Chapter 3: Blood volume
3.1 Introduction

Assessment of blood volume is of key importance in fully understanding osmoregulation in elasmobranchs, as has been previously discussed (Section 2.4). Changes in blood volume are associated with changes in blood haematocrit, as variation in plasma volume alters the relative concentration of erythrocytes. Within the literature there are numerous examples of haematocrit studies on elasmobranchs (Table 3.1.1), some of which show changes with acclimation to salinity change and some of which do not. Changes in haematocrit are important because they are suggestive of, but not evidence for, changes in blood volume associated with chronic salinity transfer. Similarly, a more constant blood haematocrit during chronic acclimation to salinity change suggests a lesser affect on blood volume. A smaller degree of haemodilution may therefore be encountered by fully euryhaline species, such as *C. leucas*, as compared to partially euryhaline species, such as *S. canicula*. Given the probable central role played by changes in blood volume in the cascade of osmoregulatory processes during acute salinity transfer, smaller changes in blood volume are descriptive of tighter regulation of osmotic and diffusional fluxes. This is intuitive, greater ability to maintain haematic parameters at different salinities are naturally associated with more euryhaline elasmobranchs. Whereas, salinity transfers of high magnitude conducted on *S. canicula* tend to result in increased mortality over a period of days, suggesting that partially euryhaline elasmobranchs fail to regulate haematic parameters completely under such conditions.
<table>
<thead>
<tr>
<th>Species</th>
<th>Reference</th>
<th>Species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. canicula</em></td>
<td>(Table 2.3.1.1)</td>
<td><em>S. canicula</em></td>
<td>(Hazon and Henderson 1984)</td>
</tr>
<tr>
<td><em>R. erinacea</em></td>
<td>(Goldstein and Forster 1971)</td>
<td><em>C. leucas</em></td>
<td>(Table 2.3.1.2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>C. leucas</em></td>
<td>(Thorson et al. 1973)</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>H. portusjacksoni</em></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td><em>T. testacea</em></td>
<td>(Cooper and Morris 1998)</td>
</tr>
</tbody>
</table>

**Table 3.1.1** – State of blood haematocrit of different species of elasmobranchs in response to chronic transfer (> 72 hours) to altered environmental salinity.
Interestingly, there are conflicting records for the haematocrit of *S. canicula* at different salinities. Hazon and Henderson (1984) recorded no significant differences in the haematocrit of animals after 14 day acclimation to a range of salinities (50 – 140% SW). However, results from this study have demonstrated significant differences after 14 day acclimations between 80, 100, and 120% SW in the same species (Table 2.3.1.1). This is probably due to the larger sample size utilised in this study.

Although numerous attempts have been made to investigate blood volume in a variety of species (Hazon, *pers. comm.*), few studies have quantitatively assessed the blood volume of elasmobranchs (Table 3.1.2). Practical assessment of blood volume typically involves the introduction of a marker substance into the subject. This then mixes within the vascular space and becomes diluted. Sampling of the blood and subsequent measurement of the marker then permits a dilution factor to be calculated. However, the nature of the chosen marker is of vital importance, particularly in elasmobranchs.
<table>
<thead>
<tr>
<th>Species</th>
<th>Blood volume (ml 100 g(^{-1}) BM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Raja binoculata</em></td>
<td>8.0</td>
<td>(Thorson 1958)</td>
</tr>
<tr>
<td><em>Raja rhina</em></td>
<td>7.2</td>
<td>(Thorson 1958)</td>
</tr>
<tr>
<td><em>N. brevirostris</em></td>
<td>7.0</td>
<td>(Thorson 1958)</td>
</tr>
<tr>
<td><em>Carcharhinus nicaraguensis</em></td>
<td>6.8</td>
<td>(Thorson 1958)</td>
</tr>
<tr>
<td><em>G. cirratum</em></td>
<td>6.8</td>
<td>(Thorson 1958)</td>
</tr>
<tr>
<td><em>S. acanthias</em></td>
<td>6.8</td>
<td>(Thorson 1958)</td>
</tr>
<tr>
<td></td>
<td>6.6</td>
<td>(Opdyke et al. 1975)</td>
</tr>
<tr>
<td><em>Hydrolagus colliei</em></td>
<td>5.2</td>
<td>(Thorson 1958)</td>
</tr>
<tr>
<td><em>S. canicula</em></td>
<td>6.8 / 4.1 *</td>
<td>(Tort et al. 1991)</td>
</tr>
</tbody>
</table>

