Kidney form and function and the role of arginine vasotocin (AVT) in three agamid lizards from different habitats in Western Australia.

STEWART S. FORD
BSc. (Hons.)

This thesis is presented for the degree of Doctor of Philosophy of The University of Western Australia

School of Animal Biology

2005
ABSTRACT

Reptiles are polyphyletic, and previous studies of renal anatomy and physiology in reptiles have covered a wide diversity of species of different phylogeny and habitat. To date, no study has examined the renal morphology and function of a group of closely related reptiles from different environments, yet this design has a number of advantages. Firstly, phylogenetic effects are reduced while adaptive specialisations in renal function or structure can be elucidated, and secondly, the variation in renal form and function between closely related species may be quantified in an effort to appreciate better the variation between more distantly related species. In this thesis, kidney morphology and renal function were studied in three Western Australian agamid lizards inhabiting environments differing in the availability of water. These key species were *Pogona minor*, *Ctenophorus nuchalis* and *Ctenophorus salinarum*. The renal anatomy of the three key lizards was characterised by determining glomerular diameter, volume density, surface area and number in each. Allometric relationships between kidney, colon and body mass were investigated in these and an additional 11 species of agamid lizard. Patterns of response to osmotic challenge were recorded by measuring renal variables such as urine flow rate, glomerular filtration rate and fractional reabsorption of filtrate among the three key species, and concurrent measurements of circulating arginine vasotocin in *P. minor* and *C. nuchalis* allowed the response of this hormone to homeostatic imbalance in these species to be gauged.

The gross morphology and the glomerular characteristics of the kidneys was remarkably similar between species. Glomerular number and other characters varied as a function of body size rather than species, contrasting with reports in the literature suggesting that a
given species has a particular number of glomeruli. Allometric analysis revealed that the size of the kidneys varied between species, with *C. nuchalis* having larger kidneys, and, therefore, a greater capacity for ion transport, than *P. minor*. Additionally, colon mass was consistently greater than kidney mass across a broad range of agamid lizards. Physiologically, *P. minor* differed fundamentally from *C. nuchalis* and *C. salinarum*. It had a greater antidiuretic response to dehydration and salt-loading than either *Ctenophorus* species and the nature of the response varied between these treatments. Dehydration in *P. minor* elicited a predominantly tubular antidiuresis (ΔGFR/ΔV = 0.37), whereas salt-loading provoked a primarily glomerular antidiuresis (ΔGFR/ΔV = 0.93) and a significant increase in relative osmolar clearance to 39% from 13% in hydrated lizards. This contrasts with *C. nuchalis* and *C. salinarum*, in which the response was balanced between the glomerulus and tubule in both treatments. Furthermore, in *P. minor*, circulating levels of AVT were elevated only in dehydrated lizards (48.8±2.4 pg.mL$^{-1}$) compared to those given an acute water-load (14.7±3.6 pg.mL$^{-1}$), whereas in *C. nuchalis* both dehydration (29.5±9.5 pg.mL$^{-1}$) and salt-loading (48.8±14.8 pg.mL$^{-1}$) resulted in significantly elevated levels of the hormone relative to hydrated lizards (9.9±5.9 pg.mL$^{-1}$).

Thus, kidney morphology is constrained among species and the response of each species to osmotic perturbation is similar. However, the mechanisms underlying antidiuresis and the hormonal control of this process differ subtly between species, and there is some evidence to suggest that *P. minor* is more adapted to a mesic environment than the other two lizards examined in this study. The hypothesis that renal form and function reflect the environment in which a lizard lives therefore receives partial support, although the reptilian bauplan is able to mitigate many of the forces that could potentially lead to renal specialisation.
ACKNOWLEDGEMENTS

My sincere thanks and appreciation to:

• My supervisors, Prof. Don Bradshaw & Dr. James O’Shea who discussed aspects of the work with me, assisted in the development of some of the techniques used in this study and reviewed draft copies of the chapters and thesis.

• Victoria Cartledge, who put up with me at home and at work, and assisted me in the lab and on lizard collection trips.

• Felicity Bradshaw, who taught me the AVT RIA.

• Tom Stewart and Patience Lindhjem who shared their histological knowledge.

• Jason Fraser, who allowed me to catch lizards on his field trips, Prof. Phil Withers, Dr. Graham Thompson, Chris Clemente and Mitch Ladyman provided some lizards, and Glen Sheill, Magdalena Zofkova and Jessica Oates assisted on collecting trips.

• Professor Phil Withers and Dr. Bob Black who gave me statistical advice.

• Professor Phil Withers and Dr. Brenton Knott, members of my supervisory panel, who read the draft thesis.

I would also like to thank The University of Western Australia, which provided me with a University Postgraduate Award and Fee-waiver scholarship and a Postgraduate Student Association President’s Scholarship. The School of Animal Biology and the Australian Geographic Society gave funding for the project.
# TABLE OF CONTENTS

ABSTRACT ........................................................................................................................................... iii  
ACKNOWLEDGMENTS......................................................................................................................... vi  
PLATES AND FIGURES........................................................................................................................ xi  
TABLES .................................................................................................................................................. xii  

CHAPTER 1: GENERAL INTRODUCTION ......................................................................................... 1  

CHAPTER 2: GENERAL METHODS ...................................................................................................... 7  

2.1.1 Species used ................................................................................................................................. 9  
2.1.2 Animal Ethics ................................................................................................................................. 9  
2.1.3 Animal Husbandry ....................................................................................................................... 10  
2.1.4 Conditioning ............................................................................................................................... 10  
2.1.5 Euthanasia .................................................................................................................................. 10  

CHAPTER 3: ALLOMETRY AND MORPHOLOGY .............................................................................. 15  

3.1 INTRODUCTION ............................................................................................................................. 17  
3.2 MATERIALS AND METHODS .................................................................................................... 23  

3.2.1 Animals ...................................................................................................................................... 23  
3.2.2 Dissection ................................................................................................................................... 23  
3.2.3 Fixation and storage .................................................................................................................... 24  
3.2.4 Processing for light microscopy ................................................................................................. 24  
3.2.5 Processing for scanning electron microscopy (SEM) ................................................................. 24  
3.2.6 Stereology .................................................................................................................................. 25  
3.2.7 Statistics ................................................................................................................................... 28  
3.3 RESULTS ....................................................................................................................................... 30  

3.3.1 Allometry: all species ................................................................................................................ 30  
3.3.2 Allometry of individual species ................................................................................................ 33  
3.3.3 Morphology .............................................................................................................................. 41  
3.3.4 Stereology ............................................................................................................................... 45  
3.4 DISCUSSION ............................................................................................................................... 52  

3.4.1 Allometry ................................................................................................................................... 52  
3.4.2 Anatomy .................................................................................................................................... 54  
3.4.3 Stereology .................................................................................................................................. 55  

CHAPTER 4: RENAL PHYSIOLOGY .................................................................................................. 61  

4.1 INTRODUCTION ............................................................................................................................. 63  
4.2 MATERIALS AND METHODS .................................................................................................... 69  

4.2.1 Animals ...................................................................................................................................... 69  
4.2.2 Conditioning ............................................................................................................................. 69  
4.2.3 Treatment groups ....................................................................................................................... 69  
4.2.4 Experimental protocol .............................................................................................................. 71  
4.2.5 Statistical analyses ................................................................................................................... 72  
4.3 RESULTS ....................................................................................................................................... 75
4.3.1 Plasma osmolality (P\text{OSM}) ................................................................. 79
4.3.2 U/P\text{OSM} .................................................................................................. 80
4.3.3 Urine Flow Rate (V) .................................................................................. 82
4.3.4 Glomerular Filtration Rate (GFR) ............................................................ 85
4.3.5 Fractional reabsorption of filtrate (F\text{RH}_{2}O) ...................................... 87
4.3.6 Osmolar clearance (C\text{OSM}) .................................................................. 88
4.3.7 Free-water clearance (C\text{H}_{2}O) ............................................................... 90
4.3.8 Relative Osmolar Clearance (C\text{OSM}/C\text{IN}) ......................................... 93
4.3.9 Relative free-water clearance (C\text{H}_{2}O/C\text{IN}) .......................................... 95
4.3.10 Haematocrit ............................................................................................. 97
4.3.11 Glomerular vs. tubular response index ................................................. 97

4.4 DISCUSSION .................................................................................................... 99
4.4.1 Treatment groups ..................................................................................... 99
4.4.2 Plasma osmolality and U/P\text{OSM} .......................................................... 100
4.4.3 Haematocrit .............................................................................................. 103
4.4.4 V, GFR and F\text{RH}_{2}O .............................................................................. 104
4.4.5 Osmolar and free water clearance ......................................................... 106
4.4.6 The role of the cloaca-colon complex .................................................... 108

CHAPTER 5: THE ROLE OF ARGinine VASOTocIN ................................................. 111
5.1 INTRODUCTION ............................................................................................... 113
5.2 MATERIALS AND METHODS ................................................................. 115
5.2.1 Lizards ................................................................................................... 115
5.2.2 Radioimmunoassay of arginine vasotocin ............................................ 115
5.2.3 Statistical analyses ................................................................................. 118
5.3 RESULTS ........................................................................................................ 119
5.3.1 Plasma AVT varied in response to osmotic challenge ............................ 119
5.3.2 Correlation of circulating AVT with plasma osmolality ....................... 121
5.3.3 Correlation with renal physiological variables ...................................... 121
5.4 DISCUSSION ................................................................................................. 132
5.4.1 Circulating AVT in response to osmotic challenge ............................... 132
5.4.2 Correlations of AVT with renal variables .............................................. 136

CHAPTER 6: GENERAL DISCUSSION ..................................................................... 139

CHAPTER 7: CONCLUSION .................................................................................... 147

REFERENCES ........................................................................................................ 149
FIGURES

Fig. 2.1: *Pogona minor* .......................................................... 11
Fig. 2.2: Distribution of *P. minor* and annual rainfall map .............. 11
Fig. 2.3: *Ctenophorus nuchalis* .................................................. 12
Fig. 2.4: Distribution of *C. nuchalis* and annual rainfall map .............. 12
Fig. 2.5: *Ctenophorus salinarum* .................................................. 13
Fig. 2.6: Distribution of *C. salinarum* and annual rainfall map .............. 13
Fig. 3.1.1: Ventral kidney of *Pogona barbata* ............................... 19
Fig. 3.1.2: Schematic diagram of kidney architecture of *P. barbata* .......... 20
Fig. 3.2.1: Demonstration of point counting method ......................... 27
Fig. 3.2.2: Completion of glomerular diameter histograms .................... 29
Fig. 3.3.1: Regression of *M_b* on SVL in 14 lizards ....................... 32
Fig. 3.3.2: Regression of *M_k* and *M_m* mass on SVL ........................ 32
Fig. 3.3.3: Regression of *M_k* and *M_m* on body condition index ............ 34
Fig. 3.3.4: *P. minor*; regression of *M_k* and *M_m* on SVL .................. 34
Fig. 3.3.5: *C. nuchalis*; regression of *M_k* and *M_m* on SVL ............... 38
Fig. 3.3.6: *C. salinarum*; regression of *M_k* and *M_m* on SVL ............... 38
Fig. 3.3.7: Regression of *M_k* on SVL in three key species .................. 39
Fig. 3.3.8: Regression of *M_k* on SVL in three key species ................. 39
Fig. 3.3.9: Regression of *M_m* on SVL in three key species ................. 40
Fig. 3.3.10: Photograph of a typical agamid kidney ......................... 40
Fig. 3.3.11: Light microscopic view of glomeruli in *P. minor* ............... 42
Fig. 3.3.12: Light microscopic view of glomeruli in *C. nuchalis* .......... 42
Fig. 3.3.13: Light microscopic view of glomeruli in *C. salinarum* .......... 43
Fig. 3.3.14: Scanning electron micrograph of *P. minor* glomeruli .......... 43
Fig. 3.3.15: LM view of *P. minor* kidney architecture ...................... 44
Fig. 3.3.16: LM view of *P. minor* kidney showing collecting duct bundle .... 44
Fig. 3.3.17: Regression of *D_max* against *M_b* ............................ 50
Fig. 3.3.18: Regression of *S_{Vg,k}* against *M_k* .......................... 50
Fig. 3.3.19: Regression of *N_{Vg,k}* against *M_k* .......................... 51
Fig. 3.3.20: Regression of *N_* against *M_k* ................................ 51
Fig. 3.4.1: Diagram of structural changes as kidney size increases ........... 57
Fig. 4.3.1: Difference in slopes between *P. minor* treatment groups ......... 92
Fig. 5.3.1: Circulating AVT in *P. minor* ..................................... 120
Fig. 5.3.2: Circulating AVT in *C. nuchalis* ..................................... 120
Fig. 5.3.3: *P_{AVT} regressed on P_{OSM} in *P. minor* ....................... 122
Fig. 5.3.4: \( P_{AVT} \) regressed on \( P_{OSM} \) in \( C. nuchalis \) ........................................................... 122
Fig. 5.3.5: \( V \) regressed on \( P_{AVT} \) in \( P. minor \) ........................................................................ 125
Fig. 5.3.6: \( V \) regressed on \( P_{AVT} \) in \( C. nuchalis \) ....................................................................... 125
Fig. 5.3.7: \( P. minor \); regression of GFR on \( P_{AVT} \) .................................................................... 126
Fig. 5.3.8: \( C. nuchalis \); regression of GFR on \( P_{AVT} \) ................................................................. 126
Fig. 5.3.9: Regression of \( FR_{H2O} \) on \( P_{AVT} \) in \( P. minor \) .......................................................... 127
Fig. 5.3.10: Regression of \( FR_{H2O} \) on \( P_{AVT} \) in \( C. nuchalis \) ......................................................... 127
Fig. 5.3.11: \( P. minor \); regression of \( U/P_{OSM} \) on \( P_{AVT} \) ......................................................... 128
Fig. 5.3.12: \( C. nuchalis \); regression of \( U/P_{OSM} \) on \( P_{AVT} \) ......................................................... 128
Fig. 5.3.13: \( P. minor \); regression of \( C_{H2O} \) on \( P_{AVT} \) ............................................................... 129
Fig. 5.3.14: \( C. nuchalis \); regression of \( C_{H2O} \) on \( P_{AVT} \) ........................................................... 129
Fig. 5.3.15: Regression of \( C_{OSM} \) on \( P_{AVT} \) in \( P. minor \) ......................................................... 130
Fig. 5.3.16: Regression of \( C_{OSM} \) on \( P_{AVT} \) in \( C. nuchalis \) ....................................................... 130
Fig. 5.3.17: \( P. minor \); regression of \( C_{H2O}/C_{IN} \) on \( P_{AVT} \) ....................................................... 131
Fig. 5.3.18: \( C. nuchalis \); regression of \( C_{H2O}/C_{IN} \) on \( P_{AVT} \) ....................................................... 131

TABLES

Table 4.3.1: Renal function in the three key lizard species ........................................... 76 & 77
Table 4.3.2: Summary of two-factor AN(C)OVA of renal function ................................ 78
Table 4.3.3: Post-hoc values for \( C. nuchalis \) \( P_{OSM} \) ................................................................. 80
Table 4.3.4: Post-hoc values for \( P. minor \) \( V \) ......................................................................... 84
Table 4.3.5: Post-hoc values for \( C. nuchalis \) \( V \) ......................................................................... 84
Table 4.3.6: Post-hoc values for \( P. minor \) GFR ................................................................. 86
Table 4.3.7: Post-hoc values for \( C. nuchalis \) GFR ................................................................. 86
Table 4.3.8: Post-hoc values for \( C. nuchalis \) \( FR_{H2O} \) ................................................................. 89
Table 4.3.9: Post-hoc values for \( C. nuchalis \) \( C_{OSM} \) ................................................................. 89
Table 4.3.10: Post-hoc values for \( P. minor \) \( C_{H2O} \) ................................................................. 92
Table 4.3.11: Post-hoc values for \( C. nuchalis \) \( C_{H2O} \) ................................................................. 94
Table 4.3.12: Post-hoc values for \( P. minor \) \( C_{OSM}/C_{IN} \) ...................................................... 94
Table 4.3.13: Post-hoc values for \( P. minor \) \( C_{H2O}/C_{IN} \) ...................................................... 96
Table 4.3.14: Post-hoc values for \( C. nuchalis \) \( C_{H2O}/C_{IN} \) ...................................................... 96
Table 4.3.15: Index of glomerular vs. tubular responses in each species ......................... 98
Table 5.3.1: Summary of relationships vs. tubular responses in each species ............... 123
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AVT</td>
<td>arginine vasotocin</td>
</tr>
<tr>
<td>BCI</td>
<td>body condition index</td>
</tr>
<tr>
<td>C&lt;sub&gt;H2O&lt;/sub&gt;</td>
<td>free water clearance</td>
</tr>
<tr>
<td>C&lt;sub&gt;H2O&lt;/sub&gt;/C&lt;sub&gt;IN&lt;/sub&gt;</td>
<td>relative free water clearance</td>
</tr>
<tr>
<td>C&lt;sub&gt;IN&lt;/sub&gt;</td>
<td>clearance of inulin</td>
</tr>
<tr>
<td>C&lt;sub&gt;OSM&lt;/sub&gt;</td>
<td>osmolar clearance</td>
</tr>
<tr>
<td>C&lt;sub&gt;OSM&lt;/sub&gt;/C&lt;sub&gt;IN&lt;/sub&gt;</td>
<td>relative osmolar clearance</td>
</tr>
<tr>
<td>D&lt;sub&gt;max&lt;/sub&gt;</td>
<td>maximum glomerular diameter</td>
</tr>
<tr>
<td>FR&lt;sub&gt;H2O&lt;/sub&gt;</td>
<td>fractional reabsorption of filtrate</td>
</tr>
<tr>
<td>GFR</td>
<td>glomerular filtration rate</td>
</tr>
<tr>
<td>Hct</td>
<td>haematocrit</td>
</tr>
<tr>
<td>M&lt;sub&gt;b&lt;/sub&gt;</td>
<td>body mass</td>
</tr>
<tr>
<td>M&lt;sub&gt;c&lt;/sub&gt;</td>
<td>colon mass</td>
</tr>
<tr>
<td>M&lt;sub&gt;k&lt;/sub&gt;</td>
<td>kidney mass</td>
</tr>
<tr>
<td>MYBP</td>
<td>millions of years before present</td>
</tr>
<tr>
<td>N&lt;sub&gt;g&lt;/sub&gt;</td>
<td>total number of glomeruli per kidney</td>
</tr>
<tr>
<td>N&lt;sub&gt;Vg,k&lt;/sub&gt;</td>
<td>numerical density of glomeruli in kidney</td>
</tr>
<tr>
<td>P&lt;sub&gt;AVT&lt;/sub&gt;</td>
<td>concentration of AVT in the plasma</td>
</tr>
<tr>
<td>P&lt;sub&gt;OSM&lt;/sub&gt;</td>
<td>plasma osmolality</td>
</tr>
<tr>
<td>S&lt;sub&gt;A&lt;/sub&gt;&lt;sub&gt;g&lt;/sub&gt;</td>
<td>total surface area of glomeruli in kidney</td>
</tr>
<tr>
<td>S&lt;sub&gt;Vg,k&lt;/sub&gt;</td>
<td>surface density of glomeruli in kidney</td>
</tr>
<tr>
<td>SVL</td>
<td>snout-vent length</td>
</tr>
<tr>
<td>THSD</td>
<td>Tukey’s honest significant difference for unequal N</td>
</tr>
<tr>
<td>U/P&lt;sub&gt;OSM&lt;/sub&gt;</td>
<td>ratio of urine osmolality to plasma osmolality</td>
</tr>
<tr>
<td>V</td>
<td>urine flow rate</td>
</tr>
<tr>
<td>V&lt;sub&gt;Vg,k&lt;/sub&gt;</td>
<td>volume density of glomeruli in kidney</td>
</tr>
</tbody>
</table>
CHAPTER 1: GENERAL INTRODUCTION
From an evolutionary perspective, Australian agamid lizards are well suited to studies by adaptationists. These small- to medium-sized, rough-skinned lizards are widespread in Australia, and their widespread radiation on the continent presents a unique opportunity to investigators interested in renal morphology and function, because these characters can be correlated with physiology, ecology and contemporary distribution. Amphiboluroid agamids are squamates (Witten 1982), Gondwanan in origin (Schulte II et al. 2003), and experienced a pan-Australian radiation, probably from a xeric refuge in the north-west (Cogger and Heatwole 1981) recently (less than 20 MYBP), as the continent became progressively drier (Bowler 1976). Previous authors have noted the ‘arid-adapted’ nature of these lizards (Witten 1993). Because the group is of close ancestry, differences between its species are likely to have arisen through adaptations to their varying environments and habitats. Their contemporary distribution encompasses mesic to arid areas, with the majority inhabiting semi-arid to arid zones (Greer 1989). This is remarkable, considering the apparent absence of features allowing these animals to cope with a lack of water. Agamid lizards, and reptiles in general, are incapable of elaborating hyperosmotic urine from the kidney (Dantzler 1989a; Bradshaw 1997), making conservation of water in arid environments inherently difficult. These lizards also lack cephalic salt-secreting glands and a urinary bladder for storage of water. How then do these lizards survive in arid areas?

Possibly, a suite of ancestral characters predispose these lizards to life in xeric environments, i.e. they are “exapted” to an arid life (Bradshaw 1988, 1997). Alternatively, derived characters that evolved during their recent radiation into arid areas may allow persistence of these animals in desert environments. Other factors important for survival, such as post-renal sites for modification of urine and an innate tolerance of osmotic loads are also important when considering osmoregulation.
(Dantzler 1989a), but this study concentrates on the structural and functional mechanisms employed by the kidneys to facilitate survival in xeric conditions.

Previous attempts at describing reptilian renal function have amalgamated fragments of information garnered from disparate species, from different habitats and evolutionary lineages, into a generalised reptilian renal system. A better model is required. This study aims to examine the link between renal form and function in closely related agamid lizards from varying habitats, and to interpret the patterns observed in terms of their adaptive significance.

The objectives of the thesis were, therefore:

• To compare the renal morphology, physiology and endocrinology of three species of agamid lizards from selected habitats varying in the availability of water.

• To quantify the efficacy of their homeostatic responses to solute deprivation and loading.

• To identify aspects of kidney structure or their allometric relations which have an effect on the renal function of each species.

• To determine whether differences in kidney structure or function between the species represent adaptations to their environment.

To do this, the allometry, morphology and physiology of the kidneys in three species of Western Australian agamid lizards from different environments were studied. Allometric relationships between kidney, colon and whole animal were examined, incorporating data from 14 species. In each of three key species, stereological analysis was used (for the first time in any reptile) to examine their kidney morphology in detail. These key species were targeted as being of particular interest because of their distributions, and their kidney morphology and renal
physiological responses to osmoregulatory challenge were documented. Concurrently, the response of the principal antidiuretic hormone in reptiles, arginine vasotocin (AVT), was measured in two species by radioimmunoassay.

The key species were *Pogona minor*, *Ctenophorus nuchalis* and *Ctenophorus salinarum*. The Western bearded dragon, *P. minor* (Sternfeld 1919), is a semi-arboreal lizard that inhabits mesic and semi-arid areas of Western Australia (Cogger 2000). Fluid distribution and plasma electrolyte concentration have been studied in this species (Bradshaw 1970), and the renal anatomy of *P. barbata* was studied in detail by Splechtna (1970). The central netted dragon, *C. nuchalis* (De Vis 1884) is a burrowing species inhabiting open, arid areas of Australia (Greer 1989), and its behaviour, ecology and endocrinology have been extensively studied (Bradshaw and Main 1968; Carpenter et al. 1970; Heatwole 1970; Pianka 1971; Bradshaw 1975; Rice and Bradshaw 1980; Bradshaw 1981, 1986, 1997). In contrast, little is known about the salt-lake dragon *C. salinarum* (Storr 1966), a small, burrowing agamid that inhabits the margin of salt-lakes in semi-arid areas of southern Western Australia (Greer 1989). *C. salinarum* shares several ecological and morphological characteristics with *Ctenophorus maculosus*, a lizard reported as being capable of excreting hyperosmotic urine by Braysher (1976). Distribution maps for each species are presented in the next chapter.

The core hypothesis of this study is:

**That each of the three species will show evidence of specialisation of its renal morphology, physiology and endocrinology indicative of adaptation to their varying environments, resulting in interspecific variation of these traits.**
Specifically, I hypothesise that *P. minor*, occupying a more mesic distribution, will have a filtration-oriented renal system and a more sensitive response of AVT to osmoregulatory perturbation. Concomitantly *C. nuchalis*, being an arid-area inhabitant, will have a largely secretion-based renal system and be more tolerant to osmoregulatory imbalance. *C. salinarum*, which occurs in semi-arid areas, will display characters intermediate between *P. minor* and *C. nuchalis*. 
CHAPTER 2: GENERAL METHODS
2.1.1 Species used

Allometric relationships were examined among 199 lizards from 14 different species. Species and numbers used were: Chlamydosaurus kingii (5), Ctenophorus caudicinctus (18), C. cristatus (2), C. fordii (1), C. isolepis (4), C. nuchalis (65), C. ornatus (28), C. reticulatus (2), C. salinarum (25), C. scutulatus (10), Lophognathus gilberti (1), L. longirostris (4), Moloch horridus (2), Pogona minor (32). The renal morphology and physiology of three key species of Western Australian agamid lizard was studied: Pogona (Amphibolurus) minor (Western bearded dragon, Fig. 2.1), C. nuchalis (central netted dragon, formerly Ctenophorus (Amphibolurus) inermis, Fig. 2.3) and Ctenophorus (Amphibolurus) salinarum (salt-lake dragon, Fig. 2.5). These three species were collected in specific localities: C. nuchalis was captured at Shark Bay between the points E113°58′31.5″ S026°53′58.5″ and E114°03′08.1″ S026°57′06.6″. C. salinarum was captured at Lake Deborah East (E119°22′59.5″ S31°08′50.3″) and at Mongers Lake near Perenjori (E117°12′20.3″ S29°14′09.4″). P. minor was captured at Bungalbin Hill and surrounds (E119°32′12.1″ S30°23′12.7″). Distribution maps for each species are shown superimposed on annual rainfall data as a basic measure of aridity, and the capture locations are indicated by a red sphere in Figs. 2.2, 2.4 and 2.6. Distribution data are from the Western Australian Museum.

