; Rand-Weaver and Swanson, 1993; Seddiki *et al.*, 1995; McCormick, 1996). GH receptors have been identified in the gill, gut and kidney of rainbow trout as well as the liver (Sakamoto and Hirano, 1991). GH has been shown to enhance SW adaptation in juvenile *O. niloticus*, similar to its effects on salmonids (Xu *et al.*, 1997). However, Auperin *et al.* (1995) found no evidence for an osmoregulatory role for GH in the same species.

Administration of GH has been shown to stimulate drinking in FW rainbow trout and Atlantic salmon post-transfer to SW, although (as with cortisol see section 1.3.2) there was no stimulatory effect whilst the fish were still in FW (Fuentes and Eddy unpublished data cited (Fuentes and Eddy, 1997).

Administration of GH has been shown to increase branchial $Na^+K^+ATPase$ activity (Madsen, 1990c; Madsen, 1990a; Sakamoto *et al.*, 1993; Nonnotte and Boeuf, 1995; Seddiki *et al.*, 1995; McCormick, 1996), and also to increase the size and number of chloride cells (Madsen, 1990c; Madsen, 1990a; Bindon *et al.*, 1994) in salmonids. The method of action of GH may be direct via receptors in the target organs including the gill, or may be indirect via insulin-like growth factors (IGF), particularly IGF-I especially in the short term (McCormick *et al.*, 1991; McCormick, 1996). GH may also act by stimulating the interrenal tissue or by increasing its sensitivity to ACTH in order to increase cortisol production (Young, 1988). However, it must be noted that no effect of GH treatment on gill $Na^+K^+ATPase$ activity was found in post-smolt Atlantic salmon (Cornell *et al.*, 1994). In tilapia, GH has been shown to increase the numbers of mitochondria and promote the extension of the tubular system of branchial chloride cells (Nonnotte and Boeuf, 1995).

GH appears to act in synergy with cortisol in promoting enhanced hypoosmoregulatory ability of a number of salmonids, by increasing $Na^+K^+ATPase$ activity and the size and number of chloride cells (Madsen, 1990b; Madsen, 1990c; Bindon et al., 1994; McCormick, 1996). This may, in part, be due to an increase in gill corticosteroid receptor abundance (Shrimpton et al., 1995).

GH receptors have been identified in the kidney of salmonids and kidney IGF-I mRNA has been shown to increase with increases in plasma GH (Sakamoto et al., 1993). However, the mechanism of action of the GH/IGF-I axis in the kidney has yet to be investigated.

1.3.4 Prolactin

Prolactin is produced by the pars distalis of the pituitary or hypophysis and is considered to be the primary FW adapting hormone. Plasma prolactin levels decrease upon transfer of FW adapted euryhaline teleosts to hyperosmotic environments and increase with transfer in the opposite direction (Suzuki and Hirano, 1991; Ayson et al., 1993; Sheperd et al., 1997). Increased ability of fish to osmoregulate in high salinity water tends to be associated with low plasma levels of prolactin and plasma prolactin concentration decreases during smoltification (Prunet et al., 1989). Generally hypophysectomy of FW teleosts results in ion depletion and death unless prolactin is administered (Pickford and Phillips, 1959; Pickford et al., 1970b). However, it should be noted that both A. anguilla (Olivereau and Chartier-Barduc, 1966) and O. mykiss (Komourdjian and Idler, 1977; Bjørnsson and Hansson, 1983) can survive in FW after hypophysectomy without prolactin replacement injections. Hypophysectomized FW O. niloticus survive better than sham operated fish when transferred to brackish water (Auperin et al., 1995) which confirms that a reduction in prolactin secretion assists adaptation of this moderately euryhaline species to brackish water.

Two different molecular forms of prolactin have been identified in teleost fish (Specker et al., 1985; Suzuki et al., 1991; Auperin et al., 1994). In tilapia there is only 69% sequence identity between the two forms, and they are denoted $tiPRL_{177}$ and $tiPRL_{188}$ as they contain 177 and 188 amino acid residues respectively (Specker et al., 1985). These two forms of prolactin have been shown to have different osmoregulatory functions in tilapia O. niloticus, with only $tiPRL_{188}$ having a clear dose-dependent ion retaining effect (Auperin et al., 1994). However, only one prolactin receptor has so far been described (Prunet and Auperin, 1994; Prunet et al., 1996).

Prolactin has been shown to stimulate the production of mucus in a number of species including A. anguilla, however this does not fully explain its ion retaining effects (Marshall, 1978).

The effect of prolactin upon ion and water transport processes has been found to show considerable variation between species. However it is generally thought to be important for FW adaptation as it reduces Na^+ and Cl^- absorption, and decreases water permeability (and hence reduces solute-linked water transport) in the intestine of *A. japonica* (Utida *et al.*, 1972), *O. mossambicus* (Mainoya, 1982) and *O. mykiss* (Morley *et al.*, 1981). Utida *et al.* (1972) found that administration of prolactin to SW *A. japonica* resulted in a decrease in ion and water absorption by the intestine to a level between that observed for FW and SW adapted eels. Injection of prolactin to FW eels had no effect due to the high levels of endogenous prolactin (Hirano and Utida, 1968). The mode of action of prolactin on intestinal transport has not been identified as yet. It may be via alteration in $Na^+K^+ATPase$ activity or by decreasing permeability to ions and water (Collie and Hirano, 1987).

Prolactin acts as the main FW adapting hormone by reducing passive ion and water permeabilities and active and passive ion secretion at the gill (Dharmamba and Maetz, 1976). These changes are thought to occur by the differentiation of the β chloride cells and the modification or dedifferentiation of the α chloride cells (Foskett *et al.*, 1983; Zadunaisky, 1984; Pisam *et al.*, 1993). Pisam *et al.* (1993) found that injection of prolactin to *O. niloticus* caused the appearance of the β chloride cells that are characteristic of FW adapted fish, and that the remaining α cells shrunk and had reduced tubular systems and shallower apical crypts.

Administration of prolactin to SW fish results in increases in plasma ion concentrations and decreased gill Na^+ efflux rates (Foskett *et al.*, 1983), and decreased branchial $Na^+K^+ATPase$ activities (Pickford *et al.*, 1970a). Prolactin results in a reduction in $Na^+K^+ATPase$ activity in FW rainbow trout and appears to antagonize the effects of GH (Madsen and Bern, 1992), although an increase in $Na^+K^+ATPase$ activity and no antagonism of the actions of GH was found for Atlantic salmon (Boeuf *et al.*, 1994).

Prolactin receptors have been identified in the gill (Prunet and Auperin, 1994; Prunet *et al.*, 1996) although the action of prolactin on these receptors and their regulation remains to be investigated. Confusion over the function of prolactin may be due to the use of heterologous hormones, or be caused by differences in developmental stage or indeed species.

Administration of prolactin to SW teleosts has been shown to reduce the permeability of the urinary bladder to water reabsorption (Johnson *et al.*, 1974). The effects of prolactin on Na^+ reabsorption however have been variable, with some authors reporting increases (Johnson *et al.*, 1974) and some reporting little or no change (Owens *et al.*, 1977), such that there may be considerable species differences (Bentley, 1987). Urinary bladder $Na^+K^+ATPase$ activity is stimulated by prolactin treatment of SW adapted *P. stellatus* (Utida *et al.*, 1974).

1.3.5 Somatolactin

Somatolactin is the third member of the GH/prolactin family of hormones and is synthesized in the pars intermedia of the pituitary (Ono *et al.*, 1990). Its physiological function has yet to be determined. It may be a multifunctional hormone, as there is circumstantial evidence to suggest a role in a diversity of biological processes including acid-base regulation (Kakizawa *et al.*, 1996), sexual maturation (Rand-Weaver and Swanson, 1993) and energy mobilization (Kakizawa *et al.*, 1995). Increases in plasma somatolactin levels are concomitant with smoltification and sexual maturation (Rand-Weaver and Swanson, 1993). However, there were no differences in plasma somatolactin levels in immature coho salmon reared in FW and SW, neither were there differences in the increase in somatolactin observed during sexual maturation in fish inhabiting FW or SW (Rand-Weaver and Swanson, 1993). In addition, another study found no difference in plasma somatolactin levels of red drum (*Sciaenops ocellatus*) post-transfer from SW to FW or distilled water (Zhu and Thomas, 1997). These findings suggest that somatolactin may not be important, at least in these species, for adaptation to water of different salinities.

1.3.6 Arginine Vasotocin

In teleost fish AVT is produced by the neurohypophysis of the pituitary (Acher, 1996). There are no consistent differences in plasma AVT levels between FW and SW stenohaline teleosts nor between euryhaline teleosts adapted to either FW or SW (Warne *et al.*, 1994; Brown and Balment, 1997; Harding *et al.*, 1997). However, plasma concentrations of AVT show a transient increase during the initial phase of acclimation when FW eels are transferred to SW (Balment *et al.*, 1993). This transient increase is associated with a similar increase in plasma ANG II levels (Balment *et al.*, 1993), although there is no evidence to suggest a stimulatory effect of ANG II on AVT secretion (Warne *et al.*, 1995). Warne and Balment (1995) found that plasma AVT was elevated when plasma osmolality was raised in SW flounder using hyperosmotic stimuli, thus suggesting that AVT secretion may be sensitive to changes in plasma tonicity. However, plasma AVT was not elevated in flounder transferred from FW to SW suggesting that AVT secretion in response to osmotic stimuli may only be expressed once plasma tonicity has exceeded a specific threshold level (Harding *et al.*, 1997). Plasma Na^+ and osmolality was also found to be significantly correlated with circulating plasma AVT concentrations in SW adapted flounder (Warne and Balment, 1995; Harding *et al.*, 1997), although such a positive relationship was not found in FW adapted fish (Balment *et al.*, 1993; Harding *et al.*, 1997). The volume status of the fish appears to modulate the sensitivity of AVT secretion to such osmotic stimuli (Harding *et al.*, 1997).

The presence of an AVT receptor has been demonstrated in fish gills (Guibbolini et al., 1988) and was designated a new type (NH_F) given its responses to the V_1 and V_2 type receptor agonists and antagonists (Guibbolini and Lahlou, 1990). The physiological function of this NH_F receptor is as yet unidentified, although AVT has been shown to effect branchial vasoconstriction (Bennett and Rankin, 1986; Warne and Balment, 1997). Branchial vasoconstriction occurs with doses of AVT higher than those considered physiologically relevant, however the gills would be exposed to concentrations higher than expected in the general circulation, due to their proximity to the pituitary (Warne and Balment, 1997).

Complications due to effects on blood pressure have also been encountered in studies on AVT and renal function. Low doses of AVT were found to be antidiuretic, whilst higher doses were diuretic in *A. anguilla* (Henderson and Wales, 1974). Later studies investigated the circulating levels of AVT in the plasma of a number of species (Warne *et al.*, 1994) and it was calculated that only the low doses of AVT and their antidiuretic action could be considered of physiological relevance (Balment *et al.*, 1993). The higher doses elicit a pressor action and the resulting diuresis can be seen only as a pharmacological effect (Balment *et al.*, 1993). The antidiuretic effects of AVT also occur when given in high dose but the pressor effect and concomitant diuresis masks this. If constant pressure is maintained, thus precluding the pressor effect of AVT, antidiuresis is caused even if administered at very high doses (Pang *et al.*, 1983).

Using the perfused trout kidney preparation to avoid vascular effects, AVT administered at physiologically relevant doses was found to elicit potent antidiuresis, mainly through reduced SNGFR together with a reduction in the populations of filtering nephrons (Amer and Brown, 1995). Administration of physiological doses of AVT to FW trout exert an antidiuretic action and it is thought that decreases in AVT secretion *in vivo* would increase the populations of filtering nephrons and hence increase urine flow if required (Brown and Balment, 1997).

As AVT is vasoconstrictive it is thought to be involved in the regulation of blood pressure and regional blood flow distribution (Warne and Balment, 1997). Decreases in blood pressure induced by papaverine, a smooth muscle relaxant, are associated with increases in plasma AVT concentrations, Warne and Balment unpublished cited (Brown and Balment, 1997). The antidiuretic action of AVT results from vasoconstriction to reduce GFR (Brown and Balment, 1997).

V_1 type receptors are thought to be present in the teleost kidney (Pang *et al.*, 1983; Mahlmann *et al.*, 1994). In addition, V_2 type receptors, previously thought to have evolved with the movement of tetrapods to land (Pang *et al.*, 1983), have also been demonstrated in trout kidney nephron segments (Sainsbury and Balment, 1991; Perrott *et al.*, 1993; Sainsbury *et al.*, 1993). There is evidence to suggest that these V_2 type receptors are located in the distal segment of the tubule (Harding and Balment, 1995).

Dispersed nephrons from 50% SW adapted trout were found to be less sensitive to AVT than trout from FW (Harding and Balment, 1995). It may be that down regulation of receptors accounts for changes between FW and SW fish as opposed to changes in plasma hormone concentrations (Harding and Balment, 1995) .

There is also evidence to suggest that AVT may also be involved in further modification of the urine at the urinary bladder, Harding and Balment unpublished observations cited (Brown and Balment, 1997).

1.3.7 Natriuretic Peptides

ANP is just one of a family of natriuretic peptides of similar structure existing in teleost fish (Takei and Balment, 1993). Three natriuretic peptides have been found in teleost fish (Takei and Balment, 1993): ANP from fish atria (Takei *et al.*, 1989), ventricular natriuretic peptide (VNP) from ventricles (Takei *et al.*, 1991) and C-type natriuretic peptide (CNP) from brain (Takei *et al.*, 1990). B-type natriuretic (BNP) the fourth natriuretic peptide, not a brain peptide but a cardiac peptide circulating in the blood, has yet to be discovered in eel as yet (Takei, 1993). VNP is secreted from both the atria and ventricles of eels (Takei and Balment, 1993). There is also evidence to suggest local synthesis of ANP and VNP in the intestine (Loretz *et al.*, 1997).

Early studies have shown that plasma ANP levels were elevated in SW as compared to FW adapted fish and vice versa (Evans, 1990; Smith *et al.*, 1991; Takei and Balment, 1993), however the assays employed in these studies were not very specific to each of the natriuretic peptides (Takei *et al.*, 1992). More recent experiments employing homologous peptides and more specific radioimmunoassay techniques have found that plasma ANP and VNP levels are not significantly different between *A. japonica* adapted to FW and SW, despite the fact that plasma Na^+ and osmolality is increased (Kaiya and Takei, 1996a).

Plasma ANP and VNP concentrations however have been noted to increase in ventral aortic blood after six hours post-transfer from FW to SW (Kaiya and Takei, 1996b). These elevated levels were not maintained and gradually returned to FW levels despite plasma Na^+ and osmolality remaining elevated (Kaiya and Takei, 1996b). Specific ANP binding sites have been identified in *A. japonica* gill by autoradiography, especially in the secondary lamellae on the side of the efferent filamental artery (Sakaguchi *et al.*, 1996). These ANP binding sites showed a tendency for down-regulation in SW as compared to FW adapted eel gills (Sakaguchi *et al.*, 1996). With transfer of SW adapted eels to FW however, there were no changes in plasma ANP or VNP concentrations despite the fact that plasma Na^+ and osmolality had decreased (Kaiya and Takei, 1996b). Plasma ANP and VNP have been shown to increase following an

acute increase in plasma osmolality after in the injection of hypertonic solutions (Kaiya and Takei, 1996c), such that elevated ANP secretion is primarily brought about via osmotic stimulation. Transfer of SW adapted eels from SW to FW would result in volume loading and not osmotic stimulation (Kaiya and Takei, 1996b).

ANP has been shown to inhibit basal drinking rates in *A. japonica* (Takei and Balment, 1993) and *A. anguilla* (Balment *et al.*, 1995), and VNP has also been shown to inhibit drinking in *A. japonica* (Takei and Balment, 1993). In *A. anguilla* administration of ANP together with angiotensins results in an attenuated dipsogenic response, suggesting that ANP may act to antagonise the dipsogenic action of the RAS (Balment *et al.*, 1995).

ANP reduces ion and water transport across the intestine of SW adapted fish (O'Grady *et al.*, 1985; Ando *et al.*, 1992; Takei and Balment, 1993; Loretz, 1995a; Loretz, 1997) and this is thought to occur by the inhibition of $Na^+K^+2Cl^-$ cotransport (O'Grady *et al.*, 1985). This inhibition is thought to be mediated by cyclic guanosine monophosphate (cGMP) which has been shown consistently to inhibit intestinal transport (Halm *et al.*, 1985b; O'Grady *et al.*, 1985; Ando *et al.*, 1992). See Collie and Hirano (1987) for a review of the role of cyclic nucleotides in intestinal transport regulation. Inhibition by ANP is more potent than that by acetylcholine, serotonin and histamine (Mori and Ando, 1991; Ando *et al.*, 1992). For a recent review of the role of ANP in the regulation of intestinal transport in vertebrates see Loretz (1995a).

ANP has been shown to stimulate cortisol secretion *in vivo* in flounder, and also to stimulate interrenal tissue in SW but not FW adapted rainbow trout *in vitro* (Arnold-Reed and Balment, 1991). This contrasts with a possible role for ANP in FW acclimation by promoting salt and water absorption by the intestine, as do reports that ANP has been shown to stimulate Cl^- secretion by the gill (Scheide and Zadunaisky, 1988; Arnold-Reed and Balment, 1991).

ANP was originally reported to be diuretic and natriuretic in rainbow trout and toadfish, however these studies had employed heterologous peptides (Duff and Olson, 1986; Lee and Malvin, 1987). Homologous ANP and VNP have since been found to be antidiuretic in *A. japonica*, whilst natriuresis was unaffected (Balment and Takei, 1993; Takei and Balment, 1993).

1.3.8 Catecholamines

Catecholamines are secreted by the chromaffin cells of the head kidney. There are four types of catecholamine receptors and the response of the target organ depends upon the receptor type present and the nature of the catecholamine-receptor complex.

Each receptor type has differential responses to each of the catecholamines. Norepinephrine (noradrenaline) is a potent stimulator of α and $\beta 1$ type receptors with only a weak effect on $\beta 2$ receptors. Epinephrine (adrenaline) on the other hand has a strong effect on $\beta 1$ and $\beta 2$ type receptors but is less effective for α receptors. See Randall and Perry (1992) for details of catecholamine production, release and actions.

Mori and Ando (1991) have shown that acetylcholine and serotonin reduce ion and water absorption in the gut of SW adapted *A. japonica*. Catecholamines such as noradrenaline have been shown to antagonize these effects of acetylcholine and serotonin (Ando and Kondo, 1993), and are thought to be important in maintaining high levels of ion and water absorption across the gut essential for survival in the marine environment. Of the catecholamines found to stimulate ion and water transport in SW eel intestine, adrenaline was found to be the most potent, followed by noradrenaline (Ando and Omura, 1993). Dopamine was less potent (Ando and Omura, 1993). Comparisons of potency of stimulation and the effects of various antagonists suggest the presence of $\alpha 2$-receptors in the SW eel intestine (Ando and Omura, 1993).

Chloride secretion across the gills is antagonistically regulated by catecholamines (Mendelson *et al.*, 1981; Mayer-Gostan *et al.*, 1987). Catecholamines affect blood flow through the gills and this will also affect ion transport (Mayer-Gostan *et al.*, 1987). Chloride secretion by the gill has been reported to be stimulated by β adrenergic mechanisms, mediated by an increase in cAMP (Mendelson *et al.*, 1981). Inhibition of chloride secretion is accomplished via α adrenergic mechanisms and does not involve alterations in cAMP or cGMP levels (Mendelson *et al.*, 1981), or Ca^{2+} mobilization (May and Degnan, 1985). Epinephrine inhibits chloride secretion at the gills via α receptors (Foskett *et al.*, 1982).

1.3.9 Other Hormones

Little is known about the function of VIP in intestinal transport in teleosts. VIP has been shown to inhibit both ion and water transport in the gut of FW adapted tilapia *O. mossambicus* (Mainoya and Bern, 1984). This inhibition may be via elevations of intracellular cAMP concentration (Mainoya and Bern, 1984), although cAMP has been shown to have a stimulatory effect on intestinal transport processes in some teleosts, see Collie and Hirano (1987) for a review. Further work on VIP in other species is required, especially since the tilapia gut is unusual in that during SW adaptation intestinal ion and water transport actually decreases (Mainoya, 1982).

VIP has been shown to stimulate Cl⁻ secretion in opercular epithelium of SW *Oreochromis mossambicus*, as does glucagon (Foskett *et al.*, 1982). Receptors for VIP

have been identified in the gut, gill and kidney (Chow, 1997), suggesting a role in osmoregulation.

UII has been shown to stimulate ion transport across the intestine of the goby *G. mirabilis* when adapted to 5% SW but not 100% SW (Loretz *et al.*, 1983; Loretz *et al.*, 1985). This increase in ion transport is thought to act via stimulation of the apical Na^+ and Cl^- coupled absorption mechanism (Loretz *et al.*, 1985).

Urotensin I (UI) stimulates ion transport by the chloride cells of the *G. mirabilis* gill, whilst UII inhibits Cl^- extrusion (Marshall and Bern, 1979; Marshall and Bern, 1981). For a review of the effects of urotensins see Larson and Bern (1987).

The caudal neurosecretory system is located proximal to the kidney and has a direct blood drainage (via the renal portal vein) to the kidney from the urophysis. The kidney is thought to be a direct target for the actions of UII, as intracellular cAMP production is stimulated by UII (Stenhouse and Balment, 1995). The bladder has also been reported as a target tissue for urotensins where both peptides have been shown to stimulate active Na^+ absorption, although UII is greatly more potent (Loretz and Bern, 1981).

Thyroid hormones, tri-iodothyronine (T_3) and thyroxine (T_4) are primarily involved in the regulation of growth, development and metabolic rate in teleost fish. There is controversy over their role in osmoregulatory processes. Some studies have found stimulatory effects of T_4 on $Na^+K^+ATPase$ activity, chloride cell proliferation and hypoosmoregulatory capability, whilst others have found no such effects, see McCormick (1995) for a review. However, there is more agreement that thyroid hormones may interact with other hormone systems such as GH and cortisol to increase $Na^+K^+ATPase$ activity and promote survival in the hyperosmotic environment (McCormick, 1995). There are increases in both T_3 and T_4 associated with the parr-smolt transformation salmonids (Prunet *et al.*, 1989). Thyroid hormone receptors have been identified in branchial tissue of *A. anguilla* and *Salmo trutta trutta* (Lebel and Leloup, 1989) but the effect of salinity on these receptors has not been investigated.

1.4 The Life History of the Eel

As a catadromous fish, *A. anguilla* spawns at sea and then migrates into FW to grow and mature before returning again to the sea to breed. The spawning grounds of *A. anguilla* were identified earlier this century from indirect evidence provided by catches of larvae in the Atlantic Ocean (Schmidt, 1922). Schmidt studied the geographical distribution of these larval catches, the size of the larvae and when they were caught. Together with information on the ocean currents of the North Atlantic he extrapolated that the spawning grounds were in the south-western Sargasso Sea (23-30°N; 48-74°W), and

that *A. anguilla* spawned in spring or early summer. He also suggested the Sargasso to be the spawning grounds of *A. rostrata*, although the two species were separated somewhat in time and in the exact area of spawning. More recently the spawning grounds of *A. japonica* were identified as being in the area of the North Equatorial Current between the Philippine and Mariana Islands (approx. 15°N; 140°E) in a similar way (Tsukamoto, 1992). *A. japonica* is currently thought to spawn in summer and take around seven months to migrate to the coast of Japan, and, as in *A. anguilla*, major ocean currents serve to transport the larvae back to the coasts from where the adults originated (Tsukamoto, 1990; Kimura *et al.*, 1994). However, these hypothesized spawning grounds cannot be proven until spawning adults and eggs are found. As the northern limit of the Sargasso is delimited by a thermal front it is thought that this may be involved in triggering spawning in *A. anguilla* (Westin, 1990). However, it is a salinity front that delimits the spawning area for *A. japonica* in the Pacific Ocean, suggesting that the physical trigger for spawning may differ between the two species (Tsukamoto, 1990; Tsukamoto, 1992).

Until 1896 the glass eel was earliest stage of the eel known (Grassi, 1896). The larvae of *A. anguilla*, called leptocephali, which hatch in the Sargasso sea had in fact been previously believed to be a separate species *Leptocephalus brevirostris* (Kaup, 1856). When they hatch, the larvae are around 2.2 mm (Lecomte-Finiger, 1994) and they then begin their migration towards the European continental shelf (Schmidt, 1922). Schmidt hypothesized that this transatlantic migration took between two and three years. However, a more recent reinterpretation of Schmidt's original data suggests that they may travel for as little as eleven months (Boëtius and Harding, 1985). Recent evidence from studies of otolith microstructure in glass eels caught around the western coasts of Morocco and Europe confirm that larvae spend less than one year in the open ocean (Lecomte-Finiger, 1992). Events throughout the glass eels' larval development are recorded on otoliths through changes in calcium mineralization as they grow, and growth increments are generally recorded daily (Pannella, 1971). The larvae are though to commence feeding four to five days post-hatching (Prokhorchik, 1986), before which they were nourished by the yolk. Although the leptocephali have large mouths and big teeth their source of nutrition during the transatlantic migration is unknown.

It was believed that the larvae simply drifted with the gulf stream to reach their destination, however, analysis of oceanographic data suggests that for water masses to be translated from the Western Atlantic to the east takes around one year. As the duration of larval migration as determined by (Lecomte-Finiger, 1992) was up to 280 days it is generally thought that they probably also swim actively (Lecomte-Finiger, 1992). It must be noted however that very recently this hypothesis was refuted by McCleave *et al.* (1998) who propose that the larvae do indeed simply drift passively to achieve transport from the spawning site to the coasts of Europe.

The leptocephali metamorphose into glass eels before entering continental shelf waters and when fully grown the leptocephali are about 7.5 cm in length (Lecomte-Finiger, 1992). During the process of metamorphosis into glass eels the eels reduce in length by about 1 cm, and also lose around three-quarters of their body weight (Schmidt, 1922). In Lecomte-Finiger's (1992) study, the duration of metamorphosis was found to range from 33 to 69 days depending upon latitude, with the more northern fish taking longer. During metamorphosis the fish undergo a period of starvation, and as well as the body taking on an eel-like shape, the digestive system undergoes reorganization and the teeth take on the adult form.

The definitions of the various early life stages of the eel are largely based upon changes in pigmentation, see Elie *et al.* (1982) for a detailed description. Essentially the leptocephalus is willow leaf-like and transparent and moves without producing any turbulence in the water (Fig. 1.8a). The glass eel is eel-like in form, it swims in an snake-like fashion and has no external pigmentation except for the caudal spot (Fig. 1.8b). In the elver, pigmentation has developed in the dorsal and ventral regions (Tesch, 1977). There is no change in body form between glass eel and elver, although the length of the fish usually decreases slightly by about 0.5 cm (Tesch, 1977; Lecomte-Finiger, 1992).

Freshwater life is generally considered to begin as the glass eel changes into an elver (Lecomte-Finiger, 1992) and there is a change in the otolith microstructure at this stage (Michaud *et al.*, 1988; Martin, 1995). Glass eels congregate around the coasts of the UK in winter before entering the estuaries in the late winter or early spring. In the Severn Estuary, where the glass eels for this study were sourced, the first catches of glass eels usually occur during late February or early March. Once they start eating and become pigmented the fish are generally thought to remain in FW. However, there is some evidence that a number of elvers stay in coastal waters around the estuaries for a second or even subsequent years (Krough, 1939; Tesch, 1977).

The timing and direction of the FW-ward migration is thought to be determined by a number of factors: temperature, salinity, tidal state and olfactory cues (Creutzberg, 1958; Creutzberg, 1959; Tongiorgi *et al.*, 1986; Tosi *et al.*, 1988; Tosi *et al.*, 1990; Martin, 1995). The dominance of a particular cue may change throughout the migration season and with latitude (Martin, 1995). Glass eels have been reported to ascend during both day and night. The glass eels migrate near the water surface at low tide and they tend to 'hug' the bank as they move upstream (Tesch, 1977). Glass eels are also known to be carried upstream by the flood current with the largest migrations occurring during spring tides (Tesch, 1977).

As they become elvers the eels become pigmented by the recruitment of black melanophores. As they feed and grow further a yellow-green pigment is deposited and they are termed yellow eels (Tesch, 1977). An adult eel is shown in figure 1.8c. The extent of upstream migration during the first year is extremely variable (Michaud *et al.*,

Fig. 1.8a: *A. anguilla* Leptocephalus Larvae

A fully grown leptocephalus is approximately 7.5 cm in length.

Fig. 1.8b: *A. anguilla* Glass Eels

Glass eels are approximately 6.5 cm long.

Fig. 1.8c: Adult *A. anguilla*

Adult eels may reach 1 m in length when fully grown.
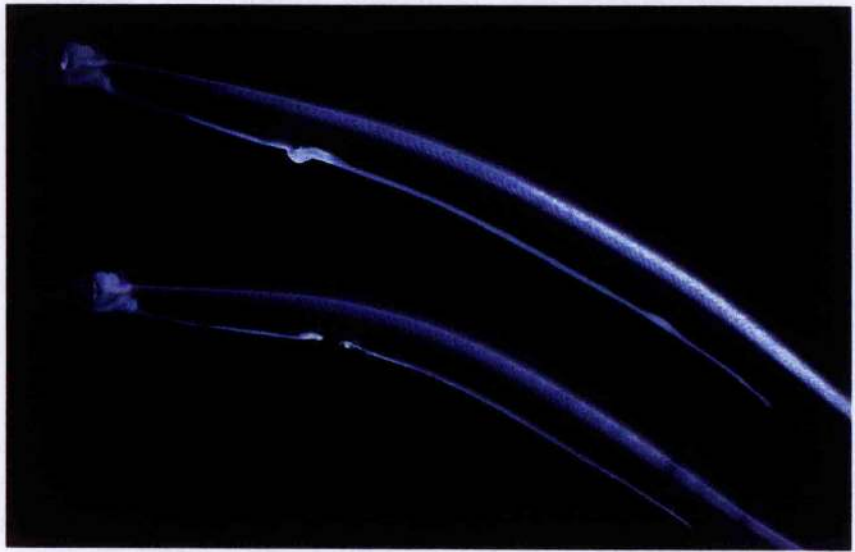
1988; Naismith and Knights, 1988; Lecomte-Finiger, 1992; Martin, 1995), and the migration upstream initiated by glass eels and elvers may be continued for several years afterwards by yellow eels (Naismith and Knights, 1988; Haro and Krueger, 1991). Yellow eels stay in FW, feeding and growing for between three and thirty years, generally longer for females (Tesch, 1977). Female eels normally migrate to SW having attained a greater body length than males, and tend to show a greater growth rate (Russell-Poole and Reynolds, 1996). The larger size of female eels at maturity results not simply through a differential growth rate, but via a combination of more rapid growth and older age (Russell-Poole and Reynolds, 1996).

Yellow eels then change into what are known as silver eels in preparation for their return journey to the Sargasso. The change in outward appearance is small, with a silvering of the skin on the ventral surface, an enlargement of the eyes, alteration in the shape of the head and a change in the size and colour of the pectoral fin. They do not eat and the gut atrophies (Tesch, 1977). Silver eels begin their 5000 km return migration from the FW systems of Europe to the Sargasso in the autumn, a journey thought to take about seven months. However, very few specimens have been caught during this migration, and then only within a few hundred km from the coast (Tesch, 1977). If silver eels are prevented from migrating their sexual development ceases, and this cannot be resumed simply by transfer to SW. There is evidence to suggest that hydrostatic pressure may be a factor involved in triggering further sexual development (Fontaine *et al.*, 1985; Fontaine, 1989).

It has been suggested that silver eels orientate themselves using geomagnetic and olfactory cues, and also by water currents (Tesch, 1977). A study using stocked eels, originally glass eels from France which were transported to the Baltic, found that they failed to migrate out of the Baltic area as adult silver eels, suggesting the importance of an imprinted cue (Westin, 1990).

Eels have been successfully treated to induce sexual maturation and spawning has been induced. Fertilization, embryonic development, hatching and early larval development have been reported for *A. japonica* (Yamamoto and Yamauchi, 1974; Yamauchi *et al.*, 1976; Ohta *et al.*, 1997). However, although the resulting larvae have never survived beyond 17 days (Satoh, 1979), these experiments contributed much to the understanding of the biology of the early larval stages of the eel. Further work is underway with the ultimate aim of establishing a method for the full cultivation of the Japanese eel. It has been assumed that eels die after spawning, however following hormonally induced maturation second and even third maturations have been induced in a number of male *A. anguilla*, complete with feeding and growing intervals between spawning episodes (Dollerup and Graver, 1985). Females have also been induced to spawn by hormone treatment, and although they do not normally survive, one has been reported to survive to spawn again for a second time (Le-Belle and Fontaine, 1987).

A number of authors have suggested that this remarkable lifecycle be tied in with Wegner's theory of continental drift such that the eels have had to travel further with time as the American and European continental plates drifted further and further apart (Tesch, 1977).

1.5 Objectives and Aims

The vast majority of work on teleost osmoregulation has been carried out on a very small fraction of the total number of species, the European eel being one of most extensively studied. However, almost all of these studies pertain to the adult eel and the acclimation to SW associated with the silvering process. The physiology underlying the migration of glass eels into FW however has been neglected almost without exception. At this early stage in the life of the eel, whilst ontogenic processes are still underway, these small fish face a massive challenge to their osmoregulatory systems as they migrate from SW into FW. This migration may be successful only after several attempts, so that they must withstand rapid and repeated alterations in the salinity of their external environment over perhaps only a matter of hours. In addition, the surface area to volume ratio of these small fish is of great magnitude, compounding the effects of this already substantial osmotic challenge.

This study investigates some of the primary physiological responses involved in osmoregulation in glass eels and elvers. It was thought by some that once the elvers had made the transition into FW they became essentially stenohaline FW fish. This study challenges that idea, by looking at some of the fundamental osmoregulatory processes at work in glass eels and elvers when subject to different environmental salinity conditions. The primary objective of this study was to establish whether or not glass eels and elvers retain osmoregulatory plasticity to enable them to adapt to environments of different salinity, post-migration to FW. Secondly, do the eels manage to grow and develop normally whilst retained under these different salinity conditions? Further aims were to investigate the responses of glass eels and elvers when osmotically challenged, and whether they elicited appropriate regulatory responses in terms of drinking and branchial $Na^+K^+ATPase$ activities. As one of the major hormones involved in the control and integration of osmoregulatory processes, cortisol content was also investigated, and the ability of these eels to maintain normal Na^+ and K^+ homeostasis was addressed.

Chapter 2

Materials and Methods

2.1   Fish

Glass eels were caught in brackish water in the Severn Estuary (Western Aquaculture, Cullompton, Devon, UK) during their inland migration in late winter, usually during the last week in February or the first week in March. They were transported to the Gatty Marine Laboratory in 33% seawater. Fish were maintained for five days in through-flowing 33% seawater in order to recover from any transportation stress before being used in any experiments.

Filtered seawater from St Andrews Bay and freshwater from the domestic supply were fed to the aquarium tanks via header tanks. Fish were held in plastic tanks of either 40 l or 20 l capacity where the water was allowed to through-flow at a rate of around 0.6 l/min and 0.3 l/min respectively, therefore there was complete turnover of water within the tanks in approximately one hour. Stocking density was no more than 45 g/l. Light in the aquarium was controlled by a timer giving a 12 h light:12 h dark cycle. Fish were kept in well aerated water.

Fish were held at ambient temperature and experiments were carried out at ambient temperature throughout. The temperatures of the water within the various holding tanks were monitored throughout the year. There was no difference in ambient temperature between SW and FW. A graph illustrating the ambient water temperature within the aquarium throughout a typical year is illustrated in figure 2.1.

Both glass eels and elvers received a maintenance diet of commercially available food (BP Nutrition), which was supplemented with freshly hatched *Artemia* larvae. The pelleted food was crushed with a mortar and pestle prior to feeding to glass eels.

2.1.1   Development

At monthly intervals fish acclimated to FW, 33% SW, 66% SW and SW (see section 2.3.1) were assessed for development by measuring the progression of pigmentation (n = 10). Fish were killed by being transferred to a 1:200 (v/v) dilution of 2-phenoxyethanol in water of the same salinity as the experimental condition. They were then washed, again in water of the same salinity as the experimental condition, before being patted dry on paper towel and laid out on their right-hand side on a glass Petri dish. The Petri dish holding the fish was placed on a light box (J. N. Campbell) below a CCD video camera (Cosmicar/Pentax). Using AnalySIS v 2.0 Image Analysis software, 'snapshots' were taken and the colour values subsequently assessed for each pixel along a transect drawn along the side of the fish. The transect began above the spinal cord level with the posterior end of the heart, and ended where the tail begins to narrow before the tip (Fig. 2.2). Colour was quantified on a scale where 0 = white and 255 = black. The mean value for each individual was noted and then overall means for each experimental group were calculated.

Fig. 2.1:   Temperature of Aquarium Water

Temperature of water within the aquarium ± 1°C, over the course of 1997.
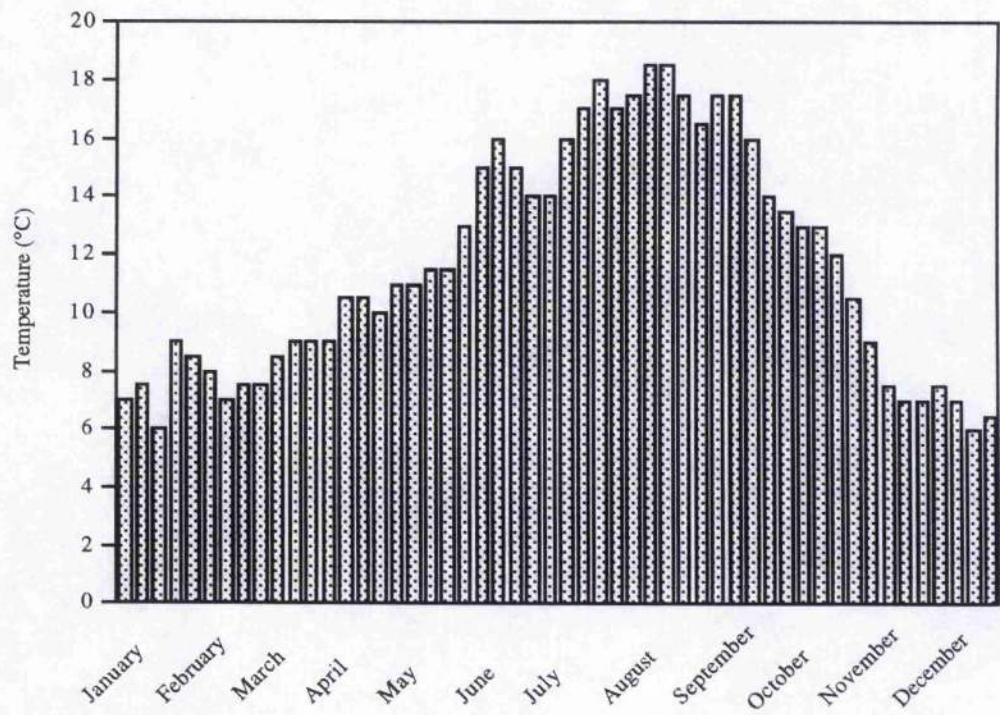
Fig. 2.2:   Transect Used for Determination of Pigmentation

The dashed line represents the transect which was drawn above the spinal cord (solid line) as used for the determination of pigmentation by image analysis. An elver is approximately 7 cm in length.

Transect

Gills

Heart

Spinal cord
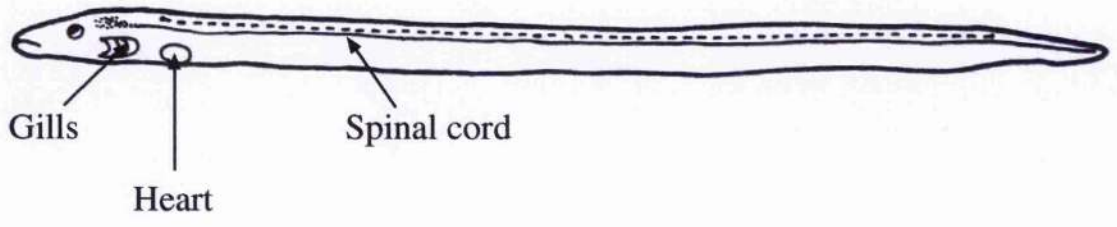
2.2 Chemicals and Equipment

All chemicals were obtained from BDH (BDH Chemicals Ltd., Poole, Dorset, UK) and were of AnalaR® grade unless otherwise stated. Other suppliers of chemicals were Sigma (Sigma Chemical Company, St Louis, USA), Fisons (Fisons Scientific Equipment, Loughborough, Leicestershire, UK) and Pharmacia (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) as specified in the text. All radiochemicals were obtained from Amersham (Amersham Life Science Ltd., Amersham, Berkshire, UK). Cortisol antiserum was obtained from the Scottish Antibody Production Unit (SAPU, Carluke, Lanarkshire, UK).