*Table 3.1.2 – Blood volume of elasmobranchs obtained using dye dilution techniques. Volumes are expressed as ml per 100 gram of body mass. (*) denotes corrected value for binding affinity of Evans blue.*
All of the studies on elasmobranch blood volume to date have used a dye dilution method utilising T-1824 (Evans blue) and back-extrapolation of the concentration/time curve. This has lead to a possible overestimation of blood volume due to the binding affinity of the dye. Tort and co-workers (1991) described the possible sources of this overestimation. Once injected into the blood Evans blue binds to the protein fraction, mostly to albumin proteins (Freedman and Johnson 1969). It is well accepted that the albumin concentration in elasmobranch blood is low (Irisawa and Irisawa 1952), although Evans blue does bind to elasmobranch globulins to a greater extent than others (Tort et al. 1991). However, overall binding of Evans blue is lower than in other vertebrates with high blood serum albumin levels, with around 40% of injected dye failing to bind to the protein fraction (Tort et al. 1991). Values for blood volume obtained via this method may therefore be unreliable, as protein binding of Evans blue may differ from species to species. For example, a protein fraction with some albumin properties has been recorded in some species of carcharhinids but not in others (Yanagisawa and Hashimoto 1984).

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

There are inherent dangers with introducing substances into the vascular space without a complete understanding of their natural occurrence. Marker dilution assessment of blood volume depends on a uniform distribution of that marker and a predictable and
quantifiable mixing within the vascular space. Any breakdown of the marker or movement from the vascular space must be accounted for. Therefore utilising protein bound dyes and labelled starch in elasmobranch species are not robust methods of assessing blood volume, unless species and salinity specific concentrations of those substances have been recorded.

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

Other than dye dilution, the other common methods of assessing blood volume in fish involve the use of radioactive markers. This can be through isotopically labelled microspheres (Kent and Olsen 1982), $^{125}$Iodine (I) bovine serum albumin (BSA) (Gingerich and Pityer 1989), or $^{51}$Chromium (Cr) labelled erythrocytes (Conte et al. 1963; Duff et al. 1987; Gingerich et al. 1987; Gingerich and Pityer 1989; Gingerich et al. 1990). Microspheres by their nature become trapped in the fine capillaries of tissues and as such are useful for assessing blood flow to specific organs. However, their use as a means of assessing total body blood volume is limited by this feature. Use of $^{125}$I-labelled BSA in elasmobranchs raises questions similar to those outlined for Evans blue, concerning the behaviour of a substance which may naturally occur in variable quantities in the blood. The level of natural occurrence for the carrier medium greatly
influences the dynamics of the label and its degree of mixing within the vascular space. Gingerich and Pityer (1989) illustrated that assessment of blood volume in the teleost Salmo gairdneri via both $^{125}$I-labelled BSA and $^{51}$Cr-labelled erythrocytes yielded different results. Whole body blood volume was significantly lower when calculated from $^{51}$Cr-labelled erythrocytes than when $^{125}$I-labelled BSA or both markers were used. They concluded that it was not clear whether this disparity was due to the distribution of erythrocyte poor blood into the secondary circulation, or the result of extravascular exchange of plasma proteins. Protein permeability is high in teleost capillary membranes, and plasma protein retention in the blood has been shown to correlate directly with blood hydrostatic pressure (Hargens et al. 1974). There is therefore large scope for error when assessing blood volume using albumin bound labels, particularly in elasmobranchs which may have highly variable amounts of albumin proteins in the blood serum.

For the reasons detailed above blood assessment in this study was conducted via the use of $^{51}$Cr-labelled erythrocytes. Not only do erythrocytes occur naturally in elasmobranch blood, but their concentration can be easily quantified by haematocrit. From a practical viewpoint $^{51}$Cr is an ideal marker to use as it has a high energy $\gamma$ emission which facilitates accurate and rapid measurements. Furthermore, the use of $^{51}$Cr-labelled erythrocytes and their relative stability in the vascular space as compared to albumin bound dyes, presents the possibility of prolonged assessment over acute salinity transfer. Once fully mixed in the vascular space any changes in marker concentration would be due to either the break down of erythrocytes or the radioactive decay of the marker, both of which remain reasonably constant over the time period. Therefore upon acute salinity transfer any variation recorded in the experimental groups which is not
recorded in the control group can be assumed to represent a tangible change in the dilution of the marker resulting from a change in blood volume. Assessment of blood volume during both chronic and acute transfer was of key importance to the study as it allowed a quantification of the concentration and dilution of body fluids associated with acclimation to salinity change. As such this would give great insight into the osmotic stress and response of *S. canicula* during both chronic and acute salinity transfer. This is the fundamental factor in elasmobranch osmoregulation at different salinities.
3.2 Materials and methods