2.1.2 Animal Ethics

All experiments were undertaken with the permission of the UWA Animal Ethics and Experimentation Committee (AEEC), licence numbers RA3/100/50 and RA3/100/233. The Department of Conservation and Wildlife (CALM) granted permission to capture wild animals for scientific use.
2.1.3 Animal Husbandry

Lizards were housed individually in ventilated, semi-opaque plastic terraria on a substrate of sand. Their diet consisted mainly of small crickets, occasionally substituted with mealworms (Magnificent Mealworms Inc.). Feeding occurred three times per week and water was provided in petri dishes. A constant temperature of 26 °C (day) and 17 °C (night) was maintained, on a 12:12 hour cycle.

2.1.4 Conditioning

Before osmotic treatment, wild-caught individuals were maintained in their containers on a normal feeding regime for one to two weeks, allowing time for them to become conditioned to their new environment. Typically, feeding resumed within one week of capture and individuals were healthy and active at the commencement of treatment.

2.1.5 Euthanasia

Lizards were euthanased by an intraperitoneal injection of 200 mg.kg\(^{-1}\) pentobarbitone sodium (Lethabarb). Toe-pinch, tail-pinch and blink reflex test were performed to ensure that the lizards were dead prior to dissection.
Fig. 2.1: *Pogona minor* basking. SVL approximately 10 cm.

Fig. 2.2: Distribution of *P. minor* superimposed on annual rainfall (mm). The point of capture of *P. minor* is marked with a red sphere.
Ctenophorus nuchalis at the entrance to its burrow. SVL approximately 9 cm.

Distribution of Ctenophorus nuchalis and total annual rainfall (mm)

Fig. 2.4: Distribution of C. nuchalis superimposed on annual rainfall (mm). C. nuchalis was caught at the locality indicated by the red sphere.
Fig. 2.5: *Ctenophorus salinarum*, not far from its burrow. SVL approximately 6 cm. (Photograph courtesy of Scott Thompson).

Fig. 2.6: Distribution of *C. salinarum* superimposed on annual rainfall (mm). *C. salinarum* was caught at the localities indicated by the red spheres.
CHAPTER 3: ALLOMETRY AND MORPHOLOGY
3.1 INTRODUCTION

Allometric relationships often provide clues to the function of structures. For example, in neonates of the lizard *Sceloporus jarrovi*, adequate osmoregulatory function requires the combined function of both the mesonephric and metanephric kidneys, thereby maintaining the allometric relationship between total kidney mass and body mass observed in adults (Beuchat and Braun 1988). Such relationships are evidence of a tight integration between form and function. Others are pervasive, existing in several vertebrate orders, such as the exponent of basal metabolic rate to body mass in mammals of 0.66 (White and Seymour 2003), the reason for which has yet to be satisfactorily explained (Hoppeler and Weibel 2005).

In this chapter, the relationship between the kidneys and colon, organs of central importance in the osmoregulation of agamid lizards (Bradshaw 1986, 1997), to scaling variables such as body mass and snout vent length is examined, as are the structural relationships between the kidneys of the three key lizard species. The anatomy of the agamid kidney has received some attention. *Pogona (Amphibolurus) barbata* kidneys were described by Splechtna (1970) (Fig. 3.1.1), and the kidney of *Ctenophorus ornatus* was examined using vascular casting and light microscopy by O’Shea et al. (1993).

In general terms, the internal organisation of the reptilian kidney is relatively uniform and simple (Fig. 3.1.2) in comparison to the specialised renal architecture of mammals and birds (Dantzler 1989a). Lizard nephrons consist of a glomerulus, ciliated neck segment, proximal tubule, ciliated short intermediate segment, and distal tubules that drain into collecting ducts. The glomeruli of agamid kidneys are closely associated with one or more distal tubules,
suggestive of a functional tubulo-glomerular feedback mechanism, and these associations are likely to be the evolutionary precursor of the juxtaglomerular apparatus (O’Shea et al. 1993). However, the nephrons of reptilian kidneys are limited in their function because they lack the loop of Henle and the functional organisation that facilitates a countercurrent multiplier mechanism in mammals and birds (Jamison and Kriz 1982). Because of this, the kidneys are of limited use in water conservation and several reptile groups possess extra-renal osmoregulatory organs, such as cephalic (nasal & lingual) salt glands or a bladder (Nagy and Medica 1986; Bradshaw 1997). The lizards studied in this chapter lack these osmoregulatory aids (Saint Girons and Bradshaw 1987) and the principal osmoregulatory organs are therefore kidney and colon.

Quantifying morphological relationships between the kidneys of the key species is made possible through stereology (quantitative microscopy), a body of mathematical methods that relates three-dimensional parameters defining structure to two-dimensional measurements obtained from sections of the structure (Weibel 1979; Elias and Hyde 1980; Bertram 2001). In practice, this allows variables such as number, volume, surface area and density of three-dimensional bodies such as glomeruli in a kidney to be estimated using a test grid overlain on multiple sections of the tissue. By counting the number of points falling on glomeruli and the number of intersections with them, and combining this with some knowledge of average glomerular diameter, calculation of these three-dimensional attributes is possible. These methods have been used to document kidney structure in mammals (Nyengaard and Bendtsen 1991; Bertram 1995; Bertram et al. 2000) and birds (Warui 1989; Casotti and Richardson 1992; Casotti et al. 1998; Casotti 2001), but to date none have been applied to reptiles.
Fig. 3.1.1: Ventral kidney of *Pogona barbata*. Arteries: striped; afferent veins: black; efferent veins: stippled; ureters: white. Adapted from Splechtna (1970).
Fig. 3.1.2: *Pogona barbata*: schematic diagram of kidney architecture and vasculature. The nephric tissue is arranged as lobules centered around an intralobular artery and vein. Between the lobules is the collecting duct system which drains into the ureter and is accompanied by interlobular veins. Arteries: striped; afferent veins: black; efferent veins: stippled; ureters: white. Adapted from Splechtna (1970).
Glomerular counts using other methods have been performed, however, and Yokota et al. (1985) found that the allometric equation relating the number of glomeruli to body mass \( (M_b) \) (grams) in lizards based on 13 different species ranging in mass between 3 g and 1 kg was:

\[
\text{No. of glomeruli} = 330 M_b^{0.803}
\]

In a study of the agamid lizard \textit{Ctenophorus ornatus}, O'Shea et al. (1993) found through examination of serial sections that two kidneys had only 380 and 570 glomeruli per kidney, rather than over 3600 glomeruli for a 20 g lizard, as predicted by the equation above (Bradshaw 1997). Based on this observation, these authors suggested that \textit{C. ornatus} had a reduced dependence on glomerular filtration rate compared with the lizard \textit{Sceloporus cyanogenys}, with 1000 glomeruli per kidney (Davis and Schmidt-Nielsen 1967), and other reptiles, according to the equation of Yokota et al. (1985). Other examples of glomerular number in reptiles include 6000 per kidney in the grass snake \textit{Anguis} (Zarnik 1910), 15000 per kidney in the European viper \textit{Coronella} (Zarnik 1910), 800 per kidney in the gecko \textit{Tarentola (=Platydactylus) mauritanicus} (Zarnik 1910), 2300 per kidney in the skink \textit{Tiliqua rugosa} (Bentley 1959) and 1250 per kidney in juvenile crocodiles (von Møllenberg 1930; quoted in Davis and Schmidt-Nielsen 1967).

The number of glomeruli in \textit{C. ornatus} does, therefore, appear to be quite low for reptiles. However, the estimates above do not take into account kidney or body mass. Indeed, the relationships between the number of glomeruli and other glomerular variables (e.g. volume in kidney, surface area, and density) to body mass, snout vent length or kidney mass have received little attention in reptiles. Allometric relationships for these variables were examined in three species of agamid lizard from different environments: \textit{P. minor}, \textit{C. nuchalis} and \textit{C}.
salinarum. Of particular interest is the means by which kidneys increase in size. Whether new glomeruli are added, whether existing glomeruli increase in size, and whether glomerular volume, surface area or density change from the juvenile to adult condition are additional questions addressed in this chapter.

With reference to the core hypothesis of this thesis, that the more mesic species (*P. minor*) would have a filtration-oriented renal system compared with a secretion-oriented system in arid-inhabiting *C. nuchalis*, the morphological specialisations I expect are that *P. minor* will have larger and more numerous glomeruli than *C. nuchalis* and a larger surface area available for filtration.
3.2 MATERIALS AND METHODS

3.2.1 Animals

199 agamid lizards of 14 species were dissected for kidney and colon mass measurements. The largest was an adult *Chlamydosaurus kingii* (533 g) and the smallest a juvenile *Ctenophorus caudicinctus* (0.49 g). Additionally, the renal morphology of the three key species was studied in detail using stereological techniques. These were *P. minor* (*N*=5), *C. nuchalis* (*N*=6) and *C. salinarum* (*N*=6). Capture locations and husbandry were presented in Chapter 2.

3.2.2 Dissection

Lizards were euthanased with an intraperitoneal or intracardiac injection of pentobarbitone sodium (Lethabarb) at a dosage of 200 mg.kg\(^{-1}\) and placed supine on ice under a dissecting microscope. A ventral incision along the length of the abdominal cavity was made and the viscera displaced to the exterior. The ventral pelvis was bisected revealing the colon-cloaca complex, and the mid-ventral surface of the tail cut until a point caudal to the kidneys was reached. The ventral pelvis then removed, and the colon/cloaca dissected from the ventral surface of the kidneys. At the anterior entrance of the colon is a sphincter; this was bisected and the colon removed (after cutting the oviducts in females), emptied and weighed. The kidneys were dissected in a caudal-ventral direction, beginning with a lateral incision just caudal to the tip of the kidneys into the tail muscle, severing the caudal vein. A mass of connective tissue marking the point of attachment of the colon to the kidneys and the ureters was grasped with forceps, allowing the joined caudal margin of the kidneys to be lifted. Successive renal arteries and the spinal veins running into
the dorsal surface of each kidney were cut. Severing the right and left renal veins allowed the kidneys to be lifted from their cradle in the pelvis and weighed after blotting away excess blood providing fresh kidney mass ($M_k$).

3.2.3 Fixation and storage

Kidneys were then placed in 10% buffered formol saline (BFS) and maintained at 4 °C for a period ranging between one week and one month before being transferred to 70% ethanol for storage at 4 °C before processing. After a week in ethanol the kidneys were weighed again, providing a fixed kidney mass. Fresh and fixed colon masses were obtained similarly to kidney mass.

3.2.4 Processing for light microscopy

Kidneys were stored for several weeks in ethanol at 4 °C following fixation before processing for light microscopy. Kidneys were paraffin-embedded for sectioning on a Spencer 820 microtome. 8 µm serial sections of the kidney tissue were cut, mounted on glass slides and run through a graded ethanol series before staining using standard haematoxylin and eosin procedures. Coverslips were applied and a semi-permanent mounting medium (DePeX, Gurr) was used.

3.2.5 Processing for scanning electron microscopy (SEM)

Vascular casts of some lizards were produced in situ using the methods of O’Shea et al. (1993). The blood was displaced with heparinised (300 IU.ml$^{-1}$) Mackenzie’s ringer (composition in mmol: NaCl, 115; NaHCO$_3$, 20; KCl, 3.2; NaH$_2$PO$_4$, 3.1; MgSO$_4$, 1.4; D(+)-glucose, 16.7; CaCl$_2$, 1.3; bubbled with 95% O$_2$ : 5% CO$_2$ at 20 °C and filtered) via a
3.2.6 Stereology

Stereological methods (Weibel 1979) were used to obtain the glomerular volume density, $V_{Vg,k}$, or the volume of glomerulus in kidney (no units: cm$^3$.cm$^{-3}$), the surface volume density of glomeruli in kidney, $S_{Vg,k}$, or the surface area in cm$^2$ of glomeruli contained in a given volume (cm$^3$) of kidney (units: cm$^{-1}$), and numerical density of glomeruli in kidney, $N_{Vg,k}$, the number of glomeruli per cm$^3$ of kidney (units cm$^{-3}$) of each kidney pair. The term ‘density’ refers to the fact that each of these measurements is expressed per cm$^3$ kidney.

The minimum number of sections required per kidney pair was determined by power analysis as described by Weibel (1979) to be 43, based on the largest glomerular diameter and an initial estimation of $V_{Vg,k}$ from 50 sections of C. nuchalis kidney. For each kidney except one of C. salinarum, more than 43 sections were analysed. Hundreds of sections were produced for each kidney, so in order to canvas the full spectrum of the kidney, rather than randomly selecting 43 sections, a random portion of every 8th section was counted. Random portions of the sections were selected as follows: the iris diaphragm was stopped
down to its smallest aperture, appearing as a tiny point of light on the surface of the slide. Randomised adjustments were made to the microscope stage until the beam changed colour from white to pink, indicating that it was passing through the marked section. Photographs were taken using a digital video camera (IAI progressive scan CV-M7) connected to a computer running ImagePro Plus (v. 4.5.0.29). This allowed the relatively rapid processing of large numbers of sections.

Glomerular volume density and glomerular surface density were calculated from point counts obtained using a custom-designed lattice grid superimposed on the images captured (see Fig. 3.2.1). The top left corner of each square was a counting point, and the intersection lines ran horizontally. The number of points falling on glomeruli, on kidney tissue and on external tissue was recorded for each slide, as were the number of intersections with glomeruli. The number of glomeruli within the test grid was counted as specified in Weibel (1979).

The technique is illustrated in the example below (Fig. 3.2.1). The counting grid is seen superimposed on a kidney section. A single glomerulus is observed in the top left-hand corner. There are two points falling on the glomerulus (black circles) and four intersecting points (blue circles). Once these counts had been conducted on all sections for a given kidney, the following formulae were applied:

\[
V_{Vg,k} = \frac{\sum_{i=1}^{n} P_g(i)}{\sum_{i=1}^{n} P_k(i)} \quad \text{Formula 3.1}
\]

\[
S_{Vg,k} = \left( \frac{2}{k \cdot d} \right) \cdot \frac{\sum_{i=1}^{n} I_g(i)}{\sum_{i=1}^{n} P_k(i)} \quad \text{Formula 3.2}
\]
where \( d \) is the length of one side of the squares making up the test lattices, \( P_g \) is the number of points falling on glomeruli, \( P_k \) is the number of points falling on kidney, and \( I_g \) is the number of intersections with a glomerular surface. In this study, \( k_1 \) was 1 and \( d \) had a physical length of 39.43 µm.

**Fig. 3.2.1:** Demonstration of the point counting method. Two line intersections (points) fall on the glomerulus (black spheres) and four points intersect with a glomerular surface (blue spheres).

The diameter of every glomerulus photographed was measured using ImageJ (v. 1.3.3, for Mac OS X) calibrated for the optical system. The histograms generated by these measurements were then completed using a method similar to that of Weibel (1979), as illustrated in Figure 3.2.2. For each, a normal distribution curve was plotted to the histograms using JMP. The location of the peak was marked and a vertical line drawn to connect to the x-axis, and a second point marked at half the height of the peak. From the second point, a line was drawn toward the origin, and the histogram was adjusted such that the class midpoints intersected with it. This corrects for profiles (glomeruli) that
were present but too small to be noticed. Assuming that glomeruli were spherical, the mean glomerular diameter, $D_g$, was calculated as:

$$
D_g = \frac{4}{\pi} \bar{d}
$$

Formula 3.3

where $\bar{d}$ is the mean glomerular diameter calculated from the completed histogram for each. $D_g$ was required for the calculation of $N_{Vg,k}$, the numerical density of glomeruli in kidney:

$$
N_{Vg,k} = \left[ \frac{1}{d^2} \cdot \frac{1}{(D_g + t - 2h_0)} \right] \sum_{i=1}^{n} \frac{N_g(i)}{P_k(i)}
$$

Formula 3.4

where $N_g$ is the number of glomeruli on section $i$, $P_k$ is the number of points on kidney tissue on section $i$, $d^2$ is the area of one of the squares of the test lattice, $t$ is the slice thickness (8 µm) and $h_0$ is the depth by which a glomerulus has to penetrate into the slice before it is detected (Weibel 1979). In this model, $h_0$ was found to be negligible and was not included.

### 3.2.7 Statistics

Regression equations in the following sections are presented in linear and power form as follows:

**linear:**

$$
\log y = \log a[\pm SE] + b[\pm SE] \log x
$$

**power:**

$$
y = ax^b
$$

where $a$ is the coefficient of proportionality and $b$ is the slope (Schmidt-Nielsen 1991).
Regressions were performed using GraphPad Prism® 4.0 for Mac OS X. The data were log-transformed before linear regression where necessary. Mass units were milligrams and lengths were in millimetres. ANOVA and ANCOVA were performed using JMP 5.1. A significance level of $P=0.05$ was used. Interaction terms in ANCOVA (testing for parallelism) were removed from the model when $P>0.25$ and Levene’s test for homogeneity of variances was used.

**Fig. 3.2.2:** Diagram showing method of completion of histograms to include missing small profiles after Weibel (1979). The peak of a normal distribution fitted to the histogram is marked (A) and a line drawn from the point marked at half this level (B). The data are adjusted so that the ‘missing’ smaller profiles are filled and the histogram is complete.
3.3 RESULTS

3.3.1 Allometry: all species

The regression line describing the relationship between log body mass in milligrams (log\(M_b\)) and log snout-vent length (log\(SVL\); mm) in 185 individuals of 14 species is shown in Figure 3.3.1 and is defined as follows:

\[
\log M_b = -0.951[\pm 0.083] + 2.695[\pm 0.044] \log SVL
\]

\[
M_b = 0.112 SVL^{2.695}
\]

\((F_{1,183}=3827, P<0.000; r^2=0.9544, S_{y.x}=0.1010)\). The weight range was 0.49 – 533 g and included adults and juveniles of both sexes. The regression of log kidney mass in milligrams (log\(M_k\), \(N=139\)) and log colon mass in milligrams (log\(M_c\), \(N=128\)) on log\(SVL\) is presented in Figure 3.3.2. As is evident from the figure, log\(M_k\) and log\(M_c\) had similar slopes \((F_{1,263}=2.18, P=0.141)\) but their elevation was significantly different \((F_{1,264}=94.1, P<0.000)\), with colonic mass being greater than kidney mass at all body sizes. The equations are defined below:

\[
\log M_k = -2.868[\pm 0.180] + 2.507[\pm 0.096] \log SVL
\]

\[
M_k = 1.355 \times 10^{-3} SVL^{2.507}
\]

\((F_{1,137}=686.1, P<0.000; r^2=0.834, S_{y.x}=0.202)\), and:

\[
\log M_c = -3.003[\pm 0.166] + 2.700[\pm 0.088] \log SVL
\]

\[
M_c = 9.931 \times 10^{-4} SVL^{2.700}
\]

\((F_{1,126}=941.8, P<0.000; r^2=0.882, S_{y.x}=0.178)\). Because the slopes of each line did not differ, a pooled slope was calculated, \(b = 2.600\).
of kidney and colon mass on body mass instead of SVL were also performed. The slopes of $M_k$ and $M_c$ on $M_b$ were not significantly different ($F_{1,263}=0.502, P=0.479$) and the pooled slope was close to unity ($b = 0.948$). The elevations were different, however, and once again, $M_c$ was greater than $M_k$ at all sizes ($F_{1,264}=96.5, P<0.000$). The equations were:

$$\log M_k = -1.991[\pm 0.128] + 0.933[\pm 0.031] \log M_b$$

$$M_k = 0.0102 M_b^{0.933}$$

$$\log M_c = -1.902[\pm 0.137] + 0.965[\pm 0.033] \log M_b$$

$$M_c = 0.0125 M_b^{0.965}$$

($M_k$: $F_{1,138}=899, P<0.000; r^2=0.867, S_{y,x}=0.181$. $M_c$: $F_{1,125}=848, P<0.000; r^2=0.872$). A regression comparing $M_k$ with $M_c$ showed that kidney mass was approximately 87% of colon mass at all body sizes across the range of agamids:

$$\log M_k = 0.0413[\pm 0.0839] + 0.870[\pm 0.040] \log M_c$$

$$M_k = 1.10 M_c^{0.870}$$

($F_{1,115}=481, P<0.000, r^2=0.807, S_{y,x}=0.223$).
Fig. 3.3.1: Regression of log $M_b$ on log SVL. Data from 14 species of agamid, comprising 185 individuals, were pooled.

Fig. 3.3.2: Regression of log $M_k$ (○) and log $M_c$ (▲) on log SVL. The slopes did not differ ($P=0.141$) but their elevations were significantly different ($P<0.000$). Data were obtained from 9 of the 14 species.
3.3.1.1 The effect of body condition on kidney and colon mass

Log\(M_k\) and log\(M_c\) were regressed against body condition index (BCI) for all lizards. BCI data were calculated as the residuals of the regression of log\(M_b\) on log\(SVL\) presented above. Condition did not have a significant effect on colon mass \((F_{1,126}=2.20, P=0.141; r^2=0.017, S_{y,x}=0.514)\) but did have a significant, albeit slight, effect on kidney mass \((F_{1,137}=4.34, P=0.039; r^2=0.031, S_{y,x}=0.486)\), which increased with body condition (Fig. 3.3.3). The line had the equation:

\[
\log M_k = 1.839\pm0.041 + 0.863\pm0.415 \text{ BCI}
\]

\[M_k = 69.02 \text{ BCI}^{0.863}\]

3.3.2 Allometry of individual species

3.3.2.1 P. minor

The regression line of log\(M_b\) on log\(SVL\) in P. minor was:

\[
\log M_b = -0.7452\pm0.2337 + 2.559\pm0.119 \log SVL
\]

\[M_b = 0.1798 SVL^{2.559}\]

\((F_{1,25}=464.5, P<0.000; r^2=0.949, S_{y,x}=0.114)\). In this lizard, the slopes of the regressions of log\(M_k\) and log\(M_c\) on log\(SVL\) were different \((F_{1,42}=5.50, P=0.024)\) (Fig. 3.3.4), unlike the slopes for these variables across all species as shown in the previous section.
Fig. 3.3.3: Regression of log $M_k$ (O) and log $M_c$ (▲) on body condition index (BCI, the residuals of a regression of log $M_b$ on log SVL) for all studied. The slopes did not differ ($P=0.807$) but elevations were significantly different ($P<0.000$). Log $M_k$ was significantly affected by BCI ($P=0.039$) but log $M_c$ was not ($P=0.141$). Data were obtained from 9 of the 14 species. Dotted line: $M_k$.

Fig. 3.3.4: Regression of log $M_k$ and log $M_c$ in *P. minor* on log SVL. The slopes of the regressions were different ($F_{1,42}=5.50, P=0.024$).
The equations of the regressions are presented below:

\[
\log M_k = -2.787 \pm 0.372 + 2.432 \pm 0.189 \log SVL
\]

\[
M_k = 1.633 \times 10^{-3} SVL^{2.432}
\]

\[(F_{1,23}=165.0, P<0.000; r^2=0.878, S_{y,x}=0.179), \text{ and:}\]

\[
\log M_c = -3.474 \pm 0.231 + 2.975 \pm 0.119 \log SVL
\]

\[
M_c = 3.357 \times 10^{-4} SVL^{2.975}
\]

\[(F_{1,19}=626.5, P<0.000; r^2=0.971, S_{y,x}=0.107).\]

3.3.2.2  *C. nuchalis*

The equation describing the relationship between SVL and *M*$_b$ in *C. nuchalis* was as follows:

\[
\log M_b = -1.109 \pm 0.327 + 2.790 \pm 0.167 \log SVL
\]

\[
M_b = 0.0778 SVL^{2.790}
\]

\[(F_{1,61}=280.8, P<0.000; r^2=0.822, S_{y,x}=0.095) (\text{Fig. 3.3.5}). \text{ The regressions of kidney and colon mass on logSVL had similar slopes}\ (F_{1,88}=1.68, P=0.199), \text{ which pooled to 2.39. The elevations were different, however}\ (F_{1,89}=38.4, P<0.000). \text{ The equations of each line are defined as follows:}\]

\[
\log M_k = -3.114 \pm 0.652 + 2.675 \pm 0.334 \log SVL
\]

\[
M_k = 7.691 \times 10^{-4} SVL^{2.675}
\]

\[
\log M_c = -1.780 \pm 0.579 + 2.098 \pm 0.296 \log SVL
\]

\[
M_c = 0.0166 SVL^{2.098}
\]
3.3.2.3  C. salinarum

The equation describing the relationship between log\(M_b\) and logSVL in C. salinarum is:

\[
\log M_b = -0.932\pm0.486 + 2.672\pm0.286 \log SVL \\
M_b = 0.117 \, SVL^{2.672}
\]

\((F_{1,23}=87.2, P<0.000; r^2=0.791, S_{y,x}=0.074)\) (Fig. 3.3.6). As in C. nuchalis, kidney mass and colon mass had similar slopes against SVL \((F_{1,42}=0.022, P=0.882)\) and had a pooled slope of 2.66. Once again, however, they had significantly different elevations \((F_{1,43}=61.5, P<0.000)\) and the mass of colonic tissue was greater:

\[
\log M_k = -3.209\pm0.730 + 2.696\pm0.430 \log SVL \\
M_k = 6.180 \times 10^{-4} \, SVL^{2.696}
\]

\[
\log M_c = -2.846\pm0.550 + 2.615\pm0.325 \log SVL \\
M_c = 1.426 \times 10^{-3} \, SVL^{2.615}
\]

\((\log M_k: F_{1,22}=39.3, P<0.000; r^2=0.641, S_{y,x}=0.111. \log M_c: F_{1,20}=64.9, P<0.000; r^2=0.764, S_{y,x}=0.081)\).

3.3.2.4  Three species comparison

The relationships between body mass, kidney and colon mass and snout-vent length were compared between P. minor, C. nuchalis and
C. salinarum. The slopes of the regressions of $\log M_b$ on $\log SVL$ between species were not significantly different ($F_{2,109}=0.705$, $P=0.050$) and the pooled slope was 2.66 (Fig. 3.3.7). However, the elevations of the lines differed ($F_{2,111}=9.00$, $P<0.000$) and an analysis of covariance examining $M_b$ between species with $SVL$ as a covariate revealed a significant difference ($F_{3,111}=717$, $P<0.000$) due to both $SVL$ ($F_{1,111}=990$, $P<0.000$) and species ($F_{2,111}=9.00$, $P<0.000$). Comparing the mean $M_b$ adjusted for $SVL$ between species revealed that C. nuchalis had a significantly greater $M_b$ than P. minor (Tukey’s honest significant difference, THSD, $P<0.05$), while C. salinarum was intermediate between the two and not significantly different to either.

