Chapter 6: Rectal gland respirometry
6.1 Introduction

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

Another method of assessing rectal gland activity is through respirometry studies and investigating the O$_2$ consumption of the gland. Such studies have been conducted on both *S. canicula* (Shuttleworth and Thompson 1980) and *S. acanthias* (Morgan et al. 1997). Shuttleworth and Thompson (1980) conducted respirometry studies on tissue from the rectal gland, spleen, and kidney of SW *S. canicula*. They discovered that whole tissue O$_2$ consumption in the spleen (94 ± 25 µl O$_2$ g$^{-1}$ h$^{-1}$) was significantly lower than that in the rectal gland (234 ± 59 µl O$_2$ g$^{-1}$ h$^{-1}$) and the kidney (248 ± 61 µl O$_2$ g$^{-1}$ h$^{-1}$). This compares to a value of 27.9 µl O$_2$ g$^{-1}$ h$^{-1}$ for whole animal O$_2$ uptake measured by Butler and Taylor (1975). The rate of O$_2$ uptake in these tissues was higher than that of the whole animal; this is due to the fact that a large proportion of body mass is associated with tissues with low O$_2$ consumption, such as skeletal elements and body fluids.

The markedly higher O$_2$ consumption rates of the rectal gland and the kidney reflect a higher metabolic rate in these tissues which is largely due to active osmolyte transport in these osmoregulatory tissues (Sections 1.5 and 6). These tissues also have higher maximal activities of Na$^+$, K$^+$-ATPase, even compared to other osmoregulatory tissues.
Conversely the low O₂ consumption seen in tissue from the spleen indicates a lower metabolic activity, reflecting its role in the storage and release of red blood cells.

Morgan and co-workers (1997) investigated the O₂ consumption in the rectal gland and gills of *S. acanthias*. It was found that O₂ consumption in the rectal gland (14.2 ± 1.2 µmol O₂ g⁻¹ h⁻¹) was significantly higher than that in the gill (9.6 ± 1.4 µmol O₂ g⁻¹ h⁻¹). Again this is consistent with the maximal activity of Na⁺, K⁺-ATPase in these tissues in *S. canicula* (Figure 5.3.2.1). The addition of 0.5 mM ouabain and the resulting inhibition of Na⁺, K⁺-ATPase were associated with a 54.9 and 21.8% reduction in rectal gland and gill O₂ consumption respectively. Furthermore, the residual levels of O₂ consumption in the two tissues did not differ (Morgan et al. 1997). This suggests that different levels of Na⁺, K⁺-ATPase activity or abundance are the cause of higher O₂ consumption in the rectal gland of *S. acanthias*.

Shuttleworth and Thompson (1980) also investigated the effects of 10⁻⁴ M ouabain on basal O₂ consumption in tissue from the rectal gland, spleen, and kidney. The inhibition of Na⁺, K⁺-ATPase with ouabain significantly reduced O₂ consumption rates in all three tissues. The proportion of whole tissue O₂ consumption associated with Na⁺, K⁺-ATPase was calculated as 22.1, 20.2, and 41.1% in the rectal gland, spleen, and kidney respectively. Given that maximal activity of the enzyme is higher in the rectal gland than in the kidney (Figure 5.3.2.1) these findings suggest that a large proportion of O₂ consumption in the rectal gland is associated with other metabolic processes. The differences in ouabain-sensitive O₂ consumption during basal secretion in the rectal glands of *S. acanthias* and *S. canicula* (54.9 and 22.1% respectively) either reflect a
difference in rectal gland structure between the two species, differences in the degree of ouabain inhibition of Na\(^+\), K\(^+\)-ATPase, or differences in the secretory activity of the glands sampled between the two species. It has been demonstrated in *S. canicula* that 10\(^{-4}\) M ouabain does not completely inhibit Na\(^+\), K\(^+\)-ATPase, unlike concentrations above 1 mM (MacKenzie 1996). This may have lead to an underestimation of the relative O\(_2\) consumption by Na\(^+\), K\(^+\)-ATPase in the rectal gland of *S. canicula*.

Supporting the finding of high maximal Na\(^+\), K\(^+\)-ATPase activity in the rectal gland is the increase in rectal gland O\(_2\) consumption following administration of cAMP (0.05 mmol l\(^{-1}\)) and theophylline (0.25 mmol l\(^{-1}\)). A 5-fold increase in O\(_2\) consumption was associated with these substances, and this was entirely abolished by the coupled administration of ouabain. Interestingly, the same administration of cAMP and theophylline had no effect on O\(_2\) consumption in either the spleen or the kidney (Shuttleworth and Thompson 1980). Similar concentrations of these substances have been shown to increase secretory activity in isolated perfused rectal glands (Silva et al. 1977; Stoff et al. 1977b), as well as increase ouabain binding (Shuttleworth and Thompson 1978). These findings suggest that cAMP and theophylline specifically stimulate the activity of Na\(^+\), K\(^+\)-ATPase in the tissues of the rectal gland. This is consistent with other studies which have suggested that cAMP activates Na\(^+\), K\(^+\)-ATPase thereby reducing cellular concentrations of Na\(^+\) in cultured rectal gland cells (Lear et al. 1992). Furthermore, O\(_2\) consumption in the rectal gland is increased by the addition of theophylline alone, to levels equivalent to those associated with the coupled administration of cAMP (Shuttleworth and Thompson 1980). Theophylline inhibits the breakdown of cAMP by phosphodiesterases. This illustrates the stimulatory effect of endogenous cAMP on Na\(^+\), K\(^+\)-ATPase in the rectal gland, as well as suggesting the
continued production of this cAMP in incubated tissue slices. These findings are contrary to those of Stoff and co-workers (1977b) who reported a synergistic effect of cAMP and theophylline on Cl\(^{-}\) secretion rates during coupled administration on isolated perfused rectal glands. Theophylline may therefore also have effects on other proteins involved in ion transport which do not require increased O\(_2\) consumption during periods of active secretion.