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

3.2.1 Chemicals and equipment

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

3.2.2 Surgical procedures

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

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

The erythrocytes were then centrifuged and washed as described above 4 times in 4 °C Ringer, each time retaining a 200 µl sample of the supernatant to measure three 50 µl replicates in a $\gamma$-counter (Minaxi auto-gamma 5000 series, Packard Instrument Company, Downers Grove, Il, USA) to check for haemolysis. Finally the cells were resuspended in 4 °C Ringer to give a final haematocrit of 17% for 100% SW, 13% for 80% SW, and 22% for 120% SW. Triplicate 50 µl samples were then measured in the $\gamma$-counter to accurately assess the activity of the final erythrocyte suspension before loading into a 1 ml syringe approximately 1 ml Kg$^{-1}$ body mass for each animal. The mass of the syringes were recorded before and after discharge to accurately calculate the volumes delivered. The specific gravity of blood from donor animals acclimated to each salinity was measured to gain salinity specific mass to volume conversion factors (Table 3.3.1.1).
400 µl of blood was drawn from the mesenteric arterial cannula for osmolyte analysis prior to injection of the labelled erythrocytes. The cannula was then flushed with 320 µl of Ringer solution and 200 IU ml\(^{-1}\) heparin and the stopper pin replaced. 200 µl of blood was removed via the coeliac arterial cannula after 0.5, 1, 2, 3, and 24 hours for basal levels and after 0, 2, 4, 6, 8, and 10 hours for acute transfer; the 24 hour basal and 0 hour transfer samples being the same sample. An equivalent volume of the appropriate salinity Ringer solution was then injected via the same cannula to replace the lost volume. In order to assess blood volume triplicate 50 µl samples of whole blood were measured for radioactivity in the \(\gamma\)-counter. Blood volume was then calculated via the method detailed below.
3.2.4 Calculation of blood volume

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

$$ y = mx + c $$

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

$$ Vol = \left[ \frac{a}{c} \right] / Mass \times 100 \text{ (ml 100 g}^{-1} \text{ body mass)} $$

Where $Vol$ represents blood volume, $a$ represents the activity injected, and $Mass$ being body mass.
Figure 3.2.4.1 – Typical regression line drawn to calculate the theoretical marker concentration at time zero assuming instantaneous mixing and the slope value associated with marker decay during blood volume assessment in *S. canicula*. 
Given that major variations in activity are removed once the labelled cells have thoroughly mixed with the systemic blood, and that the rate of decay is constant, the calculated value of the linear slope \((m)\) can also be applied to later time points to extrapolate back to time 0. Therefore once acute transfer had begun and values for activity in the blood were calculated for each serially taken blood sample, values for the resultant changes in blood volume could be calculated individually for each time point by modifying the equation of the linear regression:

\[
c = y - mx
\]

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

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

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

3.3.1 Basal levels

Blood specific gravity and volume of *S. canicula* acclimated to 80, 100, and 120% SW are presented below (Table 3.3.1.1). No significant differences were seen in the specific gravity of blood taken from animals acclimated to the three salinities. Animals acclimated to 80% SW had a significantly larger blood volume than those from 100% SW. Animals acclimated to 120% SW had a highly significantly smaller blood volume than those from 100% SW.
<table>
<thead>
<tr>
<th>Salinity (SW)</th>
<th>Blood specific gravity (g ml(^{-1}))</th>
<th>Blood Volume (ml 100g(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>80%</td>
<td>0.99 ± 0.00</td>
<td>6.3 ± 0.2 *</td>
</tr>
<tr>
<td>100%</td>
<td>1.02 ± 0.01</td>
<td>5.6 ± 0.2</td>
</tr>
<tr>
<td>120%</td>
<td>1.02 ± 0.01</td>
<td>4.6 ± 0.2 **</td>
</tr>
</tbody>
</table>

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

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

The slope values for the linear regressions performed on individual animals are presented below (Table 3.3.2.1).
Blood volume of *S. canicula* from different salinities during acute transfer to 100% SW