A similar pattern was observed when comparing the relationship of $M_k$ and $SVL$ between species. Although the slopes of the lines were not different ($F_{2,89}=0.286$, $P=0.752$), the elevations were ($F_{2,91}=7.56$, $P=0.001$) (Fig. 3.3.8). An ANCOVA using $SVL$ as covariate revealed that both species and $SVL$ had significant effects (species: $F_{2,91}=7.56$, $P<0.001$; $SVL$: $F_{1,91}=305$, $P<0.000$). The pooled slope of $\log M_k$ on $\log SVL$ across all three species was 2.50. Because the slopes were the same, the adjusted means were compared as for $M_b$. When kidney mass was adjusted using $SVL$, P. minor had a lower $M_k$ than C. nuchalis (THSD, $P<0.05$). When $M_k$ was adjusted using $M_b$ instead of $SVL$, the result was the same. C. salinarum was intermediate between C. nuchalis and P. minor and not significantly different from either. The relationship between colon mass and $SVL$ was different from those above; there was a significant difference between the slopes of the regressions of $\log M_c$ on $\log SVL$ between species ($F_{2,83}=4.69$, $P=0.012$) (Fig. 3.3.9); C. nuchalis had a lower slope than either P. minor or C. salinarum. Because the slopes were not parallel, ANCOVA and a comparison of adjusted means were not performed.
Fig. 3.3.5: Regression of log$M_k$ and log$M_c$ in *C. nuchalis* on logSVL. The elevations of the regressions were different ($F_{1,89}=38.4$, $P<0.000$) but the slopes were not. Dotted line: log$M_k$

Fig. 3.3.6: Regression of log$M_k$ and log$M_c$ in *C. salinarum* on logSVL. The elevations of the regressions were different ($F_{1,43}=61.5$, $P<0.000$) but the slopes were not. Dotted line: log$M_k$
Fig. 3.3.7: Regression of $M_b$ on SVL for $P. minor$, $C. nuchalis$ and $C. salinarum$. The slopes were the same but the elevations were different ($F_{2,111}=9.00$, $P<0.000$). Dotted line: $P. minor$, solid lines: $C. nuchalis$ and $C. salinarum$.

Fig. 3.3.8: Regression of $M_k$ on SVL for $P. minor$, $C. nuchalis$ and $C. salinarum$. The slopes were the same but the elevations were different ($F_{2,91}=7.56$, $P=0.001$). Dotted line: $P. minor$, solid lines: $C. nuchalis$ and $C. salinarum$. 
Fig. 3.3.9: Regression of $M_c$ on SVL for *P. minor*, *C. nuchalis* and *C. salinarum*. Slopes were significantly different ($F_{2,83}$=4.69, $P=0.012$). Dotted line: *P. minor*, solid lines: *C. nuchalis* and *C. salinarum*.

Fig. 3.3.10: Photograph of a typical agamid kidney, showing a pigmented peritoneum and joined mediocaudal margin. V = ventral view, D = dorsal view, RPV = renal portal veins. Anterior is at top.
3.3.3 Morphology

3.3.3.1 Gross morphology

The kidneys of all lizards studied were joined at the caudal margin and lay dorsally, surrounded by pigmented peritoneum (Fig. 3.3.10). At the anterior end, the kidneys are separated, only merging caudally at approximately 2/3 of the length of the kidney from the anterior tips. The caudal merged portion then extends past the vent and into the dorsal anterior region of the tail. Each kidney is roughly triangular, as shown in Fig. 3.3.10. From the anterior end, each kidney rapidly widens to the widest point just posterior to the entry of the iliac vein, before tapering to a point caudal to the vent as mentioned.

3.3.3.2 Microscopy

Almost all glomeruli were closely associated with one or more cross-sections of distal tubule. No macula densa was evident under the light microscope. Glomeruli consisting of several capillary loops were observed in *P. minor* and *C. nuchalis* (Figs. 3.3.11 and 3.3.12) although those of *C. salinarum* appeared simpler (Fig. 3.3.13). Scanning electron microscopy images of *P. minor* glomeruli showing three capillary loops and a clustered arrangement close to a common afferent arteriole are shown in Fig. 3.3.14. *P. minor* also showed clear associations of collecting ducts into collecting duct complexes that were distinct from the nephric tissue and bound by connective tissue (Figs. 3.3.15 and 3.3.16). These complexes were staggered through the kidneys and coincided with the lobules.
Fig. 3.3.11: Light microscopic view of glomeruli in *P. minor*. Note the close association between glomeruli and distal tubule. G = glomerulus, PT = proximal tubule, DT = distal tubule, A = arteriole.

Fig. 3.3.12: LM view of glomeruli in *C. nuchalis*. Labels as for Fig. 3.3.11.
Fig. 3.3.13: Glomerulus in *C. salinarum*. Labels as for Fig. 3.3.11.

Fig. 3.3.14: SEM image of clustered *P. minor* glomeruli displaying multiple capillary loops.
Fig. 3.3.15: Collecting duct in *P. minor* draining toward the ureter. CD = collecting duct, NT = nephric tissue, U = ureter, RPV = renal portal vein.

Fig. 3.3.16: Collecting duct in *P. minor* is associated into bundles or complexes in contact with interlobular veins. NT = nephric tissue, CD = collecting duct bundle, ILV = interlobular vein.
3.3.4 Stereology

3.3.4.1 Glomerular diameters

The glomerular diameters of six individuals of each of *C. nuchalis* and *C. salinarum* and five *P. minor* were measured as the maximum glomerular diameter, $D_{max}$, taken as the mean of the largest three observed glomeruli. When the data from all three species were pooled, the relationships between $D_{max}$ and SVL, $M_b$ and $M_k$ were highly significant:

$$\log D_{max} = 1.032 \pm 0.097 + 0.410 \pm 0.052 \log SVL$$
$$D_{max} = 10.77 SVL^{0.410}$$

($F_{1,15}=62.4$, $P<0.000$, $r^2=0.806$, $S_{y,x}=0.034$) (Fig. 3.3.17)

$$\log D_{max} = 1.162 \pm 0.074 + 0.156 \pm 0.018 \log M_b$$
$$D_{max} = 14.54 M_b^{0.156}$$

($F_{1,15}=74.0$, $P<0.000$, $r^2=0.831$, $S_{y,x}=0.032$)

$$\log D_{max} = 1.547 \pm 0.045 + 0.141 \pm 0.024 \log M_k$$
$$D_{max} = 35.25 M_k^{0.141}$$

($F_{1,15}=33.5$, $P<0.000$, $r^2=0.691$, $S_{y,x}=0.043$). $D_{max}$ differed significantly between species ($F_{2,14}=4.32$, $P=0.035$) but this effect was due to the difference in body size between species, as revealed by ANCOVA using SVL as covariate, in which the effect of species was not significant ($F_{2,13}=0.52$, $P=0.609$) but the effect of SVL was highly significant ($F_{1,13}=32.7$, $P<0.000$). Similar results were observed when $M_b$.
or $M_k$ were used as covariates instead of SVL. This suggests a strong relationship between body size and maximum glomerular diameter, independent of species (Fig. 3.3.17). $D_{\text{max}}$ did not differ between sexes ($F_{1,15}=0.002, P=0.965$).

### 3.3.4.2 Glomerular volume density

The volume density of glomeruli in kidney ($V_{Vg,k}$) did not differ between species (ANOVA, $F_{2,14}=2.24, P=0.143$). Given that size was shown to have an effect on the maximum glomerular diameter, SVL was included as a covariate for the present analysis, but this showed no difference between species (ANCOVA, $F_{3,13}=1.68, P=0.221$). Because glomerular volume should be related to mass, the analysis was conducted again using kidney mass, $M_k$, as a covariate. This analysis revealed a significant difference between glomerular volume densities (ANCOVA, $F_{3,13}=4.175, P=0.028$); however, this was a result of a significant effect of $M_k$ ($F_{1,13}=6.33, P=0.026$) and not species ($F_{2,13}=3.73, P=0.053$). Unlike other stereological variables (see below), linear regression revealed no significant relationships between $V_{Vg,k}$ and SVL, $M_b$ or $M_k$, suggesting that $V_{Vg,k}$ does not change as the animals become larger. The mean $V_{Vg,k}$ was $0.0114 \pm 0.0036$. There was no effect of sex on $V_{Vg,k}$ ($F_{1,15}=0.081, P=0.780$).

### 3.3.4.3 Glomerular surface density

Like glomerular volume density, glomerular surface density ($S_{Vg,k}$) did not differ between species in single-factor ANOVA ($F_{2,14}=2.19, P=0.149$). However, it did differ significantly when SVL was used as a covariate ($F_{3,13}=4.02, P=0.032$) because of significant effects of both species ($F_{2,13}=4.75, P=0.028$) and SVL ($F_{1,13}=6.10, P=0.028$). When $M_b$ was used as a covariate, ANCOVA was not significant ($F_{3,13}=3.11$, $P=0.053$).
However, when kidney mass was used as a covariate there was again an overall difference ($F_{3,13}=12.6$, $P<0.000$) caused by significant effects of both species ($F_{2,13}=8.07$, $P=0.005$) and $M_k$ ($F_{1,13}=25.8$, $P<0.000$). The regression of $S_{Vg,k}$ on $SVL$ was not significant but that of $S_{Vg,k}$ on $M_k$ was, showing that surface volume of glomeruli decreased as kidney mass increased (Fig. 3.3.18):

$$S_{Vg,k} = 17.19[\pm 1.42] - 0.0368[\pm 0.0109] M_k$$

($F_{1,15}=11.3$, $P=0.004$; $r^2=0.430$, $S_{y,x}=3.83$) (Fig. 3.3.18). If $C. salinarum$, all of which had similar $M_k$, is removed, the regression line for $C. nuchalis$ and $P. minor$ had a better fit:

$$S_{Vg,k} = 22.16[\pm 1.28] - 0.0622[\pm 0.0080] M_k$$

($F_{1,9}=60.2$, $P<0.000$; $r^2=0.870$, $S_{y,x}=2.17$). $S_{Vg,k}$ was independent of sex ($F_{1,15}=0.024$, $P=0.879$).

When the surface volume density is multiplied by the mass of one kidney ($0.5M_k$), an estimate of the total surface area of glomeruli in the kidney ($SA_g$) is obtained. Plotting $SA_g$ against kidney mass showed that despite a decrease in the density of glomeruli as kidneys become larger, the total surface area of the glomeruli does increase:

$$\log SA_g = -1.791[\pm 0.174] + 0.7702[\pm 0.095] \log M_k$$

$$SA_g = 0.0162 M_k^{0.770}$$

($F_{1,15}=66.2$, $P<0.000$; $r^2=0.815$, $S_{y,x}=0.169$). The equations relating $SA_g$ to $M_b$ and $SVL$ were:

$$\log SA_g = -3.718[\pm 0.312] + 0.8078[\pm 0.0757] \log M_b$$

$$SA_g = 1.194 \times 10^{-4} M_b^{0.808}$$
\[ (F_{1,15}=114, \ P<0.000; \ r^2=0.884, \ S_{y.x}=0.134), \text{ and} \]
\[ \log S_{A_g} = -4.425[\pm 0.402] + 2.144[\pm 0.214] \log SVL \]
\[ S_{A_g} = 3.758 \times 10^{-5} SVL^{2.144} \]
\[ (F_{1,15}=101, \ P<0.001; \ r^2=0.870, \ S_{y.x}=0.142). \]

### 3.3.4.4 Numerical density and number of glomeruli

The relationship between the numerical density of glomeruli in kidney \( N_{Vg,k} \) (i.e. glomerular density, glomeruli.cm\(^{-3} \) of kidney) and variables such as \( SVL, M_b \) and \( M_k \) was interesting: larger kidneys had lower glomerular density (Fig. 3.3.19):

\[ N_{Vg,k} = 208100[\pm 19220] - 654.3[\pm 148.5] M_k \]

\( (F_{1,15}=19.4, \ P=0.001; \ r^2=0.564, \ S_{y.x}=52140). \) The relationship between \( N_{Vg,k} \) and both \( M_b \) and \( SVL \) was similar, with \( N_{Vg,k} \) decreasing as \( M_b \) and \( SVL \) increased:

\[ N_{Vg,k} = 193000[\pm 22760] - 2.481[\pm 0.856) M_b \]

\[ N_{Vg,k} = 276500[\pm 42530] - 1660[\pm 501.5] SVL \]

\( (N_{Vg,k} \text{ on } M_b: F_{1,15}=8.39, \ P=0.0111; \ r^2=0.359, \ S_{y.x}=63250. \) \( N_{Vg,k} \text{ on } SVL: F_{1,15}=11.0, \ P=0.0048; \ r^2=0.422, \ S_{y.x}=60050). \) Multiplying individual glomerular densities by the mass of one kidney (0.5\( M_k \)) gives an estimate of the total number of glomeruli present in each kidney (\( N_g \)). Plotting this against kidney mass showed that even though the density of glomeruli is lower in larger kidneys, the total number of glomeruli increases as kidney mass increases (Fig. 3.3.20):
\[ \log N_g = 2.586[\pm 0.151] + 0.5603[\pm 0.0817] \log M_k \]

\[ N_g = 385.1 M_k^{0.560} \]

\((F_{1,15}=47.0, \ P<0.000; \ r^2=0.758, \ S_{y,x}=0.146)\) (Fig 3.3.20). Again, the relationship between total number of glomeruli, \(M_b\) and \(SVL\) was similar to that of \(M_k\):

\[ \log N_g = 1.329[\pm 0.361] + 0.5522[\pm 0.0877] \log M_b \]

\[ N_g = 21.32 M_b^{0.552} \]

\[ \log N_g = 0.7552[\pm 0.410] + 1.5140[\pm 0.2183] \log SVL \]

\[ N_g = 5.691 SVL^{1.51} \]

\((\log N_g \ on \ \log M_b: \ F_{1,15}=39.6, \ P<0.000; \ r^2=0.726, \ S_{y,x}=0.156. \ \log N_g \ on \ \log SVL: \ F_{1,15}=48.1, \ P<0.000; \ r^2=0.762, \ S_{y,x}=0.145)\).
Fig. 3.3.17: Maximum glomerular diameter, $D_{\text{max}}$, scaled with body size across species. $P<0.000$, $r^2=0.810$.

Fig. 3.3.18: Surface volume density of glomeruli in kidney regressed against kidney mass (mg) across species. $P=0.004$, $r^2=0.430$. 
Fig. 3.3.19: Numerical volume density of glomeruli in kidney regressed against kidney mass (mg). $P<0.001$, $r^2=0.564$.

Fig. 3.3.20: Total number of glomeruli per kidney regressed on kidney mass (mg). $P<0.000$, $r^2=0.757$. 
3.4 DISCUSSION

3.4.1 Allometry

Bradshaw and De'ath (1991) examined the allometric relationship between body mass and snout-vent length in a study of over 650 individual C. nuchalis. Their exponents for males ($b=3.066$), females ($b=2.541$) and juvenile lizards ($b=2.645$) are similar to the overall exponent of $2.695 \pm 0.044$ reported in this study. The theoretical exponent for mass-length relationships is 3 (Schmidt-Nielsen 1991) and it would appear that the agamid body form does not differ greatly from this. The exponents of kidney and colon mass to SVL were also close to 3 with 2.507 and 2.700, respectively, and are in line with the body mass relationship. Unexpectedly, there was no difference between sexes. While there may be within-species differences between sexes (Bradshaw and De'ath 1991) these are probably masked in the present analysis by interspecific variation in body size.

P. minor differed from C. nuchalis and C. salinarum, and from the pooled data from 14 agamid species, in having kidney and colon masses of different slopes when regressed against SVL. $M_k$ scaled similarly to $M_b$ in this species, but the slope of $M_c$ was higher, so that as the lizards grow, the colon increases in mass at a greater rate than kidney (see next page). This pattern was not observed when the data from all 14 species was pooled, nor in C. nuchalis or C. salinarum, in which the colon, although proportionately larger than the kidney, scaled with body size at the same rate as the kidney. Another difference between the species was the amount of variation in $M_k$ and $M_c$ explained by SVL. In P. minor, this was high at about 90% for both variables. C. salinarum was intermediate at about 75%, while in C. nuchalis only about half of the variation in $M_k$ and $M_c$ was due to SVL.
Body mass was an obvious explanatory variable, except that in *C. nuchalis*, as in *P. minor* and *C. salinarum*, body mass and SVL were collinear. All lizards were subject to the same procedures and therefore, presumably, the same measurement error, so the variation of $M_k$ or $M_c$ in *C. nuchalis* of a given body size would appear to be real. Despite this variation, *C. nuchalis* had a significantly greater kidney mass than *P. minor* when adjusted for body mass. As stated previously, *C. nuchalis* inhabits more arid areas than *P. minor* and it may be that this greater $M_k$ could be a response to greater osmotic challenges faced in nature, representing an increased capacity for secretion of excess solutes. However, this would also increase water loss from the kidney because the urine is hypoosmotic in reptiles. As discussed in Chapter 4, however, water loss to the colon is not necessarily water lost to the external environment, because of the reabsorptive abilities of the colon.

Comparing the slopes of colon mass with body size between species shows that that of *C. nuchalis* is significantly shallower than both *P. minor* and *C. salinarum*. The regression lines for *C. nuchalis* and *P. minor* intersect at a SVL of approx 85 mm suggesting that juvenile *C. nuchalis* have a larger colon than juvenile *P. minor*, but this relationship is reversed in adulthood. *C. nuchalis* is annual (Bradshaw 1981), hatching in autumn and breeding in spring and summer before dying, whereas *P. minor* appears to be long-lived, reaching maturity at two years (Davidge 1979). It is likely that *C. salinarum* survive for more than one year, because large adults in good condition have been observed in the field in late summer and early autumn (pers. obs.). Possibly, the greater colon and kidney size of juvenile *C. nuchalis* are related to increased growth rates relative to *P. minor* during the autumn and winter directly after hatching (breeding occurs in spring and summer). *C. nuchalis* grow at rates of up to 25 mm per month during spring (Bradshaw 1981); growth rates of *P. minor* are unknown, although likely to be less given that they take longer to reach maturity.
Another factor could be diet. That of adult *P. minor* includes large beetles with a hard spiny or shield-shaped exoskeleton (pers. obs.), which are voided and which would necessarily require a gut with an ability to cope with such robust prey. This could also explain why $M_c$ in *P. minor* increases at a rate greater than $M_b$ relative to SVL in this species, as mentioned previously. A further possibility for the differences between colon size of *P. minor* and *C. nuchalis* is the respective roles of kidney and colon in osmoregulation. The colon is known as a site of solute-linked reclamation of water (Braysher and Green 1970; Bradshaw and Rice 1981) and it may be that juvenile *C. nuchalis* have a larger colon for their body size than *P. minor* or *C. salinarum* to reclaim more water and solute as juveniles when rapid growth is essential.

### 3.4.2 Anatomy

Overall, the kidneys of the three key lizard species appeared similar, apart from widely varying glomerular sizes between individuals (see later). While there was no evidence of a true juxtaglomerular apparatus such as those possessed by mammals and birds, the Malphigian corpuscles of all species studied were closely associated with distal tubule as has been observed in amphibians, reptiles, mammals and birds (Stanton et al. 1984; Kriz and Kaissling 1985; Morild et al. 1985; Dantzler 1989b; O’Shea et al. 1993), suggesting that these lizards have a functional tubuloglomerular feedback system (Dantzler 1989a). The glomeruli arose in clumps arising from a branch of the intralobular artery (Ditrich and Splechtna 1990) and this grouped arrangement was noticeable in serial sections (Fig. 3.3.12) and under SEM (Fig. 3.3.14) and matches the observations of O’Shea et al. (1993). Similarly to *Pogona barbata* (Splechtna 1970), lobules were arranged around intralobular arteries and an intralobular vein leading to the efferent
renal veins, while at the periphery of the lobule afferent portal branches (interlobular veins) from the renal portal vein supplied peritubular capillary networks. O’Shea et al. (1993) noted the extensive renal portal system in the kidney of *Ctenophorus ornatus*, suggesting a bias toward secretory rather than filtratory function. While the extent of the renal portal system of the three key species was not studied, it is likely to be similar and this system warrants further attention, particularly given that the kidneys receive both arteriolar blood and portal venous blood. Varying the relative contributions of the these systems may be one mechanism by which the function of the kidney could be adjusted.

Collecting ducts drained the nephrons and connected to the ureter (Zarnik 1910; Splechtna 1970; Ditrich and Splechtna 1990), and an interesting observation was that the collecting ducts formed bundles ensheathed in connective tissue proximal to the ureter, suggesting that they may be osmotically isolated from the surrounding nephric tissue by this stage (Fig. 3.3.16).

**3.4.3 Stereology**

Stereology did not reveal significant differences between the kidney morphology of the key species, contrary to the hypothesis stated in the Introduction. However, it did show that as kidneys increase in size, it is a combination of an increase in the number of glomeruli, as well as the size of the glomeruli, that maintains a constant glomerular volume density as the kidney becomes larger. At the same time, despite an increase in the total glomerular surface area, the density of that surface area (the surface volume of glomeruli in the kidney, $S_{Vg,k}$) decreases (Fig. 3.3.18). $D_{max}$ was taken to be an adequate and more direct measure of glomerular size than $D_g$, the mean glomerular diameter. The two were closely correlated, however; $D_g$ was consistently 85% of $D_{max}$.
The estimated total glomerular surface area ($SA_g$) may have little functional relevance, because it does not take into account capillary complexity. Dantzler (1989a) notes that the capillary filtration surface area of glomeruli is unlikely to be a function of glomerular diameter, yet $SA_g$ is directly related to the mean glomerular diameter, $D_g$. Furthermore, blood flow to glomeruli is intermittent (Hartman 1971; Dantzler 1989a; Yokota and Dantzler 1990; Dantzler 1996) and it is likely that vascular beds are opened only when required. Thus, during periods of dehydration, the number of filtering glomeruli is reduced (Dantzler 1989a), which further reduces the usefulness of the estimated glomerular area, as the implicit assumption that all glomeruli are filtering is violated.

Because the total number of glomeruli $N_g$ does not scale isometrically, increasing at approximately half the rate of $M_k$ ($b=0.560$), the number of glomeruli per cm$^3$ of kidney decreases with an increase in kidney mass, as shown by the negative slope of $N_{Vgk}$ (Fig. 3.3.19). Thus, larger kidneys have more glomeruli in total and larger glomeruli, but glomerular number and surface area are reduced per cm$^3$ kidney when compared with smaller kidneys, as illustrated in Fig. 3.4.1. In the past authors have argued that low numbers of glomeruli are indicative of a morphological adaptation to an arid environment (Davis et al. 1976; O'Shea et al. 1993). However, considering that glomerular number correlates well with body size within species (as shown here) and between species (Yokota et al. 1985), these low numbers may be a function of the size of the kidneys used. Additionally, the assertion that lower glomerular number is an adaptation to an arid environment assumes that the numbers of glomeruli in adult lizards do not fluctuate, i.e. they are fixed at maturity and do not diminish in number by atrophy or some other process during ageing, or vary by sex. It
would then be possible for a species to be assigned a particular number of glomeruli for an individual of ‘average’ size.

\[
N \approx 20 \text{ glomeruli} > N \approx 5 \text{ glomeruli} \\
radius = r < radius = 2r \\
S.A. = 20[4\pi r^2] = S.A. = 5[4\pi (2r)^2] \\
Vol. = 20[(4/3)\pi r^3] < Vol. = 5[(4/3)\pi (2r)^3] \\
(S.A./Vol.) = 3/r > (S.A./Vol.) = 3/2r
\]

**Fig. 3.4.1:** Schematic diagram showing the change in glomerular size, number, surface area and volume density from small to large kidneys.

Maturation of glomerular number has been shown to occur in domestic fowl, in which the increase in glomerular number per gram kidney (density of glomeruli) was constant as kidney mass increased up to 12 weeks post-hatching, after which glomerular density declined as kidney size increased, without further addition of new glomeruli (Wideman 1989). Concurrently, the glomeruli became larger (Wideman 1989). This is the same pattern of growth as that presented here.

O’Shea et al. (1993) asserted that if a given species has fewer glomeruli than the ‘norm’, this is evidence of adaptation to a xeric environment. However, this study has shown that similarly sized adult *P. minor* and *C. nuchalis* had similar numbers and sizes of glomeruli, and adult *C. salinarum* of the same size and weight as juvenile *P. minor* and *C. nuchalis* had approximately the same size and number of glomeruli.
Adult *C. salinarum* therefore have fewer glomeruli per kidney than *P. minor* or *C. nuchalis*, but this is a function of body size, not an adaptation. Fewer glomeruli are linked to a smaller size, and without accounting for size differences, inferences about adaptation are not possible.

As outlined in the introduction, Yokota et al. (1985) derived the following equation relating number of glomeruli to body mass: $N_g = 330M_b^{0.803}$. The equivalent equation for the agamid lizards studied here, using $M_b$ (g) is: $N_g = 967M_b^{0.552}$. The slope of the line relating number of glomeruli to body size is significantly shallower than for the broad spectrum of reptiles used in Yokota’s equation. This is reminiscent of Heusner’s (1982) assertion that the (then accepted) 0.75 power of metabolic rate against body mass observed in mammals is a ‘statistical artefact’, and that the data for individual species fall on regression lines with a slope of 0.67 (Heusner 1982). The exponent 0.75 nevertheless remained a valid statistical description of the data accumulated on the metabolic rate of mammal species (Schmidt-Nielsen 1991). This study predicts that a 10 g agamid would have 3450 glomeruli per kidney, compared with 2100 glomeruli per kidney using Yokota’s equation for a general reptile. On the other hand, a 100 g agamid would have slightly fewer glomeruli (12286) than that predicted using Yokota’s equation (13320). Thus, smaller agamids would appear to have greater numbers of glomeruli than similarly sized reptiles. This is contrary to the suggestion by O’Shea et al. (1993) that *C. ornatus* has fewer glomeruli than reptiles of the same size. In their study, the kidneys of small adults ranging in size from 6.0—6.5 mm SVL were used. Based on these dimensions, the animals would have weighed approximately 5.6 g (Bradshaw, pers. comm.). Using the equation calculated for agamids in this study, they would therefore have approximately 2500 glomeruli in each kidney, significantly more than the 570 and 380 reported. Thus, it would appear that *C. ornatus*
have fewer glomeruli than even similarly sized congeners and this warrants further study.

There is much work to be done before glomerular number to body size predictions can be improved. Only three species were included in this study, which determined an approximate slope for amphiboluroid members of the same family over a limited size range, and because of the small number of lizards in each species, it was necessary to pool them. If more lizards in each species were studied, it is likely that different allometric equations for each species would emerge. C. salinarum, in particular, appears to have a different $SV_{g,k}$ than C. nuchalis and P. minor (Fig. 3.3.18). Furthermore, if additional species were studied, the equation relating $N_g$ to $M_b$ for the Agamidae would likely change. Lastly, if enough species were included from widely disparate reptilian groups, the resultant equation would probably be similar to that of Yokota et al. (1985).