All solutions and buffers were made using deionized water unless otherwise stated. This was obtained using a MilliQ® Reagent Water System (Millepore (UK) Ltd., Watford, Herefordshire, UK), where water is distilled and filtered, therefore, whenever deionized water is subsequently referred to it will be noted as MilliQ® in the text.

Full details of consumables and equipment, and their suppliers are given in Appendix 1 rather than in the text.

2.3 Experimental Rational

2.3.1 Environmental Acclimation Studies

Once the glass eels had time (5 days) to recover from transportation stress (see section 2.1) they were transferred to through-flowing freshwater (FW), seawater (SW), 33% seawater (33% SW) or 66% sea water (66% SW). After 20 days they were considered ACCLIMATED to these salinities. A series of experiments then commenced at this point termed Month 1, and these experiments were conducted on a monthly basis until the group termed Month 5. Experiments included investigations of drinking rate, branchial $Na^+K^+ATPase$ activity, whole body Na^+ and K^+ ion content, whole body cortisol content and examinations of interrenal cell morphology. During Months 1 and 2 experiments were carried out on fish acclimated to FW, 33% SW, 66% SW and SW. During Months 3, 4 and 5 only FW and SW acclimated fish were studied. It had been planned that fish from each of the four experimental salinities would be studied throughout Months 1 to 5, however an outbreak of disease in both the 33% SW and 66% SW stock tanks prevented this. Samples of moribund fish were transported to the Marine Laboratory in Aberdeen where they were identified by the Fish Pathology Service as harbouring *Trichodina* parasites around the gill lamellae. In addition, the fish were also found to be suffering from *Vibrio* and *Pseudomonas* sp. bacterial infections.

2.3.2 Drinking Rate Challenge Studies

At these same monthly intervals, from Month 1 until Month 5, FW acclimated fish were acutely transferred to SW and their initial drinking rate responses investigated. Equivalent experiments were carried out on fish acutely transferred to FW as a control. These were termed drinking rate CHALLENGE experiments.

2.3.3 FW-SW Transfer Studies

At Month 1 (see section 2.3.1) some FW acclimated fish were acutely transferred to SW, termed FW-SW. An equivalent group of fish was transferred to FW as a control, termed FW-FW. Branchial $Na^+K^+ATPase$ activity, whole body Na^+ and K^+ ion content, whole body cortisol content and interrenal cell morphology were investigated at 3, 6, 9 and 24 hours (h), and at 3, 7, 10, 14 and 21 days (d), and at 2, 3 and 4 months post-transfer. These were termed FW-SW TRANSFER studies.

2.4 Drinking

2.4.1 Evaluation of the method

In this study, drinking rates were estimated by measuring the gut accumulation of the radioactive marker ^{51}Cr-EDTA, using a modification of the technique developed by Hazon et al., (1989) which was in routine use for adult fish to enable it suitable for the much smaller elvers. Sections 2.4.1.1/2/3 detail some validations carried out.

2.4.1.1 Determining the Optimal Specific Activity of the Marker

Drinking rate studies were carried out using ^{51}Cr-EDTA at the following concentrations in the bathing medium: 50 µCi/L; 100 µCi/L; 200 µCi/L and 400 µCi/L. Obviously it was necessary to have sufficient activity to be able to detect drinking over the course of an experiment as significantly different from background counts, whilst being as economical as possible with the radiolabel due to financial constraints. Ten SW acclimated fish per group were placed in ^{51}Cr-EDTA labelled SW for six hours, and were then killed by placing them in a beaker containing a lethal dose of 2-phenoxyethanol, 1:200 (v/v), in water of the same salinity as the experimental condition. The fish were transferred to another beaker to be washed, also in water of the same salinity as the experimental condition. Anaesthetic and wash water was changed, and the beakers rinsed between different experimental groups. The fish were patted dry with tissue before being weighed to three decimal places and placed whole in PONY vials (Canberra Packard Ltd.) and counted using a Minaxi Gamma Counter (Canberra Packard Ltd.). Duplicate 1 ml samples of the labelled water were also counted.

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From the results obtained (see Fig. 3.4 in Chapter 3) 200μCi/L was deemed suitable for use in all further drinking rate investigations.

2.4.1.2 Time Course of Routine Drinking Rate Experiments

A suitable time course for the duration of incubation of fish in the radiolabelled media was determined by placing 70 SW acclimated fish in aerated ^{51}Cr-EDTA containing SW (200μCi/L). Ten fish were removed from the radiolabelled SW at the following times post-transfer: 1 h, 3 h, 4 h, 5 h, 6 h, 9 h and 12 h, and processed as described in section 2.4.1.1. The amount of accumulated water was calculated per individual and then adjusted for the weight of the fish to be expressed as μl/g.

From the resulting graph (Fig. 3.5 in Chapter 3) it was decided that three hours would be the optimum time to obtain a reliable estimation of drinking rate without the risk that counts were being voided via the anus, as this would otherwise result in an erroneously low estimation of drinking rate. As SW acclimated fish generally drink at a higher rate than fish acclimated to lower salinities, three hours was considered suitable for all salinity groups.

In order to determine whether the levelling off of the amount accumulated in SW acclimated fish was due to whole gut equilibration, where an equivalent amount of radioactivity imbibed was lost over the same time period via the anus, or was due to a cessation of drinking, the following experiment was performed. A group of SW acclimated fish (115) were transferred to radiolabelled SW and a sample of 16 individuals removed after 3 hours. The remaining fish were washed briefly in unlabelled SW before being transferred to a further beaker of unlabelled SW. Samples of fish were removed after 1 h (4 h), 2 h (5 h), 3 h (6 h), 6 h (9 h), and 7 h (10 h). The figures in brackets illustrate the time elapsed from the beginning of the experiment.

From the resulting graph (Fig. 3.6 in Chapter 3) it can be concluded that gut equilibration takes place after 3 h post-transfer, such that counts are continually ingested but are also lost via the anus at an equivalent rate. Three hours was then used as the incubation time for routine drinking rate investigations (see section 2.4.2).

2.4.1.3 Surface Contamination by the Marker

The possible problem of contamination of the surface of the fish with the radiomarker was addressed by checking the counts obtained from counting empty vials, vials containing fish direct from their stock tank, and vials containing FW acclimated fish

that had been dipped quickly into labelled FW at the usual activity (200 μCi/L) before being killed, washed and processed as detailed in section 2.4.1.1. As there was no significant difference between these treatments (ANOVA $p > 0.05$), there was no contamination of the surface of the fish to be taken into account when calculating drinking rates (see section 3.2.1.3).

On the basis of the findings of these preliminary studies a protocol for the routine measurement of drinking was established for use in all subsequent experiments, and is detailed in section 2.4.2.

2.4.2   Routine Determination of Drinking Rate

Fish were placed in beakers of aerated water containing 200 μCi/L ^{51}Cr-EDTA and left to swim freely for a given time period. Drinking rates in acclimated fish were investigated by adding 20 fish to 200 ml labelled water for a three hour period. When conducting the drinking rate challenge studies, approximately 96 FW acclimated fish were added to 600 ml labelled SW and then groups of 12 individuals were removed at time intervals of 5 min, 15 min, 30 min, 1 h, 2 h, 3 h, 4 h and 5 h. An identical experiment was carried out on FW acclimated fish acutely transferred to 600 ml labelled FW as a control. Throughout the experiments fish were kept as free from stress as possible, with the experiments taking place in a quiet room and the beakers shaded with foil.

Duplicate 1 ml samples of the radiolabelled bathing water were taken at the end of each drinking rate investigation. Fish were killed, washed and processed as previously described in section 2.4.1.1 and using the following equation drinking rates were calculated, taking into account the weight of each fish, and expressed as μl/g/h.

$$\text{Drinking rate} = \frac{C - b}{(M - b) \times T \times W} \times 1000$$

Where:

C = counts in fish (cpm)

b = background (cpm)

M = mean counts in medium, the labelled water (cpm/ml)

T = time in labelled water (h)

W = weight of fish (g)

In the drinking rate challenge studies the data is quoted as the amount of water accumulated (μl/g) by a given time-point rather than as a rate (μl/g/h), where time (T) is omitted from the calculation.

In addition to determining the volume of water imbibed, the number of fish drinking over the time period, in each group sampled, was also determined. The average background count of the gamma counter was calculated as 46.5 ± 1.10 cpm, where n =

10 (see section 3.2.1.3 in Chaper 3). Fish containing counts of 51 cpm or less were considered not to have been drinking (average background count plus 10% error of counting). Counts of between 52 and 61 cpm inclusive were deemed to indicate that the fish were probably drinking. Fish containing counts of 62 cpm or more were drinking.

2.5 Branchial Na⁺K⁺ATPase Activity

The measurement of ouabain inhibitable Na^+K^+ATPase activity was based upon the method previously described by Esmann (1988). The measurement of protein was based upon that described by Bradford (1976). These methods had been in routine use in the research laboratory working with tissue samples from adult fish. However, modifications were necessary to employ these methods to analyse the small amount of material available from glass eel and elver gills.

2.5.1 Evaluation of the Method

2.5.1.1 Concentration of Ouabain

The concentration of ouabain required to inhibit Na^+K^+ATPase activity in elver gill tissue was determined by carrying out the enzyme assay as described in section 2.5.2.2 using ouabain over a range of concentrations with a pooled sample of elver homogenates prepared as described in section 2.5.2.1.

The resulting dose response curve (Fig. 3.16 in Chapter 3) showed that 0.2 mM ouabain in the assay was sufficient to fully inhibit Na^+K^+ATPase activity.

2.5.1.2 Enzyme Activity Time Course

Again, a pooled sample of elver gill homogenates was used to determine the time course over which the activity of the Na^+K^+ATPase should be measured. The incubations were carried out as described in section 2.5.2.2 except that samples were stopped at various time points from 15 to 105 minutes.

From the resulting time-course graph (Fig. 3.17 in Chapter 3) inorganic phosphate production was found to be linear up to 105 minutes. It was therefore valid to perform the incubation step for 1 hour during all further assays.

2.5.2 Routine Determination of Na+K+ATPase Activity

2.5.2.1 Preparation of Samples

(i) Dissection

Fish were killed and washed as described in section 2.4.1.1 and kept on ice. Gills were dissected out as follows: Each fish was placed ventral surface up, on a bed of paper tissue to hold it steady. A small cut was made through the skin, across the body, behind the heart using microsurgical scissors (John Weiss & Son Ltd.). From here an incision was made through the skin in a posterior-anterior direction until level with the front of the operculum. The fish was then turned onto its right-hand side. Whilst holding the upper jaw with forceps, a No. 11 surgical blade (Swann-Morton® Ltd.) was used to cut around both gills which could then be removed together by pulling the lower jaw downwards. The excised gills and surrounding tissue were placed on a glass Petri dish and the opercular, and any other non-branchial tissue was then teased off using fine forceps and the blunt edge of the scalpel blade. This stage of the procedure was aided using an IROSCOPE binocular microscope (J. N. Campbell) at between x 20 and x 40 magnification.

All the gills (8 gill arches) from five fish (5 pairs gills) were pooled in cold 1.5 ml Safe-Lock microcentrifuge tubes (Eppendorf®) and were frozen in liquid nitrogen before being packed in dry ice prior to storage at -80°C. Gill tissue was not weighed to avoid dehydration. Surgical instruments and Petri dishes were cleaned thoroughly and scalpel blades changed between procedures on fish from different experimental groups.

(ii) Crude Membrane Preparation

Four days after sampling, the microcentrifuge tubes containing the gills were defrosted on ice, and the tissue homogenized in 500 µl homogenization buffer (50 mM Hepes; 1 mM EDTA; 0.1 mM dithiothreitol and 1 mM PMSF; pH 7.4 - see Appendix 2) using 30 double-strokes of a micropestle (Eppendorf®). After homogenization a further 500 µl of homogenization buffer was added, the sample vortex mixed, and then filtered. To minimise loss of volume, the solution was filtered through circles of surgical gauze (The Boots Company plc) four layers thick loaded into the bottom of sterile 2 ml syringes (Terumo Europe NV and Becton Dickinson UK Ltd). The homogenate was dispensed into the syringe using a Pasteur pipette, the plunger inserted and depressed to expel as much homogenate as possible into a clean ice-cold microcentrifuge tube. A new filter-loaded syringe was used for each sample. The filtered homogenate was then centrifuged at 4°C for 45 minutes at 33,000 g/16,000 rpm using a JA18.1 rotor in a J2-MC centrifuge (Beckman Instruments Inc.). After centrifugation the tubes were kept on ice and the

supernatant drawn off using a Pasteur pipette and discarded. Homogenization buffer (500 µl) containing 0.01% (w/v) sodium deoxycholate was added to the pellet which was then resuspended using a further 30 double-strokes of the micropestle.

(iii)   Bradford Protein Assay

The protein concentration of each crude membrane preparation was assessed immediately after preparation using the method described by Bradford (1976). Each sample was diluted with homogenization buffer if required, such that all the samples contained between 0.2 and 0.4 mg/ml protein. They were then stored at -80°C overnight before proceeding with the $Na^+K^+ATPase$ assay the following day.

A series of ten protein standards ranging from 20 µg/ml to 2 mg/ml using 6:4 serial dilutions of bovine serum albumin (BSA) in MilliQ®, were made in RT30 plastic assay tubes (Bibby Sterilin Ltd.) and set on ice. Samples (10 µl) from each standard or unknown were pipetted, in duplicate, into wells in sterile flat-bottomed 96 well microplates (Dynex Technologies). Quadruplicate wells containing 10 µl MilliQ® were used as blanks. Microplates loaded with blanks and standards were kept in the refrigerator until duplicate 10 µl samples of each of the unknown crude membrane preparations were also pipetted into wells. Bradford reagent (200 µl) (see Appendix 2) was then added to each well. After being shaken for 15 minutes (IKA-Labortechnik) the plates were read at 600 ± 5 nm using an MRX Microplate Reader (Dynex Technologies). Spectral response curves were performed to ensure that this was the optimal wavelength for the measurement of protein concentration (see Appendix 3). The inbuilt program, Endpoint v 1.1 was employed to subtract the average optical density (OD) value of the blank wells from all the other wells, before constructing a standard curve and calculating the protein concentration in the unknowns. Figure 2.3a shows a typical microplate as read by the plate reader for the determination of protein concentration. A typical standard curve is illustrated in Appendix 4.

2.5.2.2   Ouabain sensitive $Na^+K^+ATPase$ assay

(i)   Phosphate Standards

A series of ten phosphate standards were made in RT30 tubes using 10 mM KH_2PO_4 (BDH GPR™) as the stock. To prepare the standards 100 µl of this stock was added to 900 µl 5% trichloroacetic acid (TCA) to form the first standard of 1 mM KH_2PO_4, this was followed by a 6:4 dilution series in 5% TCA down to 10 µM KH_2PO_4. Aliquots (40 µl) from each standard were pipetted in duplicate into wells in a 96 well microplate together with quadruplicate wells containing 40 µl 5% TCA as blanks.

Fig. 2.3a: Typical Microplate Showing the Arrangement of Wells as Used for the Determination of Protein

Blanks are positioned in wells A 1-4. The standard curve, consisting of ten standards in duplicate, then runs along the remainder of row A and the entirety of row B, where wells A 5 and 6 contain the 2 mg/ml BSA standard. The remaining wells contain the unknown samples, in duplicate, along the rows.

Fig. 2.3b: Typical Microplate Showing the Arrangement of Wells as Used for the Determination of Phosphate Released

Blanks are positioned in wells A 1-4. The standard curve, consisting of ten standards in duplicate, then runs along the remainder of row A and the entirety of row B, where wells A 5 and 6 contain the 1 mM KH_2PO_4 standard. Wells C1, 2 and 3 contain plus ouabain replicates of sample 1 whilst wells C 4, 5 and 6 contain minus ouabain replicates of sample 1. Wells D 1, 2 and 3 contain plus ouabain replicates of sample 2 whilst wells D 4, 5 and 6 contain minus ouabain replicates of sample 2, and so on.

The plates loaded with blanks and standards were kept in the refrigerator whilst the Na⁺K⁺ATPase enzyme assay took place.

(ii) **Enzyme Assay**

RT 25 plastic assay tubes (Bibby Sterilin Ltd.) were set up on ice, with triplicate plus and minus ouabain tubes for each sample, containing either 10 μl 2 mM ouabain or 10 μl MilliQ® respectively. The tubes then received 10 μl assay buffer (300 mM histidine; 1.2 M NaCl; 80 mM $MgCl_2$ and 10 mM NaN_3; pH 7.2 - see Appendix 2) followed by 60 μl crude membrane preparation. To these tubes were added 10 μl 200 mM KCl and 10 μl 30 mM ATP (pH 6.8 with tris base - see Appendix 2) thus starting the enzyme reaction. Tubes were then incubated for 1 hour in a water-bath at 24°C. At the end of this time the tubes were placed back on ice and 100 μl ice cold 10% TCA added to stop the reaction. The tubes were then vortex mixed and placed in the refrigerator for 15 minutes.

The tubes were centrifuged at 1,300 g for 5 minutes at 4°C in order to spin down the precipitate (2,400 rpm using an MSE Coolspin centrifuge, Fisher Scientific UK Ltd, or 2200 rpm using a Beckman J6-MC centrifuge, Beckman Instruments Inc.). Single samples (40 μl) were then taken from each supernatant and dispensed into the remaining wells of the 96 well microplate already loaded with blanks and standards. MilliQ® (150 μl) was then added to all wells followed by 40 μl molybdate reagent (see Appendix 2). Stannous chloride solution was prepared by the addition of 50 μl of 1 M $SnCl_2$ made up in concentrated hydrochloric acid (HCl), to 2.45 ml MilliQ®, and finally 10 μl of this 20 mM stannous chloride solution was added to each of the wells.

The plate was shaken for 15 minutes before reading the optical density at 690 ± 5 nm using the MRX microplate reader. Spectral response curves were performed to ensure that this was the optimal wavelength for the measurement of phosphate concentration (see Appendix 3). As with the Bradford protein assay the average OD value of the blank wells was subtracted from all the other wells before Endpoint v 1.1 constructed a standard curve and calculated the unknowns. Figure 2.3b shows a typical microplate as read by the plate reader for the determination of phosphate concentration. A typical standard curve is illustrated in Appendix 4.

(iii) **Bradford Protein Assay 2**

The protein assay was repeated alongside the phosphate assay to check the protein concentration of the samples after dilution. This allowed the ouabain inhibitable branchial Na⁺K⁺ATPase activity to be expressed as nmoles/mg protein/h and also as the total sample as nmoles/5 pairs of gills/h.

2.6 Total Body Na⁺ and K⁺ Content

The total body Na⁺ and K⁺ contents were estimated by flame photometry following nitric acid digestion of whole fish.

2.6.1 Preparation of Samples

After fish were killed, washed, and dried as detailed in 2.4.1.1, eight fish per experimental group were wrapped together in labelled foil and stored at -80°C until processed further.

After defrosting on ice, the fish were rinsed in MilliQ®, patted dry with tissue and weighed to three decimal places. Following this, individual fish were chopped finely upon a glass microscope slide and loaded into 2 ml Safe-Lock microfuge tubes (Eppendorf®). Concentrated (sp. gr. 1.42) nitric acid (1 ml) was added, the tubes thoroughly vortex mixed, and then left overnight at room temperature in a fume hood. Even using Safe-Lock tubes, it was necessary to open and close each microfuge tube twice during the digestion process, in order to prevent the lids opening violently, due to the build up of gases.

The following day, the tubes were centrifuged at 13,000 rpm in a Biofuge A bench top microcentrifuge (Heraeus Christ) for three minutes, 400 µl of the supernatant was transferred to a clean microfuge tube and 1.6 ml MilliQ® added (1:4 v/v). These tubes were in turn centrifuged for a further three minutes, before 1.8 ml of this supernatant was transferred to a sterile Bijou vial (Bibby Sterilin Ltd.) and 1.8 ml MilliQ® added (1:1 v/v). The sample was thus a 1 in 10 dilution of the original nitric acid digest of the whole fish.

2.6.2 Determination of Na⁺ and K⁺ by Flame Photometry

As the samples were a 1 in 10 dilution of the original nitric acid digest, the standard solutions for both Na⁺ and K⁺ determinations were therefore also made up in a 1 in 10 dilution of nitric acid in MilliQ®.

Samples were read using an EEL Flame Photometer (Evans Electroselenium Ltd.). Between samples the apparatus was flushed with MilliQ® and the scale checked and adjusted if required approximately every 30 samples. The scale was set with a 1 in 10 dilution of nitric acid as zero, and the top standard, either 1 mM NaCl, 2 mM NaCl or 2 mM KCl as 100.

For the determination of Na⁺ most samples were read off using the 1 mM NaCl scale. However, some samples were off this scale, so they were read from a standard curve where the top standard was 2 mM NaCl. A double dilution series was made for each NaCl standard over the range of 31.25 µM to 1 or 2 mM, and this was used to

construct the standard curves. Likewise the 2 mM KCl stock was used to construct a standard curve down to 31.25 μM.

CurveExpert 1.34 was used to draw standard curves and determine the unknown values. Typical standard curves are supplied in Appendix 4.

2.6.3 Estimation of Total Body Water Content

Total body water content was assessed in fish acclimated to FW, 33% SW, 66% SW and SW in order to calculate the total body Na^+ and K^+ content.

After fish had been killed, washed and surface water removed as previously described in section 2.4.1.1, they were placed in individual porcelain crucibles of known weight. The crucibles containing the fish were then weighed again so that the wet weight of the fish could be established. These were placed in an oven at 60°C for 24 hours and at the end of this time weighed. They were then placed back in the oven for a further 16 hours before being weighed once more. As there had been no further reduction in weight, the 24 hour weight values were used to calculate the % water content of the fish from each of the salinity groups.

As there was no significant difference in % water content (ANOVA $p > 0.05$) between fish acclimated to each of the four salinity groups the mean for all the groups was calculated (79.5% $\pm$ 0.2%, n = 48), and this value used in the determination of total body Na^+ and K^+ content.

2.7 Cortisol Content

2.7.1 Whole Body Cortisol Extraction

The procedure employed for the extraction of cortisol was based on that previously described by Pottinger and Mosuwe (1994). Cortisol, was extracted from whole glass eels and elvers using ethyl acetate, with centrifugation to separate the cortisol-containing solvent fraction.

Fish were killed, washed and dried as previously described in section 2.4.1.1, then wrapped, eight fish together per experimental group, in labelled foil before being stored at -80°C until processed further.

After defrosting on ice, the fish were rinsed in MilliQ®, patted dry with tissue and weighed to three decimal places. Individual fish were then roughly cut into pieces approximately 2 mm thick upon glass microscope slides and placed into ice cold Universal vials (Bibby Sterilin Ltd.). They were then homogenized in MilliQ® (1:5 w/v) using a Kinematica Polytron® fitted with a 12 mm diameter probe (Kriens/Lucern GmbH), on half power for approximately 15 seconds. The probe was rinsed in MilliQ® between homogenizing individuals of the same experimental group. Between

experimental groups the probe was first rinsed in 0.1% sodium dodecyl sulphate (SDS), and then in clean MilliQ®.

Homogenates were transferred into glass centrifuge tubes and extracted with 5 volumes of ethyl acetate. After thorough vortexing for 1 minute, the samples were centrifuged at 1,500 g/2,500 rpm, for 15 minutes at 4˚C using an MSE Mistral centrifuge (Fisher Scientific UK Ltd.). The supernatant was then decanted off into glass centrifuge tubes, using clean Pasteur pipettes between samples from different experimental groups, and evaporated to dryness using a Genevac Centrifugal Evaporator (Genevac Ltd.). Once completely dry they were used in the radioimmunoassay.

2.7.2 Determination of Cortisol Content by Radioimmunoassay

The concentration of cortisol within whole body elver extracts was determined using a radioimmunoassay (RIA) method modified from that described by Waring *et al.* (1996).

2.7.2.1 RIA Components

(i) Cortisol Standard

Inert hydrocortisone (11β,17α,21-trihydroxypregn-4-ene,3,20-dione, 98%, Sigma) was used as the standard and was stored at 2mg/ml in 99.7-100% ethanol at -20˚C. Before use the standard was diluted to 10ng/ml and a series of triplicate standards made, ranging from 19.53 pg/tube contained in 2 μl, to 2500 pg/tube contained within 250 μl, in plastic LP3 assay tubes (Denley Instruments Ltd.). These standard tubes were evaporated to dryness, as with the unknown sample tubes, using the Genevac centrifugal evaporator.

(ii) Tritiated Cortisol

[1,2,6,7-^{3}H]cortisol (^{3}H-cortisol) in toluene:ethanol (9:1 v/v), with a specific activity ranging from 80-105Ci/mmol was obtained from Amersham Life Science Ltd. and stored at -20˚C. A working stock was prepared from this by diluting 50 μl in 5 ml toluene:ethanol (9:1 v/v) which was also maintained at -20˚C. This working stock contained approximately 20,000 dpm/μl. On the first day of the radioimmunoassay an aliquot of ^{3}H-cortisol working stock was dried down and resuspended in an appropriate volume of assay buffer (to contain 20,000 dpm per 200 μl) (see Appendix 2 and section iii immediately below). The usual procedure was to dry down 200 μl of ^{3}H cortisol working stock which was then re-suspended in 40 ml assay buffer. This gave ample

material for a set of eight standards, plus zero tubes and total counts tubes, all in triplicate, together with 48 samples in duplicate.

(iii)   Assay Buffer

Assay buffer was made fresh for each assay (40 mM Na_2HPO_4; 10 mM NaH_2PO_4; 0.9% (w/v) NaCl; 0.5% (w/v) BSA; pH 7.4 - see Appendix 2) and stored either in the fridge or on ice throughout.

(iv)   Cortisol Antibody

Cortisol antiserum was obtained from the Scottish Antibody Production Unit (SAPU) with specificity as follows: cortisol 100%; corticosterone 0.153%; cortisone 0.048%; 11-deoxycortisol 0.573% and deoxycorticosterone 0.037% (Manufacturers data). The antiserum was a pool of selected antisera obtained from a single sheep which had been injected with cortisol-3-0 (carboxymethyl)oxime-bovine albumin conjugate. Each vial contained the lyophilized residue from 1 ml of anticortisol serum diluted 1 in 5 with a 0.04 M phosphate buffer containing 0.5% (w/v) BSA. Vials containing lyophilized antiserum were obtained from SAPU and stored at 4°C. As required, the contents of each vial were reconstituted with 1 ml MilliQ®, and then allowed to stand for 1 hour at room temperature to ensure completely solubilized. After gentle mixing by inversion, the reconstituted antiserum was dispensed into 150 µl aliquots and stored at -20°C until needed. The antibody was diluted 1 in 300 in the ^{3}H-cortisol-assay buffer-mix (see section 2.7.2.2) before use, such that the final concentration of the antibody was 1 in 1500.

(v)   Dextran Coated Charcoal

Dextran coated charcoal (DCC) was prepared on day 1 by stirring 25 mg dextran T70 (Pharmacia) and 250 mg charcoal (BDH - Norit®) in 50 ml assay buffer, on ice for 2 hours, after which it was stored at 4°C overnight. On day 2 the DCC was stirred for a further 30 minutes on ice before use.

2.7.2.2 Routine RIA Procedure

Each lyophilized standard was re-suspended in 200 µl of a 1 in 1500 dilution of antibody in the radiolabelled cortisol containing assay buffer (20,000 dpm / 200 µl). Total counts tubes and zero tubes containing no cortisol also received 200µl of this ^{3}H-cortisol-assay buffer-antibody-mix. Dried down unknown extracts were resuspended in 500 µl of this same mixture, taking care to ensure it was passed over all the inside of the

glass tube that the extract had been in contact with. All the tubes, both standards and unknowns, were vortex mixed and then allowed to sit on ice for 30 minutes to ensure that all the cortisol, where present, had been resuspended. This was especially important for the tubes containing the dried down extracts, as samples would be taken from these into other tubes before incubation.

Duplicate 200 µl samples from each tube containing resuspended extracts were pipetted into clean assay tubes, and both these and the standard tubes were covered with laboratory film and incubated for 1 hour at 37˚C in a water bath, before being placed in the fridge at 4˚C overnight.

The following day 200 µl ice cold DCC was added to all tubes, except the total counts tubes. To maintain the same volume in all the tubes, 200 µl assay buffer held aside from day 1 was added to the total counts tubes. The total counts tubes did not receive DCC, and thus showed the total amount of radioactive counts added to all the tubes. After vortex mixing, all tubes were incubated on ice for 15 minutes, before centrifugation at 1,500 g/2,500 rpm for 15 minutes at 4˚C in the MSE Mistral Centrifuge (Fisher Scientific UK Ltd.). The supernatants from each tube were carefully decanted off into PONY Vials, and 4 ml Emulsifier Scintillator plus™ liquid scintillation cocktail added (Canberra Packard Ltd). After shaking to mix, tubes were counted for 5 minutes using a 2000 Tri-carb® Scintillation Analyzer (Canberra Packard Ltd.). Using CurveExpert v 1.34, unknown values were determined from a standard curve plotted as % bound against pg cortisol/tube. A typical standard curve is plotted in Appendix 4.

2.7.2.3 Extraction Efficiency

^{3}H-cortisol (approximately 20,000 dpm) was added to homogenized tissue samples (n = 7) before extraction as described in section 2.7.1, such that the recovery of that labelled cortisol could be assessed. Labelled cortisol working stock (see section 2.7.2.1ii) was dried down and resuspended in ethyl acetate at a concentration of 100,000 dpm/ml and 200 µl was added to each homogenized tissue sample which was then thoroughly vortex mixed and then incubated on ice for one hour. The extraction procedure was then carried out as described in section 2.7.1. After decanting off the supernatant from the first extraction, the procedure was repeated again for a second time, adding a further volume of ethyl acetate to the homogenate, before continuing as before. Duplicate 200 µl samples of the extract were counted directly and the remainder dried down using the centrifugal evaporator. Once dry, the samples were carefully resuspended in 500 µl assay buffer and set on ice to ensure fully resuspended. Duplicate 100 µl samples were counted after 15 min and 30 min. Extraction efficiencies could be calculated for the both first and second extractions, both before and after drying down and resuspension.

2.8   Interrenal Tissue Morphology

Fish were killed, washed and dried as described in section 2.4.1.1. The tails were then cut off posterior to the anus and discarded. The remaining tissue was preserved by being submerged in Bouin's solution, a microanatomical fixative (see Appendix 2). The tissues were left in Bouin's for a period of approximately 16 hours and were washed in 70% ethanol, before being stored in 70% ethanol until processed further. Four fish from each experimental group were fixed in Bouin's although only one individual proceeded through the whole procedure until the photography stage. Below is a brief outline of the protocol employed.

A piece of the fixed fish of approximately 6 mm was cut from just behind the operculum and was subjected to a dehydration series (96% ethanol, two baths each of 1 h 30 min; absolute ethanol, first bath 45 min, the second 1 h 45 min; absolute ethanol:chloroform (1:1) for 30 min; 100% chloroform, first bath 2 h 15 min, and then left in the second bath for 16 h 15 min), before being embedded in paraffin wax. Serial longitudinal sections (5 μm) were cut from the blocks, and were stained using Masson's Trichrome Stain as follows (details of reagents are given in Appendix 2).

Sections were transferred from distilled water to Celestine Blue for 10 minutes, they were rinsed in distilled water and then stained with Mayer's Haemalum for 10 minutes before being washed in running tap water. The sections were then placed in Yellow Mordant for 3 minutes before being washed again under running tap water for 3 minutes. After staining in Ponceau Acid Fuchsin for 5 minutes the sections were rinsed briefly in tap water before being placed in 1% phosphomolybdic acid for 15 minutes until the connective tissue was free from red dye. The sections were stained with Aniline Blue or Light Green for 5 minutes, rinsed in 1% acetic acid, and then were mounted onto slides.

The resulting sections showed nuclei as black, muscle/cytoplasm as red and collagen, connective tissue and mucin as blue/green.

Using 64T slide film (Eastman Kodak Company) photographs were taken of interrenal cells using a Leitz Dialux 20 microscope (E.Leitz (Instruments) Ltd.) and Wild photographic set-up (Wild Heerbrugg Ltd.). In addition to slides showing interrenal cells lying down the posterior cardinal vein adjacent to the liver (x400), a number of overlapping photographs were taken at lower power (x100) across the whole of a section. This enabled a montage to be constructed to illustrate the location of these interrenal cells within the body of the fish.

2.9   Statistics

All values are expressed as mean ± SE unless otherwise stated. Statistical tests were carried out using either StatView® v 4.02 or InStat v 2.01, both for Macintosh.

One way analysis of variance (ANOVA) was used where there were more than two groups to be compared. This was followed by Dunnett comparisons between a control group and each of the other groups if the ANOVA was considered significant. Where there were only two groups of data to be examined, these were investigated using unpaired, two-tailed t-tests.

For example, for the environmental acclimation studies (section 2.3.1) where there were fish acclimated to four different environmental salinities, an ANOVA was performed of all four groups, followed by Dunnett comparisons between the FW acclimated fish (control) and those from the other salinities. During the FW-SW transfer studies (section 2.3.3) FW acclimated fish at Month 1 were challenged with SW (FW-SW), or with FW (FW-FW) as a control, and then sampled at a number of time-points between 3 hours and 4 months post-transfer. In this case ANOVAs were performed within both the FW-FW and FW-SW groups, each including the value obtained from the FW acclimated fish at Month 1 (control). Where significant differences arose, these ANOVAs were followed by Dunnett comparisons between the FW acclimated fish at Month 1 (control) and the other time-points. Finally, within each time-point, unpaired t-tests were used to compare between FW-FW and FW-SW values. For the drinking rate challenge studies (section 2.3.2) a similar procedure was employed, using ANOVAs within the FW-FW and FW-SW groups, and t-tests between FW-FW and FW-SW groups within timepoints. In this case the control value included in the ANOVA and Dunnett calculations was the amount of water accumulated at time zero in FW acclimated fish with the appropriate background counts removed i.e. 0 $\mu l/g$.

The full results of statistical tests are noted in Appendix 5. In the text and figure legends only *p* values are given, prefixed with ANOVA, Dunnett, or t-test as required. To which statistical analyses the asterisks marked on graphs refer to is made clear in the figure legends. Significance levels are noted as follows:

When:	$p > 0.05$	not significant	no asterisks
	$p < 0.05$	significant	*
	$p < 0.01$	very significant	**
	$p < 0.001$	extremely significant	***

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Chapter 3

Results

3.1 Development

As discussed in section 1.4 the length and weight of eels has been reported to decrease slightly during the transition from glass eel to elver stages (Tesch, 1977; Lecomte-Finiger, 1992). This therefore renders these characteristics unsuitable as measures of development. However, pigmentation does increase with development (Tesch, 1977). In accordance with these findings the density of colour/pigmentation was used as an indicator of development. The colour of the fish over a transect along the body was determined using an image analysis system as described in section 2.1.1. Using an arbitrary scale where 0 = white and 255 = black, the mean colour value along the transect was determined, such that more pigmented fish had a higher colour values.

In acclimated fish at Month 1 (Fig. 3.1) pigmentation increased with increasing salinity (ANOVA $p < 0.05$), with the 66% SW group significantly more pigmented than the FW group (Dunnett's $p < 0.05$). In acclimated fish at Month 2 (Fig. 3.2) there were significant differences in pigmentation between groups (ANOVA $p < 0.0001$) such that all of the salinity groups were significantly more pigmented than the FW group (Dunnett's $p < 0.01$).

Over the course of Months 1 to 5 (Fig. 3.3) there were significant increases in pigmentation within both the FW and SW acclimated groups (ANOVA $p < 0.0001$). Within the FW acclimated group, fish at Months 3, 4 and 5 were significantly more pigmented than fish at Month 1 (Dunnett's $p < 0.01$). Within the SW acclimated group, fish at Months 2, 4 and 5 were significantly more pigmented (Dunnett's $p < 0.01$). SW acclimated fish were found to be significantly more pigmented than fish from FW at Months 1 (t-test $p < 0.05$), 2 (t-test $p < 0.001$) and 5 (t-test $p < 0.0001$).

From these results it is clear that fish acclimated to different salinities continue to develop normally, as assessed by the progression of pigmentation. In fact SW acclimated fish were more pigmented than those from FW at Months 1, 2 and 5.

3.1.1 Body Weight

There were no significant differences in body weight with changes in environmental salinity in acclimated fish (see section 2.3.1) at both Month 1 and Month 2 (ANOVA $p > 0.05$). However, over the course of Months 1 to 5 there were significant decreases in the body weight of both the FW (0.277 ± 0.011 g at Month 1 to 0.157 ± 0.009 g at Month 5), and SW acclimated (0.249 ± 0.012 g at Month 1 to 0.126 ± 0.004 g at Month 5) fish (ANOVA $p < 0.0001$). Within both salinity groups, fish from Months 3, 4 and 5 were of significantly lower weight than fish at Month 1 (Dunnett's $p < 0.01$). Body weights were significantly higher in FW as compared to SW acclimated fish at Month 5 (t-test $p < 0.01$).

Fig. 3.1:   Environmental Acclimation Studies - Pigmentation - Month 1

Mean colour values (where 0 = white and 255 = black) in fish at Month 1 acclimated to
FW, 33% SW, 66% SW and SW (n = 10). Values shown are means ± SE. * indicates
a significant difference, at $p < 0.05$ (Dunnett's), from the FW acclimated fish. See
experimental rational section 2.3.1 for details of history of these fish.