**Figure 3.3.2.1** – Blood volume of *S. canicula* acclimated to 80, 100, and 120% SW during acute transfer to 100% SW. Values are presented as means ± SEM (n = 6, 7, and 8 respectively). Statistically significant differences from the control transfer were assessed via one-way ANOVA and a Tukey post hoc test (significance was denoted as * (P < 0.05), ** (P < 0.01), and *** (P < 0.005)); statistically significant differences from values at time 0 were assessed via repeated measures ANOVA with Dunnett post hoc test (significance was denoted as † (P < 0.05), †† (P < 0.01), and ††† (P < 0.005)). Once differences had occurred they persisted throughout the transfer, although for clarity differences are only noted at the first and last instances.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Salinity (% SW)</th>
<th>80</th>
<th>100</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td>-16.2</td>
<td>-11.0</td>
<td>-87.6</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>-68.2</td>
<td>-8.8</td>
<td>-113.9</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>-113.1</td>
<td>-22.7</td>
<td>-11.0</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>-190.2</td>
<td>-191.1</td>
<td>-53.7</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>-121.4</td>
<td>-139.2</td>
<td>-34.6</td>
</tr>
<tr>
<td>6</td>
<td></td>
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<td>-103.9</td>
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</tr>
<tr>
<td>7</td>
<td></td>
<td>-6.1</td>
<td>-6.3</td>
<td>-71.3</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>-3.1</td>
<td>–</td>
<td>-27.3</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td></td>
<td>-65.8 ± 24.7</td>
<td>-69.0 ± 28.5</td>
<td>-58.4 ± 11.9</td>
</tr>
</tbody>
</table>

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

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

The blood volume of 100% SW *S. canicula* was calculated as $5.6 \pm 0.2$ ml 100g$^{-1}$ body mass (Table 3.3.1.1). This is consistent with those reported for other elasmobranchs (Table 3.1.2). It also supports the suggestion that the Evans blue method can lead to an overestimation of blood volume in elasmobranchs, although the allowance made for this may be too great. This value is slightly below the standard value ($6.8$ ml 100 g$^{-1}$ body mass), and slightly above the corrected value ($4.1$ ml 100 g$^{-1}$ body mass) reported for the species using Evans blue (Tort et al. 1991). The reasons for these differences are not clear. It is possible that they reflect the extravascular exchange of plasma proteins, as suggested by Gingerich and Pityer (1989). However, given the highly variable portion of albumin proteins in the blood of elasmobranchs and the lack of understanding of the *in vivo* behaviour of markers bound to such proteins (Section 3.1), the value reported here from a marker associated with naturally occurring, species specific erythrocytes can be considered the more accurate measurement.
It has also been shown that acclimation of *S. canicula* to 80% SW resulted in an increase in blood volume and an associated decrease in blood haematocrit. Conversely, acclimation to 120% SW resulted in a decrease in blood volume and an associated increase in blood haematocrit. This is the first ever measurement of the effects of salinity acclimation on elasmobranch blood volume. Given the central role which is believed to be played by changes in blood volume in elasmobranch osmoregulation (Shuttleworth 1983; Solomon et al. 1984; Solomon et al. 1985; Shuttleworth and Thompson 1986; Silva et al. 1996; Cooper and Morris 1998; Olson 1999; Silva et al. 1999; Anderson et al. 2002a; Anderson et al. 2002b) these findings are of great importance.

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

The blood volume of *S. canicula* was also assessed during acute salinity transfers from 80, 100, and 120% to 100% SW (Figure 3.3.2.1). The fact that there were no significant changes in the blood volume of animals undergoing the control transfer from 100% to
100% SW was as expected given the constant environmental conditions (Figures 2.3.2.1 and 2). This is also suggestive that the marker used to assess blood volume in this study is stable in vivo since there was no significant decrease in recorded activity in the blood of the control animals once complete mixing had occurred.