Future comparisons with the data presented here should therefore be aware of the limitations. This family-level investigation is valuable, however, because it provides allometric equations for a group of closely related species of a similar body form, allowing comparison with other reptiles. Currently, the number of glomeruli in agamid lizards (C. ornatus excepted) scales to the power of 0.5 relative to body mass, unlike reptiles as a whole which are closer to 1.0 (Yokota et al. 1985). This difference may be a statistical artefact such as that proposed by Heusner (1982) for mammalian metabolic rate. The importance of the number of glomeruli may be overemphasised, however, since glomerular filtration is intermittent in reptiles (Dantzler 1989a), and it is likely that the extensive renal portal system in these agamids (O’Shea et al. 1993) has an equally important role in providing ions for excretion from the kidney.
4.1 INTRODUCTION

The kidney is crucial to the maintenance of the internal environment and the conservation of water in terrestrial vertebrates. As a result, mammalian and avian kidneys have evolved specialised nephrons that include a loop of Henle and *macula densa* which, in conjunction with a highly organised layout, allow them to conserve water by producing urine that is more concentrated than the plasma (hyperosmotic) (Jamison and Kriz 1982). Reptiles lack these specialised features and are unable to produce hyperosmotic ureteral urine, yet are the most common and diverse vertebrates in the arid regions of Australia and other parts of the world (Pianka 1986). This apparent paradox has been of interest to reptile physiologists for years, and has led to much investigation and several reviews (Dantzler 1970; Bradshaw 1977a, b, 1978a; Dantzler 1982a; Yokota et al. 1985; Bradshaw 1986, 1992, 1997). Additionally, the comparative physiology of the vertebrate kidney has been studied extensively (Dantzler 1970; Dantzler and Braun 1980; Dantzler 1989a; Bradshaw 1997), as have osmoregulation (Bradshaw 1975; Shoemaker and Nagy 1977; Bradshaw 1992, 1997), glomerular function (Yokota et al. 1985) and the hormonal control of the renal system (Heller and Pickering 1961; LeBrie 1972; Bradshaw 1978a, b; Dantzler 1982b; Bradshaw 1992, 1997; Bentley 1998).

One very evident aspect of the work conducted thus far is the degree of variation in kidney function between reptiles (Dantzler and Holmes 1974; Shoemaker and Nagy 1977; Dantzler 1980), which is not surprising given the range of species studied. In this Chapter, I examine the homeostatic responses to osmotic challenge of three closely related species of agamid lizard inhabiting different environments, in an attempt to minimise the differences arising between species attributable to phylogeny. This allows the response of
each species to be interpreted in the context of its habitat and life-history (see later).

Inulin, a plant polysaccharide, was used in measurements of renal clearances by Homer Smith more than seven decades ago (Smith et al. 1938). It is freely filtered at the glomerulus, and is neither reabsorbed by the nephron nor modified metabolically in vertebrates (Shannon and Smith 1935). Classical clearance studies have utilised inulin to determine glomerular filtration rate (GFR, otherwise known as clearance of inulin, C_{IN}) based on the formula:

\[ C_{IN} = V \cdot \frac{U_{IN}}{P_{IN}} \]  
Equation 4.1.1

where \( V \) is the urine flow rate, \( U_{IN} \) is the concentration of tritiated (\(^3\)H) inulin in urine, and \( P_{IN} \) is the concentration of [\(^3\)H]inulin in the blood plasma. The GFR is the rate at which plasma is cleared of inulin as it passes through the kidney. Another useful parameter is the ratio between the urine and plasma osmolalities (U/P_{OSM}) at a given time, a measure of the concentrating ability of the kidney. The difference between the GFR and \( V \) is the fractional reabsorption of filtrate, i.e. filtrate that is not excreted but is reabsorbed by the kidney tubules (Bradshaw 1997) and is calculated as:

\[ FR_{H2O} = \frac{C_{IN} \cdot V}{C_{IN}} \cdot 100\% \]  
Equation 4.1.2

The urine excreted can be considered to consist of two components: an isoosmotic solution plus free water. If the urine is hypoosmotic, as is usually the case in reptiles, the free-water fraction is a positive quantity. Concomitantly, if the urine is hyperosmotic as in mammals and birds, the free water is a negative quantity and equals that
removed from the filtrate in the concentration process (Bradshaw 1997). Hence the formula:

$$V = C_{OSM} + C_{H2O}$$

where $C_{OSM}$ and $C_{H2O}$ are the osmotic and free-water clearances, respectively. We can calculate the osmolar clearance using the same formula as GFR (Equation 4.1.1), replacing terms relating to inulin with those for osmolytes, thus:

$$C_{OSM} = V \cdot \frac{U_{OSM}}{P_{OSM}}$$

Equation 4.1.4

By rearranging formula 4.1.3:

$$C_{H2O} = V - C_{OSM}$$

Equation 4.1.5

The relative osmolar clearance ($C_{OSM}/C_{IN}$) is the fraction of filtered osmolytes that is excreted, and reflects the extent to which osmolytes are absorbed or excreted in their passage along the tubule (Bradshaw 1997). The relative free water clearance ($C_{H2O}/C_{IN}$) gives an estimate of the amount of free water added or abstracted to the urine. In reptiles, this is usually a positive quantity because their urine is hypoosmotic; note that if $C_{H2O}/C_{IN}$ were equal to 0, the urine would be isoosmotic. If it were 1.0, the urine would be equivalent to distilled water, containing no dissolved osmolytes (Bradshaw 1997). In reptiles, the additional free-water that results in a positive $C_{H2O}/C_{IN}$ is ‘created’ by the reabsorption of osmolytes in excess of water by the tubules as a hyperosmotic reabsorbate is reclaimed from the filtrate (Bradshaw 1997); $C_{H2O}/C_{IN}$ is therefore a measure of the tubular permeability to water, and a low $C_{H2O}/C_{IN}$ is indicative of increased tubular permeability.
Reptiles share some common functional characteristics. When dehydrated or presented with a salt load causing a change in hydration or osmotic condition, they alter their whole-kidney GFR (Dantzler 1989a). Usually, the result of dehydration or salt-loading is an antidiuresis. This appears to be true of crocodilians (Schmidt-Nielsen and Skadhauge 1967; Schmidt-Nielsen and Davis 1968; Kuchel and Franklin 1998), chelonians (Dantzler and Schmidt-Nielsen 1966), and all lizards studied to date (see below). In some snakes, however, salt-loading increases GFR (Bradshaw 1997). Only in the water snake *Natrix sipedon* does GFR decrease slightly in response to a salt-load (Dantzler 1967, 1968), as occurs in other reptiles (Bradshaw 1997). Lizards respond to salt-loads with a significant reduction in GFR and V, although some species such as *Tiliqua rugosa* (Schmidt-Nielsen and Davis 1968), *Tiliqua scincoides* (Schmidt-Nielsen and Davis 1968), *Hemidactylus sp.* (Roberts and Schmidt-Nielsen 1966), *Dipsosaurus dorsalis* (Bradshaw et al. 1972), and (in one study) *Varanus gouldii* (Green 1972) showed little or no decline of GFR in response to salt-load (Bradshaw 1997).

Dehydration also elicits an antidiuretic effect in lizards. Glomerular antidiuresis is accompanied by changes in tubular function, such as an increase in the fractional reabsorption of filtrate (FR$_{H2O}$) and a decrease in both relative osmolar and free-water clearances (Bradshaw and Rice 1981). In other words, dehydration causes a reduction in the rate of filtration coupled with an increased rate of reclamation of fluid and solutes from the tubules, i.e. a combined tubular and glomerular (tubuloglomerular) response. The emphasis in lizards is therefore on conservation of fluid by solute-linked reclamation of water rather than elimination of excess solute accompanied by copious dilute urine (but see Discussion). In order to quantify the tubular versus glomerular relationship, the index of Yokota et al. (1985), which compares the
relative role of a glomerular response versus a tubular response in reducing urine flow rate, was calculated for each species. This index is defined as ‘the ratio of the fractional change in GFR between the hydrated and dehydrated conditions to the corresponding change in urine flow rate’, and is denoted $\Delta$GFR/$\Delta$V.

Apart from snakes, among reptiles a consistent pattern of response to osmotic challenge can be described. As Bradshaw (1997) stated:

“What is obvious […] when examining [renal responses in reptiles] is that they are for the most part incomplete for any one species and the classical paradigm describing renal function in reptiles has thus been cobbled together from a number of eclectic sources. This paradigm states, essentially, that all reptiles evidence an antidiuresis when deprived of water and that this invariably involves a reduction in GFR due probably to a decrease in both the number of glomeruli filtering and the single nephron filtration rate (SNGFR). […] the antidiuresis may also be assisted by associated changes in tubular function which further reduce the rate of urine production. These are an increase in fractional reabsorption of filtrate and a decrease in relative free water clearance, indicative of an increase in water permeability of the nephron which thus allows water to be reclaimed by the body.”

Within this framework, any differences observed between disparate species may be due to several factors. The most obvious is phylogenetic separation and the adaptation of different species to their particular environments. Phenotypic plasticity is also emerging as an important factor in reptilian ecology and evolution (Shine 2003) and may have important implications for renal function. Additionally, experimental conditions may vary between studies and this can have a marked effect on the results obtained. For example, in the report of Roberts and Schmidt-Nielsen (1966), the desert lizards Phrynosoma cornutum and Tropidurus sp. produced isoosmotic urine regardless of the experimental treatment to which they were subjected. This observation is probably a result of conducting the experiments at room temperature, which was likely to be lower than the preferred body temperature of the species (Bradshaw 1997).
While the case for variation between widely disparate species is well established, one question that has not been addressed to date is the degree of variation in renal function between closely related reptiles inhabiting the same or similar habitats. Quantifying this variation was one of my aims in this Chapter. My specific objectives for this chapter were:

- to compare the renal physiology of three Western Australian agamid lizards from different habitats after osmotic challenge;
- to quantify the variability of the homeostatic responses to solute deprivation and loading; and,
- to determine whether any variance observed reflects the different habitats in which each species lives.

Overall, functional patterns are expected to fit the paradigm outlined above, that is, each lizard should display the classical antidiuretic response to dehydration and salt-loading. However, I hypothesise that each species will vary in its response to dehydration and salt-loading. *P. minor*, the more mesic species, is expected to respond more to dehydration and salt-loading than *C. nuchalis*, for which water deprivation in its arid environment is normal. Under hydrated conditions, *P. minor* should have a greater rate of urine flow than *C. nuchalis*. In each case, I anticipate that *C. salinarum* will display renal functional characters intermediate between those of *P. minor* and *C. nuchalis*. 
4.2 MATERIALS AND METHODS

4.2.1 Animals

Adult C. nuchalis, C. salinarum and P. minor were used in experiments. Sexes were not discriminated and individuals were randomly assigned to treatment groups.

4.2.2 Conditioning

All lizards were wild caught, and conditioned in order to approximate physiological uniformity prior to treatment. They were maintained without interference (apart from feeding) for between one and two weeks, which was sufficient for them to become accustomed to me and resume feeding.

4.2.3 Treatment groups

Acute water-load: P. minor only.

An acute hydrating treatment in which individuals were injected intra-peritoneally (IP) with deionised distilled water (ddH$_2$O) at a dosage of 10 mL.100g$^{-1}$ (10% of their body mass) for 3 consecutive days.

Chronic water-load: P. minor and C. nuchalis.

This was a chronic water-load in which lizards were injected IP with distilled deionised water (ddH$_2$O) at a dosage of 5% $M_b$ for 7 consecutive days.
**Hydrated:** *P. minor, C. nuchalis and C. salinarum.*

This treatment was designed to induce hydration without the concomitant reduction in plasma osmolality observed with water-loading, and therefore serve as a hydrated control. Lizards were injected IP with an hypoosmotic (150 mOsm.kg⁻¹) NaCl solution at a dosage of 5% *Mₖ* per day for 3 consecutive days.

**Chronic hydration:** *C. nuchalis only.*

As above, except individuals were injected for 7 consecutive days. This allowed comparison with the shorter hydrating treatment to see if it was effective as a control.

**Dehydrated:** *P. minor, C. nuchalis and C. salinarum.*

Water and food were withheld for a period of 7 consecutive days.

**Salt-loaded:** *P. minor, C. nuchalis and C. salinarum.*

A hyperosmotic solution of 2.14 M NaCl was injected IP at a dosage of 0.2% *Mₖ* for 7 consecutive days, a similar treatment to that used by Bradshaw and Rice (1981).

Treatment groups common to all three species were the hydrated, dehydrated and salt-loaded treatments. In all treatment groups, access to free water was withheld during conditioning and food was withheld for 3 days before the animals were used in experiments, to prevent faecal material from affecting urine collection.
4.2.4 Experimental protocol

Approximately 12 hours prior to testing, individuals were injected with 0.1 mL of isoosmotic saline solution, pH 7.2-7.4, containing 0.037 MBq of $[^3]$H]Inulin (0.37 MBq.mL$^{-1}$). Experiments began between 09:00 and 11:00 hours. Lizards were weighed prior to experimentation. They were then secured to a wooden frame and placed prone in a constant-temperature cabinet at 37.0 ± 1.0 °C. After 30 min a tared glass cannula (un-heparinised haematocrit tube) was placed into the cloaca to drain the urine. This procedure collected up to 75 µL of urine. The tube was checked regularly for blockage or ineffective drainage, in which case it was cleared and/or repositioned. Once approximately 70 µL of urine had been collected, the cannula was removed, weighed and the urine sample collected. After several urine collections over a period of 1-3 hrs, a blood sample was collected by cardiac puncture and then re-weighed. Blood was centrifuged for 3 min at 6500 × g and the haematocrit recorded. Following blood sampling, the lizards were euthanased with Nembutal (pentobarbitone sodium) at a dosage of 200 mg.kg$^{-1}$. 10 µL of each urine and plasma sample was assayed for $[^3]$H]inulin using a liquid scintillation counter (Packard Tri-Carb 2300TR) with Ultima Gold (Packard) scintillant. After vortexing, samples were counted for 10 min. Urine and plasma osmolality were determined by analysing 15 µL of sample in an Osmomat freezing-point osmometer. Where 15 µL of urine was not available, smaller amounts were diluted to this volume for analysis. Osmolar clearances were calculated as for inulin and are presented in Table 4.3.1 (see Results).
4.2.5 Statistical analyses

In all analyses, a significance level $\alpha=0.05$ was considered statistically significant. Statistical packages used were StatSoft® STATISTICA (Version 4.1), JMP® (Version 3.2.1) and GraphPad Prism (v. 3.0a), all for Macintosh. Analysis of variance (ANOVA) was used to compare treatment means for the following physiological variables: plasma osmolality ($P_{OSM}$), urine/plasma osmolality ($U/P_{OSM}$), fractional reabsorption of filtrate ($FR_{H2O}$), relative free-water clearance ($%C_{H2O}/C_{IN}$), relative osmolar clearance ($%C_{OSM}/C_{IN}$) and haematocrit ($Hct$) (see Table 4.3.1). Haematocrit, $FR_{H2O}$, $%C_{H2O}/C_{IN}$ and $%C_{OSM}/C_{IN}$ were arcsine-transformed prior to analysis.

Mass-independent treatment means for urine flow rate ($V$), glomerular filtration rate ($GFR$), osmolar clearance ($C_{OSM}$) and free-water clearance ($C_{H2O}$) were compared using analysis of covariance (ANCOVA) with log body mass ($logM_b$) as the covariate, rather than dividing by body mass as has traditionally been done. This avoids the assumption that these variables scale linearly with body mass over a range of masses and is therefore preferable to traditional methods (Packard and Boardman 1999).

$V$, $GFR$, $C_{OSM}$ and $C_{H2O}$ were log-transformed prior to analysis after examining the residuals against body mass and observing an increase in variation with $M_b$. Parallelism was checked by examining the interaction term of the variable of interest with $M_b$; terms with $P\geq0.25$ were pooled. All variables used in ANOVA and ANCOVA were tested for homogeneity of variances using Levene’s test. In cases where data violated the assumptions of ANOVA or ANCOVA, nonparametric tests (Kruskall-Wallace test) were conducted to support the validity of the parametric test. Post-hoc relationships between treatment means were examined using Tukey’s honest significant difference (HSD) test.
For each variable, a two-factor ANOVA/ANCOVA was performed to examine the separate effects of species and treatment. To test the overall homogeneity of slopes, Type III sums of squares were used and the following test applied:

\[
F = \frac{\text{MS}_{\text{homogeneity of slopes}}}{\text{MS Error}_{\text{sat}}} = \frac{\text{SS}_{\text{AC}} + \text{SS}_{\text{BC}} + \text{SS}_{\text{ABC}}}{\text{SS Error}_{\text{sat}}} / \frac{\text{df}_{\text{AC}} + \text{df}_{\text{BC}} + \text{df}_{\text{ABC}}}{\text{df}_{\text{Error}_{\text{sat}}}}
\]

with numerator degrees of freedom = \text{df}_{\text{AC}} + \text{df}_{\text{BC}} + \text{df}_{\text{ABC}} and denominator degrees of freedom \text{df}_{\text{Error}_{\text{sat}}}.

In this study, AC was the interaction of species with logM_b, BC was the interaction of treatment with logM_b, and ABC was the interaction of species, treatment and logM_b. The \text{P}-value was obtained from \text{F}-tables or from a \text{P}-value calculator available on the internet at the following URL:


Interaction terms were considered to be significant when \text{P} \leq 0.05.
4.3 RESULTS

The results of this Chapter are summarised in Tables 4.3.1 and 4.3.2. Data are presented as means ± SE. Although analyses were performed on mass-independent data using $M_b$ as a covariate, the rate variables presented in Table 4.3.1 (urine flow rate, glomerular filtration rate, osmolar clearance, free-water clearance) were mass adjusted by dividing by body mass in kilograms to allow comparison with the literature. Thus, while the values shown are expressed as mL.kg.hr$^{-1}$, the statistical relationships are based on ANCOVA.

Fractional variables such as fractional reabsorption of filtrate ($\text{FR}_{\text{H}2\text{O}}$), relative osmolar clearance ($\text{C}_{\text{OSM}}/\text{C}_{\text{IN}}$), relative free water clearance ($\text{C}_{\text{H}2\text{O}}/\text{C}_{\text{IN}}$) and haematocrit (Hct) are expressed as percentages.
Table 4.3.1: Renal function in three species of agamid lizard. Rates are mass specific. Superscript indicates between-treatment significant differences calculated based on mass-independent data (post-hoc tests: Tukey’s HSD for unequal sample size; lower-case superscript, $P \leq 0.05$; upper-case superscript, $P \leq 0.001$). All data presented as mean ± SE. N is as stated unless otherwise shown.

<table>
<thead>
<tr>
<th>Species</th>
<th>Treatment</th>
<th>N</th>
<th>$P_{\text{OSM}}$ (mOsm.kg$^{-1}$)</th>
<th>$U/P_{\text{OSM}}$</th>
<th>$V$ (mL.kg$^{-1}$.hr$^{-1}$)</th>
<th>GFR (CIN) (mL.kg$^{-1}$.hr$^{-1}$)</th>
<th>FR$\text{H}_{2}\text{O}$ %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pogona minor</strong></td>
<td>Chronic water-load</td>
<td>7</td>
<td>301.4±16.8$^a$</td>
<td>0.24±0.03$^{a,b}$</td>
<td>8.00±1.01$^{A,B}$</td>
<td>18.23±3.57$^A$</td>
<td>53.09±3.39$^A$</td>
</tr>
<tr>
<td></td>
<td>Acute water-load</td>
<td>5</td>
<td>266.7±15.7$^{a,c}$ (N=4)</td>
<td>0.32±0.14$^c$</td>
<td>14.02±1.39$^{C,D}$</td>
<td>35.14±3.71$^{B,c}$</td>
<td>59.69±1.14$^b$</td>
</tr>
<tr>
<td></td>
<td>Hydrated</td>
<td>5</td>
<td>309.7±14.5$^{a}$ (N=3)</td>
<td>0.28±0.04$^d$</td>
<td>5.16±1.02$^{c,f}$</td>
<td>12.81±1.90$^d$</td>
<td>58.73±5.61$^c$</td>
</tr>
<tr>
<td></td>
<td>Dehydrated</td>
<td>4</td>
<td>330.6±5.9$^c$</td>
<td>0.63±0.10$^b$</td>
<td>0.45±0.14$^{B,D,I}$</td>
<td>3.04±0.41$^e$</td>
<td>85.47±3.38$^{A,B,C,D}$</td>
</tr>
<tr>
<td></td>
<td>Salt-loaded</td>
<td>4</td>
<td>379.1±3.7$^{a,B}$</td>
<td>0.76±0.03$^{a,c,d}$</td>
<td>0.85±0.71$^{A,C,E}$</td>
<td>2.26±1.90$^{A,B,d}$</td>
<td>59.06±3.63$^{D}$</td>
</tr>
<tr>
<td><strong>Ctenophorus nuchalis</strong></td>
<td>Chronic hydration</td>
<td>6</td>
<td>302.7±14.7$^{A,B,c}$</td>
<td>0.30±0.04$^a$</td>
<td>15.66±2.72$^{A,B}$</td>
<td>29.22±5.69$^a$</td>
<td>44.79±4.12$^{a,b}$</td>
</tr>
<tr>
<td></td>
<td>Chronic hydration</td>
<td>5</td>
<td>340.2±8.7$^d$</td>
<td>0.39±0.01$^b$</td>
<td>13.03±3.32$^{C,D}$</td>
<td>27.61±3.74$^{B,c}$</td>
<td>55.65±5.84</td>
</tr>
<tr>
<td></td>
<td>Hydrated</td>
<td>7</td>
<td>375.2±11.6$^b$</td>
<td>0.35±0.04$^e$</td>
<td>10.31±1.34$^{E,F}$</td>
<td>19.42±1.60$^{e}$</td>
<td>47.81±3.72$^{c,d}$</td>
</tr>
<tr>
<td></td>
<td>Dehydrated</td>
<td>9</td>
<td>341.2±6.4$^{a,e}$</td>
<td>0.48±0.04$^f$</td>
<td>1.87±0.48$^{B,D,F}$</td>
<td>8.88±1.59$^{d}$</td>
<td>76.25±5.73$^{b,d}$</td>
</tr>
<tr>
<td></td>
<td>Salt-loaded</td>
<td>11</td>
<td>392.9±6.2$^{A,d,e}$ (N=9)</td>
<td>0.63±0.06$^{A,B,c}$</td>
<td>1.84±0.60$^{A,C,E}$</td>
<td>7.83±2.94$^{a,c,e}$</td>
<td>73.47±3.17$^{A,C}$</td>
</tr>
<tr>
<td><strong>Ctenophorus salinarum</strong></td>
<td>Hydrated</td>
<td>8</td>
<td>340.4±9.6$^a$</td>
<td>0.64±0.05</td>
<td>10.77±2.36$^{a,b}$</td>
<td>27.68±6.33</td>
<td>57.99±5.36$^{a,b}$</td>
</tr>
<tr>
<td></td>
<td>Dehydrated</td>
<td>5</td>
<td>346.7±7.1$^b$</td>
<td>0.85±0.10</td>
<td>2.96±1.40$^a$</td>
<td>14.65±2.73</td>
<td>82.48±5.26$^a$</td>
</tr>
<tr>
<td></td>
<td>Salt-loaded</td>
<td>6</td>
<td>407.0±19.1$^{a,b}$</td>
<td>0.74±0.04 (N=4)</td>
<td>2.23±0.56$^b$</td>
<td>11.35±1.66</td>
<td>79.48±3.71$^{b}$</td>
</tr>
</tbody>
</table>
Table 4.3.1 (cont.): Renal function in three species of agamid lizard. Rates are mass specific. Superscript indicates between-treatment significant differences calculated based on mass-independent data (post-hoc tests: Tukey’s HSD for unequal sample size; lower-case superscript, $P \leq 0.05$; upper-case superscript, $P \leq 0.001$). All data presented as mean ± SE. $N$ is as stated unless otherwise shown.