Mean
colour
value

Fig. 3.2:   Environmental Acclimation Studies - Pigmentation - Month 2

Mean colour values (where 0 = white and 255 = black) in fish at Month 2 acclimated to
FW, 33% SW, 66% SW and SW (n = 10). Values shown are means ± SE. ** indicates
a significant difference, at $p < 0.01$ (Dunnett's), from the FW acclimated fish.
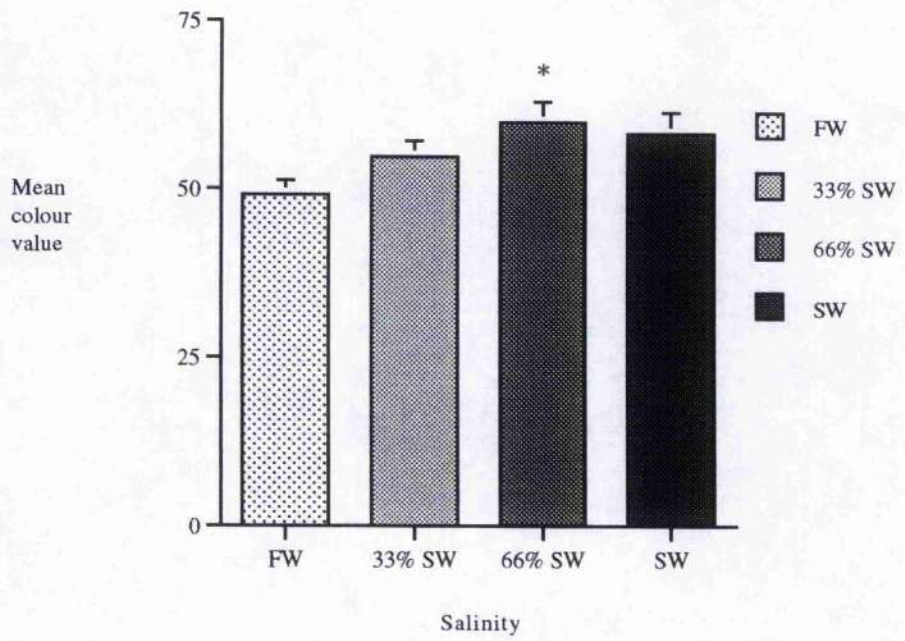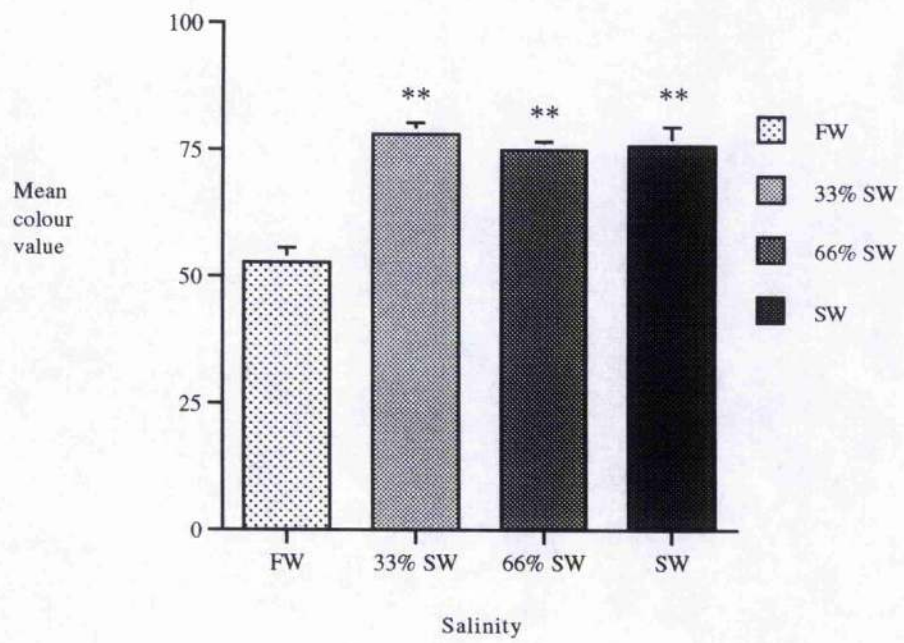
Fig. 3.3:   Environmental Acclimation Studies - Pigmentation - Months 1 to 5

Mean colour values (where 0 = white and 255 = black) in fish at Months 1 to 5 acclimated to FW and SW (n = 10). Values shown are means ± SE. * and *** indicate significant differences, at $p < 0.05$ and $p < 0.001$ respectively (t-test), between FW and SW acclimated fish within months.
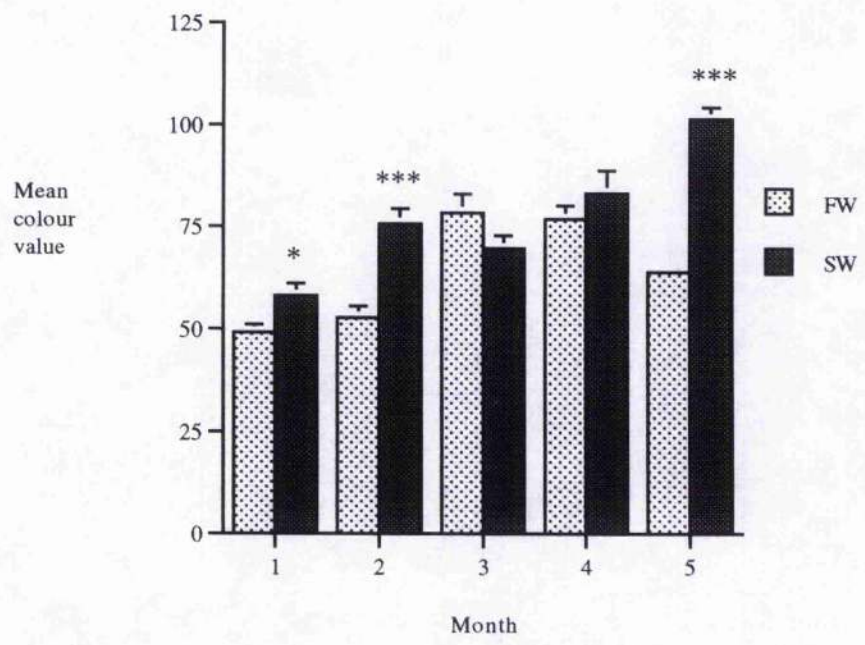
During the FW-SW transfer experiment (see section 2.3.2) there were also significant decreases in body weight over the course of the investigation, within both the FW-FW (0.249 ± 0.018 g at 3 h post-transfer, to 0.148 ± 0.012 g at 4 months post-transfer) and FW-SW (0.259 ± 0.020 g at 3 h post-transfer, to 0.121 ± 0.008 g at 4 months post-transfer) groups (ANOVA $p < 0.0001$). Within the FW-FW group the 14 d and 21 d time-points (Dunnett's $p < 0.05$), and the 2, 3 and 4 month time-points (Dunnett's $p < 0.01$) were significantly lower as compared to the control fish. Within the FW-SW group there were significant decreases in weight at 2, 3 and 4 months post-transfer (Dunnett's $p < 0.01$). The FW-FW body weights were significantly higher than those from the FW-SW group at the 24 h time-point (t-test $p < 0.01$).

From these results it is clear that there is a reduction in body weight over the course of the first six months post-migration of glass eels into FW, regardless of whether then reared in FW or SW.

3.2 Drinking Rate

3.2.1 Evaluation of the Method

3.2.1.1 Determining the Optimal Specific Activity of the Marker

Figure 3.4 shows that there was a significant increase in radioactivity per fish (cpm) with increasing specific activity of ^{51}Cr-EDTA (μCi/L) within the bathing medium (ANOVA $p < 0.01$), with values from the 200 μCi/L and 400 μCi/L groups significantly higher than background (Dunnett's $p < 0.05$ and $p < 0.01$ respectively). Bathing medium with a specific activity of 200 μCi/L ^{51}Cr-EDTA was then subsequently used for all further drinking rate investigations.

3.2.1.2 Time Course of Routine Drinking Rate Experiments

SW acclimated fish were found to increase the amount of water accumulated in the gut in a linear fashion until 3h post-transfer, after which ^{51}Cr-EDTA accumulated reached a steady state (Fig. 3.5). This saturation of radioactive accumulation can be interpreted as an equilibrium, where the rate of acquisition of counts by drinking is balanced by a loss of radioactive counts via the anus (Fig. 3.6). The radiolabel accumulated over a three hour incubation was lost when the fish were subsequently placed in isotope-free SW for the next three hours. Obviously there will be absorption of water by the intestine such that the equivalent number of radioactive counts ingested are voided by the anus in a smaller volume.

Fig 3.4: Specific Activity of ^{51}Cr-EDTA and Gut Accumulation of Counts in SW Acclimated Fish

Radioactive counts (cpm) obtained when SW acclimated elvers were incubated in ^{51}Cr-EDTA SW for 6 hours at different levels of activity and compared to background (n = 10). Values shown are means ± SE. * and ** indicate significant differences from background counts, at $p < 0.05$ and $p < 0.01$ respectively (Dunnett's).

Specific Activity (μCi/L) or background

Time Course of Water Accumulation by SW Acclimated Fish

Amount of water accumulated (μl/g) in SW acclimated elvers during incubation in ^{51}Cr-EDTA labelled SW for a number of time-periods between one and twelve hours (n = 10). Values shown are means $\pm$ SE.

Mean amount accumulated (µl/g)

Time post-transfer (h)
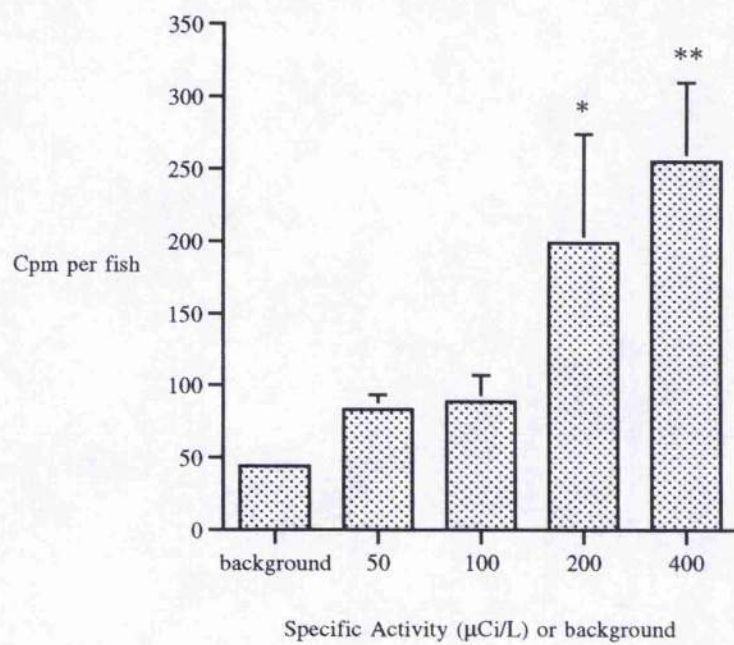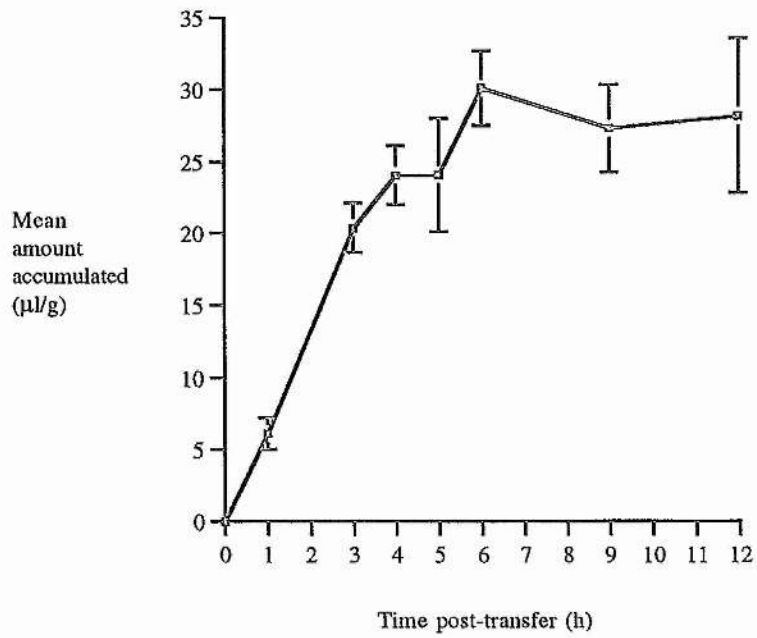
<u>Fig. 3.6:</u>   <u>Time Course of Water Accumulated and Lost by SW Acclimated Fish</u>

Amount of water accumulated (μl/g) in SW acclimated elvers post-transfer to ^{51}Cr-EDTA labelled SW for three hours. After this time they were transferred to normal SW (n = 21, except 5 h and 6 h where n = 20, 10 h where n = 17 and 3 h where n = 16). Values shown are means ± SE.
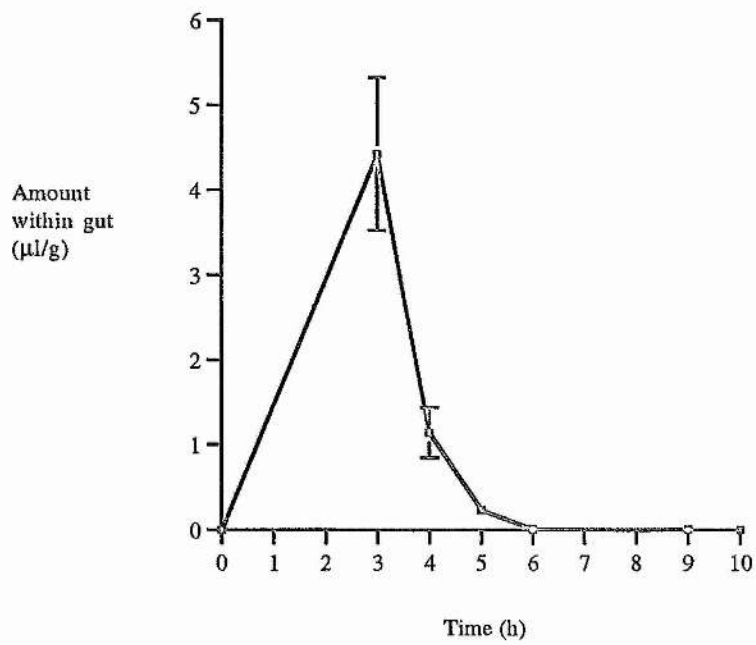
3.2.1.3 Surface Contamination by the Marker

Fish placed in ^{51}Cr-EDTA containing medium for a short period (less than ten seconds) before being washed, blotted dry and counted, exhibited no significant uptake of radiolabel as compared to background (ANOVA $p > 0.05$). There were no significant differences in counts obtained from FW acclimated fish after bathing in ^{51}Cr-EDTA labelled FW (200 μCi/L) for less than ten seconds (44.0 ± 0.81 cpm), FW acclimated fish directly obtained from the stock tank (46.2 ± 0.88 cpm), and empty counting vials (46.5 ± 1.10 cpm). Further drinking rate experiments were then performed with confidence that there was no surface contamination of the fish with the radiomarker.

3.2.2   Environmental Acclimation Studies

There was a trend towards increased drinking rate with increased environmental acclimation salinity at both Month 1 (Fig. 3.7a) and Month 2 (Fig. 3.8a) (ANOVA $p <$ 0.0001), although only the SW acclimated fish were considered significantly higher than those from FW (Dunnett's $p < 0.01$). Fish were assigned to one of three groups (as described in section 2.4.2) in order to estimate the proportion of fish drinking within each of the experimental groups. For acclimated fish at both Month 1 (Fig. 3.7b) and Month 2 (Fig. 3.8b) the proportion of fish considered drinking increased with environmental salinity, for example at Month 1: 4.8%, 14.3%, 26.3% and 75% of fish were considered drinking in FW, 33% SW, 66% SW and SW acclimated groups respectively.

Figure 3.9a illustrates drinking rates (μl/g/h) in FW and SW acclimated fish at Months 1 through to 5. Within the FW acclimated group there were significant increases in drinking rate, from 0.072 ± 0.023 μl/g/h at Month 1 to 1.121 ± 0.424 μl/g/h at Month 5 (ANOVA $p < 0.01$), with Month 5 significantly higher than Month 1 (Dunnett's $p <$ 0.05). Within the SW acclimated fish there was a more significant increase in drinking rate from 0.698 ± 0.099 μl/g/h at Month 1 to 12.854 ± 1.049 μl/g/h at Month 5 (ANOVA $p < 0.0001$), where Months 3, 4, and 5 were considered significantly higher than Month 1 (Dunnett's $p < 0.01$). Drinking rate was shown to be significantly higher, ranging from 8.5 to 13.4 times greater, in SW as compared to FW acclimated fish at all months (t-test $p < 0.001$).

There was a clear increase in the proportion of fish considered drinking in the SW acclimated groups as compared to those from FW within each month (Fig. 3.9b). In addition, within each salinity group there was a trend towards an increase in the proportion of fish considered drinking over the course of Months 1 to 5. In FW acclimated fish at Month 1 only 4.8% were considered drinking as compared to 33.3% at

Fig. 3.7a: Environmental Acclimation Studies - Drinking - Month 1

Drinking rates (μl/g/h) in fish at Month 1 acclimated to FW (n = 21), 33% SW (n = 21), 66% SW (n = 19) and SW (n = 20). Values shown are means ± SE. ** indicates a significant difference from the FW acclimated fish, at $p < 0.01$ (Dunnett's).

Fig. 3.7b: Environmental Acclimation Studies - Drinking - Month 1 - Proportion Drinking

Proportion of fish at Month 1 acclimated to FW, 33% SW, 66% SW and SW (n values as Fig. 3.7a) considered to be not drinking, probably drinking and drinking.

Drinking rate
(µl/g/h)

1.0

0.8

0.6

0.4

0.2

0.0

FW   33% SW   66% SW   SW

Salinity

**

FW
33% SW
66% SW
SW

FW   33% SW   66% SW   SW

not drinking
probably drinking
drinking

Environmental Acclimation Studies - Drinking - Month 2

Drinking rates (μl/g/h) in fish at Month 2 acclimated to FW (n = 21), 33% SW (n = 19), 66% SW (n = 20) and SW (n = 20). Values shown are means ± SE. ** indicates a significant difference from the FW acclimated fish, at $p < 0.01$ (Dunnett's).

Fig. 3.8b: Environmental Acclimation Studies - Drinking - Month 2 - Proportion Drinking

Proportion of fish at Month 2 acclimated to FW, 33% SW, 66% SW and SW (n values as Fig. 3.8a) considered to be not drinking, probably drinking and drinking.

Drinking rate
(μl/g/h)

Salinity

FW   33% SW   66% SW   SW

FW
33% SW
66% SW
SW

not drinking
probably drinking
drinking

Fig. 3.9a: Environmental Acclimation Studies - Drinking - Months 1 to 5

Drinking rates (μl/g/h) in fish at Months 1 to 5 acclimated to FW and SW (n = 21 in all cases, except SW acclimated fish at Months 1, 2, and 3 where n = 20). Values shown are means $\pm$ SE. *** indicates significant differences, at $p < 0.001$ (t-test), between FW and SW acclimated fish within months.

Fig 3.9b: Environmental Acclimation Studies - Drinking - Months 1 to 5 -
                 Proportion Drinking

Proportion of fish at Months 1 to 5 acclimated to FW and SW (n values as Fig. 3.9a) considered to be not drinking, probably drinking and drinking.
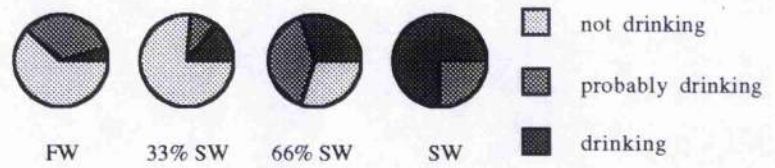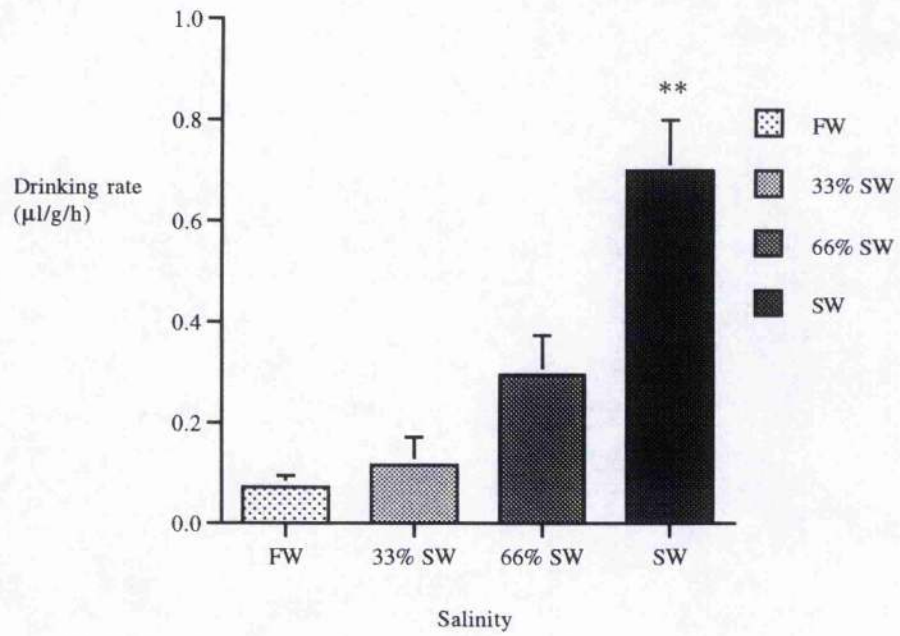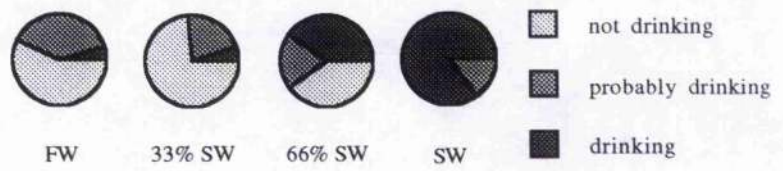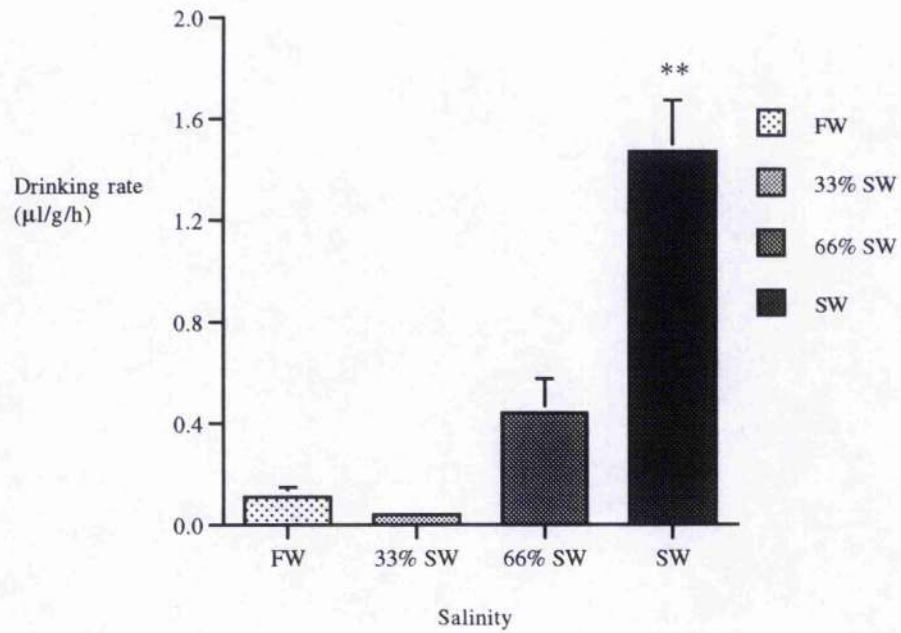
Month 1
FW   SW

Month 2
FW   SW

Month 3
FW   SW

Month 4
FW   SW

Month 5
FW   SW

not drinking

probably drinking
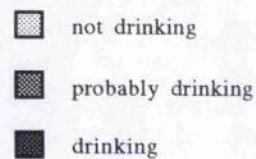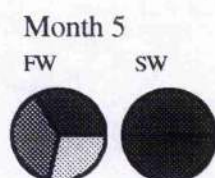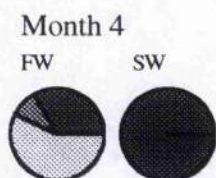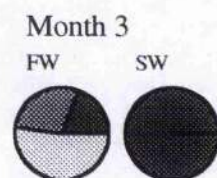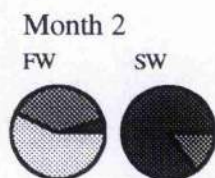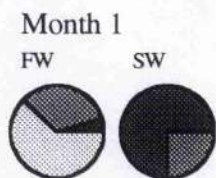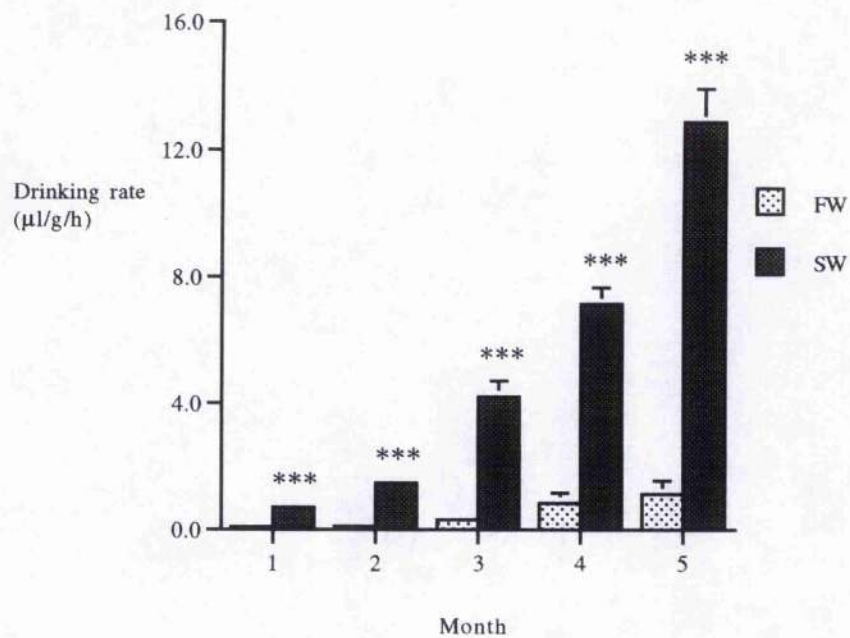
drinking

Month 5, and in SW acclimated fish 75% were considered drinking at Month 1 as compared to 100% at Month 5.

3.2.3 Monthly Drinking Rate Challenge Studies

Throughout Months 1 to 5, FW acclimated fish demonstrated an acute drinking response when challenged with SW (Figs. 3.10a to 3.14a). There was never any equivalent response in the FW-FW control groups. Note that the y-axis details the amount of water accumulated over the time-course ($\mu l/g$) rather than a drinking rate ($\mu l/g/h$). The control for the ANOVA and Dunnett analyses was considered to be the value obtained from FW acclimated fish after being placed for less than ten seconds in ^{51}Cr-EDTA labelled FW with the appropriate background counts subtracted.

At Month 1 (Fig. 3.10a) FW-SW fish accumulated greater amounts of water as compared to the FW-FW control group throughout the time-course of the investigation. Within the FW-SW challenge group there was a marked increase by 5 min post-transfer, until 1 h where it began to level off. There was no equivalent response in the FW-FW control group. Within the FW-FW control group there was a slight increase in the amount accumulated over time, although this was considered not significant (ANOVA $p > 0.05$). Within the FW-SW group differences between time-points were considered significant (ANOVA $p < 0.0001$), with all but the 5 min time-point significantly higher ($p < 0.01$). FW-SW values were significantly higher than those from the FW-FW group at all the time-points (t-test $p < 0.001$, except 5m where $p < 0.01$).

At Month 2 (Fig. 3.11a) FW-SW fish accumulated greater amounts of water as compared to the FW-FW control group throughout the time-course of the investigation. Within the FW-SW challenge group there was a marked increase by 5 min post-transfer, until 15 min when it began to level off. There was no equivalent response in the FW-FW control group. Within the FW-FW control group there was a significant increase in the amount accumulated over time (ANOVA $p < 0.0001$), although the actual volumes accumulated remained considerably lower than those from the FW-SW group (except for 5 h). Within the FW-FW group the 4 h and 5 h time-points were significantly higher than the control (Dunnett's $p < 0.01$). Within the FW-SW group differences between time-points were also considered significant (ANOVA $p < 0.0001$), with all but the 5 min time-point considered significantly higher than the control (Dunnett's $p < 0.01$). FW-SW values were significantly higher than those from the FW-FW group at all the time-points but that at 5 h post-transfer, where the FW-FW values had increased to meet those in the FW-SW group (t-test $p < 0.001$, except 5 min where $p < 0.01$).

Fig 3.10a: Drinking Rate Challenge - Month 1

Amount of water accumulated (μl/g) in FW acclimated fish at Month 1 after acute challenge with SW and FW (n = 12, except FW-SW 5 min where n = 13; FW-FW 5 h n = 15; FW-FW 30 min, FW-SW 30 min, FW-SW 1 h and FW-SW 2 h where n = 11 and FW-SW 5 h n = 10). Values shown are means ± SE. ** and *** indicate significant differences, at $p < 0.01$ and $p < 0.001$ respectively (t-test), between FW-FW and FW-SW values within time-points. See experimental rational section 2.3.2 for details of history of these fish.

Fig. 3.10b: Drinking Rate Challenge - Month 1 - Proportion Drinking

Proportion of fish at Month 1, during drinking rate challenge with SW and FW (n values as Fig. 3.10a), considered to be not drinking, probably drinking and drinking.

Amount
accumulated
(µl/g)

FW-FW
control

FW-SW
challenge

Time post- transfer (h)

FW-FW
control

FW-SW
challenge

5m   15m   30m   1h   2h   3h   4h   5h

not drinking   probably drinking   drinking

Fig 3.11a: Drinking Rate Challenge - Month 2

Amount of water accumulated (µl/g) in FW acclimated fish at Month 2 after acute challenge with SW and FW (n = 12, except FW-FW 5 min and FW-SW 15 min where n = 14; FW-FW 15 min FW-FW 30 min, FW-FW 2 h and FW-SW 5 min where n = 11; FW-SW 5 h n = 9 and FW-SW 4 h n = 7). Values shown are means ± SE. ** and *** indicate significant differences, at $p < 0.01$ and $p < 0.001$ respectively (t-test), between FW-FW and FW-SW values within time-points.

Fig. 3.11b: Drinking Rate Challenge - Month 2 - Proportion Drinking

Proportion of fish at Month 2, during drinking rate challenge with SW and FW (n values as Fig. 3.11a), considered to be not drinking, probably drinking and drinking.
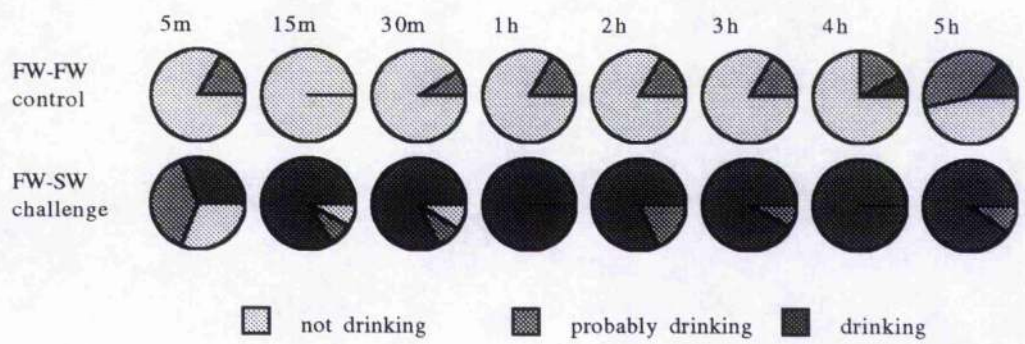
Amount accumulated (μl/g)

FW-FW control

FW-SW challenge

Time post-transfer (h)

5m 15m 30m 1h 2h 3h 4h 5h

FW-FW control

FW-SW challenge

not drinking   probably drinking   drinking

At Month 3 (Fig. 3.12a) FW-SW fish accumulated greater amounts of water as compared to the FW-FW control group throughout the time-course of the investigation. Within the FW-SW challenge group there was a marked increase by 5 min post-transfer until 1 h, when it began to level off. There was no equivalent response in the FW-FW control group. Within the FW-FW control group there was a significant increase in the amount accumulated over time (ANOVA $p < 0.05$), although actual volumes accumulated remained much lower than that of the FW-SW group. However, differences between the control and each of the FW-FW groups were not found to be significant (Dunnett's $p > 0.05$). Within the FW-SW group differences between time-points were significant (ANOVA $p < 0.0001$), with the 15 min and 30 min (Dunnett's $p < 0.05$), and the 1 h, 2 h, 3 h, 4 h and 5 h (Dunnett's $p < 0.01$) time-points considered significantly higher than the control. FW-SW values were significantly higher than those from the FW-FW group at all the time-points (t-test $p < 0.001$, except 4 h where $p < 0.01$).

At Month 4 (Fig. 3.13a) FW-SW fish accumulated greater amounts of water as compared to the FW-FW control group throughout the time-course of the investigation. Within the FW-SW challenge group there was a marked increase by 5 min post-transfer until 1 h, when it began to level off. There was no equivalent response in the FW-FW control group. Within the FW-FW control group there was a significant increase in the amount accumulated over time (ANOVA $p < 0.05$), although actual volumes accumulated remained lower than that of the FW-SW group. The FW-FW 4 h time-point was significantly higher than the control (Dunnett's $p < 0.05$). Within the FW-SW groups there were more significant increases in the amount accumulated with time (ANOVA $p < 0.0001$), with all the time-points considered significantly higher than the control (Dunnett's 5 min, 15 min, and 30 min = $p < 0.01$, and 1 h, 2 h, 3 h, 4 h, and 5 h = $p < 0.01$). FW-SW values were significantly higher than those from the FW-FW group at 5 min ($p < 0.001$), 15 min ($p < 0.001$), 30 min ($p < 0.001$), 1 h ($p < 0.01$) 2 h, ($p < 0.001$) and 5 h ($p < 0.001$) post-transfer (as determined by unpaired t-test analyses).

At Month 5 (Fig. 3.14a) FW-SW fish accumulated greater amounts of water as compared to the FW-FW control group throughout the time-course of the investigation. This experiment was carried out for only 3 h due to a shortage of experimental animals. Within the FW-SW challenge group there was a marked increase by 5 min post-transfer until 15 min, when it began to level off. There was no equivalent response in the FW-FW control group. Within the FW-FW control group there was a significant increase in the amount accumulated over time (ANOVA $p < 0.0001$), although actual volumes accumulated remained lower than that of the FW-SW group. Within the FW-FW group the 3 h time-point was considered significantly higher than the control (Dunnett's $p < 0.01$). Within the FW-SW group there were also significant increases in the amount accumulated with time (ANOVA $p < 0.0001$), however all the time-points were

Fig. 3.12a: Drinking Rate Challenge - Month 3

Amount of water accumulated (μl/g) in FW acclimated fish at Month 3 after acute challenge with SW and FW (n = 12, except FW-SW 15 min where n = 13; FW-SW 4 h n = 15; FW-FW 15 min and FW-FW 5 h where n = 11 and FW-FW 3 h n = 10). Values shown are means $\pm$ SE. ** and *** indicate significant differences, at $p < 0.01$ and $p < 0.001$ respectively (t-test), between FW-FW and FW-SW values within time-points.

Fig. 3.12b: Drinking Rate Challenge - Month 3 - Proportion Drinking

Proportion of fish at Month 3, during drinking rate challenge with SW and FW (n values as Fig. 3.12a), considered to be not drinking, probably drinking and drinking.
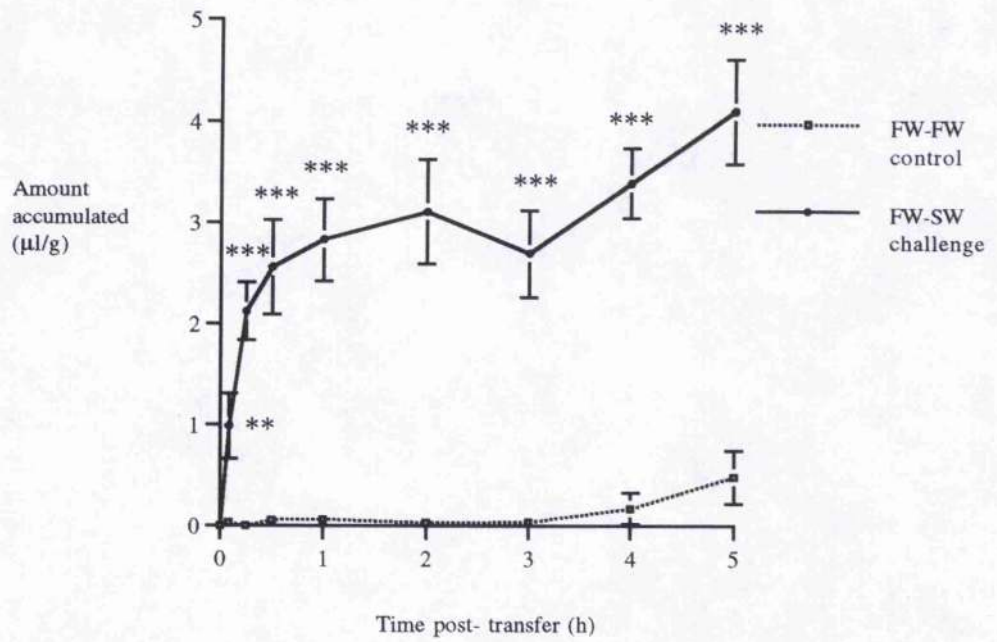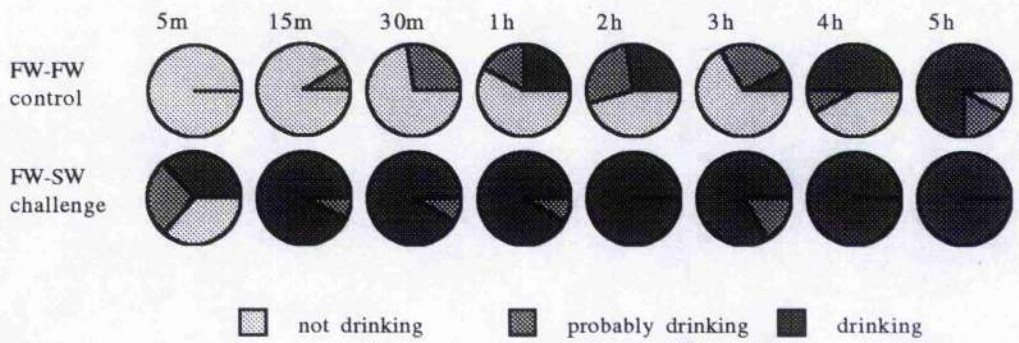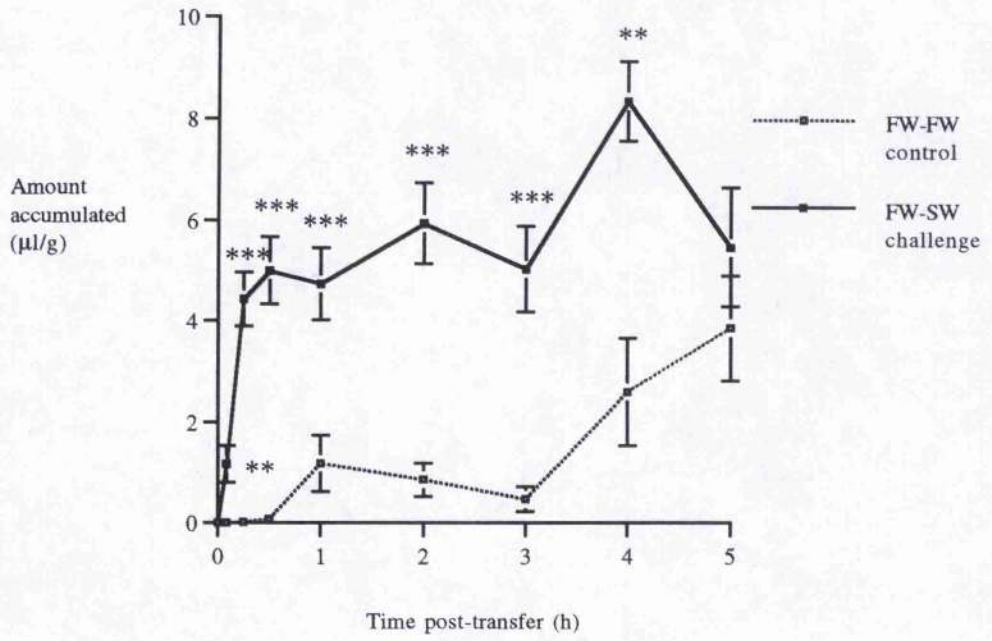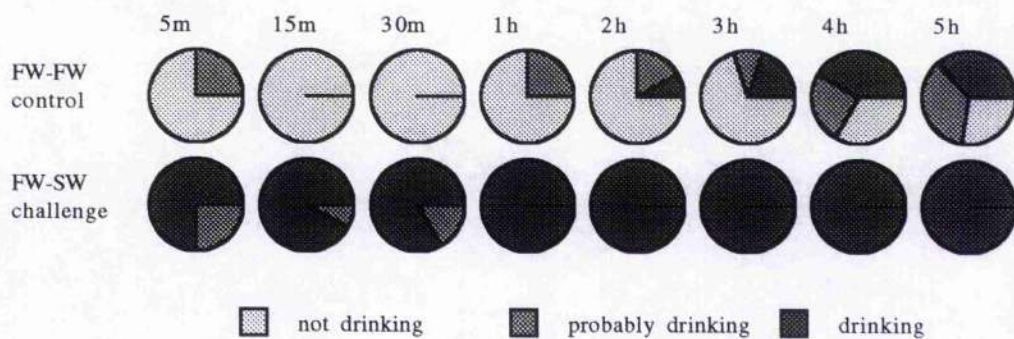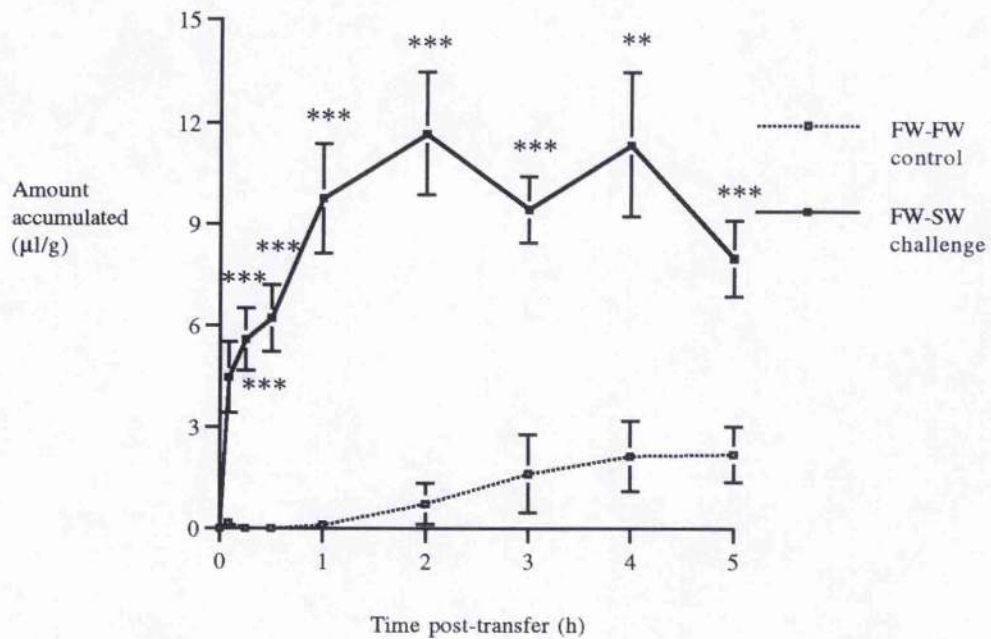
<u>Fig. 3.13a:</u>   <u>Drinking Rate Challenge - Month 4</u>

Amount of water accumulated (μl/g) in FW acclimated fish at Month 4 after acute challenge with SW and FW (n = 12, except FW-SW 1 h where n = 13; FW-FW 1 h, FW-FW 2 h, FW-FW 4 h, FW-FW 5 h, FW-SW 4 h and FW-SW 5 h where n = 14; FW-SW 30 min n = 15; and FW-FW 15 min and FW-SW 5 min where n = 11). Values shown are means ± SE. ** and *** indicate significant differences, at $p < 0.01$ and $p < 0.001$ respectively (t-test), between FW-FW and FW-SW values within time-points.