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

One such group of fast acting factors are vasopressins (such as AVT in elasmobranchs (Section 1.8.2)) which regulate urine flow rate, GFR, and tubular transport maxima for glucose in the kidney (Amer and Brown 1995; Wells et al. 2002). Similar antidiuretic hormones have been shown to increase Na\(^{+}\) entry into cells via the number of functional Na\(^{+}\) channels, and hence stimulate the action of Na\(^{+}\), K\(^{+}\)-ATPase in a variety of vertebrates (Mendoza et al. 1980; Li et al. 1982; Reznik et al. 1985).
Another group of fast acting factors are catecholamines (Section 1.12) which have been shown to induce cAMP-mediated stimulation of Na\(^+\), K\(^+\)-ATPase activity in skeletal muscle which were independent of changes in Na\(^+\), K\(^+\)-ATPase abundance (Clausen and Hansen 1977). Studies on vertebrate cerebral cell cultures have shown that catecholamine stimulation of Na\(^+\), K\(^+\)-ATPase activity can also be independent of cAMP levels (Wu and Phillips 1980). Clearly more research is required into the effects of catecholamines on Na\(^+\), K\(^+\)-ATPase, particularly in elasmobranchs.

Thyroid hormones, such as thyroxine and triiodothyronine, have also been shown to stimulate O\(_2\) consumption and active Na\(^+\) and K\(^+\) transport in number of vertebrate tissues (Gick et al. 1988).

CNP is the only natriuretic peptide in elasmobranchs (Sections 1.11.1 and 4.1) and it has been shown to bind with high affinity to two different receptors in the plasma membranes of rectal gland cells: a clearance receptor and a guanylate cyclase-linked GC-B type receptor (Gunning et al. 1993). CNP is a potent stimulant of the enzyme guanylate cyclase increasing its intracellular activity in the rectal gland of *S. acanthias* (Gunning et al. 1993). Guanylate cyclase converts guanosine triphosphate (GTP) into cyclic guanosine monophosphate (cGMP). However, perfusion of isolated rectal glands with cGMP does not stimulate chloride secretion (Silva et al. 1987). Silva and co-workers (1996) noted that exogenous cGMP had an inconsistent effect of stimulating short-circuit current in cultured rectal gland cells. Later work showed that CNP significantly increased short-circuit current in cultured rectal gland cells (Silva et al. 1999). It would appear therefore that intracellular cGMP is important in mediating the effects of CNP, although the precise action of this is unclear.
The mode of stimulation for rectal gland secretion by CNP is highly complex, although a generalised model can be drawn (Figure 6.1.1). In *S. acanthias* CNP acts as a stimulus for the release of VIP from rectal gland nerves, although CNP also has well documented direct effects on both isolated tubules (Solomon et al. 1993; Solomon et al. 1995a; Solomon et al. 1995b), and cultured rectal gland cells (Karnaky et al. 1992; Karnaky et al. 1993; Silva et al. 1999). In the same species, CNP can also act through increased activity of guanylate cyclase, as detailed above; although the actions of other natriuretic peptides have also been isolated from any increase in intracellular cGMP (Budzik et al. 1987; Barrett and Isales 1988; Lear et al. 1990). This raises the possibility of CNP acting on rectal gland cells via a pathway independent of cGMP.

One pathway via which this may occur is the inositol phosphate pathway, as work by Ecay and Valentich (1990) demonstrated that VIP increased inositol phosphate formation. Investigation conducted by Silva and co-workers (1999) suggested that at least part of the stimulatory action of CNP may occur via a similar route. Specific inhibition of protein kinase C (PKC) completely removed the cGMP-independent stimulatory effect of CNP in isolated perfused rectal glands of *S. acanthias*. However, pharmacological activation of PKC did not stimulate Cl⁻ secretion in similar preparations (Silva et al. 1999). It is possible therefore that the synergistic action of cGMP and PKC are required for increasing Cl⁻ secretion in response to CNP in the tissues of the rectal gland.
Figure 6.1.1 – Generalised model of stimulation by CNP on rectal gland secretory cells. Volume expansion causes the release of CNP from the heart. CNP binds to a guanylate cyclase B-type receptor stimulating the production of cGMP. This causes a rise in the release of intracellular stores of Ca\(^{2+}\) which increases basolateral K\(^{+}\) conductance, which in turn stimulates Cl\(^{-}\) secretion. cGMP also acts via an undetermined route to cause an increase in Cl\(^{-}\) secretion, possibly via protein kinase G (PKG). CNP also has a parallel stimulatory action on protein kinase C (PKC). In the case of *S. acanthias* CNP also stimulates release of VIP which increases cAMP levels which stimulates protein kinase A (PKA). This causes a separate increase in Ca\(^{2+}\) influx as well as having direct effects on Cl\(^{-}\) secretion (Warth et al. 1998; Silva et al. 1999).
CNP may also affect Cl⁻ secretion rate through alterations in intracellular Ca²⁺ as outlined above (Warth et al. 1998) (Figure 6.1.1). It has been demonstrated that Ca²⁺ can stimulate NaCl secretion from isolated rectal gland tubules of S. acanthias. Intracellular Ca²⁺ concentration can be elevated by carbachol stimulated store release. This release of Ca²⁺ from intracellular stores is independent of cAMP and acts to increase basolateral K⁺ conductance, thereby stimulating Cl⁻ secretion. Intracellular Ca²⁺ concentration can also be elevated in response to cAMP, by increasing the rate of transmembrane influx via protein kinase A (Warth et al. 1998). Intracellular Ca²⁺ may therefore also be important in mediating the response of the rectal gland to CNP. This also suggests that Ca²⁺ may be important in two distinct methods of stimulating rectal gland secretion (Figure 6.1.1).