<table>
<thead>
<tr>
<th>Species</th>
<th>Treatment</th>
<th>$N$</th>
<th>$C_{\text{OSM}}$ (mL.kg$^{-1}$.hr$^{-1}$)</th>
<th>$C_{\text{H2O}}$ (mL.kg$^{-1}$.hr$^{-1}$)</th>
<th>$C_{\text{OSM}}/C_{\text{IN}}$ %</th>
<th>$C_{\text{H2O}}/C_{\text{IN}}$ %</th>
<th>Haematocrit %</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pogona minor</em></td>
<td>Chronic water-load</td>
<td>7</td>
<td>1.78±0.18</td>
<td>6.16±0.91$a,b$</td>
<td>10.98±1.26$^a$</td>
<td>35.58±3.05$^{a,b}$</td>
<td>14.75±1.43</td>
</tr>
<tr>
<td></td>
<td>Acute water-load</td>
<td>5</td>
<td>4.16±1.30$^{a,b}$</td>
<td>10.12±2.58$^{c,d}$</td>
<td>13.10±5.75$^b$</td>
<td>26.84±5.17$^c$</td>
<td>no data</td>
</tr>
<tr>
<td></td>
<td>Hydrated</td>
<td>5</td>
<td>1.81±0.72$^{a,b}$</td>
<td>4.25±0.73$^{a,d}$</td>
<td>13.19±1.97$^{c}$</td>
<td>35.18±5.72$^{a,e}$</td>
<td>14.62±3.22</td>
</tr>
<tr>
<td></td>
<td>Dehydrated</td>
<td>4</td>
<td>0.30±0.09$^b$</td>
<td>0.15±0.11$^{b,d,f}$</td>
<td>9.96±2.68$^d$</td>
<td>3.89±3.05$^{b,c,e}$</td>
<td>24.75±2.17</td>
</tr>
<tr>
<td></td>
<td>Salt-loaded</td>
<td>4</td>
<td>0.63±0.51$^{a}$</td>
<td>0.22±0.20$^{a,c,e}$</td>
<td>33.86±3.79$^{a,b,c,d}$</td>
<td>7.85±6.24$^{a-d}$</td>
<td>21.50±1.50</td>
</tr>
<tr>
<td><em>Ctenophorus nuchalis</em></td>
<td>Chronic water-load</td>
<td>6</td>
<td>5.11±1.39$^{a,b}$</td>
<td>10.55±1.51$^{A,B}$</td>
<td>17.08±3.13$^b$</td>
<td>37.82±2.21$^{A,B}$</td>
<td>28.70±1.64</td>
</tr>
<tr>
<td></td>
<td>Chronic hydration</td>
<td>5</td>
<td>5.15±1.48$^{a-d}$</td>
<td>7.88±1.87$^{C,d}$</td>
<td>17.57±2.72$^c$</td>
<td>27.00±3.04$^c$</td>
<td>26.30±3.38</td>
</tr>
<tr>
<td></td>
<td>Hydrated</td>
<td>7</td>
<td>3.57±0.97$^{c,d}$</td>
<td>6.74±1.02$^{E,f}$</td>
<td>18.01±3.84$^e$</td>
<td>34.38±3.49$^{D,E}$</td>
<td>30.64±1.17</td>
</tr>
<tr>
<td></td>
<td>Dehydrated</td>
<td>9</td>
<td>0.90±0.16$^{b,d,f}$</td>
<td>0.98±0.35$^{b,d,f}$</td>
<td>11.78±2.44$^d$</td>
<td>11.64±3.73$^{B,E}$</td>
<td>26.98±1.94</td>
</tr>
<tr>
<td></td>
<td>Salt-loaded</td>
<td>11</td>
<td>1.30±0.37$^{A,C,E}$</td>
<td>0.71±0.23$^{A,C,E}$</td>
<td>15.41±1.36$^{E}$</td>
<td>8.66±7.53$^{A,C,D}$</td>
<td>24.00±1.62</td>
</tr>
<tr>
<td><em>Ctenophorus salinarum</em></td>
<td>Hydrated</td>
<td>8</td>
<td>6.40±1.26$^a$</td>
<td>4.38±1.22</td>
<td>25.11±2.87$^a$</td>
<td>16.32±3.60$^a$</td>
<td>22.00±1.10</td>
</tr>
<tr>
<td></td>
<td>Dehydrated</td>
<td>5</td>
<td>2.08±0.63$^a$</td>
<td>0.89±0.85</td>
<td>13.36±2.68$^a$</td>
<td>4.21±3.61$^a$</td>
<td>24.65±0.89</td>
</tr>
<tr>
<td></td>
<td>Salt-loaded</td>
<td>6</td>
<td>2.22±0.55$^{a}$</td>
<td>0.61±0.12</td>
<td>17.26±3.39$^a$</td>
<td>8.86±3.43$^a$</td>
<td>21.77±1.05</td>
</tr>
</tbody>
</table>
Table 4.3.2: Summary of results of two-factor ANOVA and ANCOVA\(^a\) using species and treatment as factors. \(P\)-values are presented; \(P<0.05\) are presented in bold for species and treatment effects. The interaction term was considered significant for \(P<0.05\). Relationships were defined by post-hoc tests (Tukey’s HSD for unequal \(N\)) and least-squares (adjusted) means for species or treatment. \(PM = P.\ minor;\ CN = C.\ nuchalis;\ CS = C.\ salinarum;\ H = hydrated;\ D = dehydrated;\ SL = salt-loaded.\)

<table>
<thead>
<tr>
<th>Effect</th>
<th>Species</th>
<th>Treatment</th>
<th>Species (\times) Treatment</th>
<th>Post-hoc Relationship</th>
</tr>
</thead>
<tbody>
<tr>
<td>(P_{OSM})</td>
<td>0.006</td>
<td>0.000</td>
<td>0.039</td>
<td></td>
</tr>
<tr>
<td>(U/P_{OSM})</td>
<td>0.000</td>
<td>0.000</td>
<td>0.118</td>
<td>(PM = CN &lt; CS) (H &lt; D = SL)</td>
</tr>
<tr>
<td>(V)</td>
<td>0.002</td>
<td>0.000</td>
<td>0.449</td>
<td>(PM = CN &gt; CS) (H &gt; D = SL)</td>
</tr>
<tr>
<td>GFR</td>
<td>0.001</td>
<td>0.000</td>
<td>0.050</td>
<td></td>
</tr>
<tr>
<td>FR(_{H2O})</td>
<td>0.167</td>
<td>0.000</td>
<td>0.136</td>
<td>(H &lt; D &gt; SL)</td>
</tr>
<tr>
<td>(C_{OSM})</td>
<td>0.007</td>
<td>0.000</td>
<td>0.791</td>
<td>(PM = CN &amp; CS) (CN &lt; CS) (H &gt; D = SL)</td>
</tr>
<tr>
<td>(C_{H2O})</td>
<td>0.070</td>
<td>0.000</td>
<td>0.427</td>
<td>(H &gt; D = SL)</td>
</tr>
<tr>
<td>(C_{OSM}/C_{IN})</td>
<td>0.188</td>
<td>0.001</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>(C_{H2O}/C_{IN})</td>
<td>0.008</td>
<td>0.000</td>
<td>0.125</td>
<td>(PM = CN) (CN &gt; CS) (H &gt; D = SL)</td>
</tr>
<tr>
<td>Hct</td>
<td>0.000</td>
<td>0.079</td>
<td>0.002</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)ANOVA: \(P_{OSM}, U/P_{OSM}, FR_{H2O}, Hct;\) ANCOVA with log\(M_b\) as covariate: \(V, GFR, C_{OSM}, C_{H2O}.\)
4.3.1 Plasma osmolality (POSM)

Plasma osmolality (POSM) in *P. minor* ranged from 267±16 mOsm.kg\(^{-1}\) in the acute water-load group up to 379±4 mOsm.kg\(^{-1}\) in salt-loaded lizards. ANOVA revealed an overall difference between treatment groups (*F*\(_{4,17}\)=7.01, *P*=0.002). As shown in Table 4.3.1, salt-loaded lizards had significantly greater P\(_{\text{OSM}}\) than both water-loaded groups, with Tukey’s honest significant difference for unequal *N* (THSD) *P*-values of *P*=0.001 (acute water-load) and *P*=0.027 (chronic water-load). Lizards given an acute water-load also had significantly lower P\(_{\text{OSM}}\) than dehydrated lizards (THSD, *P*=0.046).

P\(_{\text{OSM}}\) in *C. nuchalis* ranged from 303±15 mOsm.kg\(^{-1}\) to 393±6 mOsm.kg\(^{-1}\) in chronic water-load and salt-load groups, respectively. Mean P\(_{\text{OSM}}\) varied significantly between treatments (*F*\(_{4,31}\)=13.4, *P*<0.000) and there were significant post-hoc differences between several groups (Table 4.3.3). Unexpectedly, hydrated lizards had a significantly greater P\(_{\text{OSM}}\) than those presented with a chronic water-load, and dehydrated lizards’ P\(_{\text{OSM}}\) was not significantly greater than that of hydrated or chronically hydrated lizards.

P\(_{\text{OSM}}\) also varied between treatments in *C. salinarum* (*F*\(_{2,16}\)=7.64, *P*=0.005). Salt-loaded lizards had significantly higher P\(_{\text{OSM}}\) (407.0±19.1 mOsm.kg\(^{-1}\)) than dehydrated (346.7±7.1 mOsm.kg\(^{-1}\)) (THSD, *P*=0.032) or hydrated (340.4±9.6 mOsm.kg\(^{-1}\)) (THSD, *P*=0.008) lizards.
Two-factor ANOVA was used to examine the separate effects of species and treatment on POSM (Table 4.3.2). The interaction term (species × treatment) was significant ($F_{4,50}=3.46$, $P=0.014$), suggesting that POSM varied differently between species in response to the treatments. Examining the treatment groups common to all species (hydrated, dehydrated and salt-loaded groups) reveals the reason for this significant interaction. In the hydrated group, C. nuchalis had the highest POSM, followed by C. salinarum and P. minor. This differed from dehydrated and salt-loaded treatments, in which C. nuchalis POSM was intermediate between P. minor at the lower end and C. salinarum at the higher end of the range. This difference in patterns between species caused the species × treatment interaction to be significant. Because of this, post-hoc relationships among species or treatments were not examined (Table 4.3.2).

4.3.2 U/POSM

In P. minor, ANOVA revealed a significant difference between U/POSM ratios between treatment groups ($F_{4,17}=10.6$, $P<0.000$). The variances
were not homogenous (Levene’s $F_{4,17}=5.12$, $P=0.007$), however, but nonparametric (Kruskal-Wallis) ANOVA confirmed that there was an overall significant difference between treatments ($H_{4,N=22}=13.4$, $P=0.010$). As expected, $U/P_{OSM}$ was significantly higher in salt-loaded lizards (77±3%) than hydrated (THSD, $P=0.008$), acute water-loaded (THSD, $P=0.006$) and chronic water-loaded lizards (THSD, $P=0.001$), which had the lowest $U/P_{OSM}$ (24±3%). Additionally, the chronic water-load group was the only one significantly lower than dehydrated (THSD, $P=0.016$).

$U/P_{OSM}$ in *C. nuchalis* displayed a similar pattern to *P. minor*. Treatment groups differed ($F_{4,30}=6.98$, $P<0.000$), with salt-loaded lizards having a higher mean $U/P_{OSM}$ (63±6%) than both hydrated groups (THSD; hydrated: $P=0.005$; chronic hydration: $P=0.048$) as well as the chronic water-load group ($P=0.002$) which had the lowest $U/P_{OSM}$ (30±4%).

There was no significant difference between $U/P_{OSM}$ of treatment groups in *C. salinarum* ($F_{2,13}=2.23$, $P=0.147$). The highest $U/P_{OSM}$ was observed in dehydrated lizards (85±10%) compared with 64±5% in hydrated lizards (Table 4.3.1).

The results of two-factor ANOVA using species and treatment as factors showed that the interaction term for species × treatment was not significant ($F_{12,42}=1.96$, $P=0.118$), indicating that the pattern of responses to each treatment group were similar between species (Table 4.3.2). $U/P_{OSM}$ was significantly affected by both species and treatment (species: $F_{2,42}=11.3$, $P<0.000$; treatment: $F_{2,42}=13.1$, $P<0.000$), however. Therefore, while the pattern of response was similar between species, there was a treatment-independent significant difference in $U/P_{OSM}$ between them: *C. salinarum* had a significantly higher $U/P_{OSM}$ than either *C. nuchalis* or *P. minor*, which did not differ from each other.
Furthermore, there was a species-independent effect in which U/P\textsubscript{OSM} was greater in dehydrated and salt-loaded lizards than in hydrated lizards, as might be expected (Table 4.3.2).

### 4.3.3 Urine Flow Rate (V)

Treatment means of V in *P. minor* differed (whole-model ANCOVA $F_{5,19}=16.5$, $P<0.000$), with a highly significant effect of treatment ($F_{4,19}=20.3$, $P<0.000$). V was highest in the acute water-load group at 14±1 mL.kg\textsuperscript{-1}.hr\textsuperscript{-1}, followed by the chronic water-load and hydrated groups at 8.0±1.0 and 5.2±1.0 mL.kg\textsuperscript{-1}.hr\textsuperscript{-1}, respectively. V in these groups differed significantly from that in both dehydrated and salt-loaded groups, with 0.5±0.1 and 0.9±0.7 mL.kg\textsuperscript{-1}.hr\textsuperscript{-1}, respectively (Table 4.3.4). Levene’s test revealed that the treatment groups did not have equal variances ($F_{4,20}=3.64$, $P=0.022$). Nonparametric ANOVA (Kruskall-Wallis) was therefore conducted on log-transformed, mass-adjusted data which confirmed that the difference between treatment means was statistically significant ($H_{4,N=25}=20.8$, $P<0.000$). Post-hoc tests were therefore conducted, and are presented in Table 4.3.4.

In *C. nuchalis*, V differed significantly between treatments (whole-model ANCOVA $F_{5,32}=16.8$, $P<0.000$), resulting largely from the effect of treatment group, which was highly significant ($F_{4,32}=12.2$, $P<0.000$). V ranged from minima of 1.9±0.5 and 1.8±0.6 mL.kg\textsuperscript{-1}.hr\textsuperscript{-1} in dehydrated and salt-loaded groups to maxima of 16±3 and 13±3 mL.kg\textsuperscript{-1}.hr\textsuperscript{-1} in the chronic water-load and chronic hydration groups. Hydrated lizards had an intermediate V of 10±1 mL.kg\textsuperscript{-1}.hr\textsuperscript{-1} (Table 4.3.1). Post-hoc tests are presented in Table 4.3.5.

In *C. salinarum*, there was a significant difference between treatment means (ANCOVA, $F_{3,15}=8.38$, $P=0.002$) with a highly significant effect of treatment ($F_{2,15}=11.9$, $P=0.001$). Hydrated lizards had the highest V
with 11±2 mL.kg\(^{-1}\).hr\(^{-1}\) compared with significantly reduced urine flow rates of dehydrated (THSD, \(P=0.018\)) and salt-loaded (THSD, \(P=0.014\)) groups at 3.0±1.4 mL.kg\(^{-1}\).hr\(^{-1}\) and 2.2±0.6 mL.kg\(^{-1}\).hr\(^{-1}\), respectively (Table 4.3.1).

The interaction term of a two-factor ANCOVA examining the effect of species and treatment on \(V\) was not significant (\(F_{4,49}=0.94, P=0.449\)). Whole-model ANCOVA showed that treatment means differed (\(F_{9,49}=11.4, P<0.001\)). As shown in Table 4.3.2, the effect of treatment on \(V\) was highly significant (\(F_{2,49}=33.1, P=0.000\)), and there was a significant species effect as well (\(F_{2,49}=7.35, P=0.002\)). Despite removing a mass effect from the analysis by using \(M_b\) as a covariate, Table 4.3.2 shows that \(C.\) salinarum had a significantly lower \(V\) than either \(P.\) minor (THSD, \(P=0.033\)) or \(C.\) nuchalis (THSD, \(P<0.000\)) when the effect of treatment is removed, while \(P.\) minor and \(C.\) nuchalis do not differ statistically (THSD, \(P=0.182\)). Between treatments, hydrated animals had greater \(V\) than dehydrated or salt-loaded animals (THSD, \(P<0.000\) in both cases) (Table 4.3.2).
**Table 4.3.4:** Tukey’s unequal N HSD P-values for *P. minor* urine flow rate. Mass-independent data were log transformed prior to ANCOVA with logM<sub>0</sub> as covariate. Significant P-values are in bold.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Chronic water-load</th>
<th>Acute water-load</th>
<th>Hydrated</th>
<th>Dehydrated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute water-load</td>
<td>0.465</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrated</td>
<td>0.961</td>
<td>0.171</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dehydrated</td>
<td>0.002</td>
<td>0.000</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td>Salt-loaded</td>
<td>0.001</td>
<td>0.000</td>
<td>0.002</td>
<td>0.989</td>
</tr>
</tbody>
</table>

**Table 4.3.5:** Tukey’s unequal N HSD P-values for *C. nuchalis* urine flow rate. Mass-independent data were log transformed prior to ANCOVA. Significant P-values are in bold.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Chronic water-load</th>
<th>Chronic hydration</th>
<th>Hydrated</th>
<th>Dehydrated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic hydration</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrated</td>
<td>1.000</td>
<td>0.999</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dehydrated</td>
<td>0.001</td>
<td>0.001</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>Salt-loaded</td>
<td>0.000</td>
<td>0.001</td>
<td>0.000</td>
<td>0.996</td>
</tr>
</tbody>
</table>
4.3.4 Glomerular Filtration Rate (GFR)

In *P. minor*, GFR differed between treatments (whole-model ANCOVA $F_{5,19}=12.9$, $P<0.000$), with a highly significant effect of treatment group ($F_{4,19}=16.1$, $P<0.000$). However, the variances were shown to differ (Levene’s $F_{4,10}=3.14$, $P=0.037$). A nonparametric analysis was therefore conducted which showed that there were highly significant differences between treatment medians (Kruskall-Wallis $H_{4.25}=19.34$, $P=0.001$), validating the parametric model used in the analyses above. Because of this, post-hoc tests were performed, the results of which are presented in Table 4.3.6. GFR was greatest in the acute water-load group, at $35\pm4$ mL.kg$^{-1}$.hr$^{-1}$, followed by the chronic water-load and hydrated groups; all three groups had significantly greater GFR than salt-loaded lizards at $2.3\pm1.9$ mL.kg$^{-1}$.hr$^{-1}$ (Table 4.3.6). Unlike salt-loading, dehydration did not cause a marked reduction of GFR (Table 4.3.1), and only the acute water-load group had a significantly greater GFR (Table 4.3.6).

As in *P. minor*, treatment means of *C. nuchalis* varied significantly (whole-model ANCOVA, $F_{4,32}=6.61$, $P=0.001$). As expected, GFR was lowest in the salt-loaded and dehydrated groups with $7.8\pm2.9$ mL.kg$^{-1}$.hr$^{-1}$ and $8.9\pm1.6$ mL.kg$^{-1}$.hr$^{-1}$, ranging up to $29\pm6$ mL.kg$^{-1}$.hr$^{-1}$ and $28\pm4$ mL.kg$^{-1}$.hr$^{-1}$ in the chronic water-load and chronic hydration groups, while the GFR of the hydrated controls was intermediate (Table 4.3.1). However, as in *P. minor*, variances of the treatment means were not homogeneous (Levene’s $F_{4,33}=3.33$, $P=0.021$), necessitating nonparametric analysis. Kruskal-Wallace ANOVA confirmed that a significant difference existed between treatments ($H_{4,38}=23.1$, $P<0.000$) and post-hoc tests were therefore conducted (Table 4.3.7).
Table 4.3.6: Tukey’s unequal N HSD P-values for *P. minor* glomerular filtration rates. Mass-independent data were log-transformed prior to ANCOVA with logM$_b$ as covariate. Significant P-values are in bold.

<table>
<thead>
<tr>
<th></th>
<th>Chronic water-load</th>
<th>Acute water-load</th>
<th>Hydrated</th>
<th>Dehydrated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute water-load</td>
<td>0.301</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrated</td>
<td>0.996</td>
<td>0.165</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dehydrated</td>
<td>0.144</td>
<td><strong>0.004</strong></td>
<td>0.252</td>
<td></td>
</tr>
<tr>
<td>Salt-loaded</td>
<td><strong>0.001</strong></td>
<td><strong>0.000</strong></td>
<td><strong>0.002</strong></td>
<td>0.125</td>
</tr>
</tbody>
</table>

Table 4.3.7: Tukey’s unequal N HSD P-values for *C. nuchalis* glomerular filtration rate. Mass-independent data were log transformed prior to ANCOVA with logM$_b$ as covariate. Significant P-values are in bold.

<table>
<thead>
<tr>
<th></th>
<th>Chronic water-load</th>
<th>Chronic hydration</th>
<th>Hydrated</th>
<th>Dehydrated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic hydration</td>
<td>0.977</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrated</td>
<td>1.000</td>
<td>0.965</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dehydrated</td>
<td>0.052</td>
<td><strong>0.024</strong></td>
<td><strong>0.037</strong></td>
<td></td>
</tr>
<tr>
<td>Salt-loaded</td>
<td><strong>0.004</strong></td>
<td><strong>0.002</strong></td>
<td><strong>0.002</strong></td>
<td>0.677</td>
</tr>
</tbody>
</table>
Similarly to the other species, in *C. salinarum* there was an overall significant difference between the treatment means (whole-model ANCOVA: $F_{3,15}=4.25$, $P=0.023$) and a significant treatment effect ($F_{2,15}=4.52$, $P=0.029$) although these were not as great as in *P. minor* and *C. nuchalis*. In fact, post-hoc tests found no significant difference between treatment means, despite the apparent reduction of GFR in dehydrated and salt-loaded lizards shown in Table 4.3.1.

Two-factor ANCOVA showed that both species and treatment effects had significance (species: $F_{2,49}=8.01$, $P=0.001$ and treatment: $F_{2,49}=21.47$, $P<0.000$). The interaction term, species × treatment, was statistically significant ($F_{4,49}=2.56$, $P=0.050$), indicating that the effect of treatment was not consistent between species, and so post-hoc tests were not conducted. Additionally, the group mean variances were not homogeneous (Levene’s $F_{8,50}=2.90$, $P=0.010$).

### 4.3.5 Fractional reabsorption of filtrate (FR$_{H2O}$)

The treatment means of *P. minor* differed significantly ($F_{4,20}=12.5$, $P<0.000$), due largely to the high FR$_{H2O}$ of lizards in the dehydrated group, which reabsorbed $86\pm3\%$ of their filtrate, compared with values ranging between $53\pm3\%$ in the chronic water-load group and $60\pm1\%$ in the acute water-load group (Table 4.3.1). Salt-loaded animals did not increase their FR$_{H2O}$, however; in fact, post-hoc tests showed that the FR$_{H2O}$ of the dehydrated group was significantly higher than all others, including salt-loaded (THSD, $P\leq 0.001$ in each case).

In *C. nuchalis*, there was a significant difference between mean FR$_{H2O}$ of the treatment groups ($F_{4,33}=9.60$, $P<0.000$). FR$_{H2O}$ was significantly greater in dehydrated ($76\pm6\%$) and salt-loaded ($74\pm3\%$) groups than chronic water-load ($45\pm4\%$) and hydrated groups, but not the chronic hydration group ($56\pm6\%$) (Table 4.3.8).
Mean FR$_{H2O}$ varied between C. salinarum treatment groups as well ($F_{2,16}=7.52$, $P=0.005$). FR$_{H2O}$ was significantly lower in hydrated lizards at 58±5% than in dehydrated (THSD, $P=0.018$) and salt-loaded (THSD, $P=0.030$) groups at 83±5% and 80±4%, respectively (Table 4.3.1).

Two-factor ANOVA using species and treatment as factors showed that there was no treatment-independent effect of species on FR$_{H2O}$, however the species-independent treatment effect was very significant ($F_{2,50}=20.54$, $P<0.000$): dehydration caused a greater increase in FR$_{H2O}$ than both hydration and salt-loading (Table 4.3.2).

### 4.3.6 Osmolar clearance (C$_{OSM}$)

In P. minor, treatment means of C$_{OSM}$ differed significantly (whole-model ANCOVA: $F_{5,16}=6.32$, $P=0.002$) because of a strongly significant effect of treatment ($F_{4,16}=7.89$, $P=0.001$). Clearance of osmolytes was greatest in lizards given an acute water-load (4.2±1.3 mL.kg$^{-1}$.hr$^{-1}$), contrasting significantly with dehydrated (THSD, $P=0.005$) and salt-loaded (THSD, $P=0.004$) lizards, which had lower osmolar clearances of 0.3±0.1 mL.kg$^{-1}$.hr$^{-1}$ and 0.6±0.5 mL.kg$^{-1}$.hr$^{-1}$, respectively. Chronic water-load and hydrated groups were intermediate (see Table 4.3.1.).
Table 4.3.8: Tukey’s unequal N HSD P-values for *C. nuchalis* fractional reabsorption of filtrate. Percentages were arcsine transformed prior to ANOVA. Significant *P*-values are in bold.

<table>
<thead>
<tr>
<th></th>
<th>Chronic water-load</th>
<th>Chronic hydration</th>
<th>Hydrated</th>
<th>Dehydrated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic hydration</td>
<td>0.789</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrated</td>
<td>0.997</td>
<td>0.919</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dehydrated</td>
<td><strong>0.002</strong></td>
<td>0.072</td>
<td><strong>0.002</strong></td>
<td></td>
</tr>
<tr>
<td>Salt-loaded</td>
<td>0.010</td>
<td>0.237</td>
<td><strong>0.011</strong></td>
<td>0.924</td>
</tr>
</tbody>
</table>

Table 4.3.9: Tukey’s unequal N HSD P-values for *C. nuchalis* osmolar clearance. Mass-independent data were log transformed prior to ANCOVA with logM₈ as covariate. Significant *P*-values are in bold.

<table>
<thead>
<tr>
<th></th>
<th>Chronic water-load</th>
<th>Chronic hydration</th>
<th>Hydrated</th>
<th>Dehydrated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic hydration</td>
<td>0.956</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrated</td>
<td>1.000</td>
<td>0.949</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dehydrated</td>
<td><strong>0.006</strong></td>
<td><strong>0.002</strong></td>
<td><strong>0.003</strong></td>
<td></td>
</tr>
<tr>
<td>Salt-loaded</td>
<td><strong>0.016</strong></td>
<td><strong>0.006</strong></td>
<td><strong>0.009</strong></td>
<td>0.985</td>
</tr>
</tbody>
</table>
ANCOVA revealed a significant difference among treatment groups in *C. nuchalis* (whole-model $F_{5,30}=10.9$, $P<0.000$), largely because of a strongly significant effect of treatment ($F_{4,30}=7.03$, $P<0.000$). $C_{\text{OSM}}$ was significantly greater in chronic water-load and chronic hydration groups than dehydrated and salt-loaded lizards (Table 4.3.9). Hydrated lizards were intermediate between these (Table 4.3.1).

As for *P. minor* and *C. nuchalis*, whole-model ANCOVA showed a significant difference between *C. salinarum* treatment groups ($F_{3,13}=5.41$, $P=0.012$). Treatment had a significant effect ($F_{2,13}=7.26$, $P=0.008$) with the hydrated lizards having higher $C_{\text{OSM}}$ ($6.4\pm1.3$ mL.kg$^{-1}$.hr$^{-1}$) than either dehydrated ($2.1\pm0.6$ mL.kg$^{-1}$.hr$^{-1}$) or salt-loaded ($2.2\pm0.6$ mL.kg$^{-1}$.hr$^{-1}$) lizards. Dehydrated and salt-loaded groups appeared to have similar $C_{\text{OSM}}$ in Table 4.3.1, but post-hoc tests showed that only dehydrated lizards had significantly lower $C_{\text{OSM}}$ than hydrated (THSD, $P=0.038$).

Two-factor ANCOVA of $C_{\text{OSM}}$ between species among treatments revealed a strongly significant difference (whole-model ANCOVA: $F_{9,43}=6.29$, $P<0.000$) because of significant differences between species ($F_{2,43}=5.67$, $P=0.007$) and treatments ($F_{2,43}=15.2$, $P<0.000$) (Table 4.3.2). When the effect of species was examined, post-hoc tests revealed that *C. nuchalis* had significantly greater $C_{\text{OSM}}$ than *C. salinarum* ($P=0.001$), but not *P. minor* ($P=0.289$). Additionally, *C. salinarum* and *P. minor* did not differ ($P=0.253$). Among treatments, hydrated lizards had significantly greater $C_{\text{OSM}}$ than dehydrated ($P<0.000$) and salt-loaded lizards ($P<0.000$), but the latter did not differ ($P=0.851$) (Table 4.3.2).