<u>Fig. 3.13b:</u>   <u>Drinking Rate Challenge - Month 4 - Proportion Drinking</u>

Proportion of fish at Month 4 during drinking rate challenge with SW and FW (n values as Fig. 3.13a), considered to be not drinking, probably drinking and drinking.

not drinking   probably drinking   drinking
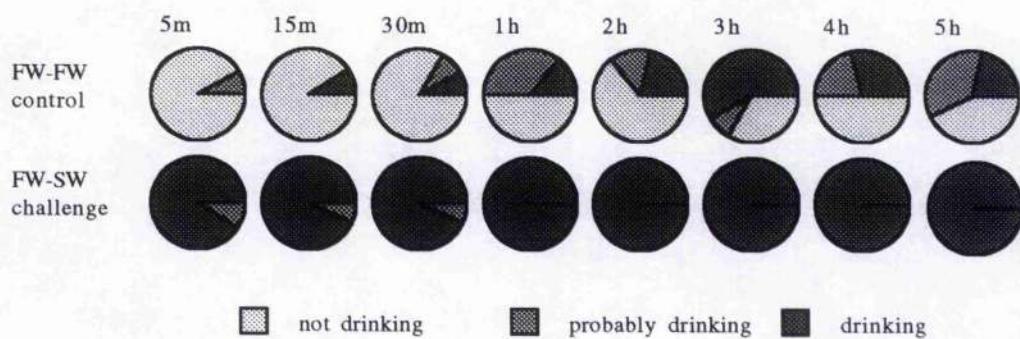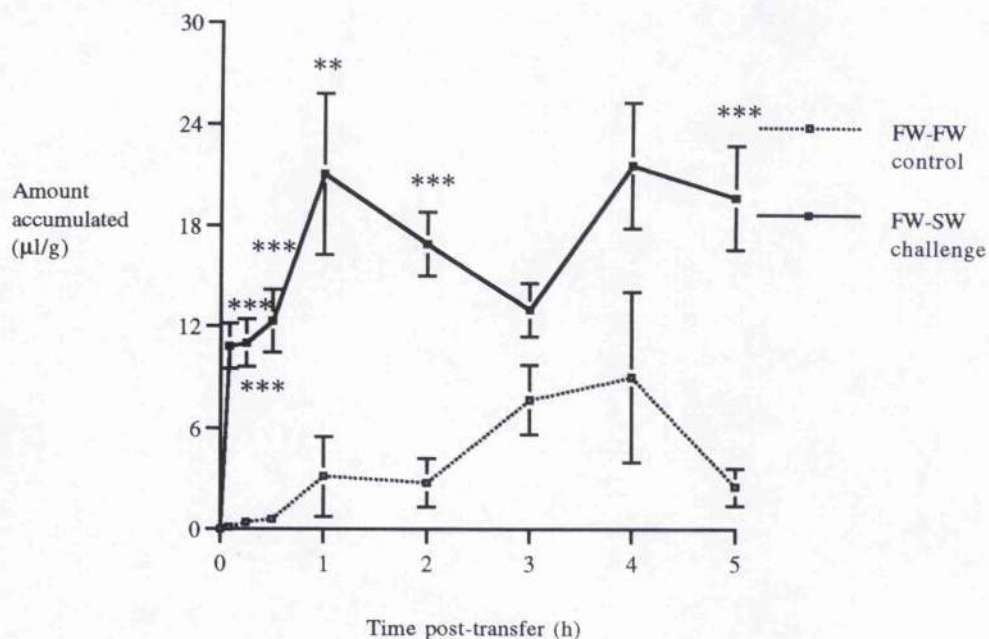
Fig. 3.14a: Drinking Rate Challenge - Month 5

Amount of water accumulated (µl/g) in FW acclimated fish at Month 5 after acute challenge with SW and FW (n = 12, except FW-FW 3 h where n = 14; FW-SW 1 h n = 15 and FW-FW 30 m, FW-FW 1 h = 11 and FW-SW 15 m where n = 10). ** and *** indicate significant differences, at $p < 0.01$ and $p < 0.001$ respectively (t-test), between FW-FW and FW-SW values within time-points.

Fig. 3.14b: Drinking Rate Challenge - Month 5 - Proportion Drinking

Proportion of fish at Month 5 during drinking rate challenge with SW and FW (n values as Fig. 3.14a), considered to be not drinking, probably drinking and drinking.
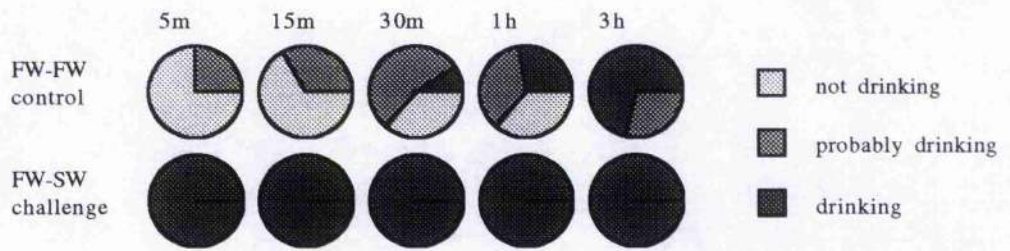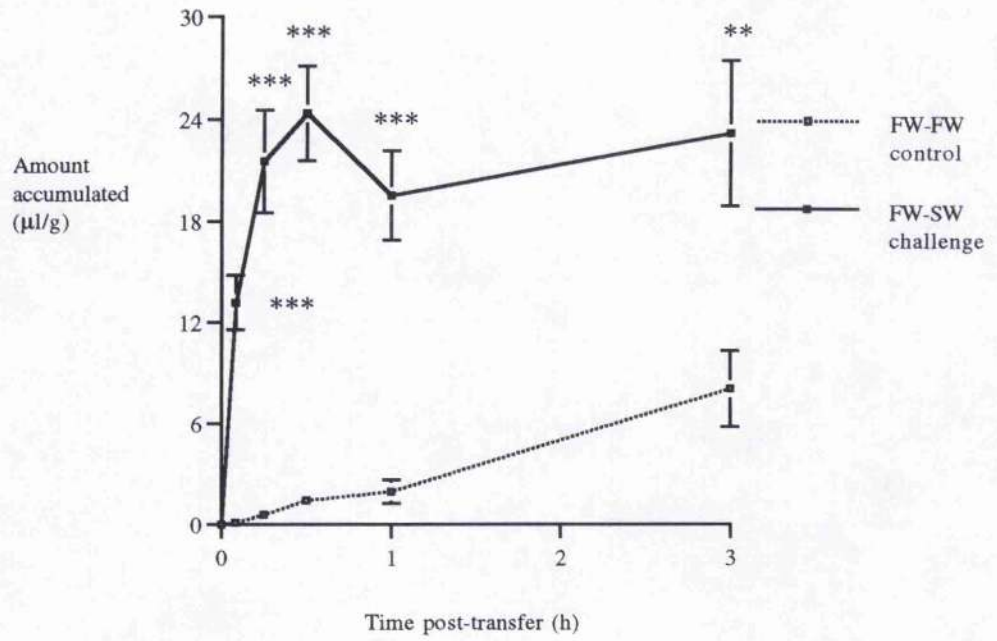
<u>Fig. 3.15:</u>   <u>Drinking Rate Challenge - Months 1 to 5 - Volume Accumulated at 15</u>
            <u>Minutes Post-transfer Calculated as Drinking Rate</u>

Drinking rates ($\mu l/g/h$) derived from the amount accumulated ($\mu l/g$) after 15m post-transfer to SW or FW at Months 1 to 5 (n = 11, except Month 1 FW-SW, Month 4 FW-SW and Month 5 FW-FW where n = 12; Month 3 FW-SW n = 13; Month 2 FW-SW n = 14 and Month 5 FW-SW where n = 10). Values shown are means ± SE. Mean (n = 20, except Months 4 and 5 where n = 21) and maximum drinking rates for SW acclimated fish at each month are also shown for comparison. *** indicates a significant difference, at $p < 0.001$, between FW-FW and FW-SW values (t-test) within months.
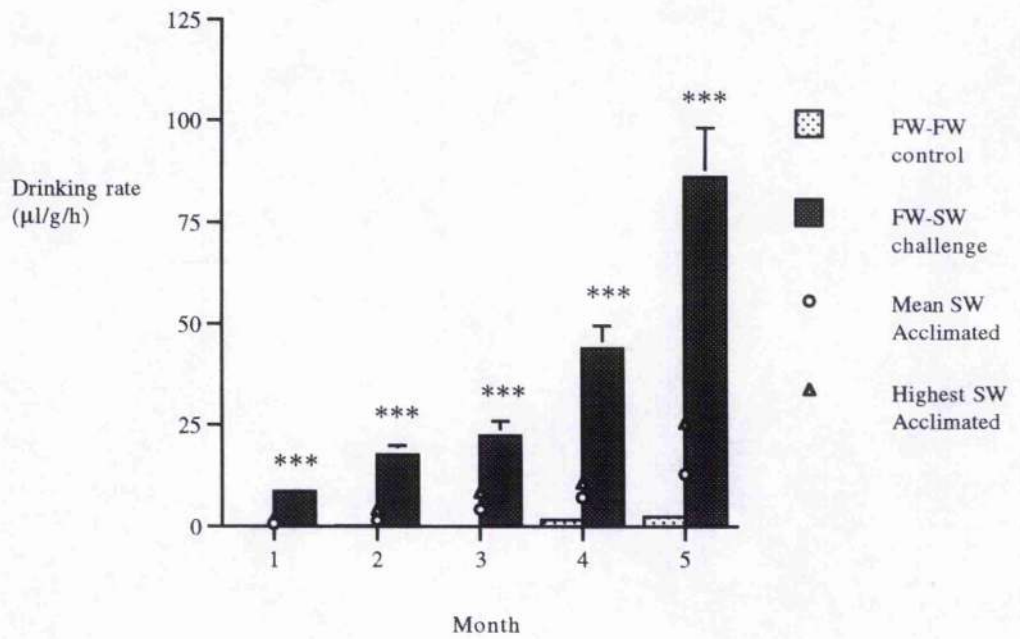
considered significantly higher than the control (Dunnett's $p < 0.01$). FW-SW values were significantly higher than those from the FW-FW group at all the time-points (t-test $p < 0.001$, except 3h where $p < 0.01$).

There was a clear increase in the proportion of fish considered definitely drinking (as described in section 2.4.2) in the FW-SW groups, as compared to the FW-FW control groups, throughout Months 1 to 5 (Fig. 3.10b to 3.14b). In addition, within the FW-SW groups, the proportion of fish considered drinking was noted to be higher, earlier in the investigation, over the course of Months 1 to 5. For example, at 5 min post-transfer to SW, 30.8%, 36.4%, 75%, 90.9% and 100% of fish were considered drinking at Months 1, 2, 3, 4 and 5 respectively.

In order to look at the acute drinking response between months, equivalent drinking rates were calculated from the 15 min post-transfer time-points for both the FW-FW and FW-SW groups (Fig. 3.15). It was clear that the FW-SW challenge groups showed an increased rate as compared to the FW-FW control group at all months. There were no significant differences between the FW-FW values over the course of Months 1 to 5 (ANOVA $p > 0.05$). Within the FW-SW group however there was a significant increase in drinking rate with time (ANOVA $p < 0.0001$), with Months 4 and 5 considered significantly higher (Dunnett's $p < 0.01$). FW-SW fish had significantly higher drinking rates than those from the FW-FW groups at all months (t-test $p < 0.001$). The FW-SW equivalent drinking rates were considerably higher than both the mean and maximum drinking rates observed in SW acclimated fish during Months 1 to 5.

3.3 Branchial Na⁺K⁺ATPase Activity

Typical standard curves used for the calculation of protein and inorganic phosphate concentrations can be found in Appendix 4.

3.3.1 Evaluation of the Method

3.3.1.1 Concentration of Ouabain

From the ouabain dose response curve (Fig. 3.16) it was found that concentrations above 10 μM within the assay incubation mixture were sufficient to maximally inhibit Na⁺K⁺ATPase activity. It was decided that a concentration of 0.2 mM would be more than adequate to entirely inhibit activity, and this was subsequently used in all further assays.

Fig. 3.16:   Ouabain Dose Response Curve

Total ATP hydrolysis with varying ouabain concentrations in the assay. Values shown
are means ± SE. Samples were incubated for 1h at 24°C.

Total ATP
hydrolysis
(nmoles/mg
protein/h)

Log dose [ouabain] (M)
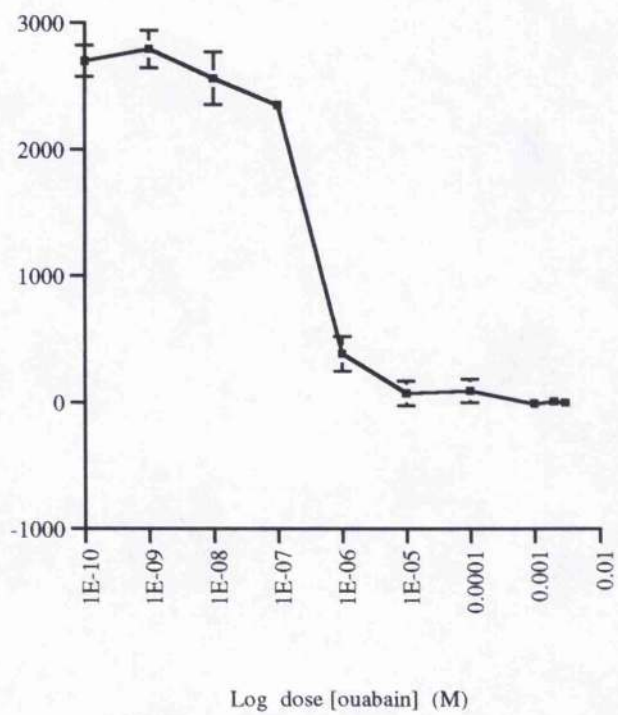
<u>Fig. 3.17:</u>   <u>Na$^+$K$^+$ATPase Assay Incubation Time Course</u>

Na$^+$K$^+$ATPase activity (nmoles/mg protein) time course showing samples incubated with 0.2 mM ouabain at 24 °C for various time-periods. Values shown are means ± SE.

Na$^+$K$^+$ATPase activity (nmoles/mg protein)

Time (m)
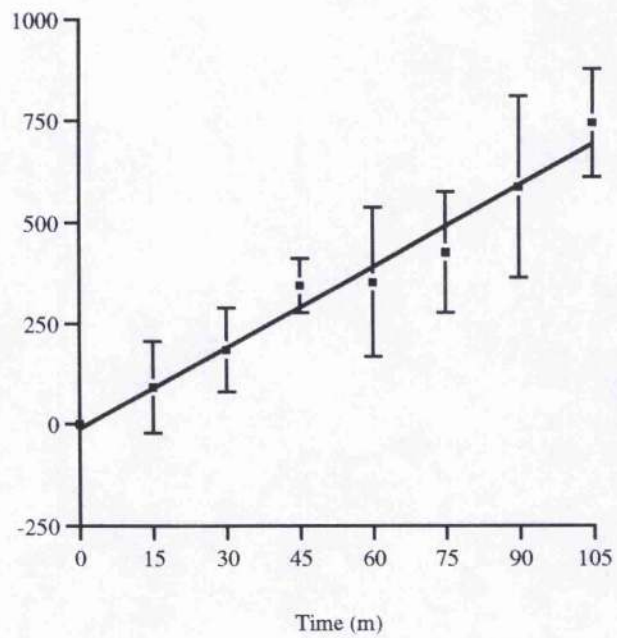
3.3.1.2 Enzyme Activity Time Course

The incubation time-course graph (Fig. 3.17) illustrates that the $Na^+K^+ATPase$ enzyme activity remains linear for at least 105 minutes. Routine experiments were therefore conducted using incubations of 1 hour with confidence that a reliable estimation of activity could be made.

3.3.2 Gill Homogenate Protein Concentration

3.3.2.1 Environmental Acclimation Studies

There were no significant changes in homogenate protein concentration with changes in environmental salinity in both Month 1 and Month 2 acclimated fish (ANOVA $p > 0.05$). (Protein concentrations from acclimated fish at Month 1 were as follows: FW = 0.287 ± 0.016, 33% SW = 0.293 ± 0.135, 66% SW = 0.304 ± 0.008 and SW = 0.311 ± 0.008 mg/ml, and at Month 2: FW = 0.265 ± 0.010, 33% SW = 0.230 ± 0.009, 66% SW = 0.248 ± 0.021 and SW = 0.270 ± 0.012 mg/ml.) All values are means $\pm$ SE.

However, over the course of Months 1 to 4 (Fig. 3.18) gill homogenate protein concentrations (mg/ml per sample) were found to alter significantly with time, within both the FW and SW acclimated groups (ANOVA $p < 0.0001$ and $p < 0.001$ respectively). Within the FW acclimated group Months 3 and 4 were significantly lower than Month 1 (Dunnett's $p < 0.01$), and within the SW acclimated group the Month 3 time-point was considered significantly lower than that of Month 1 (Dunnett's $p < 0.05$). Protein concentration was significantly higher in SW acclimated fish as compared to fish from FW at Months 3 and 4 (t-test $p < 0.05$ and $p < 0.001$ respectively).

3.3.2.2 FW-SW Transfer Studies

During the FW-SW transfer experiment gill homogenate protein concentration was also noted to vary with time, within both the FW-FW and the FW-SW groups (Fig. 3.19). Within the FW-FW group there was a significant decrease in protein concentration with time (ANOVA $p < 0.0001$), where the 4 month time-point was considered significantly lower than the control (Dunnett's $p < 0.01$). Within the FW-SW group however, there was a significant increase in protein concentration with time (ANOVA $p < 0.0001$), although the final 4 month time-point was in fact lower. Within the FW-SW group the 21 d and 2 month time-points were considered significantly higher than the control (Dunnett's $p < 0.05$). Gill homogenate protein concentration was significantly higher in FW-SW fish as compared to those from the FW-FW control group at 14 d ($p < 0.001$), 21 d ($p < 0.01$), 2 months ($p < 0.001$), 3 months ($p < 0.001$) and 4 months post-

Fig. 3.18:   Environmental Acclimation Studies - Gill Homogenate Protein
               Concentration - Months 1 to 4

Gill homogenate protein concentration (mg/ml) per sample (5 pairs gills) in fish at
Months 1 to 4 acclimated to FW and SW (n = 6 except Month 3 SW and Month 4 SW
where n = 5). Values shown are means ± SE. * and *** indicate significant differences,
at $p < 0.05$ and $p < 0.001$ respectively (t-test), between FW and SW acclimated fish
within months.

<u>Fig. 3.19:</u>   <u>FW-SW Transfer Studies - Gill Homogenate Protein Concentration</u>

Gill homogenate protein concentration (mg/ml) per sample (5 pairs gills) during the FW-SW transfer experiment (n = 6 except FW-FW 10d and FW-SW 4 Months where n = 5). Values shown are means ± SE. ** and *** indicate significant differences, at $p < 0.01$ and $p < 0.001$ respectively (t-test), between FW-FW and FW-SW values within timepoints. See experimental rational section 2.3.3 for details of history of these fish.
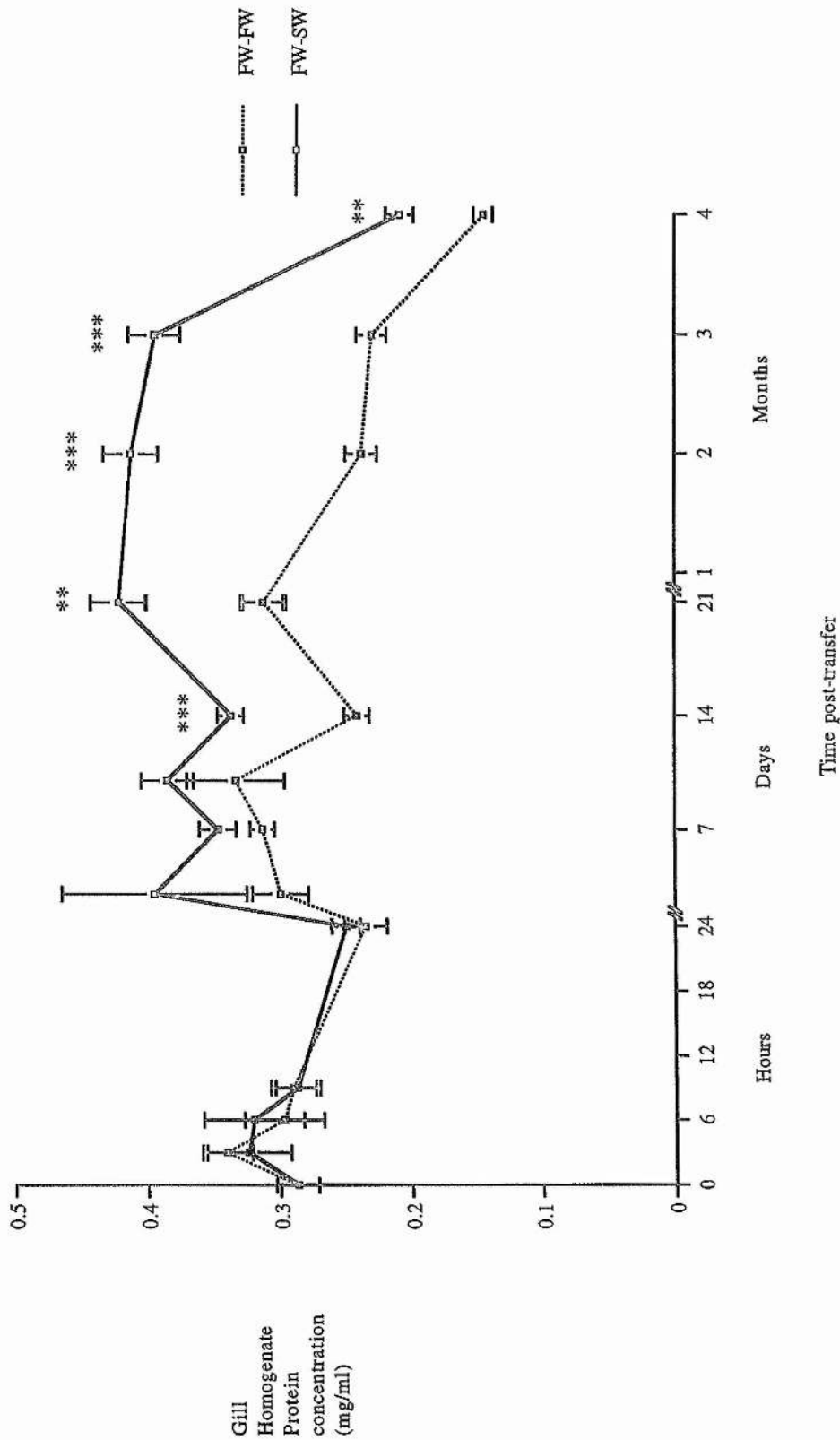
transfer ($p < 0.01$), despite the drop in the FW-SW value at 4 months post-transfer (as determined by unpaired t-test analyses).

Given these significant changes in protein concentration between FW and SW acclimated fish at Months 3 and 4, and within the FW-SW transfer experiment at all the time-points between 14 d and 4 months post-transfer, the $Na^+K^+ATPase$ results are presented here as nmoles/5 pairs gills/h, i.e. per sample. The conventional method of expression as nmoles/mg protein/h would have resulted in underestimates in differences in $Na^+K^+ATPase$ activity where there were differences in protein concentration. This will be examined further in Chapter 4, the Discussion.

3.3.3   Na⁺K⁺ATPase Activity

3.3.3.1 Environmental Acclimation Studies

Acclimated fish at Month 1 (Fig. 3.20) and Month 2 (Fig. 3.21) showed no significant changes in $Na^+K^+ATPase$ activity (nmoles/5 pairs gills/h) with changes in environmental salinity (ANOVA $p > 0.05$).

Over the course of Months 1 to 4 (Fig. 3.22) there was a significant reduction in the $Na^+K^+ATPase$ activity of FW acclimated fish after Month 1 (ANOVA $p < 0.0001$), where Months 2, 3 and 4 were found to be significantly lower than Month 1 (Dunnett's $p < 0.01$). Within the SW acclimated group there was also a reduction in $Na^+K^+ATPase$ activity after Month 1, although at Month 4 this had risen to above the Month 1 SW level (ANOVA $p < 0.0001$). This increase in activity within the SW acclimated group at Month 4 was considered significant (Dunnett's $p < 0.01$). At Month 4 the SW acclimated fish had significantly higher levels of $Na^+K^+ATPase$ activity than the FW acclimated fish (t-test $p < 0.01$).

3.3.3.2 FW-SW Transfer Studies

During the FW-SW transfer experiment (Fig. 3.23) $Na^+K^+ATPase$ activities (nmoles/5 pairs gills/h) were found to decrease significantly with time in the FW-FW control group (ANOVA $p < 0.0001$). $Na^+K^+ATPase$ activities were significantly lower than the control at 24 h, 3 d, 3 months and 4 months post-transfer (Dunnett's $p < 0.01$). Within the FW-SW group there was trend towards increased $Na^+K^+ATPase$ activity after around 7 d post-transfer which peaked at 2 months post-transfer, before returning to the original lower level by 4 months post-transfer (ANOVA $p < 0.0001$). The peak in $Na^+K^+ATPase$ activity at 2 months post-transfer was considered significant (Dunnett's $p < 0.01$). During the first 24 hours post-transfer there were fluctuations in $Na^+K^+ATPase$

<u>Fig. 3.20:</u>   <u>Environmental Acclimation Studies - Na$^+$K$^+$ATPase Activity - Month 1</u>

Na$^+$K$^+$ATPase activity (nmoles/5 pairs gills/h) in fish at Month 1 acclimated to FW, 33%
SW, 66% SW and SW (n = 6). Values shown are means ± SE. There were no
significant differences in Na$^+$K$^+$ATPase activity with changes in environmental salinity
(ANOVA $p > 0.05$).

Na$^+$K$^+$ATPase
activity
(nmoles/5 pairs
gills/h)
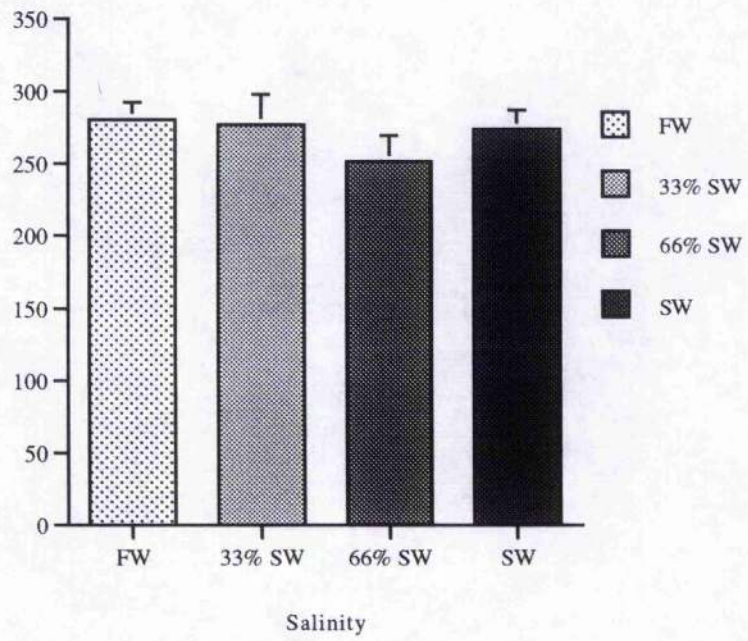
Fig. 3.21:   Environmental Acclimation Studies - Na+K+ATPase Activity - Month 2

Na+K+ATPase activity (nmoles/5 pairs gills/h) in fish at Month 2 acclimated to FW, 33% SW, 66% SW and SW (n = 6). Values shown are means ± SE. There were no significant differences in Na+K+ATPase activity with changes in environmental salinity (ANOVA $p > 0.05$).

Na$^+$K$^+$ATPase activity (nmoles/5 pairs gills/h)

Salinity

FW
33% SW
66% SW
SW
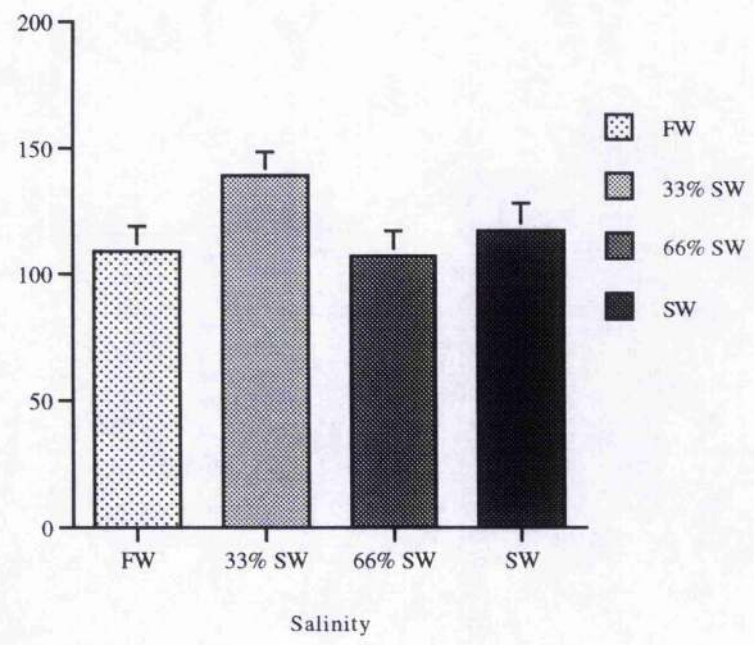
Fig. 3.22:   Environmental Acclimation Studies - Na^+K^+ATPase Activity - Months 1 to 4

Na^+K^+ATPase activity (nmoles/5 pairs gills/h) in fish at Months 1 to 4 acclimated to FW and SW (n = 6 except Month 3 SW and Month 4 SW where n = 5). Values shown are means ± SE, where error bars are not visible this is because they are too small to print at this scale. ** indicates a significant difference, at $p < 0.01$ (t-test), between FW and SW acclimated fish within months.

Na$^+$K$^+$ATPase
activity
(nmoles/5 pairs
gills/h)
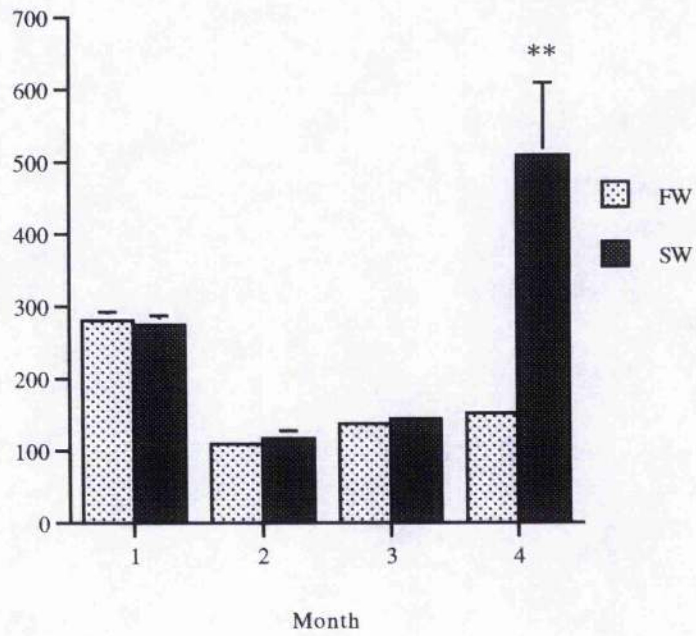
<u>Fig. 3.23:</u>   <u>FW-SW Transfer Studies - Na$^+$K$^+$ATPase Activity</u>

Na$^+$K$^+$ATPase activity (nmoles/5 pairs gills/h) during the FW-SW transfer experiment (n = 6 except FW-FW 10 d and FW-SW 4 Months where n = 5). Values shown are means ± SE. Where error bars are not visible this is because they are too small to print at this scale. *, ** and *** indicate significant differences, at $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively (t-test), between FW-FW and FW-SW values within time-points.
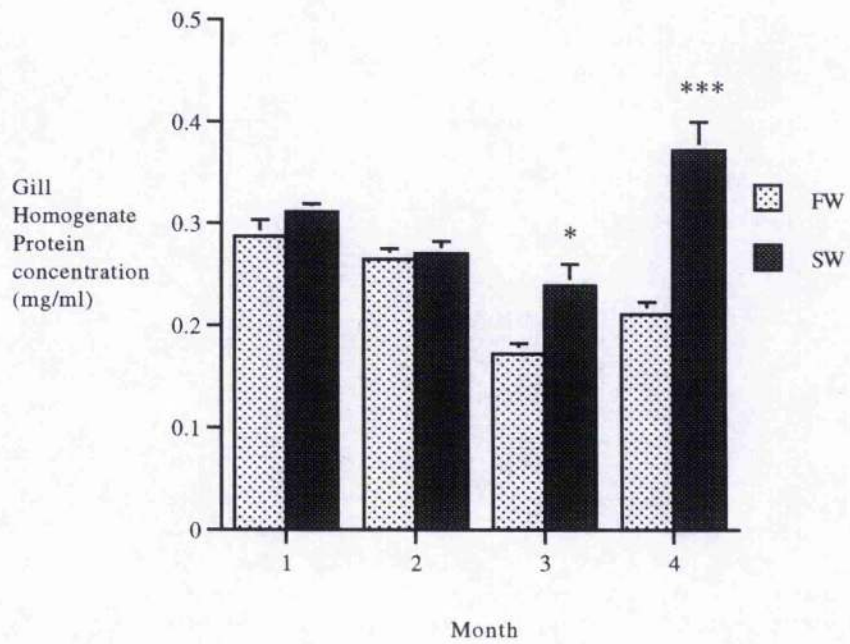
FW-FW
FW-SW

Na⁺K⁺ATPase activity (nmoles/5 pairs gills/h)

Time post-transfer

Hours   Days   Months

900
800
700
600
500
400
300
200
100
0

0   6   12   18   24   7   14   21 1   2   3   4

*
*
**
***
*
*
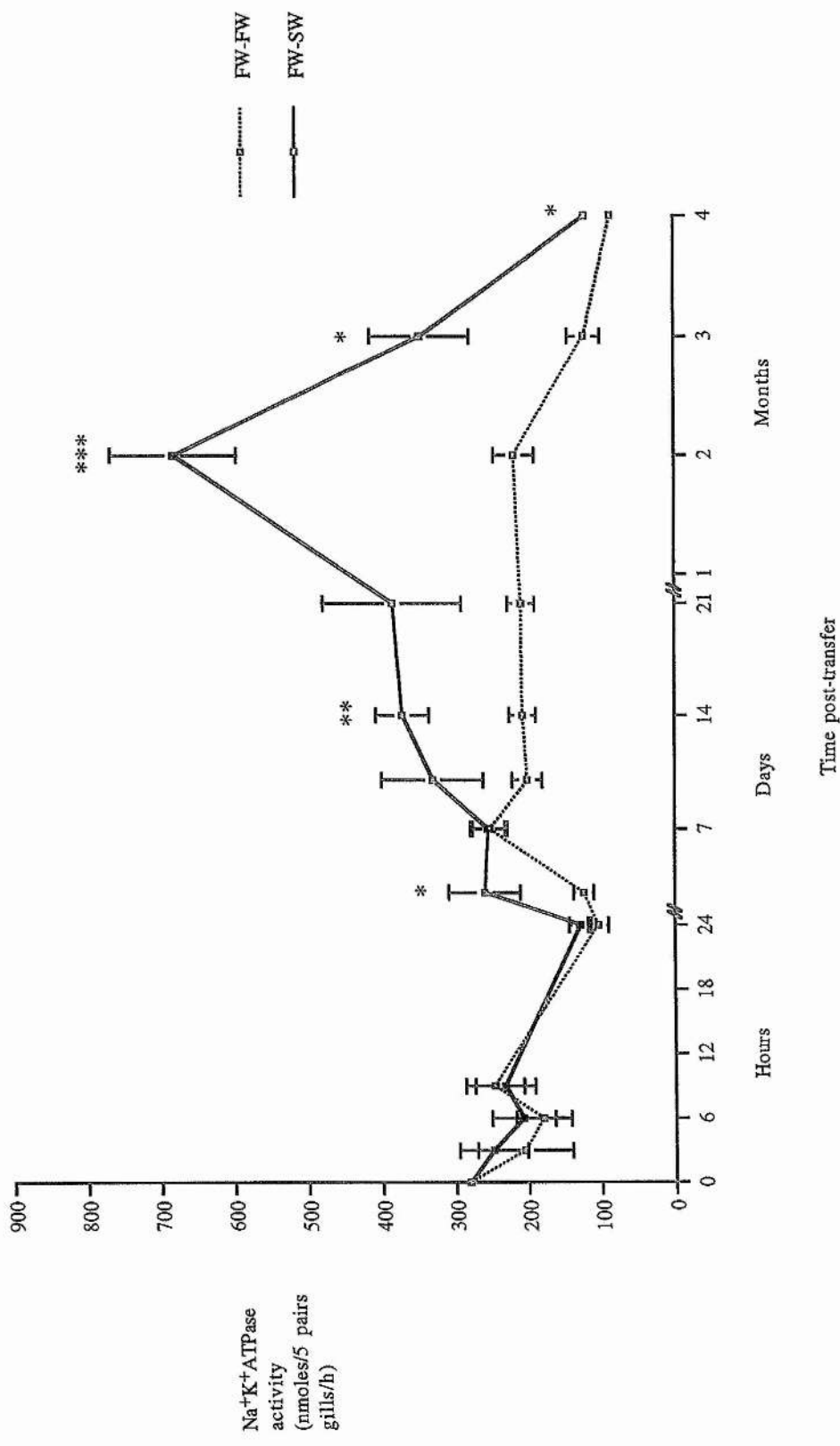
activity, with the FW-FW and FW-SW groups following each other quite closely, however the FW-SW values were significantly higher than FW-FW values at 3 d ($p < 0.05$), 14 d ($p < 0.01$), 2 months ($p < 0.001$), 3 months ($p < 0.05$) and 4 months ($p < 0.05$) post-transfer (as determined by t-test analyses).