Furthermore, Ca²⁺ has been found to directly reduce rectal gland secretion. This can occur via two distinct modes of action: constriction of the rectal gland artery and a reduction in blood perfusion, and also through a reduction in intracellular Ca²⁺ concentration by reduced influx (Fellner and Parker 2002). Ca²⁺ could play a major role in controlling the activity of the rectal gland through a combination of modifying blood flow and tubular secretion.

Despite the lack of a definitive mode of action, CNP has well documented effects of the osmoregulatory tissues of elasmobranchs (Sections 1.11.1, 4.1). There is also wide scope for the modulation of these effects. For these reasons CNP was the chosen stimulant for this study which investigated the effects of salinity acclimation on the respiratory parameters of rectal glands from the partially euryhaline S. canicula and the fully euryhaline C. leucas.
In this study the O$_2$ consumption by rectal glands was measured in a partially (*S. canicula*) and a fully (*C. leucas*) euryhaline species of elasmobranch in order to assess any differences associated with acclimation to salinity change. Furthermore, following from the study of maximal Na$^+$, K$^+$-ATPase activity in the rectal glands of both species (Chapter 5), the O$_2$ consumption associated with this enzyme was also measured. The effect of CNP on both parameters was also measured in order to gain further understanding of the role of this hormone in the endocrine control of rectal gland function in different elasmobranch species.
6.2 Materials and methods

6.2.1 Chemicals and equipment

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

Respirometry experiments on *S. canicula* were conducted at 11 °C using 1302 O$_2$ electrodes and a 928 6-channel measurement system (Strathkelvin Instruments Ltd., Glasgow). The data was analysed using 928 O$_2$ system version 2.2 (Strathkelvin Instruments Ltd.). Respirometry chambers were kept at a constant temperature via a model LTD6 refrigerated bath (Grant Instruments Ltd., Cambridge). All solutions were also kept at a constant temperature via this method and bubbled with air for 10 minutes before use.

Respirometry experiments on *C. leucas* were conducted at 23 °C using a 781 O$_2$ electrode and metre (Strathkelvin Instruments Ltd.), and a microrespirometer sampling at 4 Hz (Strathkelvin Instruments Ltd.). The analogue signal was sent to a PowerLab 4/20 (AD Instruments Pty. Ltd., Castle Hill, NSW, Australia) running Chart 5.0 software (AD Instruments Pty. Ltd.). The microelectrode was inserted into a glass respiration chamber which was water-cooled to a constant temperature via a model LTD6 refrigerated bath (Grant Instruments Ltd.). All solutions were also kept at a constant temperature via this method and bubbled with air for 10 minutes before use.
6.2.2 Tissue sampling

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

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

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

660 µl of Ringer solution of the appropriate salinity was added to the respirometry chamber (200 µl for *C. leucas*), and the O₂ consumption of the electrode was measured for 15 minutes in order to compensate for this during tissue studies. The respirometry chamber was then thoroughly rinsed with Milli Q before being refilled with Ringer and the tissue introduced. O₂ consumption was again measured for a 15 minutes period. The tissue was then removed and placed in Ringer whilst the respirometry chamber was rinsed again. The chamber was then filled with one of the following solutions: 10⁻⁸ M CNP in Ringer solution, 10⁻¹⁰ M CNP, 10⁻¹² M CNP, or just Ringer solution. The CNP used was homologous for *S. canicula* and was kindly donated by Prof. Y. Takei. Subsequent analysis of CNP in *C. leucas* demonstrated an identical amino acid sequence (Takei, *pers. comm.*). The tissue was then replaced in the chamber and O₂ consumption was again measured for 15 minutes. The tissue was then removed, the chamber rinsed, and then refilled with the same solution as before plus 2mM ouabain. The tissue was replaced in the chamber and O₂ consumption was measured for a final 15 minutes.
The concentration of CNP used in any particular electrode was rotated to avoid bias in the results (with *C. leucas* the order in which the CNP concentrations were used was rotated as a single electrode was used). The concentrations of CNP used were chosen based on previous work investigating affects on the rectal gland (Anderson et al. 2002a). The concentration of ouabain was chosen based on previous work on Na\(^+\), K\(^+\)-ATPase in the rectal gland (Pillans et al. 2005) (Chapter 5).