### 4.3.7  Free-water clearance ($C_{\text{H2O}}$)

In *P. minor*, ANCOVA revealed an overall difference (whole model: $F_{9,10}=13.99$, $P=0.001$) attributable partly to the effect of treatment
(F_{4,10}=4.88, P=0.019) but also to the effect of the covariate, logM_b (F_{1,10}=16.8, P=0.002). The interaction term (treatment × logM_b) was included in the model because it was significant (P<0.25), indicating that the treatment lines were not parallel. This was because the slope of the dehydrated and salt-loaded groups differed from that of the others when plotted against M_b (Figure 4.3.1). Additionally, variances were not homogeneous (F_{4,15}=5.04, P=0.009). As a result, a more strict alpha, α=0.01, was used in the interpretation of the results in *P. minor*. C_{H2O} was greatest in the acute water-load group, at 10±3 mL.kg^{-1}.hr^{-1} and lowest in dehydrated and salt-loaded groups, which had significantly lower free-water clearance than all other groups at 0.2±0.1 and 0.2±0.2 mL.kg^{-1}.hr^{-1}, respectively (see Table 4.3.10).

Overall, C_{H2O} differed between treatments in *C. nuchalis* (whole-model ANCOVA: F_{5,29}=13.6, P<0.000) largely due to the effect of treatment (F_{4,29}=13.2, P<0.000). As in *P. minor*, the lowest C_{H2O} were observed in dehydrated and salt-loaded lizards (Table 4.3.1), which were again significantly lower than all of the other groups (Table 4.3.12).

In *C. salinarum*, as in both *P. minor* and *C. nuchalis*, there was an overall difference between treatment groups (whole-model ANCOVA: F_{3,12}=4.48, P=0.025) and the effect of treatment was significant (F_{2,12}=6.47, P=0.012). Hydrated lizards had a C_{H2O} of 4.4±1.2 mL.kg^{-1}.hr^{-1}, higher than either dehydrated (0.89±0.85 mL.kg^{-1}.hr^{-1}) or salt-loaded (0.61±0.12 mL.kg^{-1}.hr^{-1}) groups. Despite the large apparent difference between hydrated and the other groups, no statistical difference was found.
Fig. 4.3.1: *P. minor*: Dehydrated and salt-loaded treatment groups had different slopes compared to the others.

<table>
<thead>
<tr>
<th></th>
<th>Chronic water-load</th>
<th>Acute water-load</th>
<th>Hydrated</th>
<th>Dehydrated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute water-load</td>
<td>0.865</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrated</td>
<td>0.998</td>
<td>0.789</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dehydrated</td>
<td>0.005</td>
<td>0.001</td>
<td>0.009</td>
<td></td>
</tr>
<tr>
<td>Salt-loaded</td>
<td>0.003</td>
<td>0.001</td>
<td>0.006</td>
<td>1.000</td>
</tr>
</tbody>
</table>

*Table 4.3.10:* Tukey’s unequal N HSD $P$-values for *P. minor* free water clearance. Mass-independent data were log-transformed prior to ANCOVA with logM$_b$ as covariate. Significant $P$-values ($\alpha<0.01$) are in bold.
Two-factor ANCOVA was significant overall (whole-model: $F_{9,39}=8.56$, $P<0.000$) due to a highly significant effect of treatment ($F_{2,39}=21.9$, $P<0.000$). However, there were no differences between species ($F_{2,39}=2.86$, $P=0.070$). Across species, hydrated lizards had greater $C_{\text{H2O}}$ than either dehydrated or salt-loaded treatments (THSD, $P<0.000$ in both cases) (Table 4.3.2).

### 4.3.8 Relative Osmolar Clearance ($C_{\text{OSM}}/C_{\text{IN}}$)

ANOVA revealed a significant difference in $C_{\text{OSM}}/C_{\text{IN}}$ between $P. minor$ treatments ($F_{4,17}=9.47$, $P<0.000$). In salt-loaded lizards 34±4% of osmolytes were cleared, compared with 10±3% in dehydrated lizards. (see Table 4.3.1.). All groups had similar $C_{\text{OSM}}/C_{\text{IN}}$, except salt-loaded lizards, which had a significant increased clearance of osmolytes (natriuresis) (Table 4.3.12), which was not observed in $C. nuchalis$ or $C. salinarum$.

In $C. nuchalis$, no significant differences between treatment groups were found ($F_{4,31}=0.965$, $P=0.440$). $C_{\text{OSM}}/C_{\text{IN}}$ ranged from 18±4% in hydrated lizards to 12±2% in dehydrated lizards, with all other groups intermediate (Table 4.3.1.). The mean across all groups was 15.6±1.2% ($N=36$).

Treatment means did vary in $C. salinarum$ ($F_{2,14}=4.30$, $P=0.035$). Hydrated lizards had the highest relative osmolar clearance (25±3%) and dehydrated the lowest (13±3%). However, post-hoc tests revealed no significant differences.
Table 4.3.11: Tukey’s unequal N HSD P-values for *C. nuchalis* free water clearance. Mass-independent data were log transformed prior to ANCOVA with logM\(_6\) as covariate. Significant P-values are in bold.

<table>
<thead>
<tr>
<th></th>
<th>Chronic water-load</th>
<th>Chronic hydration</th>
<th>Hydrated</th>
<th>Dehydrated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic hydration</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrated</td>
<td>0.999</td>
<td>1.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dehydrated</td>
<td>0.003</td>
<td>0.010</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td>Salt-loaded</td>
<td>&lt;0.000</td>
<td>&lt;0.000</td>
<td>&lt;0.000</td>
<td>0.515</td>
</tr>
</tbody>
</table>

Table 4.3.12: Tukey’s unequal N HSD P-values for *P. minor* relative osmolar clearances. Data were arcsine-transformed prior to ANOVA. Significant P-values are in bold.

<table>
<thead>
<tr>
<th></th>
<th>Chronic water-load</th>
<th>Acute water-load</th>
<th>Hydrated</th>
<th>Dehydrated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute water-load</td>
<td>0.990</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrated</td>
<td>0.994</td>
<td>1.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dehydrated</td>
<td>1.000</td>
<td>0.960</td>
<td>0.976</td>
<td></td>
</tr>
<tr>
<td>Salt-loaded</td>
<td>0.001</td>
<td>0.003</td>
<td>0.010</td>
<td>0.001</td>
</tr>
</tbody>
</table>
Two-factor ANOVA of $C_{\text{OSM}}/C_{\text{IN}}$ among treatment groups between species did yield a significant effect of treatment ($F_{2,44}=1.74$, $P=0.188$) but the species $\times$ treatment interaction was significant ($F_{4,44}=5.60$, $P=0.001$), indicating that treatments affected each species differently. This reflects the natriuresis observed in $P. \text{minor}$ but neither of the $Ctenophorus$ species.

### 4.3.9 Relative free-water clearance ($C_{\text{H2O}}/C_{\text{IN}}$)

ANOVA revealed a significant difference in $C_{\text{H2O}}/C_{\text{IN}}$ between treatments in $P. \text{minor}$ ($F_{4,17}=11.3$, $P<0.000$). Chronic water-load and hydrated groups had the highest $C_{\text{H2O}}/C_{\text{IN}}$, with 36±3% and 35±6%, respectively. $C_{\text{H2O}}/C_{\text{IN}}$ was lowest in dehydrated (3.9±3.1%) and salt-loaded lizards (7.9±6.2%), and acute water-load lizards were intermediate (Table 4.3.1). In general, dehydrated and salt-loaded lizards had significantly lower $C_{\text{H2O}}/C_{\text{IN}}$ than hydrated and water-loaded groups (Table 4.3.13).

Relative free-water clearances were strongly significantly different between treatment groups of $C. \text{nuchalis}$ ($F_{4,31}=17.5$, $P<0.000$). Salt-loaded lizards had a significantly lower $C_{\text{H2O}}/C_{\text{IN}}$ than all other groups except dehydrated, which had significantly lower $C_{\text{H2O}}/C_{\text{IN}}$ than chronic water-load and hydrated groups, but not chronic hydration (Table 4.3.14).

Like the two previous species, ANOVA of $C_{\text{H2O}}/C_{\text{IN}}$ in $C. \text{salinarum}$ revealed significant differences between treatment groups ($F_{2,14}=4.02$, $P=0.042$); however, post-hoc tests revealed no significant differences between treatment pairs.
**Table 4.3.13:** Tukey’s unequal N HSD P-values for *P. minor* relative free-water clearances. Data were arcsine-transformed prior to ANOVA. Significant P-values are in bold.

<table>
<thead>
<tr>
<th></th>
<th>Chronic water-load</th>
<th>Acute water-load</th>
<th>Hydrated</th>
<th>Dehydrated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute water-load</td>
<td>0.669</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrated</td>
<td>1.000</td>
<td>0.797</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dehydrated</td>
<td>0.002</td>
<td>0.024</td>
<td>0.006</td>
<td></td>
</tr>
<tr>
<td>Salt-loaded</td>
<td>0.005</td>
<td>0.075</td>
<td>0.018</td>
<td>0.976</td>
</tr>
</tbody>
</table>

**Table 4.3.14:** Tukey’s unequal N HSD P-values for *C. nuchalis* relative free-water clearance. Data were arcsine-transformed prior to ANOVA. Significant P-values are in bold.

<table>
<thead>
<tr>
<th></th>
<th>Chronic hydration</th>
<th>Hydrated</th>
<th>Dehydrated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic hydration</td>
<td>0.279</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrated</td>
<td>0.957</td>
<td>0.638</td>
<td></td>
</tr>
<tr>
<td>Dehydrated</td>
<td>&lt;0.000</td>
<td>0.067</td>
<td>&lt;0.000</td>
</tr>
<tr>
<td>Salt-loaded</td>
<td>&lt;0.000</td>
<td>0.019</td>
<td>&lt;0.000</td>
</tr>
</tbody>
</table>
Two-way ANOVA of $C_{\text{H}_2\text{O}}/C_{\text{IN}}$ revealed significant differences between species ($F_{2,44}=5.36, P=0.008$) and treatment ($F_{2,44}=28.0, P<<0.000$). The interaction term (species × treatment) was not significant ($F_{4,44}=1.91, P=0.125$), suggesting that treatments affected each species in the same way. Post-hoc analysis revealed that $C. nuchalis$ had significantly greater $C_{\text{H}_2\text{O}}/C_{\text{IN}}$ than $C. salinarum$ (THSD, $P=0.010$) but not $P. minor$ (THSD, $P=0.796$), and $C. salinarum$ and $P. minor$ did not differ significantly (THSD, $P=0.175$) (Table 4.3.2). Across species, hydrated lizards had significantly greater $C_{\text{H}_2\text{O}}/C_{\text{IN}}$ than either dehydrated or salt-loaded groups (THSD, both $P<0.000$) while the latter did not differ ($P=0.982$).

4.3.10 Haematocrit

Haematocrit data were not collected for the first treatment group trialed ($P. minor$: acute water-load). The difference between the Hct of remaining $P. minor$ treatment groups was not statistically significant (ANOVA $F_{3,11}=3.54, P=0.052$). Furthermore, Hct did not differ between treatment groups in $C. nuchalis$ ($F_{4,28}=1.93, P=0.134$) or $C. salinarum$ ($F_{2,14}=2.25, P=0.142$). Two-factor ANOVA using treatment and species as factors revealed a significant difference ($F_{8,44}=6.23, P<0.000$), however the interaction between species and treatment was significant ($F_{4,44}=4.92, P=0.002$) because $P. minor$ responded differently to the hydration treatment, preventing the use of post-hoc tests.

4.3.11 Glomerular vs. tubular response index

This index, introduced by Yokota et al. (1985), compares the relative role of the glomerular versus tubular responses to dehydration and (in this study) salt-loading (Table 4.3.15). As shown in the table, the response of $P. minor$ to dehydration is very different to its response to
salt-loading. Dehydration resulted in a predominantly tubular response compared to the glomerular response of salt-loaded lizards. In contrast, dehydration did not cause a great difference in tubular vs. glomerular response in either *C. nuchalis* or *C. salinarum*, both of which showed a balanced response to osmotic challenge. *C. nuchalis* did show a slight tubular bias, however.

Table 4.3.15: V and GFR as a percentage of hydrated controls are presented, along with the index of Yokota et al. (1985) (ΔGFR/ΔV) comparing the relative role of the glomerular vs. tubular response in each species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Dehydrated</th>
<th>Salt-loaded</th>
<th>Yokota’s Index</th>
<th>Dehydrated</th>
<th>Salt-loaded</th>
<th>Yokota’s Index</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V % of</td>
<td>GFR % of</td>
<td></td>
<td>V % of</td>
<td>GFR % of</td>
<td></td>
</tr>
<tr>
<td></td>
<td>hydrated</td>
<td>hydrated</td>
<td>Index</td>
<td>hydrated</td>
<td>hydrated</td>
<td></td>
</tr>
<tr>
<td><em>P. minor</em></td>
<td>8.7</td>
<td>23.7</td>
<td>0.37</td>
<td>16.5</td>
<td>17.6</td>
<td>0.93</td>
</tr>
<tr>
<td><em>C. nuchalis</em></td>
<td>18.1</td>
<td>45.7</td>
<td>0.40</td>
<td>17.9</td>
<td>40.3</td>
<td>0.44</td>
</tr>
<tr>
<td><em>C. salinarum</em></td>
<td>27.5</td>
<td>52.9</td>
<td>0.52</td>
<td>20.7</td>
<td>41.0</td>
<td>0.50</td>
</tr>
</tbody>
</table>
4.4 DISCUSSION

4.4.1 Treatment groups

Preliminary experiments were based on *P. minor* that were water-loaded acutely at a dosage of 10% *Mb* for 3 days. This caused the lizards distress and was therefore discontinued as a treatment group. Instead, a less acute water-load of volume 5% *Mb* for 7 days (Rice 1980) was used. Thus, *P. minor* has two water-load groups. The effect of each water-load is similar and no significant differences occur across any of the renal parameters studied.

When comparing the treatments between species some confusion may arise between hydrated and water-loaded groups. An aim of this study was to create a state of hydration in the absence of hypervolaemia and the reduction in P_{OSM} induced by water-loading. The rationale for this stems from a desire to produce a ‘control treatment’. Thus, injections of hypoosmotic sodium chloride, at a concentration of 150 mOsm.kg⁻¹, were given. Because the plasma osmolality of these animals is approximately 300 mOsm.kg⁻¹ under normal conditions, it was reasoned that a solution of half that strength would provide sufficient water and ions to rehydrate a lizard in a short period of time. In previous studies, desert iguanas (*Dipsosaurus dorsalis*) were rehydrated using hypoosmotic sodium at a concentration of 320 mOsm.kg⁻¹ (Dupré and Crawford 1985), and *Ctenophorus ornatus* were given injections of 10% *Mb* 300 mOsm.kg⁻¹ NaCl for 10 days in an examination of the role of the colon in modifying post-renal urine (Bradshaw 1986).

Two regimes were trialed in *C. nuchalis*, both consisting of injections of hypoosmotic (150 mOsm.kg⁻¹) NaCl at a dosage of 5% *Mb*, but with one
group receiving injections for 3 days and the other for 7 days. The effect of both groups was nearly identical (see post-hoc tables) despite apparently lower GFRs and FR_{H2O} in hydrated (3 days) lizards. Because of the groups’ similarity, the 3 day treatment was used as the hydrated control in interspecies comparisons.

Overall, water-loading and hydration had similar effects and there were rarely any statistically significant difference between the two groups. P. minor and C. nuchalis subjected to the chronic water-load adopted from Rice (1982) were able to maintain their plasma osmolality at ‘normal’ levels (Table 4.3.1), however, this treatment can cause the animals distress (see Discussion, Chapter 5) and a weak solution of NaCl is therefore a preferable treatment for hydration.

### 4.4.2 Plasma osmolality and U/P_{OSM}

Australian agamids comprise approximately 20% of the desert lizard fauna (Pianka 1986), yet lack a urinary bladder or cephalic salt-secreting glands (Saint Girons and Bradshaw 1987), features that in other reptiles aid in water conservation and the excretion of excess osmolytes (Nagy and Medica 1986). However, some of these lizards are capable of tolerating extremely high plasma osmolalities (hypernatraemia), up to 600 mOsm.kg\(^{-1}\) in some individual Ctenophorus ornatus (Bradshaw 1970), which can lead to anuria as the need to conserve fluid becomes paramount. Such extreme plasma osmolalities were not observed in this study, but dehydration and salt-loading were effective in raising plasma osmolality and producing a marked antidiuresis (relative to hydrated and water-loaded lizards) in each species, in accordance with the antidiuretic paradigm. As expected, P_{OSM} was significantly higher in salt-loaded groups than water-loaded groups (C. nuchalis, P. minor) or hydrated groups (C. nuchalis, C. salinarum). It was also greater than dehydrated groups in C. nuchalis
and *C. salinarum*. Because of increasing $P_{\text{OSM}}$, urine osmotic pressure increased in salt-loaded *P. minor* and *C. nuchalis* and as a further result, significant increases in $U/P_{\text{OSM}}$ were observed. Dehydrated *P. minor*, too, had significantly elevated $U/P_{\text{OSM}}$ compared to hydrated lizards. In *C. salinarum*, however, high variability meant that while $U/P_{\text{OSM}}$ increased in dehydrated and salt-loaded lizards relative to hydrated lizards, this was not statistically significant.

A meaningful examination of renal variables obtained in the laboratory needs to take into account data obtained in the field under natural conditions. Field values of plasma Na$^+$ and K$^+$ in *P. minor* give an estimated $P_{\text{OSM}}$ ranging between 273 and 300 (mOsm.kg$^{-1}$) for this species in Perth (Bradshaw 1970). This is equivalent to osmolyte levels in hydrated *P. minor* in this study, suggesting that the hydrated treatment was effective in simulating a state of hydration similar to that in the field in this species. In *C. nuchalis* this may not have been the case; wild-caught *C. nuchalis* sourced from the same locality as those in this study had $P_{\text{OSM}}$ ranging between 286 and 343 mOsm.kg$^{-1}$ with a mean of 309.5 mOsm.kg$^{-1}$ (Nagy and Bradshaw 1995), equivalent to the chronic water-loaded group, suggesting that the animals captured in the field were adequately hydrated. Similarly, Bradshaw (1997) gives field $P_{\text{OSM}}$ for *C. nuchalis* of 294 mOsm.kg$^{-1}$ (spring) and 315.6 mOsm.kg$^{-1}$ (autumn). These published values for *C. nuchalis* contrast with the value of 375 mOsm.kg$^{-1}$ observed in hydrated lizards, and suggest that the ‘hydrated’ *C. nuchalis* of this study may in fact have a higher $P_{\text{OSM}}$ than adequately hydrated lizards in the field. Unlike *P. minor*, therefore, the treatment used to hydrate the animals in the laboratory caused an elevation of plasma osmolality above levels normally experienced in the field. However, urine flow rate was markedly elevated in hydrated *C. nuchalis* relative to dehydrated and salt-loaded groups, suggesting that they were adequately hydrated.
Potential adaptive reasons for the retention of sodium in the plasma of this group are discussed later.

Like hydrated lizards, dehydrated and salt-loaded C. nuchalis may have relatively higher P\text{OSM} than those in the wild. Field records of sodium and potassium from C. nuchalis at Port Hedland give an estimated P\text{OSM} lower than that of the highest mean P\text{OSM} (392 mOsm.kg\(^{-1}\)) in this study (salt-loaded group). At this location, males tolerated P\text{OSM} of approximately 325 mOsm.kg\(^{-1}\) (Bradshaw 1970). The P\text{OSM} observed in dehydrated and salt-loaded lizards is therefore potentially higher than that reached by wild C. nuchalis. Estimates of P\text{OSM} based on sodium and potassium may be underestimates, however, because although a chloride concentration of 100 mOsm.kg\(^{-1}\) was factored in to the calculations, other osmolytes contributing to P\text{OSM} were not. It is likely that dehydrated lizards in this study, with a P\text{OSM} of approximately 340 mOsm.kg\(^{-1}\) that did not receive additional solute by injection, would be representative of field populations experiencing osmotic stress.

Finally, no field measurements of C. salinarum P\text{OSM} have been made to date, but a close relative of this species that also inhabits salt-lake margins, Ctenophorus maculosus, may serve as a comparison. Field P\text{OSM} of C. maculosus ranged between approximately 365 and 450 mOsm.kg\(^{-1}\) across a one-year period and control lizards had P\text{OSM} prior to experimentation of 379 mOsm.kg\(^{-1}\) (Braysher 1976). This range encompasses the values observed for C. salinarum, in which the highest P\text{OSM} was 407 mOsm.kg\(^{-1}\). These values are higher than those for C. nuchalis and P. minor reported in this study and the literature. Braysher’s control C. maculosus had a higher P\text{OSM} than dehydrated C. salinarum; this may be in part due to the nature of his controls, which were starved, presumably in the absence of water, for “several days” prior to experimentation; effectively the same regime as the
dehydrated group in the present study. In fact, Braysher (1976) observed no significant difference between experimental controls and salt-loaded lizards, suggesting that his controls were, in fact, significantly dehydrated.

In a similar study examining the renal function of the varanid lizard *V. gouldii*, U/POSM increased in dehydrated and salt-loaded groups as it did in this study (Bradshaw and Rice 1981). Plasma osmolalities of salt-loaded species in this study are somewhat higher than the 363 mOsm.kg\(^{-1}\) observed in *V. gouldii* subjected to an identical treatment (Bradshaw and Rice 1981) and similar to salt-loaded *Phrynosoma cornutum* which had a P\(_{\text{OSM}}\) of 401 mOsm.kg\(^{-1}\) when salt-loaded (Roberts and Schmidt-Nielsen 1966). In the desert iguana *Dipsosaurus dorsalis* P\(_{\text{OSM}}\) ranges from approximately 290 in hydrated individuals to more than 450 mOsm.kg\(^{-1}\) in salt-loaded individuals (Dupré and Crawford 1985) which encompasses the range of P\(_{\text{OSM}}\) observed here. The species in this study therefore fit well with the range of P\(_{\text{OSM}}\) observed in other lizards inhabiting arid environments.

### 4.4.3 Haematocrit

Blood haematocrit (Hct) serves as a crude indicator of hydration state. Surprisingly, however, there were no significant differences between treatment means in any species, despite apparently greater Hct in dehydrated and salt-loaded *P. minor*. Comparative data on haematocrit are difficult to obtain for desert reptiles, in which much work has focused on individual ions and the relationship between total body water (TBW) and intracellular and extracellular fluid volumes (ICFV and ECFV). Measurement of haematocrit may have been overlooked because it is a poor indicator of hydration state: haematocrit has been shown to vary widely within the desert tortoise *Gopherus agassizii* and was considered a poor indicator of physiological state by Peterson.
(2002). Additionally, wide variation in Hct (14 to 53%) was shown in a close relative of *P. minor*, *P. barbata*, although Hct was correlated with corticosterone levels (Amey and Whittier 2000) in this species which may have indicated osmotic stress.

### 4.4.4 V, GFR and FR$_{\text{H}_2\text{O}}$

As expected, each species showed the classical response to salt-loading and dehydration: a significant reduction in urine flow rate (V). This fits the paradigm describing renal function in squamates outlined in the introduction and supports the hypothesis that dehydration and salt-loading causes antidiuresis in lizards. The glomerular and tubular responses to dehydration and salt-loading varied between species, however.

In *P. minor*, V was reduced in both dehydrated and salt-loaded lizards to similar levels, at which urine flow was almost completely stopped (anuria). In dehydrated lizards, this was accomplished by a combined glomerular and tubular response: GFR was reduced from 12.8 to 3.0 mL.kg$^{-1}$.hr$^{-1}$, FR$_{\text{H}_2\text{O}}$ increased significantly from 59 to 85%, and CH$_2\text{O}$/C$_{\text{IN}}$ was markedly reduced (i.e. tubular permeability increased), resulting in a ten-fold reduction in V. The ΔGFR/ΔV index was 0.37 (Table 4.3.15), indicating that the response was more tubular in nature than glomerular. This contrasts with the lizards *Phrynosoma cornutum* (0.68), *Tropidurus spp.* (0.87), and *Gecko gecko* (0.77), all of which show a predominantly glomerular antidiuresis in response to dehydration (Yokota et al. 1985).

In salt-loaded *P. minor*, fractional reabsorption was fixed at the same level as water-loaded and hydrated treatments and the five-fold reduction in V was accomplished mainly by a glomerular response, with a ΔGFR/ΔV ratio of 0.93, although tubular permeability increased
as well. This highly glomerular response is similar to that observed in the pond-slider, *Pseudemys scripta*, in response to dehydration (Dantzer and Schmidt-Nielsen 1966), but is higher than all of the lizards mentioned in the previous paragraph. The range of $\Delta \text{GFR}/\Delta V$ between dehydrated and salt-loaded *P. minor* indicates that this species utilises very different mechanisms to accomplish antidiuresis following these treatments.

By contrast, in *C. nuchalis* and *C. salinarum*, dehydration and salt-loading both elicited a balanced glomerular vs. tubular response to reduce $V$ (Table 4.3.15). Unlike *P. minor*, their $\Delta \text{GFR}/\Delta V$ ratio did not vary greatly between treatments and in both species, the $\Delta \text{GFR}/\Delta V$ ratio was lower (more tubular) than that observed in the other lizards for which these data are available (Yokota et al. 1985). Mechanistically, the response of these species to dehydration and salt-loading differs from that of *P. minor*, although in all cases the responses resulted in an antidiuresis.