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

This overcompensatory decrease in blood volume in S. canicula undergoing acute transfer from 80% to 100% SW is not consistent with an unaltered blood haematocrit in similarly transferred animals (Figure 2.3.2.6). Such decreases in blood volume should
result in an increased blood haematocrit if the number of erythrocytes in the blood remains constant. The lack of any significant variation in the haematocrit of these animals can be explained in a number of ways. Haematocrit in this study was measured as the volume taken up by erythrocytes within a blood sample (Section 2.2.5). Given the increase in blood plasma osmolality in these animals (Figure 2.3.2.3) there may be a decrease in erythrocyte cell volume during acute transfer from 80% to 100% SW. It is possible therefore that any increase in the concentration of erythrocytes resulting from decreased blood volume is offset by a decrease in erythrocyte cell volume, and hence their proportional volume within a blood sample. In this way these two opposing factors may negate each other and thereby result in no net change in haematocrit, as measured in this study. However, these two factors also oppose each other in animals undergoing acute transfer from 120% to 100% SW, although their affects on proportional erythrocyte volume in the blood are reversed as compared to animals acutely transferring from 80% to 100% SW. The fact that there is a significant change in the haematocrit of animals undergoing acute salinity transfer from 120% to 100% SW, but not in those from 80% to 100% SW, suggests that changes in erythrocyte cell volume are not the cause for this discrepancy.

The lack of any significant changes in the haematocrit of *S. canicula* undergoing acute transfer from 80% to 100% SW is more likely due to the nature of surgical procedures performed on each group of animals and the ongoing refinement in surgical technique throughout the study. Animals used for the measurement of blood haematocrit underwent a longer period of surgery and the cannulation of 4 separate vessels: the coeliac and mesenteric arteries (Section 2.2.4), as well as the rectal gland vein and duct (Section 4.2.2). Animals used for the measurement of blood volume underwent a shorter
period of surgery, had a smaller size of initial incision, and had only the coeliae and mesenteric arteries cannulated (Section 3.2.2). Furthermore, animals used to measure haematic parameters following acute transfer from 80% to 100% SW were the first experimental group for the entire study (January 2002). Whereas animals used to measure blood volume following the same acute transfer were the penultimate experimental group for the entire study (December 2004). There was a high level of refinement in surgical techniques over this time period and the proportion of animals with visible blood clotting upon post mortem analysis was reduced. The formation of blood clots will necessarily reduce the measured haematocrit by removing erythrocytes from the circulating blood volume. This would account for the discrepancy between the haematocrit (Figure 2.3.2.6) and blood volume (Figure 3.3.2.1) of *S. canicula* undergoing acute transfer from 80% to 100% SW.

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

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

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

There were also some problems with the calculation of blood volume during the acute transfer periods. The linear regression was calculated from blood samples taken after 0.5, 1, 2, 3, and 24 hours of injecting the labelled erythrocytes. CPM values for samples taken in the first 3 hours proved to be highly variable, due to mixing of the marker in the blood system of a predominantly sedentary animal. This early variation translated into a wide variation in the calculated slope values for the linear regression lines (Table 3.3.2.1). Due to the nature of the calculation for blood volume this variation becomes
more prevalent as time (and therefore distance from time 0) increases. Therefore whilst calculations for basal blood volume are accurate, exact values expressed for the period of acute transfer may be overestimated. This problem could be removed by replacing intensive sampling in the first three hours with single samples taken further apart during the basal study period. This would lead to a more accurate calculation of the linear regression and greater confidence in later time points. However, whilst exact values during acute transfer may have been overestimated the trends in the results are accurate, as are the values calculated for basal blood volumes in chronically acclimated animals.

Assessment of blood volume in *S. canicula* has therefore given great insight into the changes in volaemic parameters associated with both chronic and acute transfer to changes in environmental salinity. This insight is of fundamental importance in understanding the osmoregulatory responses of elasmobranchs during variations in salinity. Only through quantification of the haemodilution and concentration experienced by elasmobranchs during salinity transfer *in vivo* can the influence of volaemic change on osmoregulatory mechanism *in vitro* be validated. In particular these results have provided further evidence to support the concept of increases in blood volume occurring rapidly during acute transfer and therefore coinciding with expected periods of increased rectal gland secretion. The volaemic affects on rectal gland secretion *in vitro* have been well documented (Solomon et al. 1984; Solomon et al. 1985; Olson 1999). Furthermore, it has already been shown that variations in plasma Cl\(^-\) (and Na\(^+\)) levels also occur early during acute salinity transfer (Figure 2.3.2.4). Given these two factors it is clear than an assessment of *in vivo* rectal gland activity would give a greater level of understanding in elasmobranch euryhalinity.
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; Martin 2003a; Martin 2003b; Cooper and Morris 2004b; Cooper and Morris 2004a)
References