O\(_2\) consumption was calculated via linear regression on the final 10 minutes of each 15 minute period of observation as consumption was most consistent during this period (Figure 6.2.3.1). Rates were then normalised per gram of rectal gland wet mass.
Figure 6.2.3.1 – Typical trace for O$_2$ partial pressure in respirometry chamber showing the section utilised for analysis (right of dotted line).
6.2.4 Statistical analysis

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

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

6.3.1 *S. canicula*

O$_2$ consumption by the rectal gland of 100% SW acclimated animals remained constant for the first 2 hours after excision (Figure 6.3.1.1). This time represents double that taken to measure consumption experimentally.

Whole tissue O$_2$ consumption was not significantly different between rectal glands from animals acclimated to the three environmental salinities (Figure 6.3.1.2). O$_2$ consumption was unaffected by all three concentrations of CNP in rectal glands from *S. canicula* acclimated to all three salinities.

O$_2$ consumption by Na$^+$, K$^+$-ATPase was defined as the ouabain sensitive portion of whole tissue O$_2$ consumption. Na$^+$, K$^+$-ATPase O$_2$ consumption was not significantly different between rectal glands from animals acclimated to the three environmental salinities (Figure 6.3.1.3). O$_2$ consumption by Na$^+$, K$^+$-ATPase was unaffected by all three concentrations of CNP in the rectal glands from *S. canicula* acclimated to all three salinities.
Oxygen consumption of 100% SW acclimated *S. canicula* rectal glands

**Figure 6.3.1.1** – Whole tissue O$_2$ consumption in *S. canicula* rectal gland slices from 100% SW acclimated animals. Values are presented as means ± SEM (n = 10). Statistical analysis was performed via repeated measures ANOVA and a Tukey post hoc test (significance was denoted as * (P < 0.05), ** (P < 0.01), and *** (P < 0.005)). No significant differences were recorded.
Whole tissue oxygen consumption in rectal glands of *S. canicula* acclimated to different salinities

![Graph showing oxygen consumption](image)

**Figure 6.3.1.2** – O₂ consumption in rectal glands from *S. canicula* acclimated to 80, 100, and 120% SW. Values are presented as means ± SEM (n = 8, 14, and 8 respectively). Statistically significant differences between salinities were assessed via one-way ANOVA and a Tukey post hoc test (significance was denoted as * (P < 0.05), ** (P < 0.01), and *** (P < 0.005)); within groups, statistically significant differences from basal values were assessed via one tailed paired students t-tests with Welch correction factor (significance was denoted as † (P < 0.05), †† (P < 0.01), and ††† (P < 0.005)). No significant differences were recorded.
Ouabain sensitive oxygen consumption in rectal glands of *S. canicula* acclimated to different salinities

**Figure 6.3.1.3** – O$_2$ consumption of Na$^+$, K$^+$-ATPase in the rectal glands of *S. canicula* acclimated to 80, 100, and 120% SW. Values are presented as means ± SEM (n = 7, 7, and 5 respectively). Statistically significant differences between salinities were assessed via one-way ANOVA and a Tukey post hoc test (significance was denoted as * (P < 0.05), ** (P < 0.01), and *** (P < 0.005)); within groups, statistically significant differences from basal values were assessed via one tailed paired students t-tests with Welch correction factor (significance was denoted as † (P < 0.05), †† (P < 0.01), and ††† (P < 0.005)). No significant differences were recorded.
Variance within groups was high for both whole tissue O$_2$ consumption and that associated with Na$^+$, K$^+$-ATPase. In an effort to reduce the effects of this the slice specific effects of CNP were analysed as a percentage change between basal and CNP stimulate O$_2$ consumption on each slice from each rectal gland (Figure 6.3.1.4). This removed possible sources of variation such as secretory states of glands upon excision and natural variation between individuals. CNP significantly increased whole tissue O$_2$ consumption in the rectal glands of *S. canicula* from all three salinities above basal levels. This was the case for all concentrations of CNP studied although no dose dependent effect was seen.

The proportion of whole tissue O$_2$ consumption which was ouabain sensitive was calculated, thereby giving a percentage of O$_2$ consumption associated with Na$^+$, K$^+$-ATPase which was specific to each slice (Figure 6.3.1.5). This was not significantly different between rectal glands from animals acclimated to the three environmental salinities. The proportion of O$_2$ consumed by Na$^+$, K$^+$-ATPase was unaffected by all three concentrations of CNP in the rectal glands from *S. canicula* acclimated to all three salinities.
Figure 6.3.1.4 – The effect of CNP on O$_2$ consumption in individual sections of rectal glands from *S. canicula* acclimated to 80, 100, and 120% SW. Values are presented as means ± SEM (n = 7, 7, and 5 respectively). Statistically significant differences from the control transfer were assessed via one-way ANOVA and a Tukey post hoc test (significance was denoted as * (P < 0.05), ** (P < 0.01), and *** (P < 0.005)); within groups, statistically significant differences from basal values were assessed via one tailed unpaired students t-tests with Welch correction factor (significance was denoted as † (P < 0.05), †† (P < 0.01), and ††† (P < 0.005)).
Relative ouabain sensitive oxygen consumption in rectal glands of \textit{S. canicula} acclimated to different salinities