In *V. gouldii*, Bradshaw and Rice (1981) showed that dehydrated and salt-loaded individuals reduced $V$ by reducing GFR and increasing FR$_{H2O}$ and tubular permeability. GFR (15.9 mL.kg$^{-1}$.hr$^{-1}$), FR$_{H2O}$ (52.4%) and C$_{H2O}$/C$_{IN}$ (27.3%) in water-loaded *V. gouldii* (Bradshaw and Rice 1981) are comparable to hydrated values of the three lizards of this study. *V. gouldii* also utilised control of GFR, FR$_{H2O}$ and C$_{H2O}$/C$_{IN}$ in its antidiuretic response to dehydration and salt-loading (Bradshaw and Rice 1981), although based on the data of Bradshaw (1997), tubular mechanisms played a greater role (dehydrated $\Delta \text{GFR}/\Delta V$: 0.37; salt-loaded $\Delta \text{GFR}/\Delta V$: 0.35), similar to the slightly tubular-biased response of *C. nuchalis*. Additionally, in another agamid lizard, *Ctenophorus ornatus*, the antidiuresis produced by salt-loading was a result of both glomerular and tubular effects (Bradshaw 1997). The lack of a response of *P. minor* FR$_{H2O}$ to salt-loading therefore appears to be anomalous.
4.4.5 Osmolar and free water clearance

Associated with reductions in GFR and increases in FR$_{H2O}$ was a uniform reduction in rates of free water and osmolar clearance in response to dehydration and salt-loading in each species. Salt-loaded *P. minor*, *C. nuchalis* and *C. salinarum* all reduced C$_{OSM}$ to 35% of hydrated levels. This reduction was not as great as that seen in dehydrated lizards, however, particularly *P. minor*, which reduced C$_{OSM}$ to 17% of hydrated levels; dehydrated *C. nuchalis* and *C. salinarum* reduced C$_{OSM}$ to 25 and 33% of hydrated levels, respectively. However, in salt-loaded *P. minor*, the C$_{OSM}$/C$_{IN}$ was nearly three times greater than hydrated, resulting from a significantly reduced GFR in the absence of an increase in FR$_{H2O}$. This response contrasts greatly with that of dehydrated *P. minor*, which had a C$_{OSM}$/C$_{IN}$ amounting to 75% of hydrated (not statistically different), and relates to the very different response of *P. minor* to dehydration and salt-loading as discussed previously.

Relative free water clearance was reduced in all species in response to dehydration and salt-loading, indicating an increase in the tubular permeability to water. Dehydration resulted in the greatest reduction of C$_{H2O}$/C$_{IN}$ in *P. minor* to 11% of hydrated compared with salt-loaded lizards in which C$_{H2O}$/C$_{IN}$ was reduced to 22% of hydrated. In *C. nuchalis* the pattern was reversed: salt-loaded lizards had a greater reduction in C$_{H2O}$/C$_{IN}$ than dehydrated. The large 89% reduction of C$_{H2O}$/C$_{IN}$ in dehydrated *P. minor* is typical of the increase in tubular permeability in conjunction with a markedly increased FR$_{H2O}$, which results in an overall reduction in free water loss in the face of an increase in free water production (as FR$_{H2O}$ is increased).

*V. gouldii* is one of few lizard species for which osmolar and free water clearances have been estimated under dehydrated and salt-loaded
conditions. In the previous section, it was noted that in this lizard, GFR, FR_{H2O} and C_{H2O}/C_{IN} were adjusted in a similar manner to those variables in the lizards presented here. This is also true of osmolar and free water clearances: V. gouldii C_{H2O} and C_{OSM} are reduced in dehydrated and salt-loaded lizards, and C_{H2O} is reduced more than C_{OSM}. In V. gouldii C_{OSM}/C_{IN} is lowest in dehydrated lizards and is reduced to 59% of hydrated levels (Bradshaw and Rice 1981), similar to the percentage reductions in C_{OSM}/C_{IN} in each species presented here.

Unlike P. minor and like both Ctenophorus, salt-loading in V. gouldii also reduced C_{OSM}/C_{IN}. Relative free water clearance was markedly reduced in dehydrated and salt-loaded V. gouldii to 21 and 6% of hydrated levels (Bradshaw and Rice 1981), greater reductions than those observed in the species in this study except perhaps for P. minor, with figures of 22 and 11% respectively, as outlined above.

In summary, each species followed the antidiuretic paradigm as expected, reducing urine flow rate in response to dehydration. This was accomplished by varying GFR, FR_{H2O} and C_{H2O}/C_{IN}. P. minor reduced GFR and V to a greater extent than either C. nuchalis or C. salinarum, as predicted, suggesting that it is more sensitive to water deprivation and salt-loading than either Ctenophorus. Additionally, P. minor could utilise either a predominantly tubular response, in the case of dehydration, or glomerular response, in the case of salt-loading. Additionally, the natriuresis observed in salt-loaded P. minor appears to be somewhat anomalous when compared with C. nuchalis, C. salinarum and also the goanna, V. gouldii, and a question remains: why does this natriuresis occur?
4.4.6 The role of the cloaca-colon complex

As previously noted, these lizards lack a urinary bladder and cephalic salt-secreting glands, thus, the kidneys and the cloaca-colon complex alone are responsible for osmoregulation. To date, no reptile has been found to be capable of producing hyperosmotic ureteral urine. Braysher (1976) reported that *Ctenophorus maculosus* did excrete hyperosmotic urine, but only after significant post-renal modification in the colon; ureteral urine was hypoosmotic to the plasma at all times.

The role of the cloaca-colon complex was not investigated here, but is likely to be similar to that of *C. maculosus* (Braysher 1976), *C. ornatus* (Bradshaw 1986) and *V. gouldii* (Bradshaw and Rice 1981) in aiding homeostasis by reclaiming water from the ureteral urine. In birds and reptiles, cations such as sodium and potassium become sequestered into the growing uric acid mass as the insoluble uric acid is progressively introduced to the colon from ureteral urine (McNabb 1974; Dantzler 1982b). A similar process probably occurs in lizards. These ions then no longer contribute to the osmotic potential of the colonic fluid, creating free water which can be absorbed passively along an osmotic gradient (Murrish and Schmidt-Nielsen 1970). The alternate mechanism for reclamation is by solute-linked water flow, as has been observed in the cloaca of crocodile (Schmidt-Nielsen and Skadhauge 1967), and the colon of the lizard *Agama stellio* (Skadhauge and Duvdevani 1977). As a result of these processes, very little fluid is voided (Bradshaw 1989). Thus, even though *P. minor* increases the relative clearance of osmolytes from the kidney when salt-loaded, the urine may be stored in the colon for post-processing and the ‘lost’ fluid reclaimed. This may be a better strategy than reabsorbing excess osmolytes into the blood stream and enduring hypernatraemia, as *C. nuchalis* and *C. salinarum* did when salt-loaded.
This pattern of tolerating hypernatraemia is also seen in *C. ornatus* in the field, and in that species significant hypernatraemia allows for rapid rehydration when rains fall, accompanied by the excretion of a copious sodium-rich urine (Bradshaw 1970). Such salt-retention may therefore be a strategy for survival in arid environments, and would also generate a greater osmotic gradient between the blood plasma and colonic fluid, used to reclaim free water from the colon. In *P. minor*, however, there appears to be some threshold level of plasma osmolality, between 330 and 380, at which FR$_{H2O}$ falls to basal levels and sodium excretion takes place, albeit in a reduced volume of urine, in order to reduce P$_{OSM}$. While this may be interpreted as an intolerance to salt-loads, this may in fact be an effective strategy, using post-renal mechanisms to excrete excess salt without a great loss of fluid. In a sense, therefore, *P. minor* is equally capable of dealing with a salt-load — it simply uses an alternate strategy. Thus, the contributions of the colonic system to osmoregulation appear to vary between *Pogona* and *Ctenophorus*, which use different combinations of kidney and colon to cope with salt-loading.
CHAPTER 5: THE ROLE OF ARGinine Vasotocin
5.1 INTRODUCTION

Animals attempt to maintain the gradients between their body fluids and their environment through a complex pattern of physiological events, the functions of which are integrated largely by hormones (Bentley 1998). In reptiles, the antidiuretic hormone arginine vasotocin (AVT) is of critical importance in controlling and modulating osmoregulation (Bradshaw 2003) and is widely believed to be the principal hormone responsible for regulating rates of filtration and reclamation of body water in response to changes in hydration state (McKinley 1985; Dantzler 1989a; Bradshaw 1997; Bradshaw and Bradshaw 2002). AVT is released from the pars nervosa in response to increases in plasma osmolality (Rice 1980), and causes a reduction in urine flow rate (antidiuresis) in several reptile groups (Bentley 1998).

AVT causes an antidiuresis primarily through a reduction of the glomerular filtration rate (Jard and Morel 1963; Dantzler and Schmidt-Nielsen 1966; Dantzler 1967; Butler 1972; Green 1972; Bradshaw and Rice 1981), by regulating the number of glomeruli filtering and the filtration rate of filtering nephrons (Dantzler 1989a). Relative free-water clearance may be reduced at the same time, suggesting that AVT results in an increase in nephron permeability in some groups (Bradshaw and Rice 1981; Bradshaw 1997). This is not always the case, however; Stolte et al. (1977) reported that AVT reduced GFR but had no effect on relative free water clearance in Sceloporus cyanogenys. It also increased relative osmolar clearance in the freshwater turtle, Chrysemys picta (Butler 1972). In Ctenophorus ornatus, however, relative osmolar and free water-clearances were reduced by the action of AVT (Bradshaw 1975, 1976, 1978b), and this would appear to be the more common effect of the hormone in terrestrial reptiles (Bradshaw 1997).
Circulating levels of AVT have been measured for five species of reptile to date, only one of which, *Varanus gouldii*, has been the subject of renal studies (Bradshaw and Rice 1981; Rice 1982). The others include the green sea turtle *Chelonia mydas* (Figler et al. 1989), the skink *Tiliqua rugosa* (Fergusson and Bradshaw 1991), and two snakes, the viper *Bothrops jararaca* (Silveira et al. 1992) and the tiger snake *Notechis scutatus* (Ladyman 2004). In the previous chapter, the effect of osmotic challenge on the renal physiology of three species of closely related Western Australian agamid lizards was examined. The aim of the present chapter is to complement this work by examining circulating levels of arginine vasotocin (P_{AVT}) following osmotic challenge in each of the three species, with the aim of determining the effect of AVT on aspects of each lizard’s renal physiology.

While the three species are closely related, their environments are different. This provides an opportunity to investigate whether circulating levels of AVT are related to renal function, whether the species differ in their endocrine response to osmotic challenge, and whether any differences observed may be interpreted as adaptations to their respective environments. Based on the results of previous studies, particularly that of Bradshaw and Rice (1981), I anticipate that circulating levels of AVT will correlate with glomerular filtration rate, fractional reabsorption of filtrate, relative free water clearance and, consequently, with urine flow rate. Furthermore, the more mesic species, *P. minor*, is expected to display a greater sensitivity to osmotic challenge than arid-inhabiting *C. nuchalis*, which should be reflected in a greater increase of circulating AVT in dehydrated and salt-loaded groups (relative to hydrated levels) than *C. nuchalis*. 
5.2 MATERIALS AND METHODS

5.2.1 Lizards

Circulating levels of arginine vasotocin (AVT) were assayed in the plasma taken from those lizards involved in the experiments of the previous chapter. The amount of plasma collected from individual C. salinarum was not sufficient for AVT analysis. At the conclusion of the urine collection period, a blood sample was taken for determination of [³H]inulin concentration, plasma osmolytes and plasma AVT concentration.

5.2.2 Radioimmunoassay of arginine vasotocin

5.2.2.1 Iodination

The AVT assay used in this chapter is similar to that developed by Rice (1980; 1982). 8-arginine vasotocin (AusPep.) was iodinated using the chlorammine-T oxidation method (Hunter and Greenwood 1962) adapted for AVT (Rice 1980; 1982). The following were added to the reaction vial: 2 μg AVT in 20 μL 0.2 M acetic acid, 20 μL of 0.5 M phosphate buffer (50 mL deionised distilled water containing 3.065 g Na₂HPO₄, 0.53 g NaH₂PO₄, 0.1 g NaN₃, adjusted to pH 7.5), 5 μL containing 18.5 MBq ¹²⁵I (Amersham IMS-30), and 5 μL of 0.5 mg.1⁻¹ chloramine-T made up in 0.5 M phosphate buffer. The contents were mixed continuously by pipetting and the reaction stopped 15 s after the addition of chloramine-T by the addition of 100 μL 25% bovine serum albumin (BSA, Sigma Aldrich). After mixing, the reaction mixture was transferred to a vial containing 150 mg Dowex-2-2x8-50 in 1 mL water and mixed continuously for 10 min to remove unreacted
free iodine before being transferred to a 30 cm Sephadex G25 (fine) (Pharmacia Biotech) column equilibrated in 0.1% BSA in 0.2 M acetic acid and primed with 0.5 mL normal rabbit serum (NRS, Strategic Biosolutions). The reaction mixture was eluted with 0.2 M acetic acid containing 0.1% BSA. Fractions of approximately 1 mL were collected and 10 µL aliquots counted on a Prias Autogamma Counter (Packard) or Cobra II Autogamma (Packard). The three post-peak fractions of iodinated AVT were pooled and rechromatographed in the same manner on a second column. One mL fractions were again collected and the first and second post-peak fractions pooled and stored at -20 °C as 50 µL aliquots.

5.2.2.2  Assay standards

AVT (AusPep) dissolved in 0.2 M acetic acid and 0.1% BSA was used for the standards in the assay. This was stored as 1 mL aliquots of 640 pg.mL⁻¹ AVT at -20 °C. The concentrations used for standard curves were 0.25, 0.5, 1, 2, 4, 8, 16, and 32 pg.mL⁻¹. Standards were prepared freshly for each assay.

5.2.2.3  Extraction procedure

Plasma volumes ranging between 50 and 100 µL were thawed after being frozen and stored at -20 °C immediately after each experiment. The extraction procedure is based on the adsorption of plasma proteins on octadecasilyl-silica and subsequent elution with acetonitrile. SEP-PAK (Waters division of Millipore, MA) cartridges were pre-wet with 5 mL 100% methanol before rinsing with 10 mL distilled deionised water (ddH₂O). Samples were acidified with 1.0 M HCl amounting to one-tenth of their volume before being loaded onto the cartridge. Because of the small volume of plasma, care was taken loading each
sample. The sample was allowed to feed slowly into the matrix initially, followed by addition of a small volume of 4% acetic acid, which was also allowed to run in. The sample was washed with 15 mL of 4% acetic acid, and eluted into a glass tube using 6 mL of 75% acetonitrile (AR grade) in 4% acetic acid. Between samples, cartridges were washed using 5 mL of 8 M urea, followed by 10 mL distilled deionised H₂O before the methanol rinse. Cartridges were used a maximum of four times and at no point did the rate of fluid flow through the cartridge exceed 1 mL per second (typical flow rates were 0.3–0.5 mL sec⁻¹). Tubes containing the extracted samples were air dried in a water-bath at 37 ºC for between 4 and 6 hours. Once each tube had dried down, extracts were reconstituted in 450 or 500 µL of 0.1 M phosphate buffer (1 L of deionised distilled water containing 1.226 g Na₂HPO₄, 0.195 g KH₂PO₄, 8.775 g NaCl, and 1 g NaN₃, adjusted to pH 7.5) and vortexed for one minute. Reconstituted extracts were either processed or stored at 4 ºC for assay the next day. The recovery of AVT was not measured but previous use of this protocol has demonstrated a consistent post-extraction recovery of 85% (Fergusson and Bradshaw 1991).

5.2.2.4 Assay protocol

Plasma AVT concentrations were measured using a preincubation, double-antibody assay system (Rice 1980). A rabbit antibody raised against 8-arginine vasotocin (Strategic Biosolutions) was used at a final antibody dilution of either 1:5000 or 1:7500. 50 µL aliquots of each standard and 200 µL of each sample were incubated in duplicate along with total radioactivity (TR) and non-specific binding tubes (NSB) at 4 ºC in plastic 3DT tubes. Added to each sample tube were ten µL of 0.1 M phosphate buffer containing 0.07% rabbit gamma globulin, 50 µL of AVT antibody, 200 µL of extract and a phosphate buffer (0.01 M, pH
7.5) to a final volume of 450 µL. 50 µL (42-50 Bq) of monoiodinated AVT were added 24 hr later. Tubes were vortexed gently for 3 sec and incubated at 4 °C for a further 72 hr. Free and bound hormone were separated by adding 200 µL of a second antibody, donkey anti-rabbit serum (DARS) diluted to 1:24 in 0.1 M phosphate buffer. After vortexing gently, tubes were incubated at 4 °C for a final 24 hr before being centrifuged at 4500 RPM (2000 × g, Beckman Centrifuge). The supernatant was aspirated and the tubes counted on a Prias Autogamma counter (Packard) or a Cobra II Autogamma (Packard).

5.2.2.5 Assay error estimation

Duplicate 100 µL aliquots of plasma from two agamid lizards were included in each analysis to estimate the inter-assay variation: the first unknown had a mean concentration of 138.4 ± 18.3 pgAVT.mL⁻¹ (13.2% variation) and the second had a mean concentration of 60.3 ± 4.3 pgAVT.mL⁻¹ (7.1% variation). Intra-assay variation was assessed as the coefficient of variation of duplicate samples (Chard 1995) and was 4.6%. Because 100 µL was the standard sample volume, parallelism of the assay was not examined.

5.2.3 Statistical analyses

An α=0.05 was used as the level of significance. Statistical packages used were StatSoft STATISTICA (v. 4.1), SAS Institute Inc. JMP (v. 3.2.1) and GraphPad Prism (v. 3.0a), all for Macintosh. ANOVA was used to compare levels of AVT between treatments, and the data were log-transformed where appropriate. Linear regression analysis was used to examine correlations between circulating AVT levels and physiological variables (e.g. plasma osmolality).
5.3 RESULTS

5.3.1 Plasma AVT varied in response to osmotic challenge

The effects of each treatment group on circulating AVT (P_{AVT}) for *P. minor* and *C. nuchalis* are presented in Figures 5.3.1 and 5.3.2. There was a significant difference between treatment groups in *P. minor* ($F_{4,17}=3.77$, $P=0.023$), largely because of the higher $P_{AVT}$ in dehydrated lizards. Post-hoc tests revealed no significant differences between plasma AVT concentration in acute water-load (15.7±3.6 pg.mL$^{-1}$), chronic water-load (23.5±4.9 pg.mL$^{-1}$), hydrated (20.4±6.4 pg.mL$^{-1}$) or salt-loaded (31.0±6.7 pg.mL$^{-1}$) treatments; however, the acute water-load treatment had significantly reduced $P_{AVT}$ compared to dehydrated lizards (48.8±2.4 pg.mL$^{-1}$) (THSD, $P=0.026$) (Fig. 5.3.1).

Plasma AVT data for *P. minor* were not log-transformed because the variance did not increase with the mean and the data conformed to a normal distribution (Komogorov-Smirnov test, $d=0.101$, $P>0.05$). This was not the case for *C. nuchalis*; variance increased with the mean and the data were therefore log-transformed before analysis.

$P_{AVT}$ of *C. nuchalis* differed significantly between treatments ($F_{4,21}=4.204$, $P=0.012$). Post-hoc tests revealed that chronic water-load (35.1±8.7 pg.mL$^{-1}$), dehydrated (29.5±9.5 pg.mL$^{-1}$) and salt-loaded (48.8±14.8 pg.mL$^{-1}$) lizards had significantly higher $P_{AVT}$ than hydrated (9.9±5.9 pg.mL$^{-1}$) lizards (THSD, water-loaded: $P=0.040$; dehydrated: $P=0.041$; and salt-loaded: $P=0.020$). The $P_{AVT}$ of the chronic hydration group (15.0±8.0 pg.mL$^{-1}$) did not differ significantly from any other (Figure 5.3.2).
Fig. 5.3.1: Circulating AVT concentrations differed between *P. minor* treatment groups; ($F_{4,17}=3.77$, $P=0.023$). Significant post-hoc differences are denoted by paired alphabetic characters (THSD for unequal $N$).

Fig. 5.3.2: Circulating AVT concentrations differed between *C. nuchalis* treatment groups ($F_{4,21}=4.204$, $P=0.012$). Significant post-hoc differences are denoted by paired alphabetic characters (THSD for unequal $N$).
5.3.2 Correlation of circulating AVT with plasma osmolality

In *P. minor* alone, $P_{AVT}$ was loosely correlated with $P_{OSM}$ across treatment groups ($F_{1,17}=6.56; P=0.020$), with an $r^2=0.278$ ($S_{y,x}=13.2$) (Fig. 5.3.3). The equation relating $P_{OSM}$ to $P_{AVT}$ is:

$$P_{AVT} = 0.169 \pm 0.169 P_{OSM} - 27.53 \pm 21.34$$

Individual treatments showed no significant correlations with plasma osmolality. Unlike *P. minor*, AVT was not correlated with $P_{OSM}$ across treatment groups in *C. nuchalis* ($F_{1,23}=0.169, P=0.685$) (Fig. 5.3.4). Additionally, there were no significant correlations of AVT regressed on $P_{OSM}$ in individual treatment groups.

5.3.3 Correlation with renal physiological variables

A summary of the results of regressions of each physiological variable on circulating AVT is presented in Table 5.3.1. Urine flow rate was reduced significantly in both *P. minor* and *C. nuchalis* exhibiting elevated $P_{AVT}$ (*P. minor*: $F_{1,20}=6.20, P=0.022$; *C. nuchalis*: $F_{1,24}=5.69, P=0.025$) (Figs. 5.3.5 & 5.3.6).

GFR was not reduced in *P. minor* with higher $P_{AVT}$ ($F_{1,20}=2.46, P=0.132$) (Fig. 5.3.7) but was in *C. nuchalis* ($F_{1,24}=4.66, P=0.041$) (Fig. 5.3.8). Thus, increased levels of $P_{AVT}$ were correlated with a reduction in GFR in *C. nuchalis* but not *P. minor*.

$FR_{H2O}$ in *P. minor* showed a strong positive correlation with circulating levels of AVT ($F_{1,20}=22.9, P<0.000$) (Fig. 5.3.9), however, this relationship was not evident for *C. nuchalis* ($F_{1,24}=3.59, P=0.070$) (Fig. 5.3.10).
Fig. 5.3.3: Circulating levels of AVT in *P. minor*, regressed on plasma osmolality
\( F_{1,17}=6.56; \ P=0.020 \)

Fig. 5.3.4: Circulating levels of AVT in *C. nuchalis*, regressed on plasma osmolality.
\( F_{1,23}=0.169, \ P=0.685 \)
Table 5.3.1: Slope, intercept and significance of the regression of renal physiological variables against \( P_{AVT} \) are presented. Significant \( P \)-values are in bold.

<table>
<thead>
<tr>
<th>Variable</th>
<th>( P. ) minor</th>
<th></th>
<th></th>
<th></th>
<th>( C. ) nuchalis</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Slope</td>
<td>Intercept</td>
<td>( P )</td>
<td>Slope</td>
<td>Intercept</td>
<td>( P )</td>
<td>Slope</td>
<td>Intercept</td>
</tr>
<tr>
<td>( \log V )</td>
<td>(-0.0249)</td>
<td>1.072</td>
<td>0.022</td>
<td>( -0.0074 )</td>
<td>0.902</td>
<td>( 0.025 )</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>( \pm 0.010 )</td>
<td>( \pm 0.303 )</td>
<td></td>
<td>( \pm 0.003 )</td>
<td>( \pm 0.132 )</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \log GFR )</td>
<td>(-0.0149)</td>
<td>1.263</td>
<td>0.132</td>
<td>( -0.0046 )</td>
<td>1.292</td>
<td>( 0.041 )</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>( \pm 0.009 )</td>
<td>( \pm 0.287 )</td>
<td></td>
<td>( \pm 0.002 )</td>
<td>( \pm 0.091 )</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \text{asin} ) FR(_{H2O} )</td>
<td>0.0098</td>
<td>0.411</td>
<td>(&lt;0.000)</td>
<td>0.0029</td>
<td>0.595</td>
<td>( 0.070 )</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>( \pm 0.002 )</td>
<td>( \pm 0.062 )</td>
<td></td>
<td>( \pm 0.002 )</td>
<td>( \pm 0.065 )</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \text{asin}(U/P_{OSM}) )</td>
<td>0.0090</td>
<td>0.247</td>
<td>0.070</td>
<td>0.0017</td>
<td>0.404</td>
<td>( 0.137 )</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>( \pm 0.005 )</td>
<td>( \pm 0.141 )</td>
<td></td>
<td>( \pm 0.001 )</td>
<td>( \pm 0.049 )</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \log C_{H2O} )</td>
<td>(-0.0397)</td>
<td>1.261</td>
<td>0.006</td>
<td>( -0.0092 )</td>
<td>0.696</td>
<td>( 0.021 )</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>( \pm 0.012 )</td>
<td>( \pm 0.364 )</td>
<td></td>
<td>( \pm 0.0037 )</td>
<td>( \pm 0.161 )</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \log C_{OSM} )</td>
<td>(-0.0182)</td>
<td>0.419</td>
<td>0.045</td>
<td>( -0.0056 )</td>
<td>0.481</td>
<td>(&lt;0.05 )</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>( \pm 0.008 )</td>
<td>( \pm 0.256 )</td>
<td></td>
<td>( \pm 0.003 )</td>
<td>( \pm 0.117 )</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \text{asin}(C_{H2O}/C_{IN}) )</td>
<td>(-0.0059)</td>
<td>0.395</td>
<td>0.025</td>
<td>( -0.0020 )</td>
<td>0.298</td>
<td>( 0.041 )</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>( \pm 0.002 )</td>
<td>( \pm 0.073 )</td>
<td></td>
<td>( \pm 0.001 )</td>
<td>( \pm 0.040 )</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \text{asin}(C_{OSM}/C_{IN}) )</td>
<td>(-3.72 \times 10^{-5})</td>
<td>0.168</td>
<td>0.984</td>
<td>(-3.87 \times 10^{-4})</td>
<td>0.170</td>
<td>( 0.486 )</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Neither species' U/POSM varied significantly with \( P_{AVT} \) (\( P. \) minor: \( F_{1,17}=3.75, P=0.070; \) C. nuchalis: \( F_{1,23}=2.37, P=0.137 \)), as shown in Figs. 5.3.11 and 5.3.12.

Free water clearance (\( C_{H2O} \)) was inversely correlated with an increase in \( P_{AVT} \) in both species (\( P. \) minor: \( F_{1,15}=10.2, P=0.006; \) C. nuchalis: \( F_{1,23}=6.14, P=0.021 \)), shown in Figs. 5.3.13 and 5.3.14. Similarly to this, \( C_{OSM} \) decreased with elevated \( P_{AVT} \) in both species (\( P. \) minor: \( F_{1,17}=4.69, P=0.045; \) C. nuchalis: \( F_{1,23}=4.29, P<0.050 \)) (Figs. 5.3.15 and 5.3.16).

Relative free water clearance (\( C_{H2O}/C_{IN} \)) was lower in \( P. \) minor with elevated \( P_{AVT} \) (\( F_{1,17}=6.00, P=0.025 \)) (Fig. 5.3.17), and the same was true for C. nuchalis (\( F_{1,23}=4.72, P=0.041 \)) (Fig. 5.3.18).