3.4   Na$^+$ and K$^+$ Content

Typical standard curves used for the calculation of Na$^+$ and K$^+$ concentrations can be found in Appendix 4.

3.4.1   Environmental Acclimation Studies

There were no significant differences in either total Na$^+$ content (Fig 3.24a), nor total K$^+$ content (Fig 3.24b) of fish (μmoles/g), with changes in environmental salinity (ANOVA $p > 0.05$) in acclimated fish at Month 1.

Total body Na$^+$ content (μmoles/g) in fish at Month 2 (Fig 3.25a) acclimated to FW, 33% SW, 66% SW and SW was found to increase significantly with salinity (ANOVA $p < 0.0001$), with the 66% SW and SW groups considered significantly higher than fish from FW (Dunnett's $p < 0.01$).

There were no significant differences in total K$^+$ content (μmoles/g) with changes in environmental salinity (ANOVA $p > 0.05$) in acclimated fish at Month 2 (Fig 3.25b).

Total body Na$^+$ content (μmoles/g) within the FW acclimated fish was found to increase significantly (ANOVA $p < 0.0001$) over the course of Months 1 to 5 (Fig. 3.26a). Values obtained at Month 4 (Dunnett's $p < 0.01$) and Month 5 (Dunnett's $p < 0.05$) were considered significantly higher than those from Month 1. Within the SW acclimated fish Na$^+$ concentrations also increased significantly with time (ANOVA $p < 0.0001$), with the Month 3, 4 and 5 time-points considered significantly higher than Month 1 (Dunnett's $p < 0.01$). The SW acclimated fish were found to have a significantly higher total Na$^+$ content as compared to those from FW at Months 2 ($p < 0.0001$), 3 ($p < 0.01$) and 5 ($p < 0.001$), as determined by unpaired t-test analyses.

Total body K$^+$ content (μmoles/g) within the FW acclimated group was found to increase significantly (ANOVA $p < 0.0001$) over the course of Months 1 to 5 (Fig. 26b). Values obtained from fish at Months 4 and 5 were considered significantly higher than those from Month 1 (Dunnett's $p < 0.01$). Within the SW acclimated group K$^+$ content also increased significantly over the course of Months 1 to 5 (ANOVA $p < 0.0001$), with Months 3, 4 and 5 considered significantly higher than Month 1 (Dunnett's $p < 0.01$).

Fig. 3.24a: Environmental Acclimation Studies - Na$^+$ Content - Month 1

Total body Na$^+$ content (μmoles/g) in fish at Month 1 acclimated to FW, 33% SW, 66%
SW and SW (n = 8 except 66% SW where n = 6). Values shown are means $\pm$ SE.
Where error bars are not visible this is because they are too small to print at this scale.
There were no significant differences in Na$^+$ content with changes in environmental
salinity (ANOVA $p > 0.05$).

Fig. 3.24b: Environmental Acclimation Studies - K$^+$ Content - Month 1

Total body K$^+$ content (μmoles/g) in fish at Month 1 acclimated to FW, 33% SW, 66%
SW and SW (n = 8 except 66% SW where n = 6). Values shown are means $\pm$ SE.
Where error bars are not visible this is because they are too small to print at this scale.
There were no significant differences in K$^+$ content with changes in environmental
salinity (ANOVA $p > 0.05$).
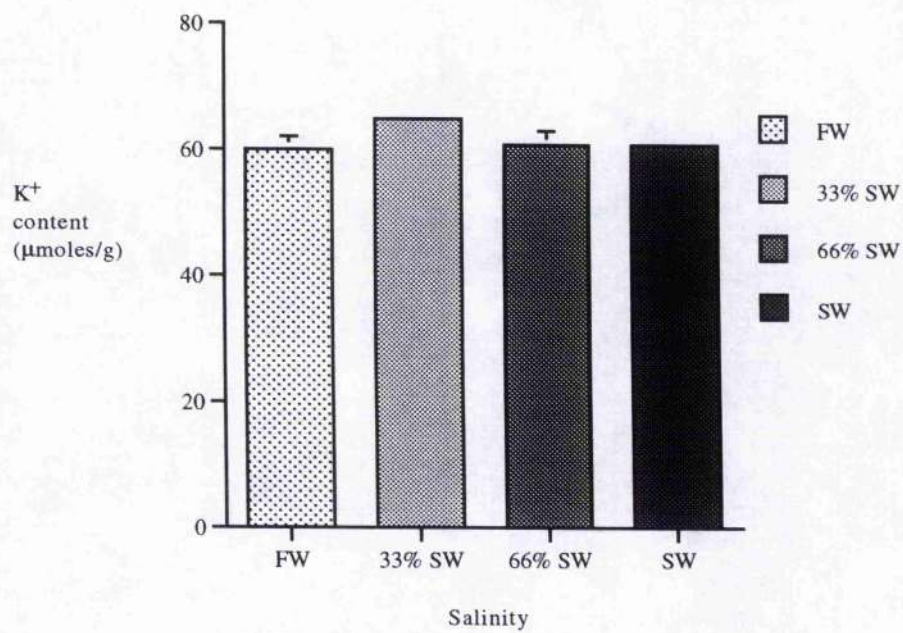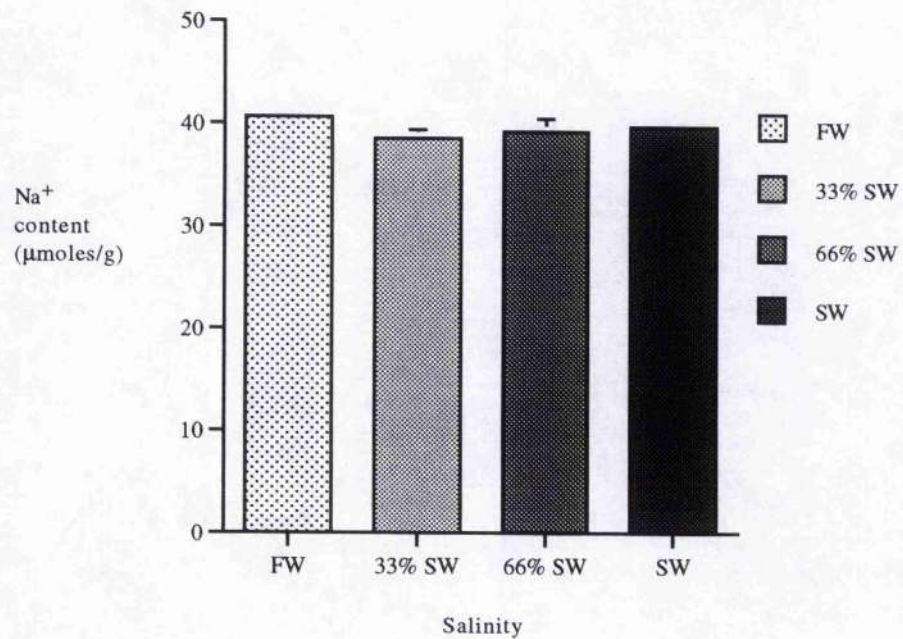
<u>Fig. 3.25a:</u>   Environmental Acclimation Studies - Na$^+$ Content - Month 2

Total body Na$^+$ content (μmoles/g) in fish at Month 2 acclimated to FW, 33% SW, 66% SW and SW (n = 8). Values shown are means ± SE. Where error bars are not visible this is because they are too small to print at this scale. ** indicates a significant difference, at $p < 0.01$ (Dunnett's), when compared to FW acclimated fish.

<u>Fig. 3.25b:</u>   Environmental Acclimation Studies - K$^+$ Content - Month 2

Total body K$^+$ content (μmoles/g) in fish at Month 2 acclimated to FW, 33% SW, 66% SW and SW (n = 8). Values shown are means ± SE. Where error bars are not visible this is because they are too small to print at this scale. There were no significant differences in K$^+$ content with changes in environmental salinity (ANOVA $p > 0.05$).
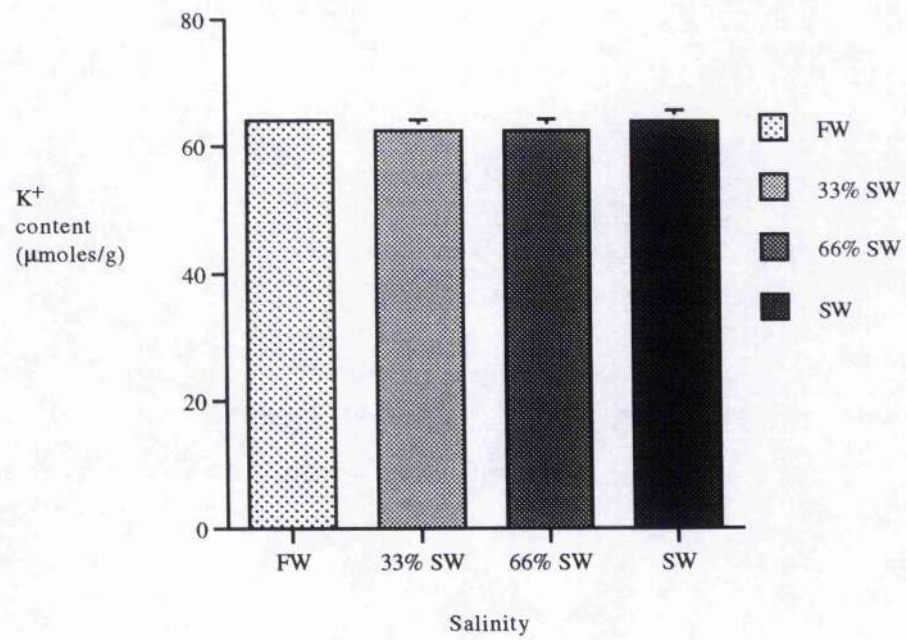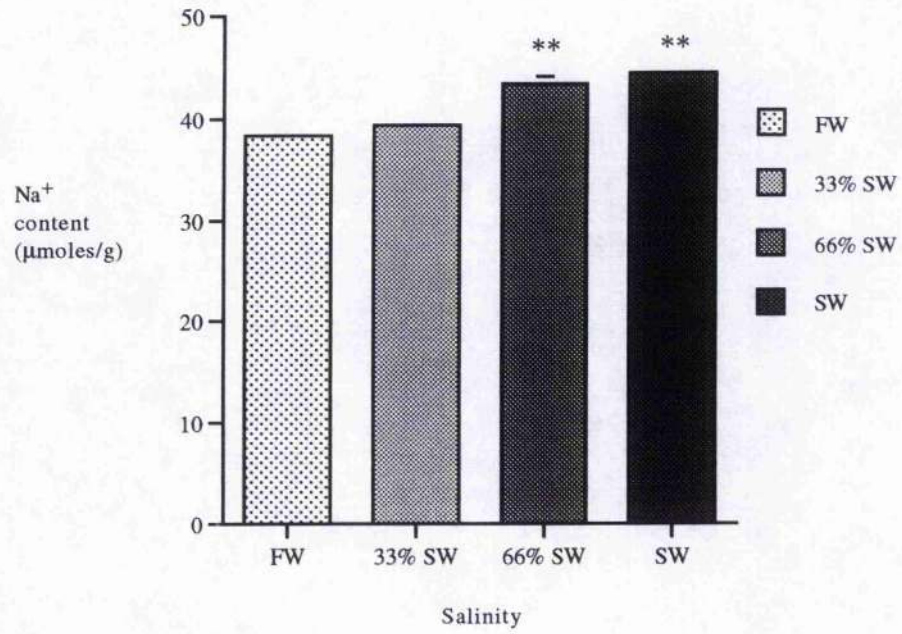
Fig. 3.26a: Environmental Acclimation Studies - Na$^+$ Content - Months 1 to 5

Total body Na$^+$ content (μmoles/g) in fish at Months 1 to 5 acclimated to FW and SW (n = 8 except Month 4 SW where n = 7). Values shown are means ± SE. Where error bars are not visible this is because they are too small to print at this scale. ** and *** indicate significant differences, at $p < 0.01$ and $p < 0.001$ respectively (t-test), between FW and SW acclimated fish within months.

Fig. 3.26b: Environmental Acclimation Studies - K$^+$ Content - Months 1 to 5

Total body K$^+$ content (μmoles/g) in fish at Months 1 to 5 acclimated to FW and SW (n = 8 except Month 4 SW where n = 7). Values shown are means ± SE. Where error bars are not visible this is because they are too small to print at this scale. * indicates a significant difference, at $p < 0.05$ (t-test), between FW and SW acclimated fish within months.
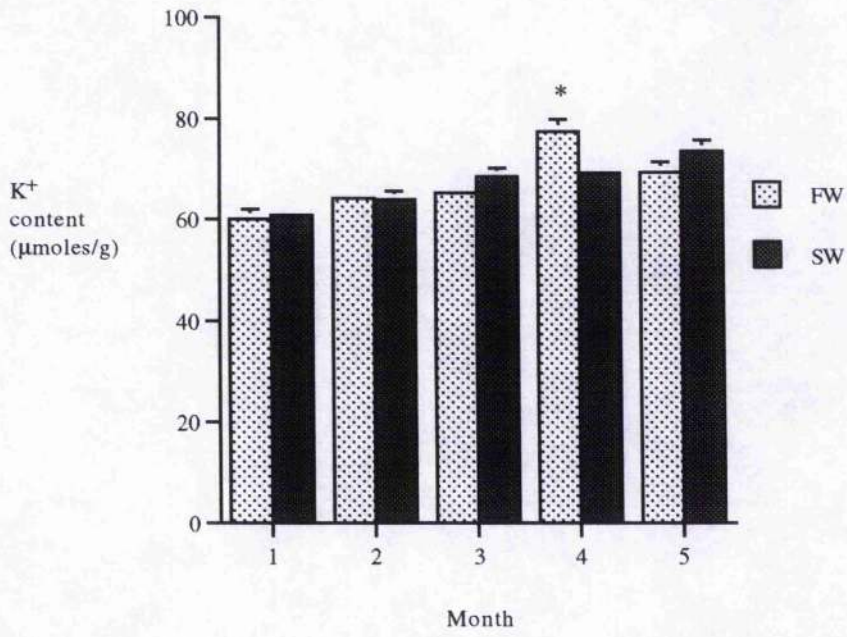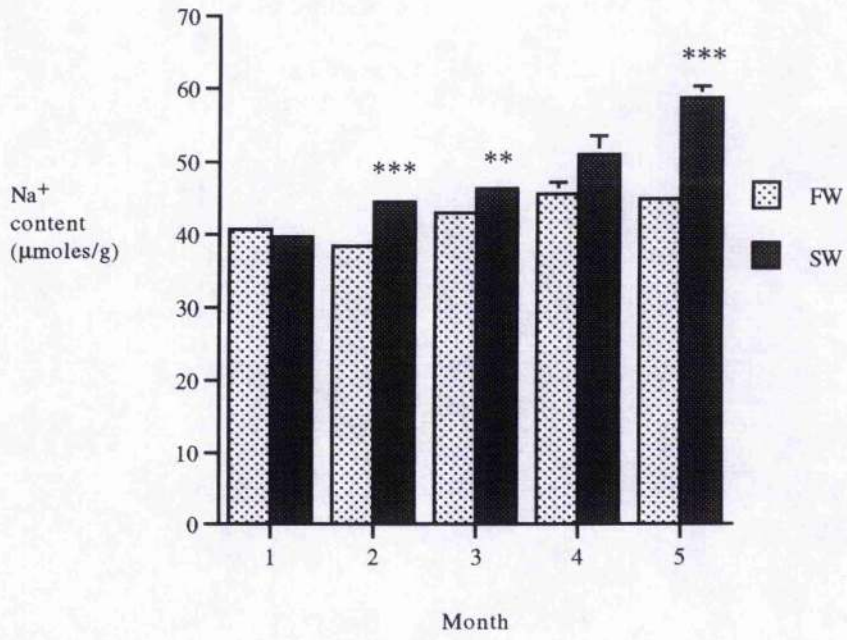
Fig. 3.27:   FW-SW Transfer Studies - Na+ Content

Total body Na+ content (µmoles/g) during the FW-SW transfer experiment (n = 8, except
FW-SW 3 h; FW-SW 6 h; FW-SW 7 d; FW-SW 10 d and FW-SW 2 Months where n =
7, and FW-FW 6 h and FW-SW 24 h where n = 6). Values shown are means ± SE.
Where error bars are not visible this is because they are too small to print at this scale. *,
** and *** indicate significant differences, at $p < 0.05$, $p < 0.01$ and $p < 0.001$
respectively (t-test), between FW-FW and FW-SW values within time-points.

Na+ content (μmoles/g) vs Time post-transfer (Hours, Days, Months)
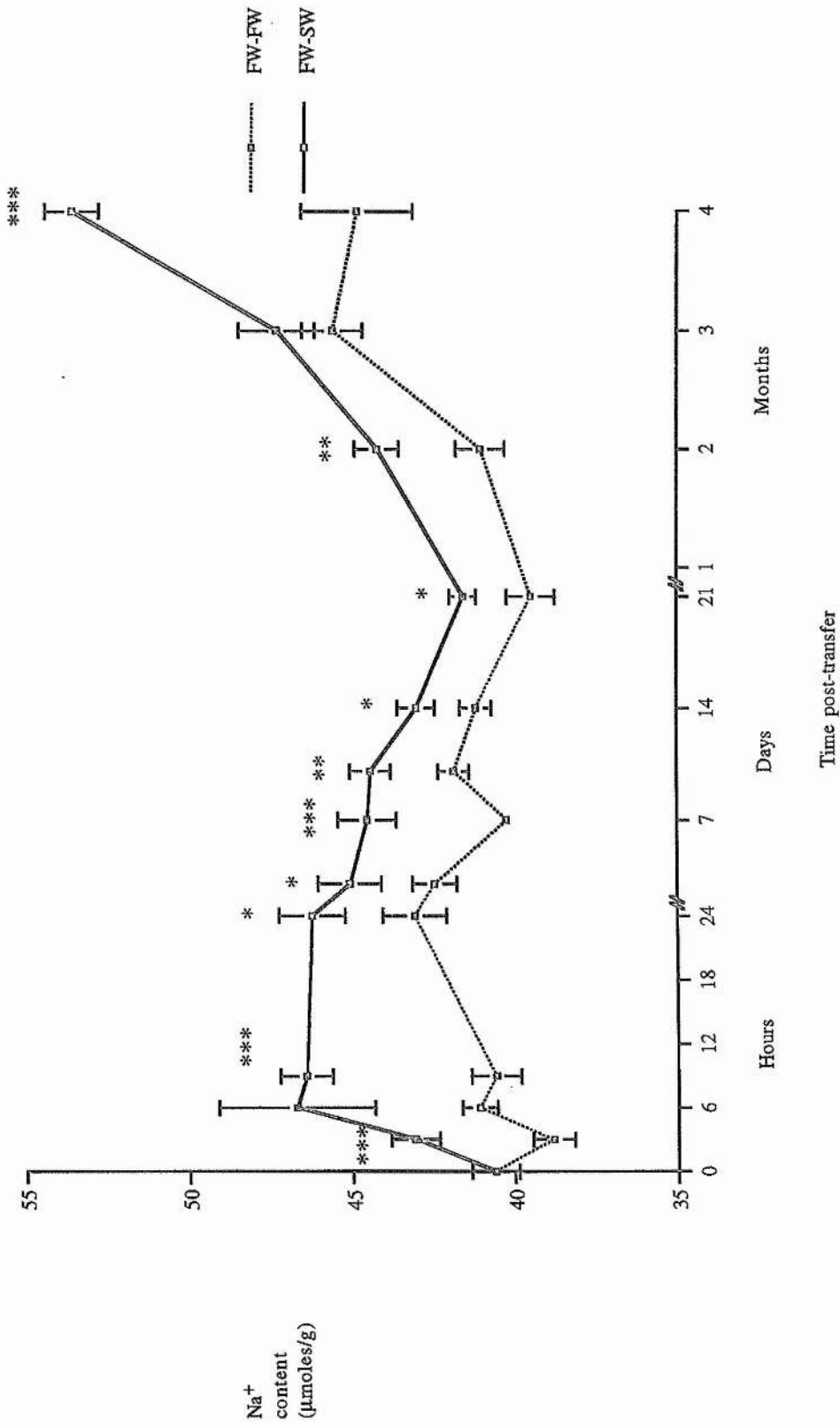
FW-FW
FW-SW

<u>Fig. 3.28:</u>   <u>FW-SW Transfer Studies - K⁺ Content</u>

Total body K^+ content (μmoles/g) during the FW-SW transfer experiment (n = 8, except FW-SW 3 h; FW-SW 6 h; FW-SW 7 d; FW-SW 10 d and FW-SW 2 Months where n = 7, and FW-FW 6 h and FW-SW 24 h where n = 6). Values shown are means ± SE. * and *** indicate significant differences, at $p < 0.05$ and $p < 0.001$ respectively (t-test), between FW-FW and FW-SW values within time-points.
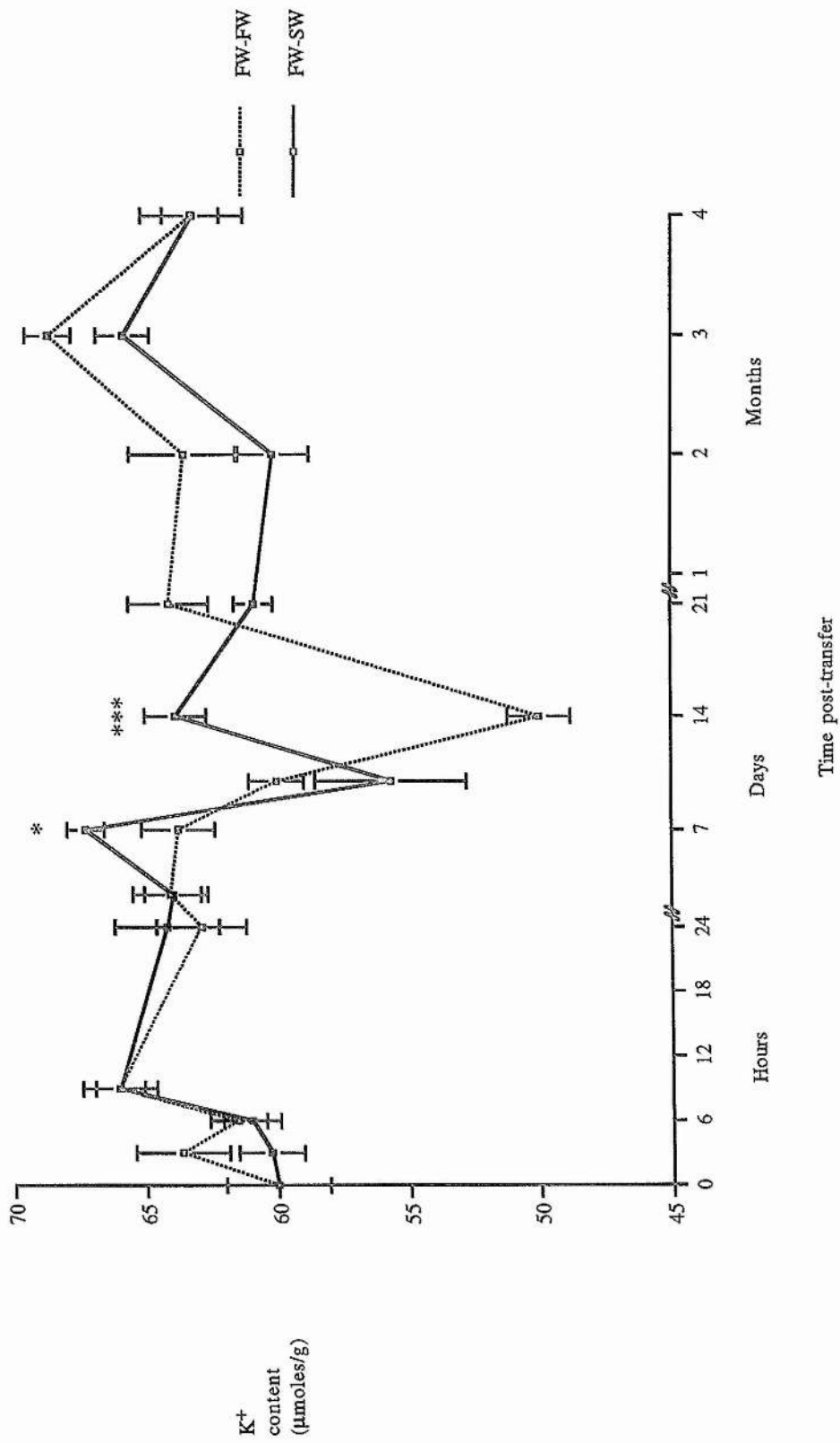
At Month 4 the FW acclimated group was found to have a significantly higher total K^+ content than the SW acclimated fish (t-test $p < 0.05$).

3.4.2 FW-SW Transfer Studies

During the FW-SW transfer experiment (Fig. 3.27) total body Na^+ content (μmoles/g) was found to increase with time within the FW-FW group (ANOVA $p < 0.0001$), with the 3 and 4 month time-points considered significantly higher than the control (Dunnett's $p < 0.01$). Within the FW-SW group there was an increase in Na^+ content during the first 24 h post-transfer, followed by a decrease until 21 d, which was then followed by a further, more marked increase until 4 months post-transfer (ANOVA $p < 0.0001$). Values for the 6 h ($p < 0.01$), 9 h ($p < 0.01$), 24 h ($p < 0.01$), 3 d ($p < 0.05$), 3 month ($p < 0.01$) and 4 month ($p < 0.01$) time-points were considered significantly higher than the control (as determined by Dunnett's analyses). Na^+ content was found to be significantly higher in the FW-SW group as compared to the FW-FW group at 3 h ($p < 0.001$), 9 h ($p < 0.001$), 24 h ($p < 0.05$), 3 d ($p < 0.05$), 7 d ($p < 0.001$), 10 d ($p < 0.01$), 14 d ($p < 0.05$), 21 d ($p < 0.05$), 2 months ($p < 0.01$) and 4 months ($p < 0.001$) post-transfer (as determined by unpaired t-test analyses).

During the FW-SW transfer experiment there were no clear trends in total body K^+ content (μmoles/g) within either the FW-FW nor FW-SW groups (Fig. 3.28), although differences within each of the groups were considered significant (ANOVA $p < 0.0001$). Within the FW-FW group there was a significant decrease in K^+ content at 14 d post-transfer (Dunnett's $p < 0.01$), and also significant increases at 9 h and 3 months post-transfer (Dunnett's $p < 0.05$ and $p < 0.01$ respectively). Within the FW-SW group there were significant increases in K^+ content at 9 h ($p < 0.05$), 7 d ($p < 0.01$) and 3 months ($p < 0.05$) post-transfer (as determined by Dunnett's analyses). FW-SW values were considered significantly higher than those from the FW-FW group at 7 d and 14 d post-transfer (t-test $p < 0.05$ and $p < 0.0001$ respectively).

3.5 Cortisol Content

A typical standard curve used for the calculation of cortisol concentrations can be found in Appendix 4.

3.5.1 Extraction Efficiency

Recovery of cortisol from the homogenate (see section 2.7.2.3 for details of the procedure) was determined as 94.79 ± 1.48 % from the first extraction (n = 7), and 3.81 ± 0.32 % from the second extraction (n = 7). After resuspension in the RIA assay buffer the extraction efficiency was determined as 90.92 ± 2.45 % from the first extraction (n =

6) and 3.87 ± 0.44 %, from the second extraction (n = 7). As, even after resuspension for use in the RIA, the first extraction efficiency was around 90%, the second extraction was not performed in subsequent preparations.

3.5.2 Environmental Acclimation Studies

There was a significant trend for whole body cortisol content (pg/g) to increase with environmental salinity in acclimated fish at Month 1 (Fig. 3.29) (ANOVA $p < 0.0001$). Cortisol contents from the 66% SW and SW groups were considered significantly higher than those from the FW acclimated fish (Dunnett's $p < 0.01$).

However, in acclimated fish at Month 2 (Fig. 3.30) there were no significant differences in whole body cortisol content with increased salinity (ANOVA $p > 0.5$). All the groups had levels similar to the 33% SW acclimated fish at Month 1.

Within both the FW and SW acclimated groups there were no clear trends in cortisol content over the course of Months 1 to 5 (Fig. 3.31). Within the FW group there was an increase in cortisol content from Months 1 to 3 inclusive, which then dropped and rose again during Months 4 and 5 respectively. These differences within the FW acclimated group were considered significant (ANOVA $p < 0.001$), and the Month 3 and Month 5 values were considered significantly higher than those obtained for FW acclimated fish at Month 1 (Dunnett's $p < 0.01$). Within the SW acclimated group there was an initial drop in cortisol content after Month 1 which then rose again over Months 2, 3 and 4 to stay elevated at Month 5. These differences between Months were also considered significant (ANOVA $p < 0.01$), although Dunnett comparisons of each SW time-point with SW acclimated fish at Month 1 SW considered none to be significantly different ($p > 0.05$). Whole body cortisol content was found to be significantly elevated in SW acclimated fish as compared to FW acclimated fish at Months 1 (t-test $p < 0.0001$) and 4 (t-test $p < 0.001$).

3.5.3 FW-SW Transfer Studies

During the FW-SW transfer experiment there were significant increases in cortisol content by 3 h post-transfer within both the FW-FW and FW-SW groups as compared to the control (Fig. 3.32). Within both the FW-FW and FW-SW groups there were significant differences in cortisol content between time-points (ANOVA $p < 0.0001$), although the response was more marked in the FW-SW group. Cortisol content then began to decrease in both groups after 9 h post-transfer, and then fluctuated around the 24 h level until the 4 month time-point. Within the FW-FW group cortisol concentration was significantly higher than the control at 3 h ($p < 0.01$), 6 h ($p < 0.01$), 9 h ($p < 0.01$), and 24 h ($p < 0.05$) post-transfer (as determined by Dunnett's analyses).

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Fig. 3.29:   Environmental Acclimation Studies - Cortisol Content - Month 1

Cortisol content (pg/g) in fish at Month 1 acclimated to FW, 33% SW, 66% SW and SW
(n = 8). Values shown are means ± SE. Where error bars are not visible this is because
they are too small to print at this scale. ** indicates a significant difference from the FW
acclimated fish, at $p < 0.01$ (Dunnett's).

Cortisol content (pg/g)
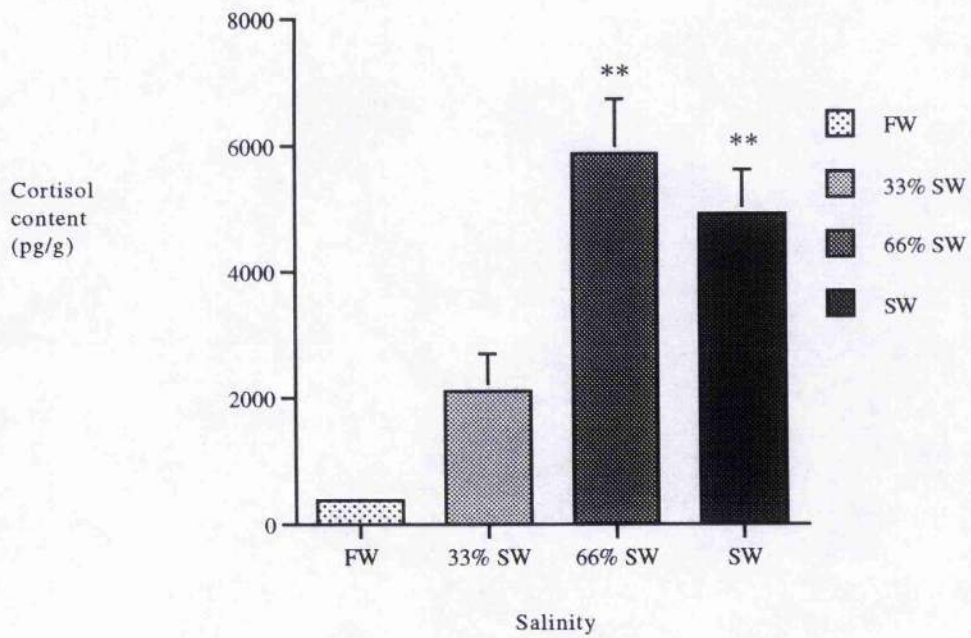
Fig. 3.30:   Environmental Acclimation Studies - Cortisol Content - Month 2

Cortisol content (pg/g) in fish at Month 2 acclimated to FW, 33% SW, 66% SW and SW
(n = 8). Values shown are means ± SE. There were no significant differences in cortisol
content with changes in environmental salinity (ANOVA $p < 0.05$).

Cortisol
content
(pg/g)

Salinity
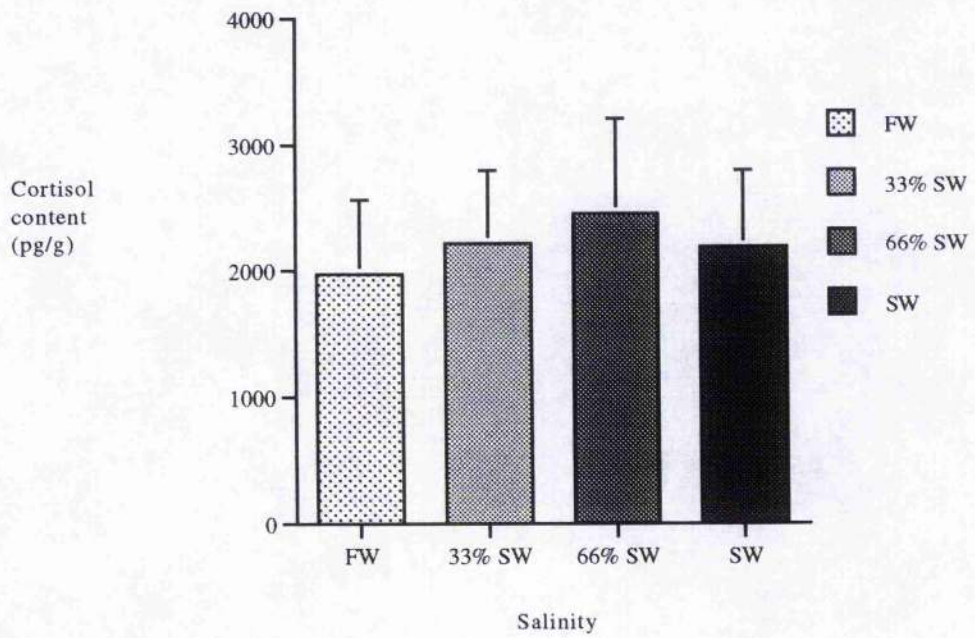
Fig. 3.31:   Environmental Acclimation Studies - Cortisol Content - Months 1 to 5

Cortisol content (pg/g) in fish at Months 1 to 5 acclimated to FW and SW (n = 8).
Values shown are means ± SE. Where error bars are not visible this is because they are
too small to print at this scale. *** indicates a significant difference, at $p < 0.001$ (t-test),
between FW and SW acclimated values within months.
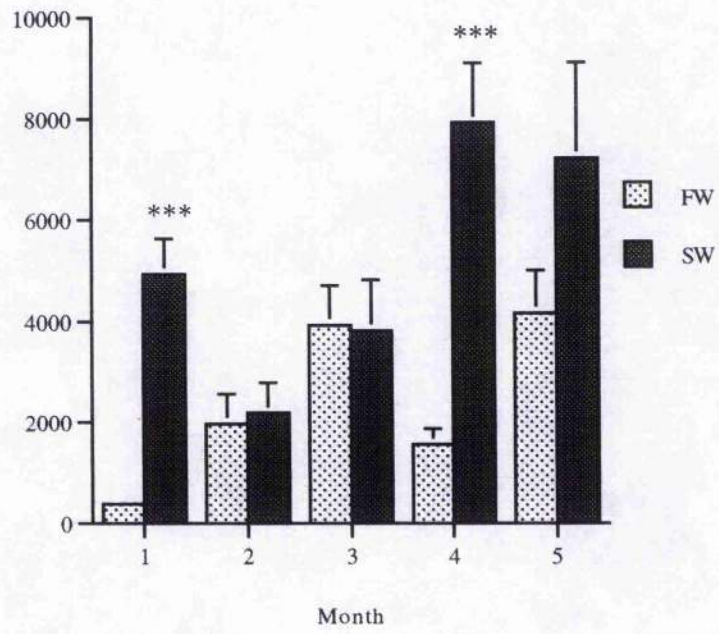
<u>Fig. 3.32:</u>   <u>FW-SW Transfer Studies - Cortisol Content</u>

Cortisol content (pg/g) during the FW-SW transfer experiment (n = 8, except FW-FW 3
h; FW-FW 9 h; FW-FW 3 d; FW-SW 3 h and FW-SW 3 Months where n = 7). Values
shown are means ± SE. * indicates a significant difference, at $p < 0.05$ (t-test), between
FW-FW and FW-SW values within time-points.
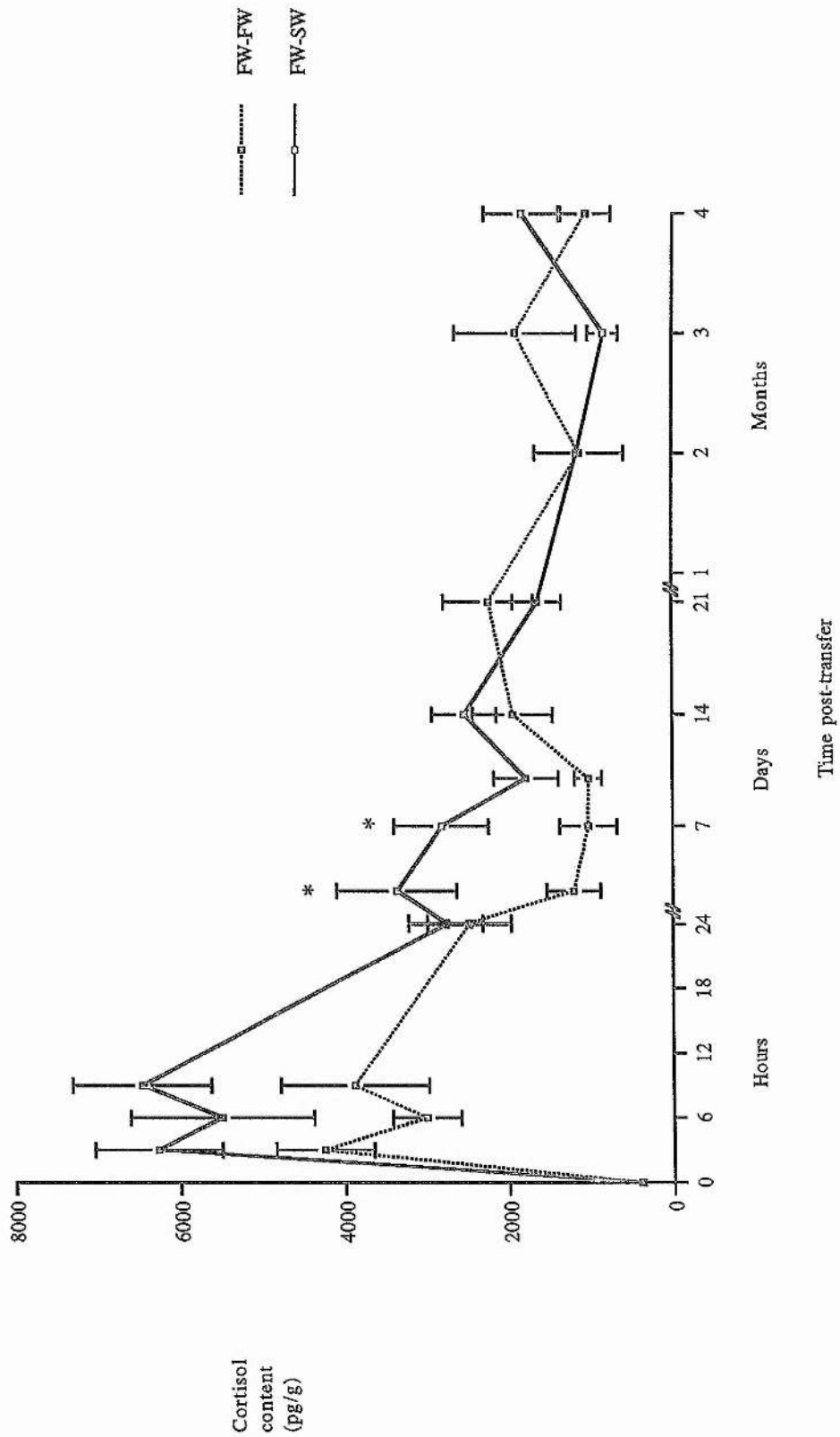
Within the FW-SW group the 3 h ($p < 0.01$), 6 h ($p < 0.01$), 9 h ($p < 0.01$), 24 h ($p <$ 0.05), 3 d ($p < 0.01$) and 7 d ($p < 0.05$) time-points were considered significantly higher than the control (as determined by Dunnett's analyses). Cortisol content in the FW-SW group was considered significantly higher than the FW-FW group at 3 d and 7 d post-transfer (t-test $p < 0.05$).