**Figure 6.3.1.5** – Relative O$_2$ consumption of Na$^+$, K$^+$-ATPase in the rectal glands of \textit{S. canicula} acclimated to 80, 100, and 120% SW. Values are presented as means ± SEM ($n = 7$, 7, and 5 respectively). Statistically significant differences from the control transfer were assessed via one-way ANOVA and a Tukey post hoc test (significance was denoted as * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.005$)); within groups, statistically significant differences from basal values were assessed via one tailed paired students t-tests with Welch correction factor (significance was denoted as † ($P < 0.05$), †† ($P < 0.01$), and ††† ($P < 0.005$)). No significant differences were recorded.
6.3.2 *C. leucas*

O₂ consumption in the rectal glands of both FW and SW acclimated animals remained constant for up to 4 hours after excision (Figure 6.3.2.1). This represents a greater amount of time than that taken to perform all experiments on excised tissue.

Consumption of O₂ in glands from FW acclimated animals was significantly higher than that of SW (Figure 6.3.2.2), and remained so for up to 4 hours (Figure 6.3.2.1). The three concentrations of CNP had no significant effects on the O₂ consumed by rectal glands of FW and SW acclimated animals. However, the variation in consumption within each salinity group was increased sufficiently to remove any significant differences between FW and SW glands.

The O₂ consumption associated with Na⁺, K⁺-ATPase was not significantly different between FW and SW acclimated animals (Figure 6.3.2.3). The O₂ consumed by Na⁺, K⁺-ATPase was unaffected by all three concentrations of CNP in both FW and SW acclimated *C. leucas*. Variance within salinity groups was again increased with the addition of CNP to the respirometry chamber.
Figure 6.3.2.1 - Whole tissue O₂ consumption in *C. leucas* rectal gland slices from FW and SW acclimated animals. Values are presented as means ± SEM (*n* = 6). Statistical analysis within groups was performed via repeated measures ANOVA and a Tukey post hoc test (significance was denoted as † (*P* < 0.05), †† (*P* < 0.01), and ††† (*P* < 0.005)). No significant differences were recorded. Statistical analysis between FW and SW values was performed via a two-tailed unpaired student’s t-test with Welch correction factor (significance was denoted as * (*P* < 0.05), ** (*P* < 0.01), and *** (*P* < 0.005)).
Whole tissue oxygen consumption in rectal glands of *C. leucas* acclimated to different salinities

**Figure 6.3.2.2** – O$_2$ consumption in the rectal glands of *C. leucas* acclimated to FW and SW. Values are presented as means ± SEM (*n* = 6). Statistically significant differences between salinities were assessed via two-tailed unpaired student’s t-tests with Welch correction factor (significance was denoted as * (*P* < 0.05), ** (*P* < 0.01), and *** (*P* < 0.005)); within groups, statistically significant differences from basal values were assessed via one tailed paired students t-tests with Welch correction factor (significance was denoted as † (*P* < 0.05), †† (*P* < 0.01), and ††† (*P* < 0.005)).
Ouabain sensitive oxygen consumption in rectal glands of *C. leucas* acclimated to different salinities

![Oxygen consumption graph](image)

**Figure 6.3.2.3** – O₂ consumption of Na⁺, K⁺-ATPase in the rectal glands of *C. leucas* acclimated to FW and SW. Values are presented as means ± SEM (n = 6). Statistically significant differences between salinities were assessed via two-tailed unpaired student’s t-tests with Welch correction factor (significance was denoted as * (P < 0.05), ** (P < 0.01), and *** (P < 0.005)); within groups, statistically significant differences from basal values were assessed via one tailed paired students t-tests with Welch correction factor (significance was denoted as † (P < 0.05), †† (P < 0.01), and ††† (P < 0.005)). No significant differences were recorded.
So as to minimise the effects of variance between individual glands, the data was again analysed for specific effects of CNP on each slice in each gland (Figure 6.3.2.4). CNP caused significant increases in whole tissue O\textsubscript{2} consumption in the rectal glands of both FW and SW acclimated \textit{C. leucas}. This was seen after administration of all concentrations of CNP, although no dose dependent response was recorded.

The O\textsubscript{2} consumed by Na\textsuperscript{+}, K\textsuperscript{+}-ATPase was again analysed as a proportion of that consumed by the whole gland (Figure 6.3.2.5). The relative O\textsubscript{2} consumption of Na\textsuperscript{+}, K\textsuperscript{+}-ATPase in the rectal glands of FW acclimated \textit{C. leucas} was not significantly different to that in SW acclimated animals. In FW acclimated animals the relative O\textsubscript{2} consumption of Na\textsuperscript{+}, K\textsuperscript{+}-ATPase was unaffected by the three concentrations of CNP. However, significant increases were seen in the relative O\textsubscript{2} consumption of Na\textsuperscript{+}, K\textsuperscript{+}-ATPase in the rectal glands of SW acclimated \textit{C. leucas} in response to CNP. Administration of 10\textsuperscript{-8} M CNP resulted in a near 3-fold increase in the percentage of O\textsubscript{2} being consumed by Na\textsuperscript{+}, K\textsuperscript{+}-ATPase; whilst 10\textsuperscript{-10} M CNP produced a near doubling of the same parameter. These concentrations of CNP also increased the relative O\textsubscript{2} consumption of Na\textsuperscript{+}, K\textsuperscript{+}-ATPase in the rectal glands of SW acclimated \textit{C. leucas} to significantly higher levels than those of SW acclimated glands under the same conditions. It can therefore be stated that CNP has a significant effect on the proportional O\textsubscript{2} consumption in the rectal glands of SW acclimated \textit{C. leucas}, but has no effect on FW animals.
Change in whole tissue oxygen consumption in rectal glands of *C. leucas* acclimated to different salinities in response to CNP