Finally, in both \( P. \) minor and C. nuchalis, \( C_{OSM}/C_{IN} \) was the variable that responded least to \( P_{AVT} \), and appeared to be constant over the range of \( P_{AVT} \) in both species (\( P. \) minor: \( F_{1,17}=0.00, P=0.984; \) C. nuchalis: \( F_{1,23}=0.50, P=0.486 \)), yielding \( r^2=0.000 \) and 0.021, respectively (Table 5.3.1).
Fig. 5.3.5: *P. minor*: regression of log-transformed $V$ on circulating AVT. ($F_{1,20}=6.20, P=0.022$)

Fig. 5.3.6: *C. nuchalis*: regression of log-transformed $V$ on circulating AVT. ($F_{1,24}=5.69, P=0.025$)
Fig. 5.3.7: *P. minor*: regression of log-transformed GFR on circulating AVT. 
\( F_{1,20}=2.46, \ P=0.132 \)

Fig. 5.3.8: *C. nuchalis*: regression of log-transformed GFR on circulating AVT. 
\( F_{1,24}=4.66, \ P=0.041 \)
Fig. 5.3.9: *P. minor*: regression of arcsine-transformed FR$_{H2O}$ on circulating AVT. ($F_{1,20}=22.9$, $P<0.000$)

Fig. 5.3.10: *C. nuchalis*: regression of arcsine-transformed FR$_{H2O}$ on circulating AVT. ($F_{1,24}=3.59$, $P=0.070$)
Fig. 5.3.11: *P. minor*: regression of arcsine-transformed U/P_{OSM} on P_{AVT}. ($F_{1,17}=3.75, P=0.070$)

Fig. 5.3.12: *C. nuchalis*: regression of arcsine-transformed U/P_{OSM} on P_{AVT}. ($F_{1,23}=2.37, P=0.137$)
Fig. 5.3.13: *P. minor*: regression of log-transformed $\text{C}_{\text{H}_2\text{O}}$ on $P_{\text{AVT}}$.
($F_{1,15}=10.2, \ P=0.006$)

Fig. 5.3.14: *C. nuchalis*: regression of log-transformed $\text{C}_{\text{H}_2\text{O}}$ on $P_{\text{AVT}}$.
($F_{1,23}=6.14, \ P=0.021$)
Fig. 5.3.15: *P. minor*: regression of log-transformed $C_{\text{OSM}}$ on $P_{\text{AVT}}$. ($F_{1,17}=4.69$, $P=0.045$)

Fig. 5.3.16: *C. nuchalis*: regression of log-transformed $C_{\text{OSM}}$ on $P_{\text{AVT}}$. ($F_{1,23}=4.29$, $P<0.050$)
Fig. 5.3.17: *P. minor*: regression of arcsine-transformed \( \frac{C_{H2O}}{C_{IN}} \) on \( P_{AVT} \).
\( (F_{1,17}=6.00, P=0.025) \)

Fig. 5.3.18: *C. nuchalis*: regression of arcsine-transformed \( \frac{C_{H2O}}{C_{IN}} \) on \( P_{AVT} \).
\( (F_{1,23}=4.72, P=0.041) \)
5.4 DISCUSSION

5.4.1 Circulating AVT in response to osmotic challenge

Circulating AVT levels differed between treatments in both *P. minor* and *C. nuchalis* as a result of osmotic challenge. In *P. minor*, dehydrated lizards had a significantly higher *P<sub>AVT</sub>* than lizards given an acute water-load while in *C. nuchalis*, dehydrated, salt-loaded and chronically water-loaded lizards had a higher *P<sub>AVT</sub>* than the hydrated group. Only one other lizard has been studied in a similar manner: the sand goanna *Varanus gouldii* (Rice 1982). In this lizard *P<sub>AVT</sub>* was highest in salt-loaded lizards, followed by dehydrated lizards and hydrated lizards (Rice 1982). This is similar to the observations of *C. nuchalis* except that the plasma AVT of salt-loaded *C. nuchalis* was not significantly greater than dehydrated.

The observation of a high *P<sub>AVT</sub>* for water-loaded *C. nuchalis* (Fig. 5.3.2) was unexpected, because following chronic injections with water the lizards should have been adequately hydrated, resulting in basal *P<sub>AVT</sub>* levels, as AVT is released in response to an increase in plasma osmolality (see Introduction). The results of the renal physiological study of Chapter 4 support the hypothesis that this group was, in fact, adequately hydrated, because it had the lowest *P<sub>OSM</sub>*, *U/P<sub>OSM</sub>* and FR<sub>H2O</sub> and highest V and GFR (Table 4.3.1) – features consistent with a water-load and an overall state of hydration.

One possible explanation for the unexpectedly high *P<sub>AVT</sub>* in water-loaded *C. nuchalis* is that AVT may act on the cloaca-colon complex of *C. nuchalis* to increase reabsorption of sodium, i.e. a natriferic effect. In *V. gouldii* seemingly conflicting data regarding the effect of AVT on the colon have been presented. A pharmacological dose of AVT (100
ng.kg\(^{-1}\)) enhanced sodium and water reabsorption from the cloaca (Braysher and Green 1970), yet an increase in circulating AVT was correlated with a decrease in the reabsorption of sodium from the colon of saline-loaded goannas (Bradshaw and Rice 1981). At the same time, aldosterone concentrations and the concentration of the colonic reabsorbate decreased with salt-loading by more than 50% (Bradshaw and Rice 1981).

Aldosterone has a natriferic effect in V. gouldii (Bradshaw 1997) and the reduction in reabsorption of sodium with saline loading may be linked to a reduction of aldosterone. However, in dehydrated lizards, high aldosterone levels are maintained and one would expect greater reabsorption of sodium as a result, but this was not the case (Bradshaw 1981). Thus, aldosterone alone cannot account for the reduced reabsorption of sodium under dehydrated and salt-loaded conditions, and equally unclear is the role of AVT in the colonic function of this lizard. It is therefore unlikely that the increase in P\(_{AVT}\) observed in water-loaded C. nuchalis was for post-renal reclamation of salt from the dilute urine presented to the colon; this is supported by the observation that \textit{in vitro} preparations of \textit{Ctenophorus ornatus} colon, while showing transmural transport of sodium, did not respond to administered AVT (Bentley and Bradshaw 1972).

A more likely cause is that the elevated P\(_{AVT}\) of water-loaded C. nuchalis was due to stress. The pH of the deionised distilled water used in the treatments was low (5.5) and stress responses such as stilting and biting of the abdomen were observed in some water-loaded individuals; similar observations were made in acutely water-loaded \textit{P. minor}, and that treatment was therefore abandoned. Additionally, some individuals displayed more acute fright/flight responses (increased fear/aggression) as treatment progressed suggesting that levels of the adrenal stress hormones epinephrine and corticosterone
may have increased. The potential effect of these hormones on AVT is unknown, although in amphibians, catecholamines (such as epinephrine) can reduce GFR and block the effects of neurohypophysial hormones (Bentley 1998). In birds, heat stress was shown to increase plasma AVT (Wang et al. 1989), while in the rainbow trout plasma levels of AVT in physically disturbed lizards exceeded those of lizards that were osmotically stressed (Kulczykowska 2001). These studies indicate that circulating AVT in the lizard C. nuchalis may increase in response to a variety of stressors, and provide a potential explanation for the otherwise anomalous observation presented here.

Dehydrated P. minor had higher $P_{AVT}$ than hydrated individuals. Salt-loaded lizards did not differ significantly from any group, however, mostly likely because of the high variation in $P_{AVT}$ among individuals of this group. Particularly interesting was the higher $P_{AVT}$ observed in dehydrated lizards, which matches the significantly greater $FR_{H2O}$ observed in this group relative to the others (Chapter 4); this and the close relationship between circulating AVT and $FR_{H2O}$ in this species (see below) suggest that not only is the nephron of P. minor sensitive to circulating AVT, but $FR_{H2O}$ may be closely linked to $P_{AVT}$ in this species. Whereas salt-loaded C. nuchalis had significantly greater $P_{AVT}$ relative to hydrated, P. minor did not show this trend. Why did salt-loading not induce an elevation in $P_{AVT}$ in P. minor?

As hypothesised, P. minor is a more mesic species than C. nuchalis, a true arid species. Between periods of rainfall, wild P. minor would encounter periods of dehydration. As shown in Chapter 4, dehydration resulted in significant reclamation of osmolytes from the nephron, while salt-loading resulted in excretion of excess solutes. It was suggested that there may be a threshold level of plasma osmolality above which P. minor ceases to reabsorb osmolytes above basal levels,
instead relying on post-renal modification of the urine and reabsorption of solutes from the colon. A hydrated lizard, presented with the immediate burden of a high salt-load following the initial injection during treatment may respond by allowing excess sodium to pass from the kidneys into the colon. It has not been subjected to the slow process of dehydration and can afford, physiologically speaking, to allow the excess sodium to flow into the colon. As we know, the fluid excreted is not lost as it can be reclaimed later by solute-linked water flow (Schmidt-Nielsen and Skadhauge 1967; Bradshaw and Rice 1981).

In contrast, a dehydrated lizard experiences a slow increase in plasma osmolality as it loses water through evapotranspiration, a chronic treatment during which $P_{\text{AVT}}$ may rise under natural control. Salt-loading is an acute treatment that demands an urgent physiological response. In this case, this immediate response seems to be primarily glomerular, with a $\Delta \text{GFR}/\Delta V = 0.93$ (Chapter 4). In this sense, C. nuchalis appears to possess an innate tolerance to the salt-load, as evidenced by the more balance response ($\Delta \text{GFR}/\Delta V = 0.44$) and significant increase in $P_{\text{AVT}}$. As discussed in Chapter 4, which strategy is more effective for dealing with the salt load is difficult to determine without an analysis of the contribution of each species’ colon to osmoregulation. Such a study, employing methods similar to those of Bradshaw (1986) in C. ornatus, would help to answer this question.

$P. \text{minor}$ and C. nuchalis may use different strategies for dealing with salt load, but this does not explain the lack of response of circulating AVT to salt-loading in $P. \text{minor}$. The duration over which salt-loading occurs (7 days) should be sufficient for $P_{\text{AVT}}$ levels to increase, as they did in C. nuchalis (Fig. 5.3.2) and V. gouldii (Rice 1982). GFR was significantly reduced in salt-loaded lizards relative to hydrated, and the tubular permeability increased. These responses are thought to be
the effects of AVT acting through V₁ and V₂ type receptors (Cowley 2000; Bradshaw and Bradshaw 2002), but in the absence of a significant increase in the hormone, it may be that these responses are a results of the action of another, complementary hormone. In Chapter 3 the close association between the glomeruli and distal tubules of the nephron was noted, suggesting that a functional tubuloglomerular feedback system exists and it may be this system, presumably through the action of nitric oxide as in mammals (Blantz et al. 2002; Wilcox 2003), that responds quickest to the salt-load in P. minor. The reason for the lack of an AVT response over the longer term, however, remains a mystery.

5.4.2 Correlations of AVT with renal variables

Plasma AVT was expected to correlate with plasma osmolality, as P_{AVT} is generally considered to respond proportionately to P_{OSM} in birds (Koike et al. 1977; Gray and Erasmus 1989; Roberts 1991) and to dehydration and the resultant increase in P_{OSM} in reptiles; this response has been demonstrated in the goanna, V. gouldii (Bradshaw and Rice 1981; Rice 1982), and two snakes, Bothrops jararaca (Silveira et al. 1992; Silveira et al. 1998), and Notechis scutatus (Ladyman 2004). In the present study, however, a correlation between P_{OSM} and P_{AVT} was observed only for P. minor (Fig 5.3.3), and not C. nuchalis (Fig 5.3.4), although as discussed above, the chronic water-load group a higher P_{AVT} than expected, mostly likely due to stress. This, coupled with the high P_{OSM} of hydrated animals (discussed in Chapter 4) that showed a reduced P_{AVT}, is the likely cause of this discrepancy. In fact, Fig. 5.3.2 showed that apart from the chronic water-load lizards, P_{AVT} did increase significantly in dehydrated and salt-loaded animals, as expected.

The ratio of urine to plasma osmolality (U/P_{OSM}) was not correlated with P_{AVT} in either species. Because AVT increased the tubular
permeability to water in both species (Figs. 5.3.17 and 5.3.18), water-linked solute flow was increased and the luminal volume was reduced without an increase in osmolality (which would require impermeability of the nephron), resulting in maintenance of the U/P_{OSM} ratio. Dantzler (1989a) identifies the intermediate segment or early distal tubule as a potential location for dilution of the luminal fluid, and V₂-like AVT receptors are located in the intermediate segment of *Ctenophorus ornatus* nephrons, making this a likely site for increasing tubular permeability in response to increases in circulating AVT (Bradshaw and Bradshaw 2002).

FR_{H2O} was significantly correlated with P_{AVT} in *P. minor* but not *C. nuchalis*, while the reverse relationship was observed for GFR (Table 5.3.1). However, in both species, urine flow rate was reduced and tubular permeability increased in individuals with higher plasma AVT. Thus, while increasing AVT was associated with antidiuresis in both species, as expected, the mechanism of action of the hormone may differ subtly between them, mirroring the observations discussed in Chapter 4.

Despite the discrepancy in the response of GFR to P_{AVT} between species, both reduced GFR in response to dehydration and salt-loading. Similarly, *C. nuchalis* significantly increased FR_{H2O} in dehydrated and salt-loaded treatments, which at the same time experienced significant increases in P_{AVT}. This supports the idea that both GFR and FR_{H2O} are influenced by circulating AVT, as in *V. gouldii* (Bradshaw 1997) and suggests that AVT is acting as a physiological antidiuretic hormone in both lizard species.

AVT may be the principal antidiuretic hormone, but its actions and the osmoregulatory response may be augmented and influenced by several other hormones, such as aldosterone, the renin-angiotensin
system, natriuretic peptides and paracrine substances such as nitric oxide. That such a complex, redundant system exists is hardly surprising given the critical role of the kidney in the maintenance of the internal environment, and it is perhaps erroneous to accredit one hormone in particular with the total control of a suite of renal responses to osmoregulation. The interactions between these hormones are little known in reptiles and provide a wealthy source of future study.

With reference to the objectives of this chapter, the endocrine responses of *P. minor* and *C. nuchalis* to osmotic perturbation do differ, but the outcome in both cases is an antidiuresis. There was some evidence to suggest that in both lizard species, AVT was acting to reduce GFR and increase FR\textsubscript{H2O}, concomitantly reducing tubular permeability, as expected based on previous studies. *P. minor* responded to salt-loading in a very different manner to *C. nuchalis*, but fluid loss would presumably be reduced by post-renal modification of the urine. Finally, based on circulating AVT data, there is no convincing evidence to suggest that the endocrine system of *P. minor* is more mesic-adapted (or less arid-adapted) than *C. nuchalis*. 
CHAPTER 6:  GENERAL DISCUSSION
In this study I examined the relationships between renal morphology, function and control in three species of agamid lizards from differing habitats, in an effort to understand better the effect of factors such as habitat or phylogeny on the renal systems of each species. The species were chosen for their relatedness. Calculations based on a phylogenetic reconstruction of the Australian Agamidae by Melville et al. (2001) suggest that ancestral *Ctenophorus* diverged from ancestral *Pogona* between 14.6 and 14.2 MYBP; this was followed relatively quickly (13.1 MYBP) by a divergence between the lizards that would become *C. nuchalis* and *C. salinarum*. Any differences observed between the species are therefore more likely to be a result of adaptations to differing habitat and varying aridity occurring over the past 13 million years than the initial phylogenetic divergence; this is a central assumption of this study.

Morphologically, there was little difference between the kidneys of each species. Because of the small number of animals examined using stereological morphometry, it is not possible at this stage to distinguish significant differences between the species, especially as glomerular characteristics appear to be remarkably consistent. This is counter to the expectation that there would have been clearly definable differences between the glomerular size, number and density in the kidneys of each species. More mesic-inhabiting *P. minor* was expected to have both larger and more glomeruli than the arid-inhabiting *C. nuchalis*, in line with the expectation that in *P. minor* the kidney would operate more through filtration than secretion. Concomitantly, *C. nuchalis* was expected to be more capable of secretion of excess wastes in the absence of a large, and potentially wasteful, filtered load. *C. salinarum*, living in an environment judged to be intermediate between the two other species, was expected to have characteristics intermediate between them.
Concurrent with the morphological expectations discussed above were hypotheses linking expectations about each lizard’s renal morphology to their renal function, taking into account the aridity of the habitats occupied by each species. Specifically, the suggestion that *P. minor* would have a higher urine flow rate and GFR than *C. nuchalis*, concomitant with its more mesic distribution and greater access to water. *C. salinarum*, inhabiting an intermediate environment was expected to have a GFR and V intermediate between the two other lizards. However, as Table 4.3.2 shows, once corrected for body mass, both *P. minor* and *C. nuchalis* had a significantly higher V than *C. salinarum*, and did not differ significantly from one another. Thus, the morphological and physiological evidence obtained do not support the hypothesis that the kidneys of arid agamids operate in a ‘secretory’ mode and more mesic agamids operate in a ‘filtratory’ mode.

However, *P. minor* has smaller kidneys for its body size than either *Ctenophorus* species, suggesting that it may have a reduced capacity for solute reabsorption (see below). *P. minor* also demonstrated a remarkable ability to vary the relative contribution of the glomerular vs. tubular response (ΔGFR/ΔV), which neither species of *Ctenophorus* appears to possess (Table 4.3.15). Furthermore, *P. minor* utilised a different strategy to cope with salt-loading, presumably relying on the cloaca-colon complex for reclamation of fluid rather than reabsorbing fluid by solute-linked water flow in the kidney; this may be linked to the smaller kidney mass, and resultant reduced capacity for solute reabsorbance of this species. Finally, in *P. minor* the AVT profile in response to osmotic challenge was different to that of *C. nuchalis*. Thus, while the kidneys are morphologically similar, they possess different capacities for ion transport, and their control and function varies subtly within the confines of a generalised reptilian response to osmotic challenge.
Given that these lizards are closely related and radiated relatively recently, we might expect that their renal morphology and function would be similar. The three key species in this study belong to a group of lizards known as the amphiboluroids (Witten 1982; Greer 1989; Witten 1993), which have been noted for their arid-adaptedness. It has been suggested that ancestral amphiboluroids occupied a xeric refuge in the Pilbara area of Western Australia (Bradshaw 1986), which is postulated to have been arid since the mid-Miocene (Cogger and Heatwole 1981). Then, as the rest of Australia became progressively drier during the last 20 million years (Bowler 1976), they radiated across the continent, rapidly diversifying as they adapted to different ecological niches and modes (e.g. burrowing, arboreal, shrub/hummock sheltering) (Melville et al. 2001). Hence, the lack of differentiation between the renal anatomy may stem from their relatively recent radiation and arid-adapted heritage. In this study, therefore, attempting to minimise phylogenetic divergence by choosing closely-related species has also reduced the potential for each species to evolve specialisations to their respective environment. Furthermore, the environments inhabited by the different species may not be sufficiently variable in their availability of water to elicit such specialisations, and the subtle differences observed between the species in this study may reflect equally subtle differences in environmental conditions. A similar study to this, comparing congeners inhabiting more greatly variable environments such as desert and rainforest would, logically, increase the likelihood of finding differences between kidney morphology and function while still minimising phylogenetic difference.

Another factor working against specialisation in the kidney and the renal system in reptiles is the reptilian bauplan itself. Reptiles are masters of the frugal existence, in general having low energy requirements and efficient biomass production (Pough 1980; Bradshaw
1986), characteristics that enable them to survive when resources are scarce. Metabolism in reptiles, measured by resting metabolic rate, is between 10 and 20% of that of similarly-sized mammals or birds (Bennet and Dawson 1976; Bennet and Nagy 1977; Andrews and Pough 1985), but reptiles can alter this by changing their body temperature as required. These features, coupled with their ability to tolerate hypernatraemia (Bentley 1959; Bradshaw 1970), reduce a lizard’s need for a kidney capable of a high degree of water conservation. Furthermore, as discussed in Chapter 4, the cloaca-colon complex is a crucial osmoregulatory organ, and plays a vital role in the reclamation of water (Bentley and Schmidt-Nielsen 1965; Junqueira et al. 1966; Schmidt-Nielsen and Skadhauge 1967). Ureteral urine is always hypoosmotic in reptiles, but voided urine has been modified in the colon and may be hyperosmotic (Braysher 1976), conserving much of the water excreted by the kidneys. Thus, despite lacking cephalic salt-glands or a bladder, selection pressure on the renal system of agamid lizards is mitigated by their reptilian characteristics and efficient post-renal modification of urine, balancing the need for specialisations in renal form and function to cope with apparently extreme environments.

Some populations, such as that of *C. nuchalis* at Shark Bay where the lizards in this study were sourced, are semelparous, with almost all adults succumbing to extremes of temperature and aridity if rain does not fall (Bradshaw et al. 1991). The population is continued by hatchlings, which emerge in mid-summer and grow rapidly during the following spring such that they are mature by their first summer (Bradshaw et al. 1991), and die after breeding (Bradshaw 1997). Clearly it would be advantageous from an evolutionary perspective for adults to survive to the following summer to breed again, so the fact that 99% of the adults do not (Bradshaw et al. 1991) suggests that survival in this environment is extremely difficult. However, because of the high
reproductive potential of these lizards, the females of which may lay up to three clutches (Bradshaw 1997), the genes of individual lizards are likely to survive through to the next year regardless of whether the lizards die post-breeding, buffering selection pressure for better osmoregulatory systems. Thus, the renal system of *C. nuchalis* need only function well enough and long enough to allow the lizards to grow rapidly in spring and breed. During this time, prey is readily available and the animals are in good condition (Bradshaw and De’ath 1991; Nagy and Bradshaw 1995) and the daily activity window of the lizards is much longer than in summer (Bradshaw 1986) — the environment is hardly ‘extreme’. Definitions of an environment as extreme or mild, therefore, cannot be based solely on rainfall and temperature data. In this case, knowledge of the breeding biology of *C. nuchalis* has shown that in fact, during the most critical periods of the lizards’ life (growth and breeding), the kidneys are not required to cope with chronic dehydration or significant hypernatraemia.

In contrast, *P. minor* and *C. salinarum* are longer lived, and while little is known of their breeding biology, adults that survive to breed in successive years would have a selective advantage. Thus, despite their more mesic distributions relative to *C. nuchalis*, the renal systems of these species are required to function for the life span of the individual, and their absence in the truly arid environments inhabited by *C. nuchalis* may reflect an inability to do so.
CHAPTER 7: CONCLUSION

The central thesis that the renal systems of each species would vary in conjunction with their environment was not well supported. Contrary to this thesis, in Chapter 3 I demonstrated that the renal morphology of the three key species was remarkably similar and largely dependent on body size. However, *C. nuchalis* has a larger kidney than *P. minor*, which implies a greater capacity for reabsorption or excretion of ions and therefore an increased ability to modify the urine renally, rather than relying on post-renal modification by the cloaca-colon complex.

This suggestion is supported by the observation, presented in Chapter 4, that *P. minor* when salt-loaded excretes the excess salt, unlike *C. salinarum* and *C. nuchalis* which increase reabsorption of salt in order to reclaim as much fluid as possible from the urine prior to excretion. As a result, urine flow rate in salt-loaded *P. minor* is reduced primarily through a reduction of glomerular filtration rate. However, the response of dehydrated *P. minor* is very different, being primarily tubular as fractional reabsorption is increased markedly relative to all other groups. This tubular response is greater than that of dehydrated and salt-loaded *C. salinarum* and *C. nuchalis*, in which a combination of a reduction of GFR, an increase in FR$\text{H}_2\text{O}$, and an increase in tubular permeability are combined to effect an antidiuresis.

The mechanisms in each species are effective at producing an antidiuresis, and it is difficult to say which may be better suited to a particular environment, than the other. Support for the central hypothesis, that *P. minor* is more adapted to mesic conditions, is found in the greater sensitivity displayed by these lizards to osmotic challenge, since *P. minor* displays an antidiuresis of greater magnitude than *C. salinarum* or *C. nuchalis*. Thus, while there are differences in the
renal function of each species, there is only limited evidence to support the hypothesis that these differences are adaptive and related to the different environments inhabited by each species.

In Chapter 5, the response of circulating AVT to dehydration and salt-loading in P. minor and C. nuchalis was shown to differ. While there was an increase in $P_{AVT}$ of both salt-loaded and dehydrated C. nuchalis, only dehydrated P. minor showed a significant increase in $P_{AVT}$. This was correlated with a significant increase in $FR_{H2O}$ in this species. $P_{AVT}$ was also correlated with increased tubular permeability and a decrease in urine flow rate in both P. minor and C. nuchalis and it therefore appears that this hormone is acting in its traditional role as a physiological antidiuretic hormone. However it appears that above a threshold level of $P_{OSM}$ induced by salt-loading, tubular $FR_{H2O}$ and $P_{AVT}$ in P. minor remain at basal levels and the cloaca-colon complex is utilised as the major site for fluid reabsorption. This deficiency is not apparent in C. salinarum or C. nuchalis and suggests that the renal system of P. minor may be less able to cope with the higher $P_{OSM}$ experienced in more arid areas. This is further evidence suggesting that P. minor may be adapted to more mesic environments.

In conclusion, the thesis that the difference between the renal systems of these species are a result of adaptation to their different environments, while not disproved, is not conclusively supported. This may have been expected, based on the arid heritage of the group as a whole, the relatively recent divergence of the group, and reptilian plasticity in terms of breeding biology and behaviour, all of which serve to mitigate the selective pressure for divergence between the renal systems of these lizards. These factors, and the differences that exist between these closely-related lizards, are worth noting in the context of previous studies that, for the most part, seek to compare renal form and function in more widely disparate reptilian species.
REFERENCES


Bentley, PJ and Bradshaw, SD (1972). Electrical potential difference across the cloaca and colon of the Australian lizards *Amphibolurus ornatus* and *A. inermis*. *Comparative Biochemistry and Physiology* 42A: 465-471.


Bradshaw, SD, Shoemaker, VH and Nagy, KA (1972). The role of adrenal corticosteroids in the regulation of kidney function in the desert lizard *Dipsosaurus dorsalis*. *Comparative Biochemistry and Physiology* 43A: 621-635.


In: The kidney: physiology and pathophysiology. Eds. Seldin, D and Giebisch, G.
New York, Raven.


Kulczykowska, E (2001). Responses of circulating arginine vasotocin, isotocin, and melatonin to osmotic and disturbance stress in rainbow trout (Oncorhynchus mykiss). Fish Physiology and Biochemistry 24: 201-206.