3.6   Interrenal Morphology

Figure 3.33 is a montage covering a whole section to show the location of the interrenal cells relative to other organs. Interrenal cells were found to line the posterior cardinal vein and and were distributed along its length. Interrenal cells from the area adjacent to the liver were photographed for comparison between experimental groups (Figs. 3.34 and 3.35). Interrenal cells were also found in the pronephric area of the fish where they lay to the anterior and posterior of a glomerulus.

There were no clear and consistent visible differences in the overall size or cytoplasmic/nuclear area ratio between fish acclimated to FW and SW (Fig. 3.34), however, the cells did appear reduced in size with time. Similarly, during the FW-SW transfer experiment (Fig. 3.35) there were no clear differences between interrenal cells from the FW-FW and FW-SW groups. However, the interrenal cells from both groups did seem smaller at 3 and 4 months post-transfer.

Fig. 3.33: Montage of Images Showing a Whole Vertical Longitudinal Section of a Glass Eel

A very thin line of interrenal cells can be seen to lie dorsal to the liver, along the edge of the posterior cardinal vein. This figure allows the positionof these cells to be seen in relation to the organs of the liver, gut, heart, and pancreas (vertical longitudinal section, X100). Scale bar = 200 μm.

Anterior

Ventral

Posterior

Heart

Liver

Pancreas

Gut

Spleen

Muscle

Posterior
cardinal
vein

Spinal cord
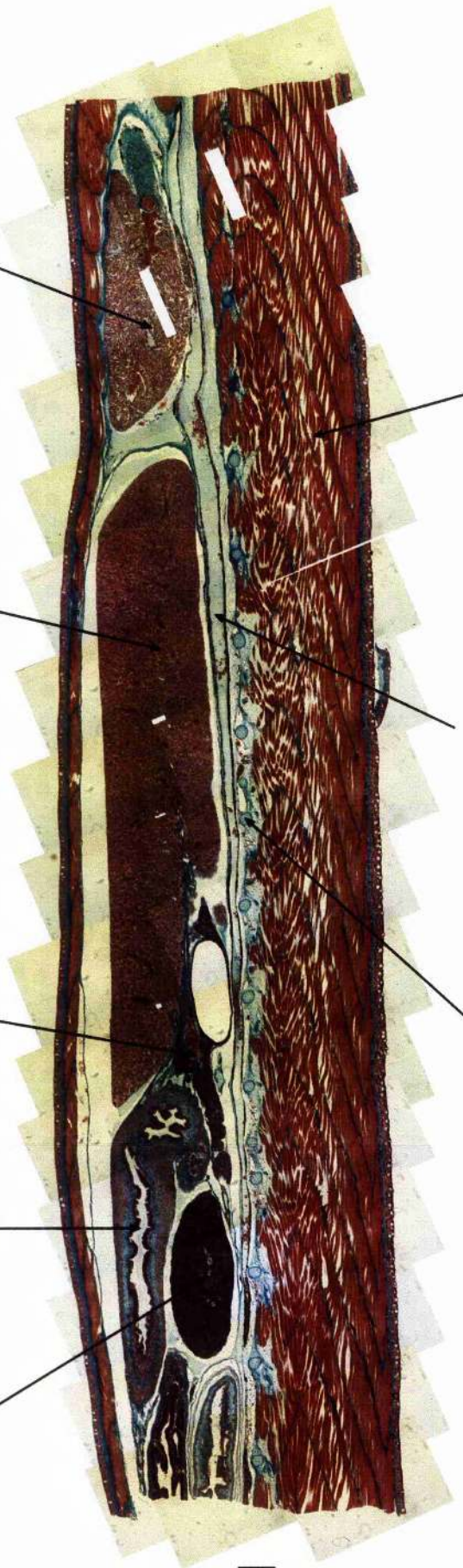
Fig. 3.34:   Environmental Acclimation studies - Interrenal Cells

The interrenal cells are coloured pale purple with a large nucleus and can be seen lying in strips one or two cells deep. Some figures also show the distinctive red blood cells for comparison. Interrenal cells of FW and SW acclimated fish, left and right respectively, at Months 1 to 5, from top to bottom (vertical longitudinal sections, X400). Scale bar = 20 µm.
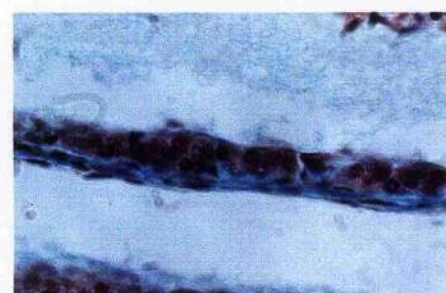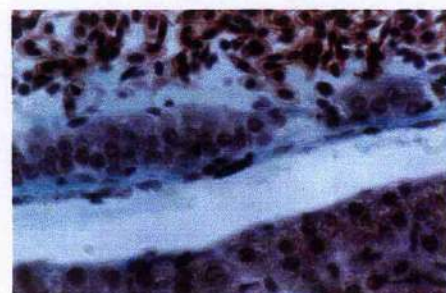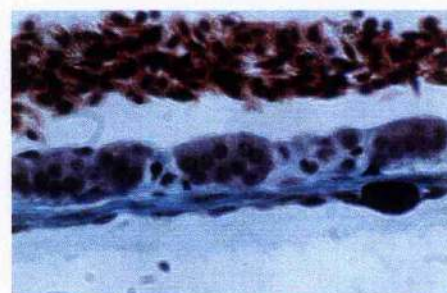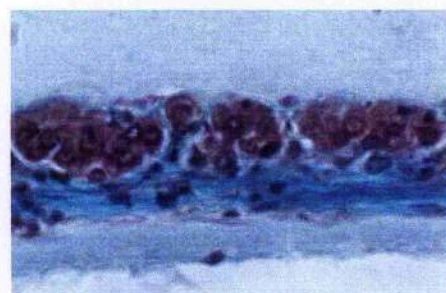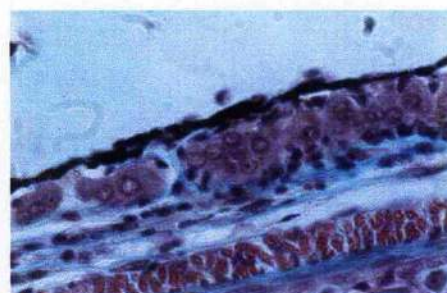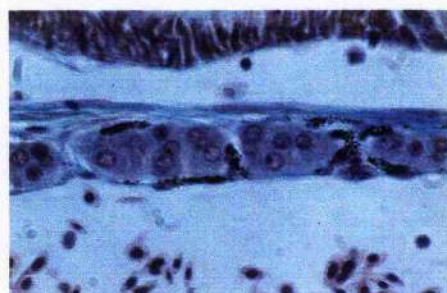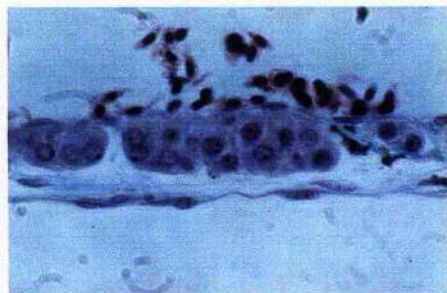
Fig. 3.35:   FW-SW Transfer Studies - Interrenal Cells

The interrenal cells are coloured pale purple with a large nucleus and can be seen lying in strips one or two cells deep. Some figures also show the distinctive red blood cells for comparison. Interrenal cells of FW-FW and FW-SW fish, left and right respectively, at 3 h, 3 d, 14 d, 2 months and 4 months post-transfer of FW acclimated glass eels to SW, from top to bottom (vertical longitudinal sections, X400). Scale bar = 20 μm.
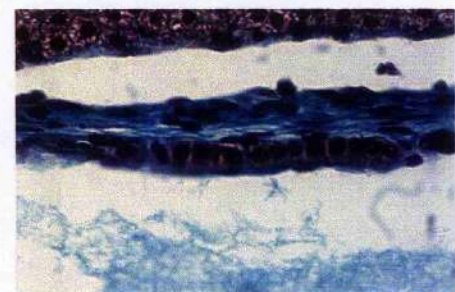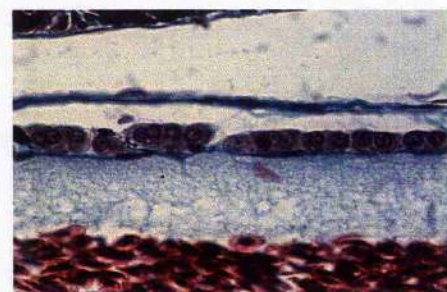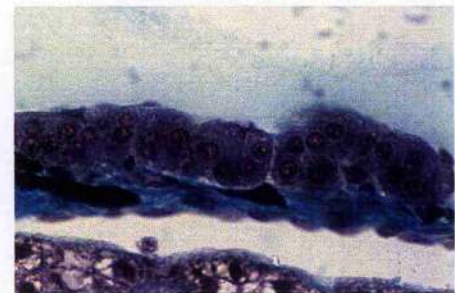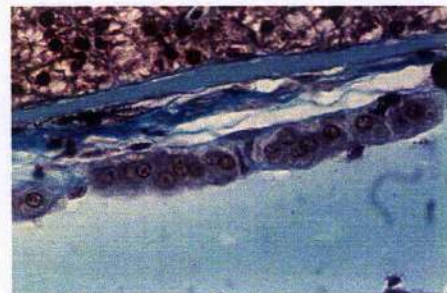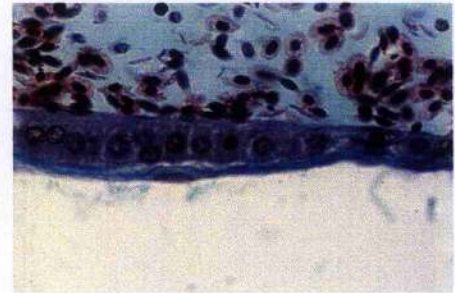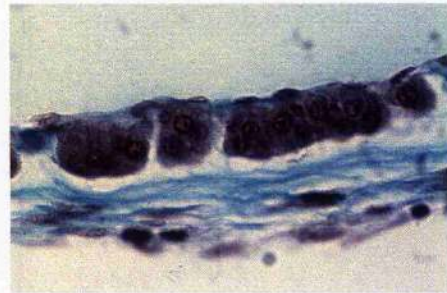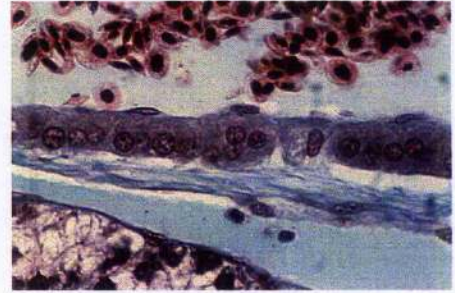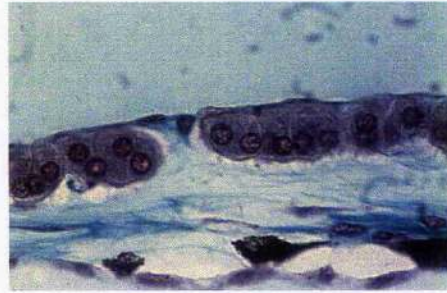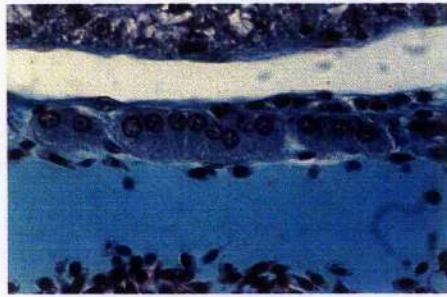
Chapter 4

Discussion

Although it has not been examined in this study, it seems likely that the larval stages of *A. anguilla* are physiologically restricted to the SW environment. There must be a time when the eels acquire euryhalinity in order to make the passage into FW. Given that migration into FW may take a number of attempts, it seems reasonable to hypothesize that there must be a window of osmoregulatory plasticity to allow for return to SW by unfavourable water currents etc. Both yellow and silver eels can withstand acute transfer from FW to SW, however silver eels are generally regarded as being pre-adapted to SW (Thomson and Sargent, 1977). Once glass eels had successfully made the migration from SW into FW they were regarded to have become essentially FW stenohaline fish by the scientific community (Hazon, personal communication). The window of euryhalinity was thought to expire soon after the glass eels had moved into FW. However, a number of circumstantial reports suggested that glass eels could in fact remain in the SW environment, continue to develop into elvers, and then migrate during the next or even subsequent seasons, along with the next migration of glass eels (Krough, 1939).

The aim of the work presented here was to investigate the possibility of such osmoregulatory plasticity in the glass eel and elver stages of the *A. anguilla* life-cycle, and to investigate some of the processes that enable survival in different osmotic environments. Fundamental processes associated with euryhalinity in teleosts, such as the modification of drinking rate, branchial $Na^+K^+ATPase$ activity, whole body cortisol content and interrenal function were investigated, alongside total body Na^+ and K^+ content as an indicator of adaptational state.

Although a number of authors have studied the behaviour of glass eels during their upstream migration, the literature is almost devoid of information pertaining to the osmoregulatory mechanisms of glass eels during this migration, or during subsequent development into elvers. Glass eels and elvers are neither larval nor adult fish, and considerable technique development was therefore necessary in order to adapt established larval and/or adult methods to these stages of the life-cycle.

In adult studies, a great deal of information can be obtained from the sequential sampling of blood for determination of ion contents and/or hormone concentrations. The small size of glass eels and elvers meant that such sequential measurements were impossible. Attempts were made to take single blood samples from these small fish, however the volumes obtained were very small and could not be reliably taken from sufficient numbers of fish to be viable for experimental purposes. This necessitated the use of whole body preparations for the measurement of cortisol and Na^+ and K^+ content. In addition, gill samples had to be pooled together from five individuals in order to determine $Na^+K^+ATPase$ activity, even when using a scaled down micro-assay.

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4.1 Drinking in Acclimated Fish

One method of studying drinking in adult fish is to cannulate the oesophagus and measure the volume of water drunk directly by connecting the other end of the cannula to a drop-counter (Hirano, 1974; Takei *et al.*, 1979; Hirano and Hasegawa, 1984; Takei *et al.*, 1988). The great advantage of this technique is that it allows continuous measurements to be made, however the results must be treated with caution. As the ingested water does not reach the stomach, the fish will continue to suffer dehydration such that the drinking response in reinforced. However, more recently workers have modified the technique such that the water drunk is subsequently replaced with an equivalent volume of water introduced to the stomach (Takei and Balment, 1993). In both cases such invasive techniques are likely to cause some degree of disturbance to the fish. In glass eels and elvers it was obviously impossible to employ these cannulation methods to measure drinking due to their small size, and initially techniques developed for measuring drinking rates in larval fish were investigated.

Drinking has been measured in larval fish including cod, *Gadus morhua* (Mangor-Jensen and Adoff, 1987; Tytler and Blaxter, 1988b), plaice, *Pleuronectes platessa* (Tytler and Blaxter, 1988b), herring, *Clupea harengus* (Tytler and Blaxter, 1988b; Tytler and Ireland, 1994), turbot, *Scophthalmus maximus* (Brown and Tytler, 1993; Tytler and Ireland, 1994) and halibut, *Hippoglossus hippoglossus* (Tytler and Blaxter, 1988a) in seawater, and rainbow trout (Tytler *et al.*, 1990) in freshwater. Drinking rates in larval cod (Mangor-Jensen and Adoff, 1987) and rainbow trout (Tytler *et al.*, 1990) have been found to increase with larval age over the first week post hatching and this was noted to be inversely related to the amount of yolk remaining. This increase in drinking rate may reflect the need to obtain water from outwith the larvae as the water available from the yolk is depleted, or it may represent the evolution from larval to adult osmoregulatory processes (Mangor-Jensen and Adoff, 1987). The modification of drinking rate with external salinity, in a similar way to that shown for adult fish has been demonstrated in post yolk-sac stages of cod, herring and plaice larvae (Tytler and Blaxter, 1988b; Tytler and Ireland, 1994).

Drinking has been shown in some of these larval studies by epifluorescence microscopical measurements of the uptake of fluorescein isothiocyanate (FITC) dextran (Tytler and Blaxter, 1988a; Tytler and Blaxter, 1988b). This technique allows the progression of ingested fluid down the gut to be followed visually (Tytler and Blaxter, 1988b). The possibility of using the FITC dextran technique in glass eels and elvers was investigated, however a microscope with a suitable objective and epifluorescence was not available in St Andrews. Attempts were made to view the fish using a UV transilluminator, however considerable autofluorescence obscured any from the ingested FITC dextran.

In 1930 Smith first demonstrated drinking in adult seawater fish using phenol red as a non-absorbable marker. The technique was later modified to allow quantitative measurements of drinking to be made (Maetz and Skadhauge, 1968; Oide and Utida, 1968; Kobayashi *et al.*, 1983; Okawara *et al.*, 1987). This involved a series of washing procedures and centrifugation steps to separate the marker from the gut which was then measured spectrophotometrically. However, the phenol red technique has now given way to more accurate, reliable and less complicated methods employing non-absorbable radiolabelled compounds. The use of three different radiomarker compounds are widely reported in the literature: ^{51}Cr-EDTA, ^{125}I-PVP (polyvinyl pyrolidone) and ^{3}H-dextran, and these have been used to measure drinking in both adult (Evans, 1968; Gaitskell and Chester-Jones, 1971; Skadhauge and Lotan, 1974; Perrott *et al.*, 1992; Tierney *et al.*, 1993; Carroll *et al.*, 1994; Balment *et al.*, 1995; Carroll *et al.*, 1995; Tierney *et al.*, 1995a; Fuentes *et al.*, 1996a; Fuentes *et al.*, 1996b) and larval (Mangor-Jensen and Adoff, 1987; Tytler and Blaxter, 1988b; Brown and Tytler, 1993; Tytler and Ireland, 1994) fish.

In this study drinking rates were estimated in glass eels and elvers using a modified technique employing one of these radioactive markers, ^{51}Cr-EDTA (Hazon *et al.*, 1989). By using such an non-invasive technique there is minimal disturbance to the fish. EDTA is an inert molecule too large to be absorbed through the gut wall and into the body and ^{51}Cr^{2+} is an ideal radiolabel for drinking rate studies. As a medium energy gamma emitter with the half-life of 27.7 days it can be counted directly using a gamma counter and the activity is maintained over the course of a small set of experiments without being highly active over long time periods, therefore waste can be disposed of with ease. The possible problem of the isotope leaving the carrier through the action of the intestine or simple dissociation must be addressed. It has been demonstrated that there is no degradation of ^{51}Cr-EDTA over at least six hours (Hazon *et al.*, 1989). Over the course of the drinking rate experiments detailed in this study the ^{51}Cr-EDTA carrier remains intact.

In adult fish the problem of losing radioactive counts via the anus can be addressed by looking at the radioactivity present in the most distal segment of the gut, as it is portioned up for counting anyway (Maetz and Skadhauge, 1968; Skadhauge and Lotan, 1974; Carrick and Balment, 1983; Hazon *et al.*, 1989; Perrott *et al.*, 1992). In adult fish most experiments have been carried out over a six hour time period (Carrick and Balment, 1983; Hazon *et al.*, 1989; Perrott *et al.*, 1992; Carroll *et al.*, 1994), double that used here for glass eels and elvers Earlier work on adult fish was sometimes carried out over even longer periods (24 and 48 hours), although the rectum was ligatured to prevent loss of radioactivity via the anus (Gaitskell and Chester-Jones, 1971). In elvers, equilibration of counts, such that the rate of acquisition of counts by drinking was balanced by the loss of counts by voiding form the gut, was found to occur after three hours (Fig. 3.6). It was hoped that this equilibration could be substantiated by

autoradiographical visualization of counts with the gut after various time periods, however the activity of the radiolabel was too low to be detected upon analysis by this method.

Glass eels and elvers acclimated to SW showed significantly higher drinking rates than those acclimated to FW over the course of Months 1 to 5 (Fig. 3.9a), such that drinking rates were around ten-fold higher in SW acclimated fish than those from FW at any given time-point. Similar results have been found for a range of adult fish, including *A. anguilla* (Maetz and Skadhauge, 1968; Gaitskell and Chester-Jones, 1971; Perrott *et al.*, 1992; Tierney *et al.*, 1993; Balment *et al.*, 1995; Tierney *et al.*, 1995b). In one of the very few papers published pertaining to the osmoregulatory processes of glass eels Ciccotti *et al.* (1993) found that the oesophagus of FW acclimated fish showed the typical multilayered mucosa of a hyperosmoregulating eel (section 1.2.1.2i). In SW acclimated glass eels the oesophageal epithelium showed areas of simple columnar epithelium together with marked intracellular spaces indicative of ion transport activity (Ciccotti *et al.*, 1993). These morphological observations are consistent with the ability of glass eels and elvers to alter their drinking behaviour to suit the salinity of the environment.

Drinking rates in glass eels at Month 1 acclimated to FW and SW in this study were 0.07 ± 0.02 and 0.70 ± 0.10 $\mu l/g/h$ respectively (Fig. 3.7a), and these are not dissimilar to the values reported for adult *A. anguilla*, measured by other workers using the same ^{51}Cr-EDTA accumulation technique (Tab. 4.1). There has been no study of mass specific effects of drinking in eels, as has been done for other species (Carroll *et al.*, 1995; Fuentes and Eddy, 1997), where they investigated the effect of weight of over two orders of magnitude. However, given the very small range in weights of the fish used in this study (approximately 0.1 - 0.3 g) there is no requirement to account for any such effect.

Drinking Rate (ml/kg/h)		Temperature	Weight	Authors
FW	SW			
0.07 ± 0.02	0.70 ± 0.10	9 °C	0.26 g	This thesis - Month 1
1.12 ± 0.42	12.85 ± 1.05	18 °C	0.14 g	This thesis - Month 2
0.06 ± 0.02	0.51 ± 0.07	8-14 °C	250-800 g	Tierney *et al.* (1995b)
0.09 ± 0.04	1.00 ± 0.26	10-12 °C	-	Perrott *et al.* (1992)
0.05 ± 0.01	0.90 ± 0.20	-	-	Balment *et al.* (1995)

Tab. 4.1: Drinking rates in adult and elver *A. anguilla* adapted to FW and SW using the ^{51}Cr-EDTA gut accumulation technique. Values are means ± SE.

By Month 5 (Fig. 3.9a) drinking rates in this study had elevated to 1.12 ± 0.42 $\mu l/g/h$ and 12.85 ± 1.05 $\mu l/g/h$ in FW and SW acclimated elvers respectively. The SW value is of a similar order to the 11 $\mu l/g/h$ described for *Aphanius dispar* which was of

slightly larger weight (0.4 - 1 g), and also acclimated to SW, but at 20°C (Skadhauge and Lotan, 1974).

It is clear from figure 3.9a that drinking rates increased significantly in both the FW and SW acclimated groups over Months 1 to 5, although drinking rates in SW acclimated fish were always greater than in fish acclimated to FW. As the glass eels and elvers in this study were held at ambient temperature throughout the course of the experiment there was a considerable increase in temperature between Month 1, where the temperature was approximately 9°C, to Month 5 were the temperature had elevated to 18°C (Fig. 4.1).

The effect of temperature on drinking in teleosts has been studied in adult fish. Drinking rates have been shown to increase with temperature, when acclimated within a range encountered by the fish within its environment, with a Q10 value of around 2 (Isaia, 1972; Maetz and Evans, 1972; Motais and Isaia, 1972; Skadhauge and Lotan, 1974; Carroll et al., 1995). Most studies have been carried out on fish in SW although a similar positive relationship between temperature and drinking rate has also been reported in FW acclimated A. anguilla (Gaitskell and Chester-Jones, 1971). The effect of temperature has been shown to vary with species, and with acute and chronic adaptation (Carroll et al., 1994). In addition, drinking rate has been found to increase with temperature in turbot larvae in SW and rainbow trout larvae in FW (Tytler et al., 1990; Tytler and Ireland, 1994).

As development and temperature are inextricably linked, it was impossible to control for the effect of temperature throughout the series of experiments reported in this thesis. Fish were held at ambient temperature throughout in order to allow development to take place at as similar a rate to the natural as possible. It is clear that the increases in drinking rate observed over the course of Months 1 to 5 would have been influenced by temperature, in addition to any effects due to development and salinity.

There was a trend towards increased drinking rate with environmental salinity at Months 1 (Fig. 3.7a) and 2 (Fig 3.8a) although only the SW acclimated fish were considered to have statistically significantly higher drinking rates than those from FW. Drinking rate has been shown to increase in adult eels with stepped increases in external salinity from FW to SW (Hazon, unpublished observations), and to double-strength SW (Maetz and Skadhauge, 1968).

Measurable drinking rates were obtained from glass eels and elvers acclimated to FW throughout the study. As noted in section 1.2.1.1, although it was once believed that fish in FW did not drink (Smith, 1930; Shedadeh and Gordon, 1969), later studies showed that fish in FW did drink, albeit at a considerably lower rate than fish acclimated to SW (Maetz and Skadhauge, 1968; Gaitskell and Chester-Jones, 1971; Hirano, 1974; Carrick and Balment, 1983; Balment and Carrick, 1985; Perrott et al., 1992; Tierney et al., 1993; Balment et al., 1995; Tierney et al., 1995b). It seems that with the

Fig. 4.1:   Water Temperature During the Experimental Period

Ambient water temperature (˚C) throughout the experimental period coincident with both the environmental acclimation studies and the FW-SW transfer studies.

Temperature (°C)

Days

Months

Time post-transfer
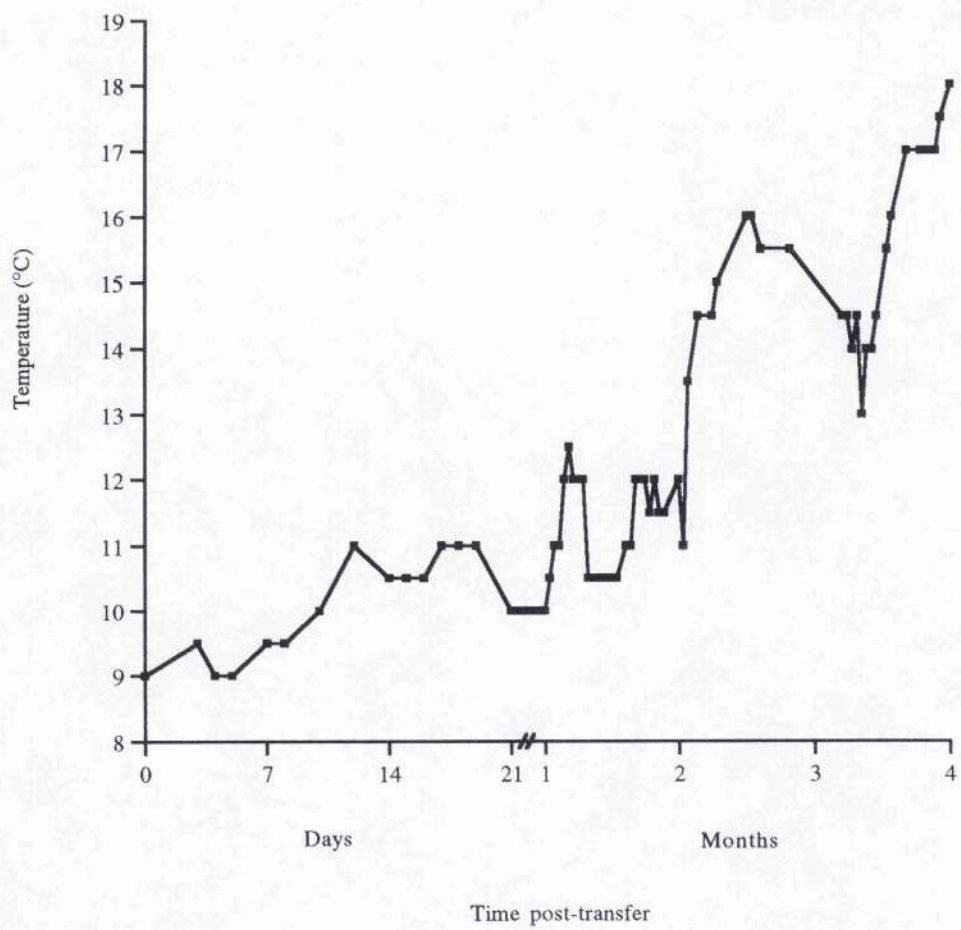
development of better, non-invasive radiochemical techniques, smaller drinking rates have become measurable. The physiological role for drinking in FW however remains to be elucidated. Larval fish tend to drink at even greater rates, such that FW larval rainbow trout may drink at a rate of 3.2 µl/g/h (Tytler *et al.*, 1990), more than double the rate observed for FW acclimated elvers at Month 5. In this diluting environment, taking in water would, in terms of osmoregulation appear maladaptive. However, drinking in FW has been shown to occur in a number of species, and may be of particular importance in juvenile fish, as FW larvae and juveniles drink at similar rates to adult fish in SW (Fuentes *et al.*, 1996b). It has been suggested that drinking in FW may be associated with calcium uptake or feeding in alevins and larvae (Tytler *et al.*, 1990). At Month 2 the 33% SW fish actually had a lower drinking rate than those from FW, although this was not significant. It appears that the drinking rate in 33% SW was reduced by Month 2 as compared to Month 1, rather than the FW drinking rate having risen. It might be expected that in 33% SW drinking would be negligible, as the osmotic difference between the internal body fluids of the fish and those of the external environment would be minimal.

In order to determine not only the mean drinking rate for each group of fish, but the frequency distribution of the volume drunk, the proportion of fish drinking within each group was estimated. A fairly conservative methodology was applied in order to assign fish to each of the categories: not drinking, probably drinking and drinking, as described in section 2.4.2. The first point of note is that in FW acclimated fish there was a higher proportion of fish considered not drinking than in SW, whist the converse was true of fish considered drinking - in SW there was a higher proportion than in FW (Fig. 3.9b). A further point is that over the course of Months 1 to 5 the proportion of fish considered drinking increased in both the FW and SW groups. In addition, the proportion of fish considered drinking increased with increasing salinity of the adaptation medium, at both Month 1 and Month 2 (Fig. 3.7b and Fig. 3.8b respectively).

4.2 Development

In this laboratory glass eels and elvers have shown their ability to survive and develop in FW, 33% SW, 66% SW and full SW for at least six months post-migration. In addition they do in fact continue to survive and grow when maintained in SW for over one year. In one of the few published studies pertaining to osmoregulation in glass eels, Ciccotti *et al.* (1993) also found no difficulty in acclimating freshly caught glass eels to FW and SW, and they reported similar survival rates for both salinities over a subsequent four month period.

During the course of the study reported here, the glass eels showed a significant reduction in wet weight in both FW and SW acclimated fish, and in FW-FW and FW-SW

transferred fish. A reduction in the weight and length of glass eels during the transition into elvers has previously been reported, and is common during experimental rearing (Tesch, 1977; Lecomte-Finiger, 1992; Ciccotti *et al.*, 1993). Glass eels were found by Ciccotti *et al.* (1993) to show an initial reduction in length and weight over the first two months post-migration, even when maintained at the constantly elevated temperatures of 15°C and 25°C (the normal aquaculture temperature), and despite maximal feeding and continual grading. Therefore, it is not surprising that the glass eels and elvers reported in this study continued to exhibit a reduction in weight for a longer period when maintained in water at ambient temperatures in St Andrews (Fig. 4.1), where they were given only a maintenance diet, and were subject to only minimal grading to remove the very largest and smallest fish.

Pigmentation is an accepted way of assessing development in glass eels and elvers (Boëtius, 1976; Elie *et al.*, 1982). Using the colour measured by an image analysis system the intensity of pigmentation was used as an indicator of development in glass eels and elvers. The purpose of this was to investigate whether fish maintained in salinities other than FW would pigment and develop as those held in FW, considered the 'normal' environment for these fish post-migration. The results obtained show that fish in 33% SW, 66% SW and full SW do indeed continue to become more pigmented with time, and that development does not appear to be arrested or slowed by being held in these higher salinity environments. In fact the SW adapted fish were found to be significantly darker than those from FW at Months 1, 2 and 5, and were not significantly different from FW adapted fish at Months 3 and 4 (Fig. 3.3). Figure 4.2 shows images of typical elvers at Month 1 and 5, together with a recently migrated glass eel and an elver approximately one year post-migration for comparison, as used for the determination of development via the measurement of colour intensity.

Obviously further work would have to be undertaken, with more emphasis on mortality rates and feeding to conclude whether or not these fish do in fact develop faster in SW than FW, a finding that would have important implications for aquaculture. If the glass eels wean earlier in SW this could save considerable growing time which obviously translates into financial benefits.

4.3   Drinking Rate Challenge

Hirano (1974) reported that when FW acclimated adult *A. japonica* were transferred to SW, drinking began immediately, and then stopped immediately upon transfer back to FW. At each month from Month 1 to Month 5, FW adapted glass eels and elvers were transferred either to FW (FW-FW) as a control, or from FW to SW (FW-SW), and drinking behaviour was monitored over the first five hours post-transfer (Figs.

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<u>Fig. 4.2:</u>   <u>Pigmentation at Various Stages of the Lifecycle</u>

Typical images of glass eels and elvers at various stages post-migration into FW as used for image analysis. From top to bottom: a glass eel soon after migration; an elver one year post-migration; glass eels at Month 1 acclimated to FW and SW respectively and elvers at Month 5 acclimated to FW and SW respectively. Fish were approximately 7 cm in length.

3.10a to 3.14a). Even by the first time-point at five minutes post-transfer there was a significantly greater amount water accumulated in the gut by the FW-SW group than the FW-FW control group. It is important to note that the values are plotted as the amount accumulated ($\mu l/g$) rather than as drinking rate expressed per unit time ($\mu l/g/h$). Throughout Months 1 to 5 the same acute stimulation of drinking upon transfer to SW can be seen, although the actual values obtained increase over the time-course. Transfer from FW to SW stimulates a rapid increase in the amount of water accumulated whilst there was no equivalent response in the FW-FW control groups.

Mean drinking rates obtained from SW acclimated fish were considerably less than the equivalent rate of drinking over the first 15 minutes post-transfer of FW acclimated fish to SW (Fig. 3.15). The FW-FW control fish also showed an increase in equivalent drinking rates over the course of Months 1 to 5, although the rates were always considerably lower than those for the FW-SW group. It is clear from the results presented here that glass eels and elvers retain an excellent acute drinking response upon immediate exposure to SW, even when the fish had been maintained in FW for more than five months.

When Kirsch and Mayer-Gostan (1973) looked at drinking in adult *A. anguilla* they found a three-fold increase in drinking rate over the first hour post-transfer from FW to SW as compared to FW. Unfortunately they did not look at drinking at earlier time-points before one hour had expired, and as such they probably underestimated the rate of drinking immediately post-transfer. In this study drinking rates calculated over the first 15 minutes showed much larger differences between FW-FW control fish and FW-SW fish. For example at Month 5, FW-SW drinking rates calculated over the first 15 minutes were 37-fold higher than the equivalent FW-FW control rates.

The challenge of FW acclimated fish to SW throughout Months 1 to 5 shows that the euryhalinity of the glass eels and elvers is such that they can respond immediately to the hyperosmotic environment with an appropriate drinking response similar to that shown for adult eels. These drinking rate challenge experiments also substantiate the presence of low rates of active drinking in FW acclimated fish. Fig. 3.10a shows a steady increase in the amount accumulated over time in the FW-FW control group. Over time there is an increase in the actual quantities imbibed by both groups and, as has already been discussed, in section 4.1 there will be an effect of temperature over this time scale.

During the acute FW-SW drinking rate challenge experiments (Figs. 3.10a to 3.14a) the amount of water accumulated within the gut was shown to level off in the FW-SW groups, certainly by 1 hour and in some cases as early as 15 minutes post-transfer. There are two possible explanations for the apparent cessation of accumulation of counts within the gut. The first option is that the fish are continuing to drink at the elevated rate but that counts are being voided by the anus such that there appears to be an equilibration.

The second option is that the fish stopped drinking soon after transfer and held the counts within their gut until at least until beyond 5 hour post-transfer. To investigate this possibility a series of 30 minute 'mini-drinking rate' studies were carried out at intervals post-transfer of fish from FW to SW, and FW as a control (Fig. 4.3). Full results of statistical tests are given in Appendix 5.

These results show that drinking does in fact decrease markedly after the first 30 minutes, and still further after one hour post-transfer, and suggests that the levelling out observed in figures 3.10a to 3.14a does in fact result from a reduction in drinking rate. Once the stomach is full of ingested SW it is likely that further drinking is prevented, perhaps by stretch receptors in the stomach, or drinking may be controlled by the concentration of Cl⁻ ions reaching the intestine, as has been suggested for adult *A. japonica* (Ando and Nagashima, 1996). Ando and Nagashima (1996) suggest that a high concentration of Cl⁻ in the intestine acts to reduce drinking, thus allowing further desalination of the ingested water before more SW is ingested. The renin angiotensin system plays an important role in the control drinking in adult teleost fish, although not immediately post transfer of fish from FW to SW (this is reported in section 1.3.1). From figure 4.3 further elevated rates of drinking in elvers did not occur until at least after 12 hours. A similar pattern has been found in adult fish. Between 4 and 24h post-transfer from FW to SW drinking rates in adult *A. anguilla* were found to decrease to FW levels (Kirsch and Mayer-Gostan, 1973).

In the short term Luke *et al.* (1994) showed drinking in *A. anguilla* to increase until 90 minutes after transfer before declining, but drinking rates were still elevated above control levels at 4 h post-transfer. Longer-term studies showed a bi-phasic response in drinking. Tierney *et al.* (1993) found that there was a slight peak in drinking rate by 2 days post-transfer, this was followed by a decline until 5 days post-transfer before increasing more markedly to stabilize at a new higher level between 7 and 14 days post-transfer, were values where still significantly higher than in FW. Stabilization of drinking to SW adapted levels appears to take between one and two weeks in adult *A. anguilla*. Preliminary experiments on elvers at Month 5 have shown that drinking rates are the same in fish after one (11.60 ± 1.10 μl/g/h, n = 12), three (11.41 ± 0.68 μl/g/h, n = 15) and seven (10.87 ± 1.02 μl/g/h, n = 12) days post-transfer from FW to SW, and that these rates were not significantly different from drinking rates established for fish that had been in SW for nearly six months (12.85 ± 1.05 μl/g/h, n = 21). This shows that even after nearly six months in FW, when elvers were transferred to SW they attained drinking rates characteristic of fish that had been in SW for six months, by only one day post-transfer. Elvers appear to be much more readily able to regulate drinking to suit the salinity of the environment than adult fish. In adult eels drinking rates characteristic of SW acclimated fish are not achieved until after at least a week post-transfer from FW to SW (Tierney *et al.*, 1993).

<u>Fig. 4.3:</u>   <u>FW-SW Drinking Rate Challenge - 'Mini-Drinking Rate' Experiments</u>

30 minute mini-drinking rates after transfer of FW acclimated elvers to SW for various time-periods up to 12 hours (n = 11, except FW-FW 0-30 min, FW-FW 1-1.5 h and FW-FW 5.5-6 h where n = 10, FW-FW 2.5-3 h and FW-SW 0-30 min where n = 12, FW-SW 11.5-12 h where n = 13 and FW-FW 11.5-12 h where n = 18). Values shown are means ± SE. Where error bars are not visible this is because they are too small to print at this scale. * and ** indicate significant differences, at $p < 0.05$ and $p < 0.01$ respectively (t-test), between FW-FW and FW-SW values within time-points.