**Figure 6.3.2.4** - The effect of CNP on O$_2$ consumption in individual sections of rectal glands from *C. leucas* acclimated to FW and SW. Values are presented as means ± SEM (*n* = 6). Statistically significant differences between salinities were assessed via two-tailed unpaired student’s t-tests with Welch correction factor (significance was denoted as * (P < 0.05), ** (P < 0.01), and *** (P < 0.005)); within groups, statistically significant differences from basal values were assessed via one tailed unpaired students t-tests with Welch correction factor (significance was denoted as † (P < 0.05), †† (P < 0.01), and ††† (P < 0.005)).
Relative ouabain sensitive oxygen consumption in rectal glands of *C. leucas* acclimated to different salinities

Figure 6.3.2.5 – Relative O$_2$ consumption of Na$^+$, K$^+$-ATPase in the rectal glands of *C. leucas* acclimated to FW and SW. Values are presented as means ± SEM (*n* = 6). Statistically significant differences between salinities were assessed via two-tailed unpaired student’s t-tests with Welch correction factor (significance was denoted as * ($P$ < 0.05), ** ($P$ < 0.01), and *** ($P$ < 0.005)); within groups, statistically significant differences from basal values were assessed via one tailed paired students t-tests with Welch correction factor (significance was denoted as † ($P$ < 0.05), †† ($P$ < 0.01), and ††† ($P$ < 0.005)).
6.4 Discussion

The recorded values for O$_2$ consumption in the rectal glands of *S. canicula* and *C. leucas* were comparable to those published previously for *S. canicula* and *S. acanthias* (Shuttleworth and Thompson 1980; Morgan et al. 1997). Ouabain sensitive O$_2$ consumption was significantly lower than that of the whole tissue for both species in all salinities. This is also consistent with previous respirometry experiments on elasmobranch rectal glands (Shuttleworth and Thompson 1980; Morgan et al. 1997). The lower concentration of ouabain used by Shuttleworth and Thompson (1980) as compared to the present study did not lead to any large scale changes in the estimation of the ouabain sensitive proportion of O$_2$ consumption by the rectal gland of *S. canicula*.

Whole tissue O$_2$ consumption in the rectal gland of *S. canicula* was unchanged by acclimation to 80 and 120% SW (Figure 6.3.1.2). This is consistent with previous results which have demonstrated that both RGF volume and Cl$^-$ clearance rate from the rectal gland are also unchanged by similar salinity acclimations (Table 4.3.1). This is again suggestive that there is no increase in rectal gland activity *in vivo* associated with long term acclimation to salinity transfer in *S. canicula*. It is possible that the magnitude of these salinity changes are not sufficient to elicit any long term changes in rectal gland O$_2$ consumption. However, it must again be stated that salinity transfers of larger magnitude increase animal mortality and this is highly suggestive that changes in rectal gland O$_2$ consumption are unlikely to be recorded with such transfers.

The ouabain-sensitive portion of rectal gland O$_2$ consumption in *S. canicula* was also unaffected by acclimation to hypo- or hypersaline conditions (Figures 6.3.1.3 and 5).
This is consistent with previous results which have shown that the maximal activity of Na\(^+\), K\(^+\)-ATPase is not significantly different between the three environments (Figure 5.3.2.1).

Whole tissue O\(_2\) consumption in the rectal glands of *C. leucas* acclimated to FW was significantly higher than that of SW acclimated animals (Figure 6.3.2.2). This seems counter intuitive due to the reversal of the concentration gradients for Na\(^+\) and Cl\(^-\) between *C. leucas* and the environment as the animals acclimate from FW to SW. In FW the gradient is for the efflux of these ions and so there is an associated requirement for the retention of Na\(^+\) and Cl\(^-\) in the body fluids. Conversely, in SW the gradient is for the influx of these ions and so there is an associated requirement for the active secretion of Na\(^+\) and Cl\(^-\) from the rectal gland. The rectal glands of SW animals also have significantly higher maximal activity of Na\(^+\), K\(^+\)-ATPase (Figure 5.3.2.2) (Pillans et al. 2005), and presumably higher rates of RGF secretion and Cl\(^-\) clearance, in order to facilitate this need for active rectal gland secretion. The fact that whole tissue O\(_2\) consumption is higher in the rectal glands of FW acclimated animals could therefore be the result of higher activity of some other metabolic process or processes in the tissue. It could be explained by a higher overall metabolic rate in FW acclimated animals due to the requirement to maintain a larger disparity between internal osmolality and that of the surrounding environment. Indeed, the maintenance of such large concentration gradients for Na\(^+\), Cl\(^-\), urea, TMAO, and other plasma osmolytes must result in a substantial energy demand. This energy demand may be met by a higher basal metabolic rate in FW acclimated *C. leucas*. 