Amount
accumulated
(µl/g)

20

15

10

5

0

**

*   *   **

0-30m   30m-1h   1-1.5h   2.5-3h   5.5-6h   8.5-9h   11.5-12h

Time post-transfer

FW-FW
control

FW-SW
challenge
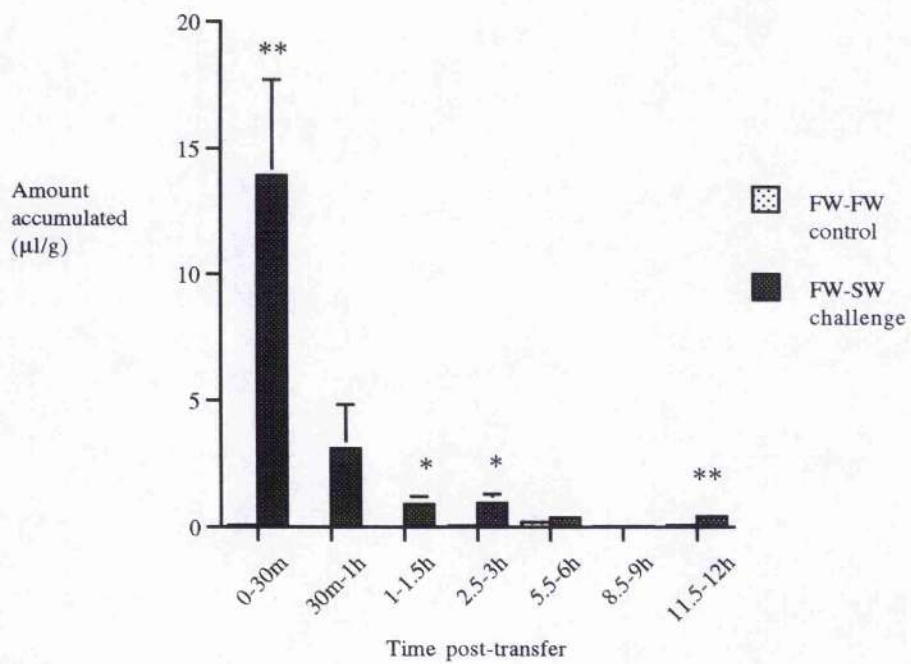
4.4   Branchial Na+K+ATPase Activity

Branchial Na+K+ATPase is widely reported to be higher in stenohaline SW as compared to stenohaline FW teleosts, and is also higher in SW acclimated as compared to FW acclimated euryhaline teleosts (Kamiya and Utida, 1969; Jampol and Epstein, 1970). Eels have been fairly well studied in this regard, with similar reports for *A. anguilla* (Motais, 1970; Milne *et al.*, 1971; Sargent *et al.*, 1975; Thomson and Sargent, 1977; Mayer-Gostan and Lemaire, 1991), *A. japonica* (Kamiya and Utida, 1968; Utida *et al.*, 1971; Ho and Chan, 1980) and *A. rostrata* (Jampol and Epstein, 1970; Epstein *et al.*, 1971; Epstein and Kormanik, 1980). In adult eels it is widely reported that there is an increase in branchial Na+K+ATPase activity of around two to four-fold after adaptation to sea water (Kamiya and Utida, 1968; Kamiya and Utida, 1969; Jampol and Epstein, 1970; Forrest (Jr.) *et al.*, 1973a; Thomson and Sargent, 1977; Ho and Chan, 1980; Luke *et al.*, 1994). However, Kirschner (1969) found no difference in gill Na+K+ATPase activity between FW and SW adapted *A. anguilla*.

It is very difficult to compare actual values between studies as many different techniques have been used in the preparation of the gill samples, with some more purified than others. In addition, many differences in assay techniques are also apparent. A simple adult eel gill homogenate may have an activity of around 4-8 μmoles/mg protein/h (Bornancin and de-Renzis, 1972; Forrest (Jr.) *et al.*, 1973a; Ho and Chan, 1980) whilst a membrane preparation may result in values of 40-50 μmoles/mg protein/h (Kamiya and Utida, 1968; Utida *et al.*, 1971; Bornancin and de-Renzis, 1972; Butler and Carmichael, 1972; Sargent *et al.*, 1975; Mayer-Gostan and Lemaire, 1991).

It is conventional to express Na+K+ATPase activities per milligram of protein. Although there were no significant differences in gill homogenate protein concentration between fish acclimated to each of the four environmental salinities at Month 1 and Month 2, by Months 3 and 4 the homogenate protein concentrations were significantly higher in the SW acclimated fish as compared to fish from FW (Fig. 3.18). If the overall wet weight of fish was compared, there was no difference between the fish at Months 1 and 2, nor at Months 3 and 4 (section 3.1). This suggests that although fish at Month 3 and 4 were similar in weight whether acclimated to FW or SW, the SW acclimated fish expressed higher levels of protein in their gills. It appears that there is more protein contained within the gill tissue with increasing time of residence in SW, although the fish are losing weight overall. This may reflect an increase in protein in all cell types, or may, in part, reflect an increase in the number, size and organizational structure of chloride cells required in SW. During the FW-SW transfer experiment the FW-SW group also exhibited significant increases in gill homogenate protein concentration as compared to the FW-FW control group at all time-points from 14 d post-transfer until the end of the

experiment (Fig. 3.19). Expressing the activities as per sample (5 pairs gills) allowed for the significant differences between the total gill protein. If the proportion of $Na^+K^+ATPase$ did not increase in proportion with the other gill protein then increases in $Na^+K^+ATPase$ activity could be masked by increases in general gill protein content if expressed as μmoles /mg protein/h. In order to avoid potential problems due to protein changes, all the $Na^+K^+ATPase$ activities in this study were expressed as per sample of five pairs of gills.

Branchial $Na^+K^+ATPase$ activity was not significantly different between FW and SW acclimated fish at Months 1, 2 and 3 (Fig. 3.22). At Month 4 however, the SW acclimated elvers showed significantly higher $Na^+K^+ATPase$ activities than fish acclimated to FW. In the literature SW adapted adult *A. anguilla* are generally reported to have higher gill $Na^+K^+ATPase$ activities than FW adapted fish (Motais, 1970; Milne *et al.*, 1971; Sargent *et al.*, 1975; Thomson and Sargent, 1977; Mayer-Gostan and Lemaire, 1991; Luke *et al.*, 1994).

Gill $Na^+K^+ATPase$ activities significantly decreased by about half in both FW and SW acclimated fish between Month 1 and Month 2, and remained low at Month 3. At Month 4 the FW value remained low, whilst the SW value increased by more than 100% (Fig. 3.22). One might expect glass eels and elvers to retain high levels of $Na^+K^+ATPase$ activities post-migration to FW to ensure they were able to regulate should they be returned to SW. As $Na^+K^+ATPase$ activities only give an indication of the maximal possible activity, the results can be difficult to interpret. Pumps may be present but not actually functioning in the animal. The drop in activity at Month 2 in both the FW and SW acclimated groups is difficult to explain.

Branchial $Na^+K^+ATPase$ activities have been shown to increase with increasing salinity of the external environment in both *A. rostrata* (Butler and Carmichael, 1972) and *A. japonica* (Utida *et al.*, 1971). There were no such changes observed here for fish acclimated to FW, 33% SW, 66% SW and SW at Month 1, nor Month 2 (Figs. 3.20 and 3.21).

During the FW-SW transfer experiment the difference in $Na^+K^+ATPase$ activity between the FW-FW control group and the FW-SW group was most pronounced at 2 months post-transfer where FW-SW values were more than 100% greater than those from the control group (Fig. 3.23). Up until 2 months post-transfer differences in $Na^+K^+ATPase$ activity were largely due to an increase in FW-SW values however after the 2 month time-point there was also a decrease in the FW-FW control values. During the FW-SW transfer experiment the initial increase in $Na^+K^+ATPase$ activity after six hours observed in adult *A. anguilla* (Luke *et al.*, 1994), as discussed in section 1.2.2, was not observed for glass eels. There were considerable fluctuations in activity over the first 24 h post-transfer where both FW-FW and FW-SW values tended to follow each other. After 3 days however, there appears to be a trend for increased $Na^+K^+ATPase$

activity in the FW-SW group, which peaks at 2 months post-transfer and then returns to nearer FW-FW control levels, although still significantly higher. A trend for increased activity until 21d was noted in adult *A. anguilla* where activities thereafter decreased such that six-month acclimated fish in SW had slightly higher values than those from FW (Luke *et al.*, 1994).

Other workers have found slightly different time-tables of changes in Na+K+ATPase activity post-transfer of FW eels to SW. Bornancin and de-Renzis (1972) found that Na+K+ATPase activity in *A. anguilla* increased after two days post-transfer from FW to SW to reach a maximum after two weeks post-transfer. Thomson and Sargent (1977) studying the same species also found maximal levels after 2 weeks, however they noted little change over the first five days. Na+K+ATPase activities also reached their maximum two weeks after transfer of *A. japonica* from FW to SW, however, when the reverse transfer back to FW was carried out the decrease in activity was more gradual and took four weeks to return to previous FW control levels (Utida *et al.*, 1971).

The boost in Na+K+ATPase activity at 2 months post-transfer may reflect changes in other parameters such as the percentage of fish weaned to the diet. Similar patterns were observed in the expression of the α1 subunit Na+K+ATPase protein when the same samples were analysed by Western Blotting (Cramb and Sterling, personal communication).

4.5   Total Body Na+ and K+ Content

Unfortunately due to the small size of the fish it was not possible to measure plasma Na+ and K+ concentrations, therefore whole body determinations were carried out. When measuring whole body Na+ and K+ contents, the results will include all intracellular ions including those from the organs such as the liver, those from the plasma, and those contributing to structural features within the fish such as bone. However, given that intracellular ion concentrations are usually maintained within fairly strict limits, and that exchanges of Na+ between the bone and other body compartments are small, total body measurements can be used as an indicator of ion balance.

The whole body Na+ values reported for glass eels and elvers ranged between 40 and 60 μmoles/g. These values are of a similar order to those reported for plaice and dab, *Limanda limanda*, although the values for these flatfish were larger at between 65 and 100 μmoles/g (Carroll *et al.*, 1995).

At Month 1 there was no significant difference between total body Na+ content in fish acclimated to FW, 33% SW, 66% SW and SW (Fig. 3.24a), however at Month 2 fish acclimated to 66% SW and SW had significantly higher Na+ contents than those acclimated to FW (Fig. 3.25a). Over the course of Months 1 to 5 there was a trend for SW acclimated fish to have higher Na+ contents than fish acclimated to FW, although this

was not significant for Month 4 (Fig. 3.26a). This difference was mainly due to an increase in Na^+ content in SW adapted fish, rather than a decrease in Na^+ content of FW acclimated fish. This probably reflects adaptation of the body fluids to the different salinities. In adult *A. anguilla* plasma Na^+ is usually higher in SW as compared to FW adapted fish, whether at the 'yellow' or 'silver' stage of the lifecycle (Sharratt *et al.*, 1964; Butler, 1966; Chan *et al.*, 1967).

Over the course of the FW-SW transfer experiment the FW-SW Na^+ values were almost always significantly higher than the FW-FW control group (Fig. 3.27). There was an initial increase in Na^+ content of the FW-SW group immediately post-transfer which then levelled off after 6 hours and slowly declined after 1 day post-transfer. After 21d post-transfer however there was an increase in Na^+ content of both the FW-SW and the FW-FW control groups and by 4 months post-transfer the FW-SW group had continued to increase, although the FW-FW groups had steadied. In adult *A. anguilla* a rapid increase in plasma Na^+ was observed post-transfer from FW to SW, attaining maximal levels of about 130% of the FW level after 2-3 days post-transfer, which then stabilized after 7 days to a level characteristic of SW adapted eels at around 115% of the FW value (Bornancin and de-Renzis, 1972). Therefore the increase in whole body Na^+ during the first hours post-transfer is similar to that occurring in plasma in adult fish. However, the second increase in Na^+ observed in elvers after 21 days post-transfer remains unexplained. It may be due to some developmental effect or as result of rapidly increasing temperature (Fig. 4.1).

During the course of Months 1 to 5 there was a significant trend for both FW and SW acclimated total body K^+ contents to increase although there were no clear differences between FW and SW for any given time-point (Fig. 3.26b). There were no obvious changes observed in total body K^+ concentration during the FW-SW transfer experiment (Fig. 3.28). Although there were considerable fluctuations in both the FW-FW control and the FW-SW groups there were no clear trends either with time or between groups.

There appears to be no major disturbances in total body Na^+ and K^+ levels, suggesting osmoregulatory balance. Despite increases in drinking, as a result of increasing environmental salinity, the Na^+K^+ATPase activities are no different between FW and SW groups during Months 1 to 3 of the acclimated experiment, and within the FW-SW transfer experiment Na^+K^+ATPase activities do not differ until after a week post-transfer from FW to SW.

4.6 Total Body Cortisol Content and Interrenal Tissue Morphology

Whole body cortisol measurements were made on ethyl acetate extracts of whole glass eels and elvers, employing a modified method of that previously reported by Pottinger and Mosuwe (1994). Other workers have used diethyl ether as an extraction solvent, freezing or centrifuging the samples to separate the fractions (Hwang et al., 1992; Hiroi et al., 1997). Within the research group at the Gatty Marine Laboratory, both solvents have been assessed as a method of extracting cortisol from whole fish. However, the ethyl acetate method consistently proved to be the most efficient method, especially for eel tissue.

The extraction efficiencies determined for the work reported here were around 95% and were similar to those reported by others using a similar extraction procedure (Pottinger and Mosuwe, 1994). In order to assess to what extent the measurement of whole body cortisol gave an accurate measure of the cortisol response of fish, Pottinger and Mosuwe (1994) compared values of plasma levels in fingerling trout, to results obtained from whole body extractions. The rainbow trout from the Pottinger and Mosuwe study (1994) weighed approximately 3g and were therefore larger than the glass eels and elvers investigated here. Data from whole body preparations must be interpreted with care. Plasma hormone concentrations can generally be taken as indicative of the hormone concentration to which the target organ is exposed. However, neither plasma nor whole body measurements reflect the activity of the interrenal tissue. A more accurate indication is obtained by looking at the secretory dynamics of the interrenal tissue. Blood production rates and metabolic clearance rates tend to be higher in SW adapted eels despite the fact plasma cortisol levels are the same (Tierney, 1993). Transfer of adult eels from FW to SW stimulates a further transient increase in metabolic clearance rate and blood production rate, in addition to the initial increase observed in plasma cortisol concentrations (Tierney, 1993). Whole body preparations are further complicated by the presence of potentially cross-reactive substances from other body compartments rather than just the plasma. Although investigating the cortisol stress response, rather than osmoregulatory effects on cortisol, Pottinger and Mosuwe (1994) demonstrated that whole body cortisol measurements provided strong evidence of corticosteroidogenic activity. However, it is obvious that the dynamics of the cortisol response cannot be commented upon from analyses of whole body cortisol contents alone. In order to gain some insight as to the interrenal activity of glass eels and elvers a morphological approach was necessary. Measurement of steroid secretory dynamics by adult methods using sequential blood samples was obviously precluded due to the small size of glass eels and elvers.

Functional interrenal cells were identified in glass eels and elvers from all of the experimental groups throughout the duration of the study. Reduction in the activity of

interrenal cells is exhibited by a decrease in cytoplasmic versus nuclear size and atrophy, whilst the opposite is true of interrenal cells that are stimulated (Chester-Jones and Mosley, 1980). Total volume of interrenal tissue also increases when stimulated (Chester-Jones and Mosley, 1980). Ideally interrenal cells from many fish would be observed and the nuclear area to cytoplasmic area ratios calculated, in order to obtain some quantitative information on the function of the interrenal. Although the interrenal cells appeared to reduce in size with time, through both the environmental acclimation and the FW-SW transfer studies, there were no clear differences between salinity groups. Due to limitations of time the single samples were not expanded upon and conclusions regarding the activity of the interrenal cannot be made.

In adult fish plasma cortisol content is not significantly different between eels acclimated to FW and SW (Forrest (Jr.) *et al.*, 1973b; Tierney, 1993). At Month 1 glass eels in SW had a significantly higher whole body cortisol content than those acclimated to FW, as did those acclimated to 66% SW (Fig. 3.29). Fish acclimated to 33% SW had intermediate cortisol contents that were not significantly higher than those obtained for the FW acclimated fish. By Month 2 cortisol contents were not significantly different between fish acclimated to FW, 33% SW, 66% SW and SW (Fig. 3.30). The FW content had increased as compared to Month 1, whilst the 66% SW and SW levels had decreased such that all the groups had whole body cortisol contents similar to the 33% SW levels observed at Month 1. At Month 3 cortisol levels were not significantly different between FW and SW acclimated fish, but both had increased as compared to Month 2 (Fig. 3.31). At Month 4 the SW acclimated fish had significantly higher cortisol contents than fish from FW, but at Month 5 although the same pattern was present, the increase was not significant.

In adult *A. rostrata* there is an abrupt increase in plasma cortisol concentration within 1 day post-transfer from FW to SW to reach a maximum after 2 days post-transfer (Forrest (Jr.) *et al.*, 1973b). Cortisol concentrations then decline, although they are still above FW control levels by seven days post-transfer (Forrest (Jr.) *et al.*, 1973b). However, fully acclimated fish at two weeks post-transfer showed no differences in plasma cortisol concentration between FW and SW fish (Forrest (Jr.) *et al.*, 1973b). Similar results have also been shown for adult *A. anguilla* by Tierney (1993). When glass eels acclimated to FW were transferred to SW there was an immediate increase in whole body cortisol content (Fig. 3.32). Whole body cortisol content was significantly elevated in the FW-SW group as compared to the fish before transfer, however there was also a significant transitory increase in the FW-FW control group, although this was not as marked. The elevated values were maintained until after 9 h post-transfer. Differences between the FW-FW control and the FW-SW groups at 3 h, 6 h and 9 h were considered not quite significant by statistical tests (t-tests). After 24 h post-transfer cortisol levels then fell and fluctuated around this lower level until the end of the experiment at 4

months, although the FW-SW group was significantly higher than the FW-FW control group at 3 and 7 days post-transfer. It seems that in addition to a salinity effect there is a stress effect caused by transferring the fish to their new tank, which affected both groups of fish. Whilst the transient increases in cortisol content post-transfer may simply reflect a stress response, the higher increase exhibited by the FW-SW group may indicate that a component is related to osmoregulation.

Forrest (Jr.) *et al.* (1973b) found that plasma Na^+ levels paralleled the transitory increases in plasma cortisol that they observed in *A. rostrata*, and cortisol appeared to be important in stimulating $Na^+K^+ATPase$ activity in the gill in order to increase Na^+ secretion to regain osmoregulatory balance. The situation in this study is much less clear, as cortisol levels are low at 2, 3, and 4 months post-transfer (Fig. 3.32) whilst total body Na^+ levels are increasing (Fig. 3.27).

4.7 Further Work

The glass eels and elvers investigated in this study appeared well able to elicit the appropriate drinking behaviour depending upon environmental salinity. However, it is not drinking *per se* that relieves the dehydration of living in SW, but the absorption of water from the gut by solute-linked water transport. As time was limited, water absorption rates were not measured in this study. Obviously measurements of water absorption, in conjunction with determinations of drinking rate would be an essential part of further work. Water absorption rates by the gut of adult fish have been reported to be in the region of 65-80% of the volume ingested, and have been shown to vary with environmental salinity. Water absorption rates in adult rainbow trout have been shown to decrease from 80% in full SW to 66% in 33% SW (Kristensen and Skadhauge, 1974). In herring larvae, water absorption rates have also been shown to vary with environmental salinity, along and directly with drinking (Tytler and Ireland, 1994). However, in turbot larvae water absorption rates were altered but drinking rates were unaffected by changes in environmental salinity (Brown and Tytler, 1993).

A considerable amount of information on the drinking behaviour of glass eels and elvers is reported in this study - pertaining to acclimated fish, and fish in the few hours immediately post-transfer from FW to SW. However further work might look at the process of acclimation over an intermediate time course, perhaps using similar time-points to the FW-SW transfer experiment up until one month. Preliminary results suggest that drinking may stabilize much earlier in elvers, although further studies would have to be carried out to confirm this.

In connection to water absorption by the gut, it would also be informative to perform measurements of intestinal $Na^+K^+ATPase$ activity. In adult eels intestinal $Na^+K^+ATPase$ activity has been shown to increase with acclimation to SW (Jampol and Epstein, 1970). With further work the $Na^+K^+ATPase$ activity assay could probably be

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scaled down further in order to obtain measurement from single fish gills, which would allow an increase in the n value per group. Perhaps with larger sample sizes the inter-sample variability could be reduced.

Although attempts were made to sample blood from glass eels and elvers, a reliable method was not found. With further time investment it should be possible to obtain blood samples which could be used to determine ion concentrations, osmolalities and perhaps even circulating hormone concentrations from pooled samples. It is possible to measure osmolalities in very small volumes of blood, and this has been performed for fish larvae (Brown and Tytler, 1993), however highly specialized equipment is required. Further work would also include isotopic techniques to measure whole body Na^+, Cl^- and water fluxes. Initial attempts were made in this area however technical problems were encountered and a lack of time prevented their full resolution. In the light of the results from the FW-SW transfer experiment in terms of cortisol content, fish would be allowed further time to acclimate to new tanks after transfer, before salinity changes were undertaken. The results from the cortisol investigations appear to be complicated by an experimental stress effect due to the movement of fish to a different tank.

A section on further work would not be complete without mentioning the use of molecular techniques. Considerable information could be gained from molecular expression studies on glass eels and elvers as molecular techniques are perhaps less restrained by the small size of these fish as compared to traditional biochemical methods.

Further experiments also ought to look at the effects of maintaining glass eels in waters of varying salinity for much longer time periods, under aquaculture conditions of temperature and feeding, and to investigate the effect of rearing salinity on the rate of growth and on mortality rates.

4.8 Conclusions

Glass eels and elvers continue to survive when maintained in SW and appear to develop at a similar, if not faster rate than those fish kept in FW. This study has made only the very initial steps to elucidating the osmoregulatory processes that allow the remarkable migration of glass eels from SW into FW. Glass eels can control their drinking with changes in environmental salinity in a manner similar to that reported for adult fish, and also exhibit a strong rapid drinking response when faced with an acute osmoregulatory challenge. What is perhaps more remarkable is that with further development into elvers, when the fish have spent a number of months in FW, this rapid dipsogenic response remains just as strong. In terms of branchial $Na^+K^+ATPase$ activity, the results are not so clear, although with transfer to SW, $Na^+K^+ATPase$ activities did increase with time. These fish appear to have well developed interrenal tissue and elicit increases in cortisol content post-transfer to SW, as might be expected for adult fish. In

many respects therefore it seems that these small glass eels and elvers have all the osmoregulatory capacity of adult eels, they can adapt to environments of differing salinity with ease, and most definitely retain considerable osmoregulatory plasticity during the first six months post-migration, whilst ontogenic processes are still underway.

The glass eels and elvers were simple to maintain in laboratory conditions although their small size did present considerable challenges in terms of technique development. As they are truly remarkable fish, it is surprising that their osmoregulation has been neglected for so long, and with further time invested in technique development, they represent an excellent subject for study. With further work, conclusions may be derived that have considerable implications for their aquaculture.

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Appendices

Appendix 1

Suppliers

Consumables

Assay tubes: RT30 and RT25 (Bibby Sterilin Ltd., Stone, Staffordshire, UK) and LP3 (Denley Instruments Ltd., Billingshurst, West Sussex, UK).

Counting vials: PONY vials and Superpolyethylene counting vials (Canberra Packard Ltd., Pangbourne, Berkshire, UK).

Cuvettes: 0.5-2 ml semi-micro cuvettes (BDH Chemicals Ltd., Poole, Dorset, UK).

Filters: Whatman® No. 1 (Whatman International Ltd., Maidstone, Kent, UK).

Fish food: BP Nutrition, Alness, Highland, UK.

Laboratory film: Parafilm (American National Can™, Greenwich, Connecticut, USA) and Nescofilm (Nippon Shiji Kaisha Ltd., Japan).

Liquid scintillation cocktail: Emulsifier Scintillator plus™ (Canberra Packard Ltd., Pangbourne, Berkshire, UK).

Microcentrifuge tubes: 1.5ml and 2ml Safe-Lock microtest tubes (Eppendorf®, Netheler-Hinz GmbH, Hamburg, Germany).

Micropestles: Eppendorf®, Netheler-Hinz GmbH, Hamburg, Germany.

Microplates: Dynatech M29A sterile, flat-bottomed 96 well microplates (Dynex Technologies, Billingshurst, West Sussex, UK).

Photographic film: 64T slide film (Eastman Kodak Company, Rochester, NY, USA).

Surgical blades: Nos. 10, 11 and 24 (Swann-Morton® Ltd., Sheffield, South Yorkshire, UK).

Surgical gauze: Sterile absorbent gauze BP (The Boots Company plc, Nottingham, UK).

Syringes: Terumo (Terumo Europe NV, Leuven, Belgium) and B-D™ Plastipak™ (Becton Dickinson UK Ltd, Cowley, Oxford, UK).

Vials: Sterile Universal vials and Bijou vials, both Sterilin (Bibby Sterilin Ltd, Stone, Staffordshire, UK).

Equipment

Bench top centrifuges: MSE Microcentaur (Fisher Scientific UK Ltd., Loughborough, Leicestershire, UK) and Biofuge A Heraeus Christ, Osterode, Germany).

Centrifugal evaporator: Genevac SF50 with CVP 100 pump (Genevac Ltd., Sproughton, Ipswich, Suffolk, UK).

Centrifuges: MSE Coolspin 10-36 and MSE Mistral 3000 (Fisher Scientific UK Ltd., Loughborough, Leicestershire, UK), and Beckman J6-MC and Beckman J2-MC (Beckman Instruments Inc., Pala Alto, California, USA).

Flame photometer: EEL (Evans Electroselenium Ltd., Halstead, Essex, UK).

Gamma counter: Minaxi γ Auto-gamma 5000 series (Canberra Packard Ltd., Pangbourne, Berkshire, UK).

Graticules: 100 x 0.1 = 10mm and 100 x 0.01 = 1mm (Graticules Ltd, Tonbridge, Kent, UK).

Light box: Jessop 1723 (J. N. Campbell, Dundee, UK).

Liquid scintillation counter: 2000 Tri-carb® Scintillation Analyzer (Canberra Packard Ltd., Pangbourne, Berkshire, UK).

Microplate reader: Dynatech MRX (Dynex Technologies, Billingshurst, West Sussex, UK).

Microscopes: Iroscope NZ-14B (J. N. Campbell, Dundee, UK) and Leitz Dialux 20 (E. Leitz Instruments Ltd., Luton, UK).

Microsurgical scissors: John Weiss & Son Ltd., London, UK.

Multipipettes: Eppendorf® Multipette® 4780 with Combitips®, and Eppendorf® Multipette® plus with Combitip® plus (Eppendorf®, Netheler-Hinz GmbH, Hamburg, Germany).

Photographic set-up: Wild MPS photoautomat and Wild MPS 51S shutterpiece (Wild Heerbrugg Ltd., Heerbrugg, Switzerland).

Shaker: IKA-VIBRAX-VXR, (IKA-Labortechnik, Janke and Kunkel GmbH and Co. KG, Werk, Staufen).

Spectrophotometer: PU 8620 UV/VIS/NIR (Philips, Pye Unicam Ltd., Cambridge, UK).

Tissue homogenizer: Polytron® PT10-35 with PTA10S probe (Kinematica, Lucern GmbH, Switzerland).

Video camera: CCD (Cosmicar/Pentax, Japan).

Computing Applications

All computing applications were for Macintosh unless otherwise stated.

Curve fitting: CurveExpert v 1.34 © 1995-1997 (Daniel Hyams, Starkville, Mississippi, USA) for PC.

Graphs: CA-Cricket graph III v 1.5 © 1992-1993 (Computer Associates International Inc., Islandia, NY, USA).

Image Analysis: AnalySIS v 2.0 © 1986-1994 (Soft Imaging Software GmbH, Münster, Germany).

Image handling: Adobe Photoshop® v 4.0 © 1989-1996 (Adobe Systems Inc., Mountain View, California, USA) and Macromedia Freehand® v 5.5 © 1988-1995 (Macromedia Inc., Richardson, Texas, USA).

References: EndNote plus v 2.0.1 © 1988-1994 (Niles and Associates Inc., Berkeley, California, USA).

Spreadsheets: Microsoft Excel v 4.0 © 1985-1992 (Microsoft Corporation, Redmond, Washington, USA).

Statistics: StatView® v 4.02 © 1992-1993 (Abacus Concepts Inc., Berkeley, California, USA) and Instat v 2.01 for MacIntosh © 1992-1993, GraphPad, San Diego, California, USA).

Appendix 2

Reagents

Na⁺K⁺ATPase

- ### Homogenization Buffer

50 mM hepes; 1 mM EDTA; 0.1 mM dithiothreitol and 1 mM PMSF; pH 7.4

First a 50 mM Hepes (N-[2-hydroxythyl]piperazine-N-[2-ethyanesulfonic acid, Sigma) and 1 mM EDTA (free acid, purified grade, Sigma) solution was made by dissolving 6.51 g Hepes and 0.146 g EDTA in 400 ml MilliQ® which was then brought to pH 7.4 with the drop-wise addition of 5 M NaOH. This was then made up to 500 ml with MilliQ®, autoclaved and then stored in a refrigerator. On the day of homogenization 0.015 g dithiothreitol (DTT, Sigma) was dissolved in 10 ml MilliQ® to make a 10 mM DTT solution and 0.024 g phenylmethanesulfonyl fluoride (PMSF, Sigma) was also dissolved in 4 ml absolute ethanol resulting in a 34 mM solution. Prior to commencing homogenization 0.5 ml of the 10 mM DTT and 1.5 ml of the 34 mM PMSF solutions were added to 48 ml of the 50 mM Hepes/1 mM EDTA stock to make the homogenization buffer. The components and the final homogenization buffer were, as far as possible, kept on ice throughout.

- ### BSA Stock Solution

Bovine serum albumin (BSA), fraction V was dissolved at 0.080 g per 40 ml MilliQ® and then split into 1 ml aliquots and stored at -20°C before being defrosted as required.

- ### Bradford Reagent

Coomassie Brilliant Blue G250 (Sigma) (0.1g) was dissolved in 50ml 95% ethanol before the addition of 100ml 85% orthophosphoric acid, and the solution made up to 1L with MilliQ®. The solution was then filtered using Whatman No. 1 filter circles and stored protected from light.

- ### Na⁺K⁺ATPase Assay Buffer

300 mM histidine; 1.2 M NaCl; 80 mM MgCl₂ and 10 mM NaN₃; pH 7.2

Histidine (L-A-amino-ß-imidazolepropionic acid) (300mM) was first dissolved in 20ml MilliQ®. This required stirring with heat. 1.75 g NaCl and 0.203 g MgCl was then added, the pH checked (pH 7.2) and then made up to 25 ml with MilliQ®. Once cooled to room temperature 0.016 g NaN₃ was added. It is important to allow to cool to avoid

the production of noxious gas. The assay buffer was stored for 1-2 weeks protected from light at room temperature.

◦ **ATP**

Aliquots of 30mM ATP (disodium salt, Sigma) were stored at -20°C and defrosted as required. Before freezing the ATP solution had been adjusted to pH 7.4 with 1M TRIS base (tris(hydroxymethyl)methylamine).

◦ **Molybdate Reagent**

Ammonium molybdate (Fisons) (2 g) was dissolved in 100 ml MilliQ®. In a fume hood, on ice, 22.2 ml concentrated sulphuric acid was carefully added drop-wise, and then made up to 200 ml.

◦ **Stannous Chloride**

Stock 1 M $SnCl_2$ solution was made by dissolving 2 g stannous chloride in 10 ml concentrated hydrochloric acid. This was stored at -20°C.

Cortisol Content

◦ **RIA Assay Buffer**

40 mM Na_2HPO_4; 10 mM NaH_2PO_4; 0.9% (w/v) NaCl; 0.5% (w/v) BSA; pH 7.4

A stock phosphate solution was made containing 400 mM Na_2HPO_4 and 100 mM NaH_2PO_4 and stored at room temperature. On the day of the assay 10 ml of this stock phosphate solution was added to 80 ml MilliQ® and then 0.9 g NaCl and 0.5 g BSA added. The solution was adjusted to pH 7.4 with 5 m NaOH before being made up to 100 ml with MilliQ®.

Interrenal Tissue Morphology

◦ **Bouin's Fluid**

Saturated picric acid (750 ml), 240 ml 40% formaldehyde and 50 ml glacial acetic acid were mixed together in a fume hood and stored at room temperature protected from light.

- **Celestine Blue B**

   Celestine Blue B (0.5 g) was added to 100 ml 5% iron alum and boiled for three minutes. Once cool this was filtered and 14 ml glycerine added.

- **Mayer's Haemalum**

   Haematoxylin (1 g) was dissolved in 10 ml absolute ethanol and added to 1 L distilled water. Aluminium potassium sulphate (50 g) and 0.2 g sodium iodate were then added and the solution allowed to stand overnight. The following day 50 g chloral hydrate and 1 g citric acid were added before boiling for five minutes. Once cooled the solution was filtered and stored at room temperature.

- **Yellow Mordant**

   Orange G (0.4 g) was added to 160 ml saturated picric acid in 96% ethanol (A). In a separate vessel 0.4 g Lissamine Fast Yellow was dissolved in 40 ml distilled water (B). A and B were combined together and stored at room temperature. A working solution was then prepared for use by diluting 30 ml of this stock (A+B) in 70 ml 75% ethanol.

- **Ponceau Acid Fuchsin**

   Ponceau 2R (2 g) and 1 g Acid Fuchsin were dissolved in 200 ml distilled water and then 3 ml glacial acetic acid added.

Appendix 3

Spectral Response Curves

Protein Spectral Response Curves
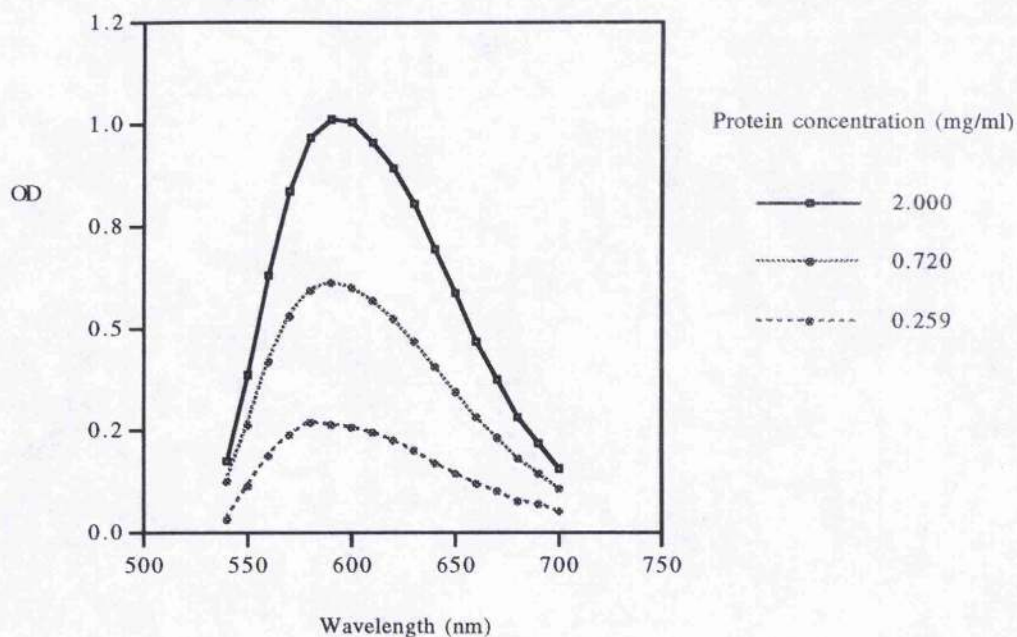
Spectral response curve for the protein assay using the standards as illustrated in the legend.

Phosphate Spectral Response Curves
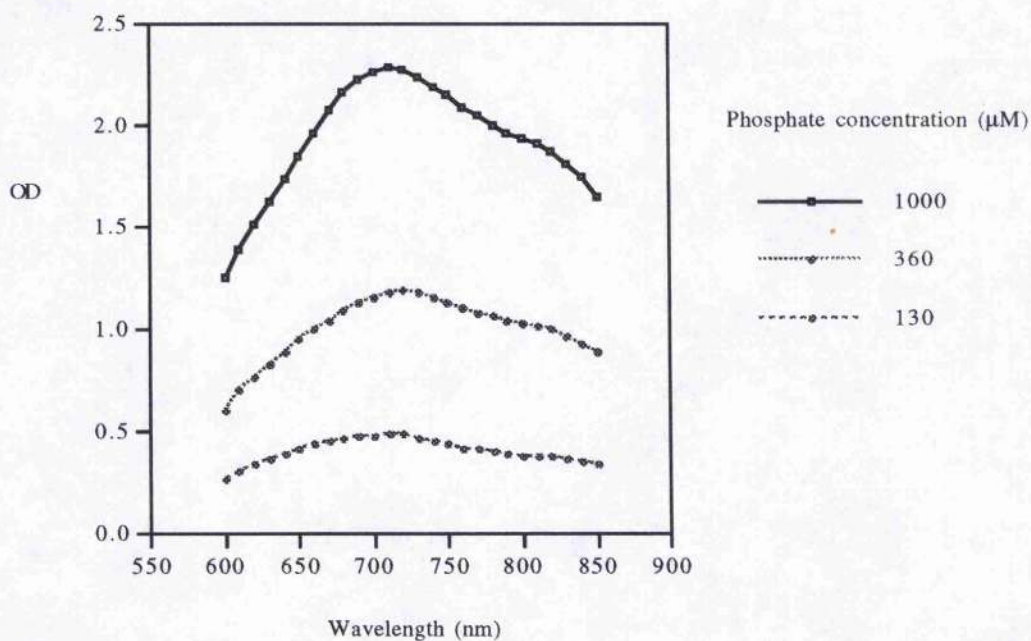
Spectral response curve for the phosphate assay using the standards as illustrated in the legend.

Appendix 4

Standard Curves

Protein Standard Curve

Optical density vs. Protein concentration (mg/ml)
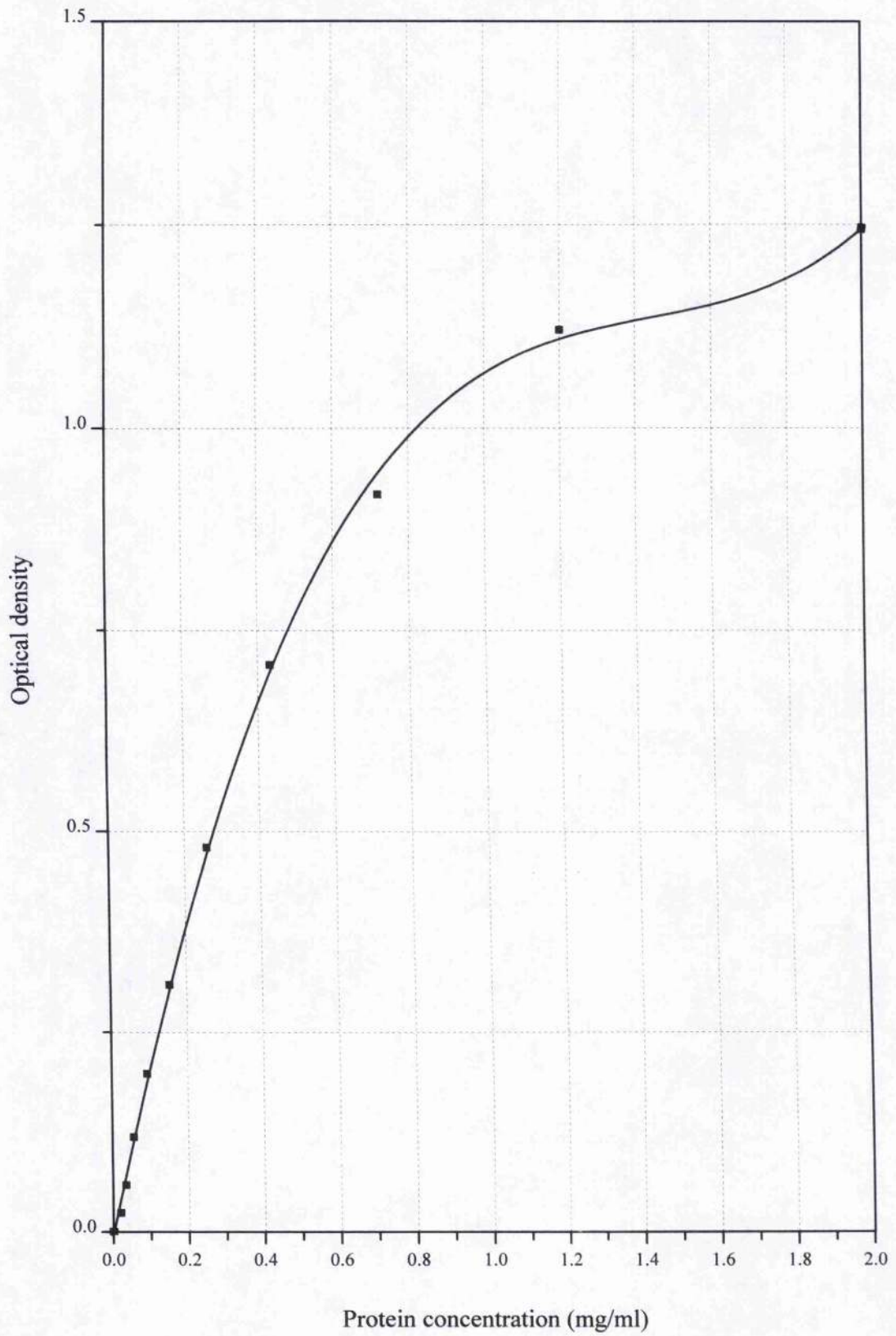
Phosphate Standard Curve
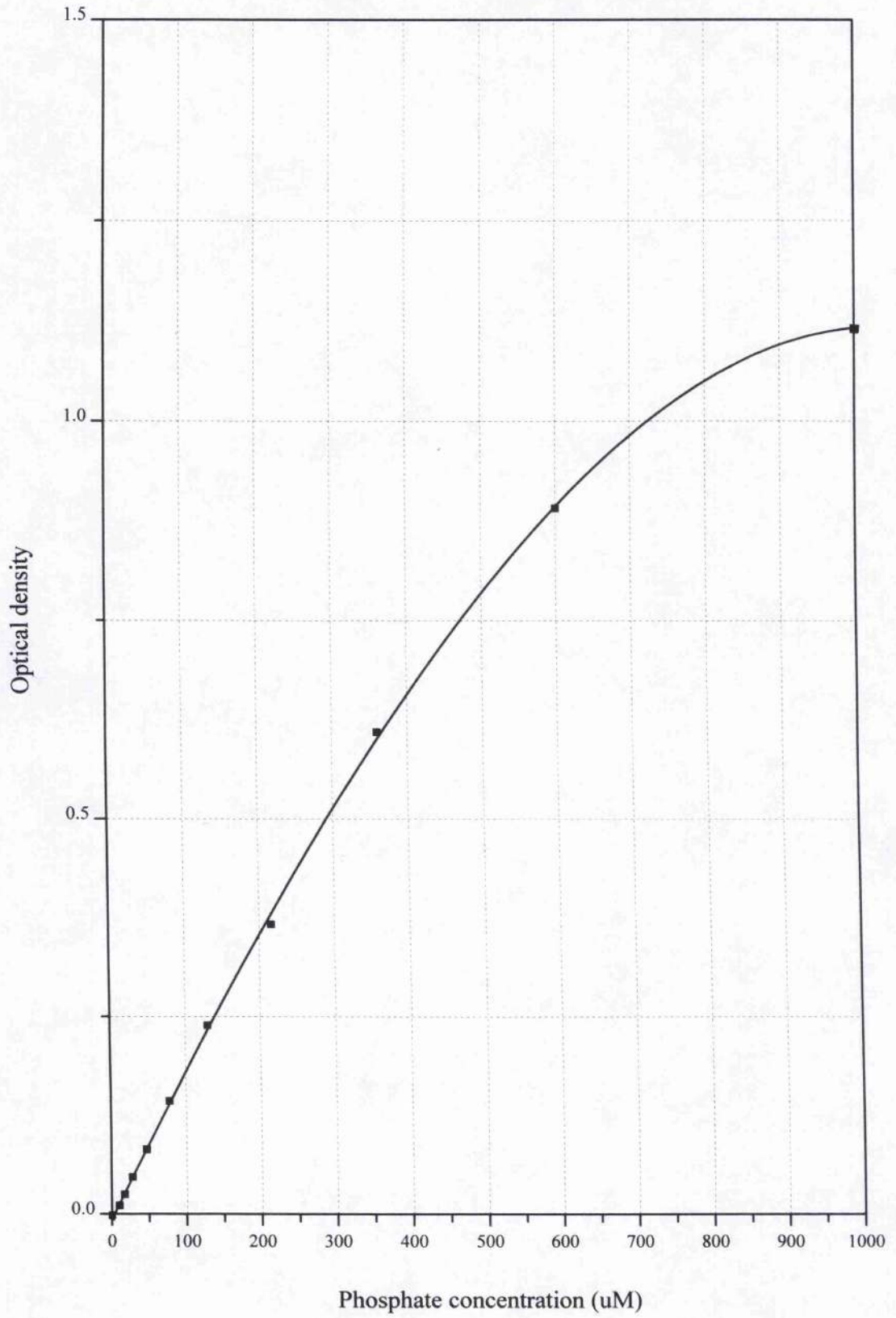
1mM Na+ Standard Curve
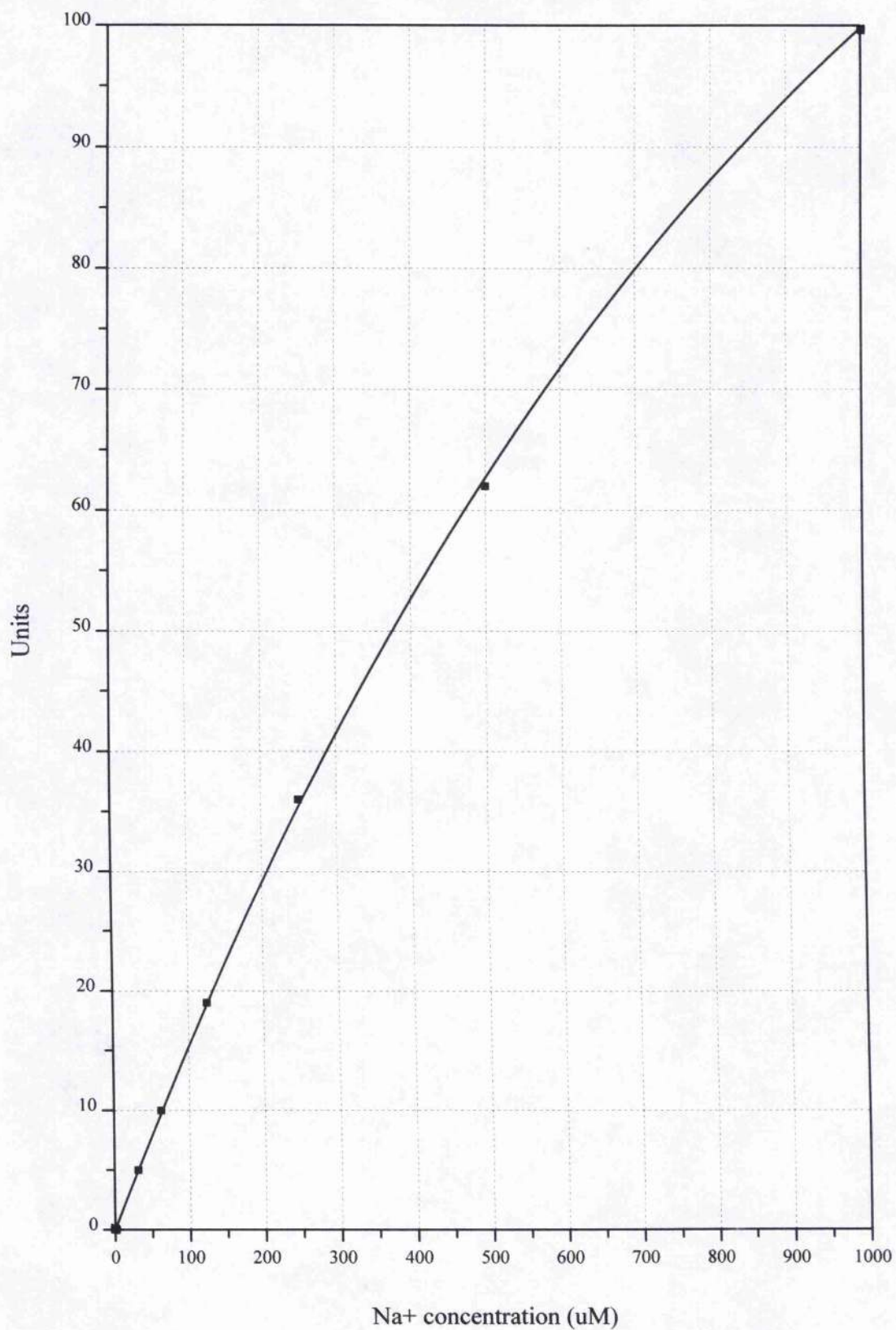
2mM Na+ Standard Curve

Na+ concentration (uM)

2mM K+ Standard Curve

Units

K+ concentration (uM)
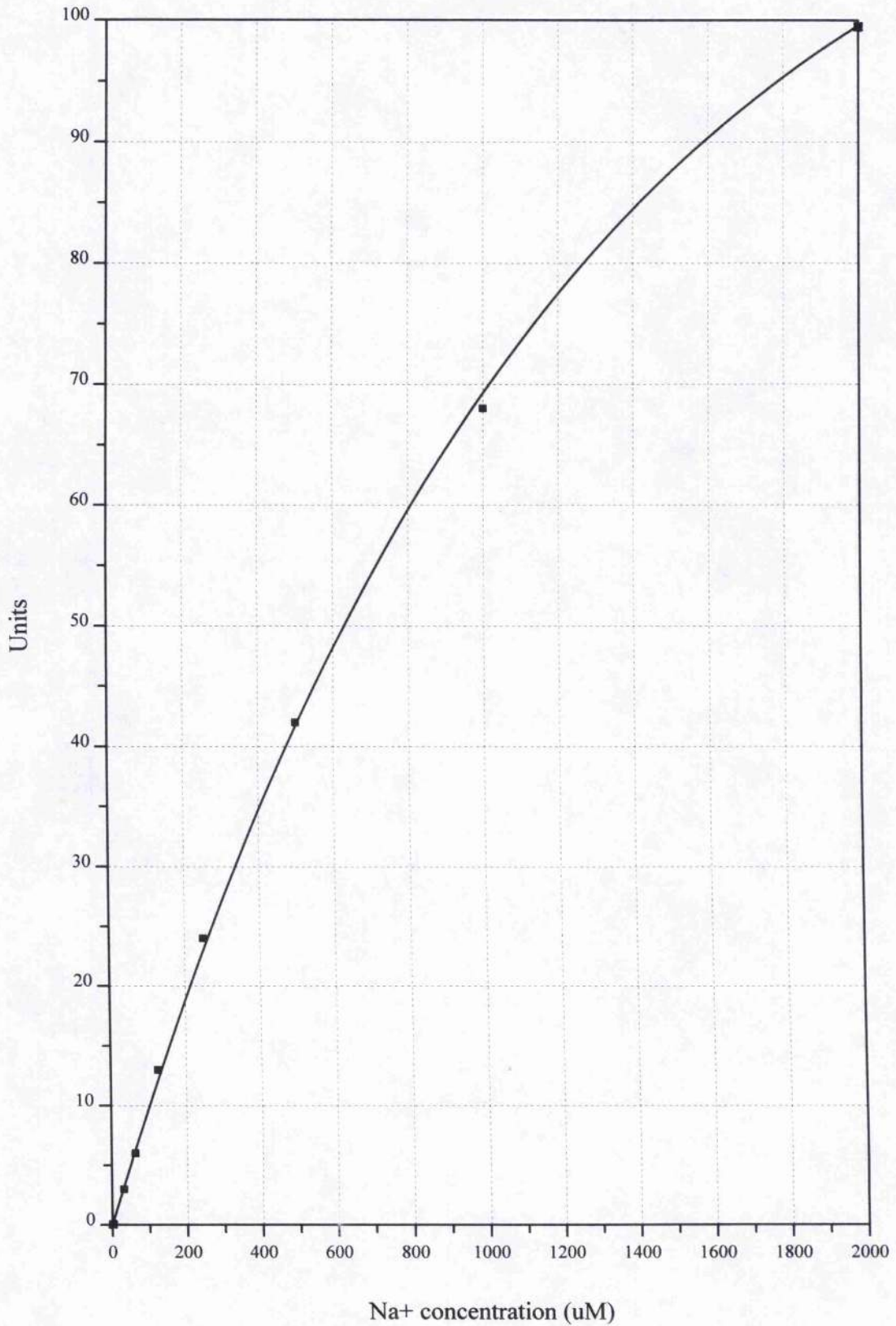
Cortisol Standard Curve
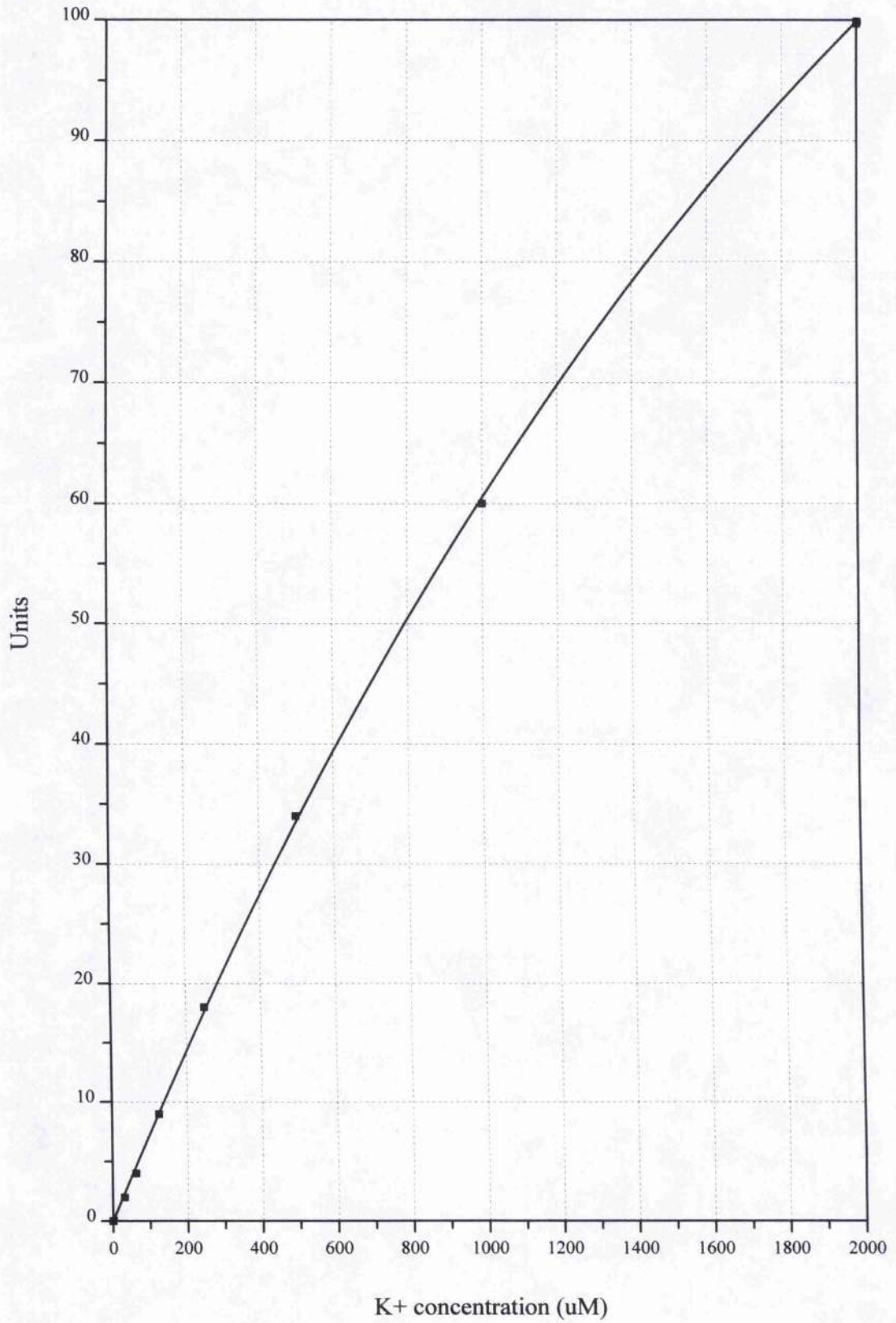
Cortisol concentration (pg/tube)
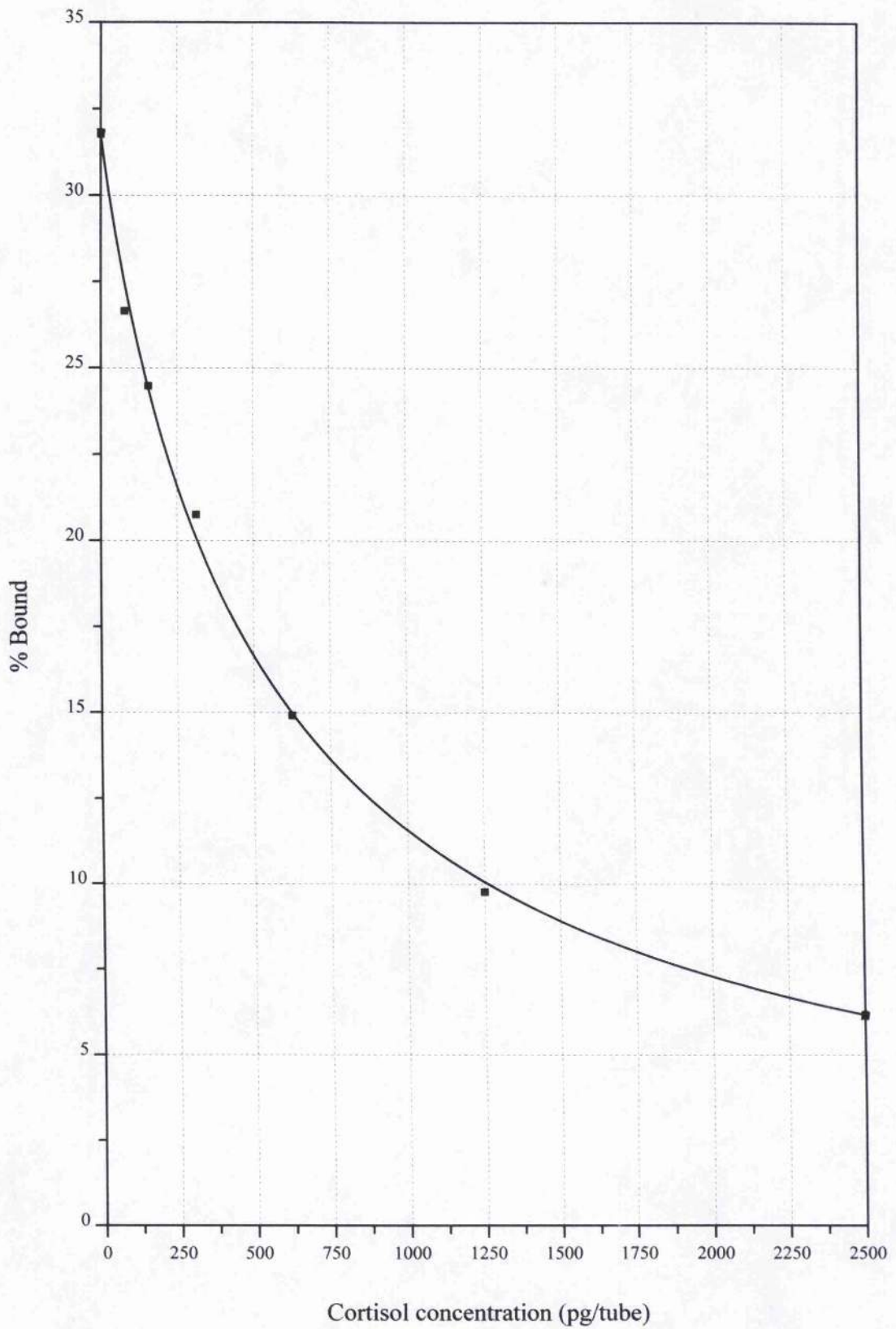
Appendix 5

Results of Statistical Tests

The following pages detail the results of statistical tests as referred to in chapter 3, Results. They are shown on a figure by figure basis (when appropriate) where df = degrees of freedom, F = ANOVA F statistic, t = t value and p = probability value. Significance levels were noted in the text as follows:

$p > 0.05$ = not significant, $p < 0.05$ = significant, $p < 0.01$ = very significant and $p <$ 0.001 = extremely significant.

Development - Environmental Acclimation Studies

Fig. 3.1	df	F	p
Month 1 - Acclimated	39	3.1285	= 0.0375

Fig. 3.2	df	F	p
Month 2 - Acclimated	39	18.157	< 0.0001

Fig. 3.3	df	F	p
Months 1 to 5 - FW Acclimated	49	18.559	< 0.0001
Months 1 to 5 - SW Acclimated	49	17.572	< 0.0001

	df	t	p
Month 1 - FW vs. SW Acclimated	18	2.4330	= 0.0256
Month 2 - FW vs. SW Acclimated	18	4.8365	= 0.0001
Month 3 - FW vs. SW Acclimated	18	1.5713	= 0.1335
Month 4 - FW vs. SW Acclimated	18	0.9687	= 0.3455
Month 5 - FW vs. SW Acclimated	18	10.672	< 0.0001

Wet Weight - Environmental Acclimation Studies

	df	F	p
Month 1 - Acclimated	145	1.4940	= 0.2188

	df	F	p
Month 2 - Acclimated	143	0.3568	= 0.7843

	df	F	p
Months 1 to 5 - FW Acclimated	184	27.828	< 0.0001
Months 1 to 5 - SW Acclimated	181	31.262	< 0.0001

	df	t	p
Month 1 - FW vs. SW Acclimated	71	1.7191	= 0.0899
Month 2 - FW vs. SW Acclimated	71	0.5097	= 0.6119
Month 3 - FW vs. SW Acclimated	71	1.9077	= 0.0605
Month 4 - FW vs. SW Acclimated	72	0.7664	= 0.4459
Month 5 - FW vs. SW Acclimated	72	3.1375	= 0.0025

Wet Weight - FW-SW Transfer Studies

	df	F	*p*
FW-FW Transfer	228	11.166	< 0.0001
FW-SW Transfer	228	12.358	< 0.0001

	df	*t*	*p*
3h - FW-FW vs. FW-SW	30	0.3793	= 0.7072
6h - FW-FW vs. FW-SW	30	0.8684	= 0.3921
9h - FW-FW vs. FW-SW	30	1.2188	= 0.2324
24h - FW-FW vs. FW-SW	30	2.2955	= 0.0289
3d - FW-FW vs. FW-SW	30	0.5728	= 0.5710
7d - FW-FW vs. FW-SW	30	0.2135	= 0.8324
10d - FW-FW vs. FW-SW	30	1.1765	= 0.2486
14d - FW-FW vs. FW-SW	30	0.9737	= 0.3380
21d - FW-FW vs. FW-SW	30	1.6519	= 0.1090
2 months - FW-FW vs. FW-SW	30	0.3868	= 0.7016
3 months - FW-FW vs. FW-SW	30	0.4650	= 0.6453
4 months - FW-FW vs. FW-SW	30	1.9171	= 0.0648

Drinking Rate - Evaluation of the Method

Fig. 3.4	df	F	p
Specific Activity of ^{51}Cr-EDTA	49	4.3600	= 0.0046

Drinking Rate - Environmental Acclimation Studies

Fig. 3.7	df	F	p
Month 1 - Acclimated	80	17.372	< 0.0001

Fig. 3.8	df	F	p
Month 2 - Acclimated	76	27.561	< 0.0001

Fig. 3.9	df	F	p
Months 1 to 5 - FW Acclimated	104	3.5495	= 0.0094
Months 1 to 5 - SW Acclimated	101	70.429	< 0.0001

	df	t	p
Month 1 - FW vs. SW Acclimated	39	6.2845	< 0.0001
Month 2 - FW vs. SW Acclimated	39	6.6213	< 0.0001
Month 3 - FW vs. SW Acclimated	39	7.5751	< 0.0001
Month 4 - FW vs. SW Acclimated	40	10.231	< 0.0001
Month 5 - FW vs. SW Acclimated	40	10.374	< 0.0001

Drinking Rate - Monthly Drinking Rate Challenge Studies

Fig. 3.10a	df	F	p
Month 1 FW-FW Drinking Rate Challenge	108	1.8271	= 0.0806
Month 1 FW-SW Drinking Rate Challenge	103	10.664	< 0.0001

	df	t	p
Month 1 - 5 min - FW-FW vs. FW-SW	23	2.8292	= 0.0095
Month 1 - 15 min -FW-FW vs. FW-SW	21	7.4147	< 0.0001
Month 1 - 30 min - FW-FW vs. FW-SW	20	5.3254	< 0.0001
Month 1 - 1 h - FW-FW vs. FW-SW	21	7.0359	< 0.0001
Month 1 - 2h - FW-FW vs. FW-SW	21	6.2114	< 0.0001
Month 1 - 3h - FW-FW vs. FW-SW	22	6.1995	< 0.0001
Month 1 - 4h - FW-FW vs. FW-SW	22	8.4827	< 0.0001
Month 1 - 5h - FW-FW vs. FW-SW	23	6.9137	< 0.0001

Fig. 3.11a	df	F	p
Month 2 FW-FW Drinking Rate Challenge	106	6.1321	< 0.0001
Month 2 FW-SW Drinking Rate Challenge	100	11.592	< 0.0001

	df	t	p
Month 2 - 5 min - FW-FW vs. FW-SW	23	3.5461	= 0.0017
Month 2 - 15 min - FW-FW vs. FW-SW	23	7.2262	< 0.0001
Month 2 - 30 min - FW-FW vs. FW-SW	21	7.0405	< 0.0001
Month 2 - 1 h - FW-FW vs. FW-SW	22	3.8955	= 0.0008
Month 2 - 2h - FW-FW vs. FW-SW	21	5.7278	< 0.0001
Month 2 - 3h - FW-FW vs. FW-SW	22	5.1826	< 0.0001
Month 2 - 4h - FW-FW vs. FW-SW	17	3.7537	= 0.0016
Month 2 - 5h - FW-FW vs. FW-SW	19	1.0113	= 0.3246

Fig. 3.12a	df	F	p
Month 3 FW-FW Drinking Rate Challenge	103	2.5708	= 0.0139
Month 3 FW-SW Drinking Rate Challenge	111	7.395	< 0.0001

	df	t	p
Month 3 - 5 min - FW-FW vs. FW-SW	22	4.0719	= 0.0005
Month 3 - 15 min - FW-FW vs. FW-SW	22	5.5804	< 0.0001
Month 3 - 30 min - FW-FW vs. FW-SW	22	6.3095	< 0.0001
Month 3 - 1 h - FW-FW vs. FW-SW	22	5.9157	< 0.0001
Month 3 - 2h - FW-FW vs. FW-SW	22	5.7553	< 0.0001
Month 3 - 3h - FW-FW vs. FW-SW	20	5.1920	< 0.0001
Month 3 - 4h - FW-FW vs. FW-SW	25	3.5963	= 0.0014
Month 3 - 5h - FW-FW vs. FW-SW	21	4.1374	= 0.0005

Fig. 3.13a	df	F	p
Month 4 FW-FW Drinking Rate Challenge	114	2.1599	= 0.0364
Month 4 FW-SW Drinking Rate Challenge	114	6.3070	< 0.0001

	df	t	p
Month 4 - 5 min - FW-FW vs. FW-SW	21	8.4376	< 0.0001
Month 4 - 15 min - FW-FW vs. FW-SW	21	7.0094	< 0.0001
Month 4 - 30 min - FW-FW vs. FW-SW	25	5.4808	< 0.0001
Month 4 - 1 h - FW-FW vs. FW-SW	25	3.4525	= 0.0020
Month 4 - 2h - FW-FW vs. FW-SW	24	6.0638	< 0.0001
Month 4 - 3h - FW-FW vs. FW-SW	22	2.0337	= 0.0542
Month 4 - 4h - FW-FW vs. FW-SW	26	2.0027	= 0.0557
Month 4 - 5h - FW-FW vs. FW-SW	26	5.2763	< 0.0001

Fig. 3.14a	df	F	*p*
Month 5 FW-FW Drinking Rate Challenge	71	8.0117	< 0.0001
Month 5 FW-SW Drinking Rate Challenge	71	11.746	< 0.0001

	df	*t*	*p*
Month 5 - 5 min - FW-FW vs. FW-SW	22	8.1149	< 0.0001
Month 5 - 15 min - FW-FW vs. FW-SW	20	7.5710	< 0.0001
Month 5 - 30 min - FW-FW vs. FW-SW	21	7.7721	< 0.0001
Month 5 - 1 h - FW-FW vs. FW-SW	24	5.6048	< 0.0001
Month 5 - 3h - FW-FW vs. FW-SW	23	3.3140	= 0.0030

Fig. 3.15	df	F	*p*
Months 1 to 5 FW-FW Challenge as Rate	55	1.4122	= 0.2432
Months 1 to 5 FW-SW Challenge as Rate	60	28.580	< 0.0001

	df	*t*	*p*
Month 1 - FW-FW vs. FW-SW	21	7.4147	< 0.0001
Month 2 - FW-FW vs. FW-SW	23	7.2262	< 0.0001
Month 3 - FW-FW vs. FW-SW	22	5.5804	< 0.0001
Month 4 - FW-FW vs. FW-SW	21	7.0094	< 0.0001
Month 5 - FW-FW vs. FW-SW	20	7.5710	< 0.0001

Gill Homogenate Protein Concentration - Environmental Acclimation Studies

	df	F	p
Month 1 - Acclimated	23	0.8167	= 0.4997

	df	F	p
Month 2 - Acclimated	23	1.7613	= 0.1869

Fig. 3.18	df	F	p
Months 1 to 4 - FW Acclimated	23	17.484	< 0.0001
Months 1 to 4 - SW Acclimated	21	9.8391	= 0.0005

	df	t	p
Month 1 - FW vs. SW Acclimated	10	1.3442	= 0.2086
Month 2 - FW vs. SW Acclimated	10	0.2875	= 0.7796
Month 3 - FW vs. SW Acclimated	9	3.0052	= 0.0148
Month 4 - FW vs. SW Acclimated	9	5.6687	= 0.0003

Gill Homogenate Protein Concentration - FW-SW Transfer Studies

Fig. 3.19	df	F	p
FW-FW Transfer	76	8.5770	< 0.0001
FW-SW Transfer	76	5.1281	< 0.0001

	df	t	p
3h - FW-FW vs. FW-SW	10	0.4196	= 0.6837
6h - FW-FW vs. FW-SW	10	0.4938	= 0.6321
9h - FW-FW vs. FW-SW	10	0.1386	= 0.8925
24h - FW-FW vs. FW-SW	10	0.7947	= 0.4452
3d - FW-FW vs. FW-SW	10	1.3085	= 0.2200
7d - FW-FW vs. FW-SW	10	2.0180	= 0.0712
10d - FW-FW vs. FW-SW	9	1.2799	= 0.2326
14d - FW-FW vs. FW-SW	10	7.0627	< 0.0001
21d - FW-FW vs. FW-SW	10	4.0642	= 0.0023
2 months - FW-FW vs. FW-SW	10	6.7941	< 0.0001
3 months - FW-FW vs. FW-SW	10	7.8153	< 0.0001
4 months - FW-FW vs. FW-SW	9	3.5952	= 0.0058

Na+K+ATPase Activity - Environmental Acclimation Studies

Fig. 3.20	df	F	p
Month 1 - Acclimated	23	0.6430	= 0.5963

Fig. 3.21	df	F	p
Month 2 - Acclimated	23	2.0834	= 0.1346

Fig. 3.22	df	F	p
Months 1 to 4 - FW Acclimated	23	61.924	< 0.0001
Months 1 to 4 - SW Acclimated	21	14.377	< 0.0001

	df	t	p
Month 1 - FW vs. SW Acclimated	10	0.3736	= 0.7165
Month 2 - FW vs. SW Acclimated	10	0.5461	= 0.5970
Month 3 - FW vs. SW Acclimated	9	0.6904	= 0.5074
Month 4 - FW vs. SW Acclimated	9	3.9396	= 0.0034

Na+K+ATPase Activity - FW-SW Transfer Studies

Fig. 3.23	df	F	p
FW-FW Transfer	76	4.3808	< 0.0001
FW-SW Transfer	76	7.0173	< 0.0001

	df	t	p
3h - FW-FW vs. FW-SW	10	0.5376	= 0.6026
6h - FW-FW vs. FW-SW	10	0.4768	= 0.6437
9h - FW-FW vs. FW-SW	10	0.2434	= 0.8126
24h - FW-FW vs. FW-SW	10	1.2041	= 0.2563
3d - FW-FW vs. FW-SW	10	2.6485	= 0.0244
7d - FW-FW vs. FW-SW	10	0.0849	= 0.9340
10d - FW-FW vs. FW-SW	9	1.6492	= 0.1335
14d - FW-FW vs. FW-SW	10	4.0763	= 0.0022
21d - FW-FW vs. FW-SW	10	1.8274	= 0.0976
2 months - FW-FW vs. FW-SW	10	5.1569	= 0.0004
3 months - FW-FW vs. FW-SW	10	3.1540	= 0.0103
4 months - FW-FW vs. FW-SW	9	2.6537	= 0.0263

Total Body Na+ and K+ Content - Environmental Acclimation Studies

Fig. 3.24a (Na+)	df	F	p
Month 1 - Acclimated	29	1.3254	= 0.2875

Fig. 3.24b (K+)	df	F	p
Month 1 - Acclimated	29	2.0174	= 0.1361

Fig. 3.25a (Na+)	df	F	p
Month 2 - Acclimated	31	24.174	< 0.0001

Fig. 3.25b (K+)	df	F	p
Month 2 - Acclimated	31	0.3205	= 0.8104

Fig. 3.26a (Na+)	df	F	p
Months 1 to 5 - FW Acclimated	39	8.5440	< 0.0001
Months 1 to 5 - SW Acclimated	38	28.562	< 0.0001

	df	t	p
Month 1 - FW vs. SW Acclimated	14	1.2274	= 0.2399
Month 2 - FW vs. SW Acclimated	14	7.0594	< 0.0001
Month 3 - FW vs. SW Acclimated	14	3.0070	= 0.0094
Month 4 - FW vs. SW Acclimated	13	1.8205	= 0.0918
Month 5 - FW vs. SW Acclimated	14	7.1259	< 0.0001

Fig. 3.26b (K^+)	df	F	p
Months 1 to 5 - FW Acclimated	39	14.807	< 0.0001
Months 1 to 5 - SW Acclimated	38	9.3579	< 0.0001

	df	t	p
Month 1 - FW vs. SW Acclimated	14	0.3357	= 0.7421
Month 2 - FW vs. SW Acclimated	14	0.1150	= 0.9101
Month 3 - FW vs. SW Acclimated	14	1.7275	= 0.1061
Month 4 - FW vs. SW Acclimated	13	2.9536	= 0.0112
Month 5 - FW vs. SW Acclimated	14	1.3785	= 0.1897

Total Body Na^+ and K^+ Content - FW-SW Transfer Studies

Fig. 3.27 (Na^+)	df	F	p
FW-FW Transfer	101	5.7425	< 0.0001
FW-SW Transfer	96	11.093	< 0.0001

	df	t	p
3h - FW-FW vs. FW-SW	13	4.3753	= 0.0008
6h - FW-FW vs. FW-SW	11	2.1278	= 0.0568
9h - FW-FW vs. FW-SW	14	5.1992	= 0.0001
24h - FW-FW vs. FW-SW	12	2.1843	= 0.0495
3d - FW-FW vs. FW-SW	14	2.1892	= 0.0460
7d - FW-FW vs. FW-SW	13	4.8920	= 0.0003
10d - FW-FW vs. FW-SW	13	3.2998	= 0.0057
14d - FW-FW vs. FW-SW	14	2.4332	= 0.0290
21d - FW-FW vs. FW-SW	14	2.4755	= 0.0267
2 months - FW-FW vs. FW-SW	13	3.0979	= 0.0085
3 months - FW-FW vs. FW-SW	14	1.1463	= 0.2709
4 months - FW-FW vs. FW-SW	14	4.6194	= 0.0004

Fig. 3.28 (K+)	df	F	p
FW-FW Transfer	101	8.8293	< 0.0001
FW-SW Transfer	96	4.4723	< 0.0001

	df	t	p
3h - FW-FW vs. FW-SW	13	1.5150	= 0.1537
6h - FW-FW vs. FW-SW	11	0.3393	= 0.7408
9h - FW-FW vs. FW-SW	14	0.0063	= 0.9951
24h - FW-FW vs. FW-SW	12	0.5008	= 0.6255
3d - FW-FW vs. FW-SW	14	0.0596	= 0.9533
7d - FW-FW vs. FW-SW	13	2.134	= 0.0497
10d - FW-FW vs. FW-SW	13	1.4984	= 0.1579
14d - FW-FW vs. FW-SW	14	8.2565	< 0.0001
21d - FW-FW vs. FW-SW	14	1.9176	= 0.0758
2 months - FW-FW vs. FW-SW	13	1.3054	= 0.2144
3 months - FW-FW vs. FW-SW	14	2.1215	= 0.0522
4 months - FW-FW vs. FW-SW	14	0.0186	= 0.9854

Total Body Cortisol Content - Environmental Acclimation Studies

Fig. 3.29	df	F	p
Month 1 - Acclimated	31	16.200	< 0.0001

Fig. 3.30	df	F	p
Month 2 - Acclimated	31	0.0952	= 0.9621

Fig. 3.31	df	F	p
Months 1 to 5 - FW Acclimated	39	7.3633	= 0.0002
Months 1 to 5 - SW Acclimated	39	4.1519	= 0.0074

	df	t	p
Month 1 - FW vs. SW Acclimated	14	6.4379	< 0.0001
Month 2 - FW vs. SW Acclimated	14	0.2490	= 0.8069
Month 3 - FW vs. SW Acclimated	14	0.0836	= 0.9346
Month 4 - FW vs. SW Acclimated	14	5.1969	= 0.0001
Month 5 - FW vs. SW Acclimated	14	1.4849	= 0.1597

Total Body Cortisol Content - FW-SW Transfer Studies

Fig. 3.32	df	F	p
FW-FW Transfer	100	5.3558	< 0.0001
FW-SW Transfer	101	11.994	< 0.0001

	df	t	p
3h - FW-FW vs. FW-SW	12	2.0708	= 0.0606
6h - FW-FW vs. FW-SW	14	2.0912	= 0.0552
9h - FW-FW vs. FW-SW	13	2.0894	= 0.0569
24h - FW-FW vs. FW-SW	14	0.4219	= 0.6795
3d - FW-FW vs. FW-SW	13	2.5650	= 0.0235
7d - FW-FW vs. FW-SW	14	2.6545	= 0.0189
10d - FW-FW vs. FW-SW	14	1.7703	= 0.0984
14d - FW-FW vs. FW-SW	14	0.9495	= 0.3585
21d - FW-FW vs. FW-SW	14	0.9515	= 0.3575
2 months - FW-FW vs. FW-SW	14	0.0613	= 0.9520
3 months - FW-FW vs. FW-SW	13	1.3051	= 0.2145
4 months - FW-FW vs. FW-SW	14	0.1768	= 0.1768

Drinking Rate - Monthly Drinking Rate Challenge Studies

Fig. 4.3	df	F	p
FW-FW Transfer	81	1.0306	= 0.4123
FW-SW Transfer	79	9.5692	< 0.0001

FW-SW all time-points were (Dunnett's) $p < 0.001$ as compared to 0-30m

	df	t	p
0-30 min - FW-FW vs. FW-SW	20	3.3147	= 0.0035
30 min - 1 h - FW-FW vs. FW-SW	20	1.7950	= 0.0879
1-1.5h - FW-FW vs. FW-SW	19	2.7440	= 0.0129
2.5-3 h - FW-FW vs. FW-SW	21	2.4402	= 0.0236
5.5-6 h - FW-FW vs. FW-SW	19	0.5018	= 0.6216
8.5-9 h - FW-FW vs. FW-SW	20	0.1638	= 0.8715
11.5-12 h - FW-FW vs. FW-SW	29	3.0544	= 0.0048