240
Similar studies on teleost species have shown varied results. Some species show a decrease in whole animal $O_2$ consumption associated with acclimation to decreased salinities (Wood et al. 2002b; Sardella et al. 2004), whilst others show no change in either whole animal $O_2$ consumption (Morgan and Iwama 1998) or that of the gills (Stagg and Shuttleworth 1982). This is illustrative of the interspecies variation that can occur when measuring salinity induced changes in biological parameters.

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

One source of high $O_2$ consumption could be the constriction of rectal gland blood vessels, so as to minimise secretory output from the gland in FW. Attempts at isolated perfused rectal gland studies in FW $C. leucas$ proved difficult due to constriction/blockage of the rectal gland artery and erratic pressure and activity within the gland (unpublished finding). Blood flow to the rectal gland of $S. canicula$ has been shown to be modified during periods of active secretion (Anderson et al. 2002a). It is possible therefore that the higher $O_2$ consumption in rectal glands of FW acclimated $C. leucas$ is at least in part due to constriction of blood vessels.
Another possible cause of higher rectal gland O₂ consumption in FW is the smooth muscle layer surrounding the gland. Work conducted by Evans and Piermarini (2001) demonstrated that this layer is responsive to contractile stimuli. It is also possible therefore that the increased O₂ consumption in rectal glands of FW acclimated *C. leucas* is at least in part due to contraction of this smooth muscle layer as a means of further restricting blood supply to the gland.

The ouabain-sensitive portion of rectal gland O₂ consumption was not significantly different between FW and SW acclimated *C. leucas* (Figures 6.3.2.3 and 5). This may appear to be inconsistent with previous studies which have shown that maximal activity of Na⁺, K⁺-ATPase in the rectal gland is significantly higher in SW acclimated animals (Figure 5.3.2.2) (Pillans et al. 2005). However, such studies represent maximal activities and it is possible that Na⁺, K⁺-ATPase activity, and hence ouabain-sensitive O₂ consumption, only differ between rectal glands of FW and SW acclimated *C. leucas* during periods of active secretion.

Natriuretic peptides such as CNP have been shown to have stimulatory effects on the secretory action of the elasmobranch rectal gland (Sections 1.11.1 and 6.1). However, whilst whole tissue O₂ consumption was significantly increased by CNP (Figure 6.3.1.4), the ouabain-sensitive portion of this in the rectal gland of *S. canicula* was unaffected by administration of three concentrations of CNP (Figures 6.3.1.5). Therefore it can be stated that any increase in O₂ consumption upon administration of CNP is not associated with a proportional increase in the activity of Na⁺, K⁺-ATPase. If indeed there is any increase in the activity of Na⁺, K⁺-ATPase, is also coupled with a proportional increase in other metabolic processes.
The effects of pharmacological agents on the tissues of the rectal gland have been shown to be highly dependent on the preparations used. Equimolar concentrations of CNP and VIP have roughly similar effects on Cl⁻ secretion in intact glands (Solomon et al. 1992a), but in dispersed tubules CNP produces less than half the respiratory stimulation of VIP (Solomon et al. 1993; Solomon et al. 1995a; Solomon et al. 1995b). Also, elevated intracellular concentrations of cGMP result in a slow increase in short-circuit current in isolated cells (Karnaky et al. 1991), but perfusion of isolated glands with high concentrations of cGMP showed no such increase (Silva et al. 1999). Furthermore, Stoff and co-workers (1977b) reported a synergistic effect on active Cl⁻ transport with administration of theophylline and cAMP in isolated perfused glands, where as treatment with cAMP caused no further increase in O₂ consumption of rectal gland sections after administration of theophylline (Shuttleworth and Thompson 1980).

In light of such discrepancies the results for ouabain-sensitive O₂ consumption in the rectal gland of *S. canicula* following administration of CNP do not seem extraordinary. It has already been suggested that the stimulatory effect of CNP via guanylate cyclase may require the coupled activation of PKC (Section 6.1). It is possible that the mode of administration of CNP in these studies effects glandular response. Introducing CNP into the surrounding medium of an isolated tissue section is not equivalent to perfusing an isolated gland. It is possible that administered CNP must pass through the circulatory system in order to have a stimulatory action on active secretion. However, this is unlikely given the documented stimulatory action of CNP on cultured rectal gland cells from *S. acanthias* (Karnaky et al. 1992; Karnaky et al. 1993). Therefore, although these results for *S. canicula* are not extraordinary, they are unexpected and unclear.
As well as being a potent stimulus for rectal gland secretion, CNP also has proven vasodilatory effects (Bjenning et al. 1992; Anderson et al. 2002a). Therefore, if the high O$_2$ consumption recorded in the rectal glands of FW acclimated *C. leucas* is in part due to vasoconstriction, administration of CNP could be expected to reverse this effect. However, CNP significantly increased the O$_2$ consumption of rectal glands from FW acclimated animals (Figure 6.3.2.4). It is possible that any decrease in O$_2$ consumption resulting from vasodilation was masked by a larger increase in consumption due to the stimulatory effect of CNP on the secretory tubules of the rectal gland. However, administration of CNP did not significantly alter ouabain-sensitive O$_2$ consumption (Figure 6.3.2.3), or its proportion in whole tissue consumption (Figure 6.3.2.5). If therefore, the high O$_2$ consumption of rectal glands from FW acclimated *C. leucas* is the result of vasoconstriction, either such constrictions are non-responsive to CNP, or they are coupled with an increase in O$_2$ consumption from another aspect of rectal gland function.

Not only did CNP affect whole tissue O$_2$ consumption in the rectal gland of SW acclimated *C. leucas* (Figure 6.3.2.4), it also had effects on the proportion of ouabain-sensitive O$_2$ consumption (Figure 6.3.2.5). This is consistent with the increased maximal activity of Na$^+$, K$^+$-ATPase in the rectal glands of these animals (Figure 5.3.2.2) (Pillans et al. 2005). It is also consistent with the increase in the abundance and activity of Na$^+$, K$^+$-ATPase in the rectal glands of SW acclimated *D. sabina* (Piermarini and Evans 2000).

The fact that administration of CNP results in an increase in the proportion of whole tissue O$_2$ consumption associated with Na$^+$, K$^+$-ATPase, but does not result in any
significant increase in the ouabain-sensitive O$_2$ consumption of SW rectal glands is intriguing. This is a reflection of the amount of variation in these parameters between individual animals acclimated to SW upon administration of CNP. Only through a proportional analysis do any trends appear. This variation could be explained by different states of activity in the glands upon excision, as well as natural variation in the species. It could be that a gland excised during a period of active secretion shows a greater response to CNP than a gland excised during a quiescent period. If this is the case it would also explain the relative lack of response in glands from FW acclimated *C. leucas* which are presumably quiescent for a much larger amount of time.

The fact that the O$_2$ consumption associated with Na$^+$, K$^+$-ATPase increases disproportionately to whole tissue consumption in SW acclimated *C. leucas* but not in FW animals, and that both animals have similar basal values, suggests a modification in the response of the rectal gland to CNP during acclimation to increased salinity. This could be achieved through alterations in the abundance and/or sensitivity of CNP receptors in the membranes of rectal gland secretory cells. There is no requirement for active rectal gland secretion in FW *C. leucas* and so increasing the number of receptors for this stimulatory hormone during SW acclimation seems plausible. An increased abundance of hormone receptors in response to changes in salinity has been reported in teleosts. SW acclimated *A. anguilla* have a three-fold higher Ang II receptor concentration than those acclimated to FW (Marsigliante et al. 1997). Furthermore, Katafuchi and co-workers (1994) showed that CNP-specific receptor expression was enhanced in FW *A. japonica*. Increased CNP receptor expression can therefore be induced by changes in environmental salinity in teleosts. Given that CNP is the most highly conserved of all the natriuretic peptides (Takei 1999), and is the only one
recorded in elasmobranchs (Schofield et al. 1991; Suzuki et al. 1991a; Suzuki et al. 1994), elasmobranchs may well increase receptor expression during long term acclimation to different salinities.

It is also possible that whilst the rectal gland of SW acclimated *C. leucas* respond to CNP via both cGMP and PKC pathways (thereby facilitating a synergistic action on Cl⁻ secretion), rectal glands of FW acclimated *C. leucas*, and those of *S. canicula* acclimated to the three environmental salinities do not respond via one of these. The fact that whole tissue O₂ consumption is still increased in this latter group suggests that these glands are responsive to CNP and that one of the pathways is probably functional. However, the lack of a disproportionate increase in Na⁺, K⁺-ATPase O₂ consumption suggests that either there is no increase in Cl⁻ secretion associated with administration of CNP in these glands, or that any increase is directly proportional to the stimulatory effects of CNP on other active metabolic processes. However, this variation in response to CNP seen in *C. leucas* represents a tangible difference in the endocrine physiology of fully euryhaline elasmobranchs when compared to a partially euryhaline species. The significance of this finding cannot be underestimated when assessing the nature of euryhalinity in elasmobranch fish.
Ambiguous citations

(Smith 1931b; Smith 1931a; Silva et al. 1977; Stoff et al. 1977a; Stoff et al. 1977b; Silva et al. 1979; Silva et al. 1983; Silva et al. 1985; Silva et al. 1987; Silva et al. 1990; Lacy and Reale 1991b; Lacy and Reale 1991a; Suzuki et al. 1991a; Suzuki et al. 1991b; Solomon et al. 1992a; Solomon et al. 1992b; Armour et al. 1993a; Armour et al. 1993b; Hentschel et al. 1993; Hentschel and Zierold 1993; Silva et al. 1993; Anderson 1995; Anderson et al. 1995a; Anderson et al. 1995b; Cornelius 1995a; Cornelius 1995b; Solomon et al. 1995a; Solomon et al. 1995b; Silva et al. 1996; Hazon et al. 1997a; Hazon et al. 1997b; Silva et al. 1997; Silva et al. 1999; Anderson 2001; Anderson et al. 2001; Anderson et al. 2002a; Anderson et al. 2002b; Silva and Epstein 2002; Wells 2002; Wells et al. 2002; Wood et al. 2002a; Wood et al. 2002b; Cooper and Morris 2004b; Cooper and Morris 2004a)
References


